Application Notebook

Mass Spectrometry Applications for Food Safety Analysis

- Pesticides
- Drug Residues
- Mycotoxins
- Marine Toxins
- Additional Contaminants
- Natural Compounds and Food Additives

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Mass Spectrometry Applications for Food Safety Analysis

Table of Contents

Application Notebook

Pesticides

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Rapid Analysis of Pesticides in Difficult Matrices Using GC/MS/MS

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Key Words

- Fruit Preserve/Jam
- GC-MS/MS
- Multi-residue Method
- Pesticide Residues
- Selected Reaction Monitoring

Introduction

Pesticides are widely used in agriculture to protect crops and to improve efficiency of production. Consequently, governments, food producers and food retailers have a duty to ensure that any residues occurring in foods for human consumption are at or below Statutory Maximum Residue Levels (MRLs). Regulation EC 396/2005 adopted in the European Union sets MRLs for more than 500 different pesticides in over 300 different food commodities.[1] Many of these MRLs are set at a default value of 0.01 mg/kg, the typical limit of determination of routine analytical methods. Thus, there is a requirement for residue laboratories to test a wide array of foods for a large number of pesticides at concentrations at or below 0.01 mg/kg, with low costs and fast turnaround times (often < 48 hours). This is most often achieved using multi-residue methods based on the use of a combination of LC/MS/MS and GC/MS techniques to determine pesticide residues in a single generic solvent extract of the sample. One such example is the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) procedure, which is based on acetonitrile extraction and dispersive solid phase extraction.[2] Since acetonitrile is readily compatible with LC/MS/MS and allows analysis of several hundred pesticides this approach has gained in popularity. Many of the less polar semi-volatile pesticides not amenable to LC/MS can be analysed using GC/MS. Unfortunately the analysis of acetonitrile by GC/MS is more problematic. The main issues are:

- Degradation of the GC-column phase by the polar solvent
- Poor focusing of chromatographic peaks due to the high polarity and hence the inability to create a well defined starting zone on less polar column films
- Vapor overload of the insert liner with expansion of the sample beyond insert liner dimensions due to the high thermal expansion coefficient
- Contamination of the system by co-extractives which are not removed by the simple dispersive solid phase extraction clean-up employed in QuEChERS.
- Low crop concentration in the final extracts employed by QuEChERS requiring concentration of the extracts.

Because of the above problems, the detection limits for some pesticides can be too high for analysis at the MRL. It is a common practice to overcome these difficulties by concentration of the extract by evaporation, exchanging acetonitrile to a more appropriate GC solvent, or by using large volume injection techniques. However there is the potential to lose volatile analytes (e.g. dichlorvos) during evaporation and solvent exchange. Also the use of large volume injections can lead to more rapid contamination of the injection inlet as well as degradation of the analytical column.

An alternative approach is to make use of the high sensitivity and selectivity of the Thermo Scientific TSQ Quantum XLS GC-MS/MS instrument which can achieve the 0.01 mg/kg target reporting limits even with relatively low volume injections. This also overcomes the problems associated with the thermal expansion of acetonitrile and reduces the amount of matrix injected.

This application note describes the analytical methodology for a fast multi-residue pesticide determination in a difficult matrix (fruit jam), using the QuEChERS extraction/clean-up procedure in combination with the TSQ Quantum XLS™ GC-MS/MS system as the detection system.

Experimental Conditions/Methods

Sample Preparation

The strawberry jam samples were extracted using the citrate-buffered QuEChERS procedure.[3] Homogenized sample (10 g) was mixed with water (10 mL) and acetonitrile (10 mL). After the addition of Internal Standard (triphenylphosphate, TPP) the mixture was shaken for 1 minute. Then $MgSO_4$ (4 g), NaCl (1 g), disodium hydrogen citrate (0.5 g) and trisodium citrate (1 g) were added and the mixture shaken for 1 minute and then centrifuged for 5 minutes at 3000 U/min. An aliquot of the acetonitrile portion (2 mL) was transferred to a new tube and $MgSO_4$ (300 mg) and primary-secondary amine (PSA) sorbent (50 mg) added. The mixture was shaken (1 minute) and centrifuged for 5 minutes at 3000 U/min. An aliquot of the supernatant (1 mL) was immediately transferred into a GC vial and acidified (10 µL of 5 % formic acid in acetonitrile). Then 1.0 µL of extract was injected into the GC-MS/MS system. The final concentration of sample was 1 g/mL of extract.

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Instrument Setup and Conditions

Determination of pesticides was carried out using a TSQ Quantum XLS GC-MS/MS system, equipped with a split/splitless injector and Thermo Scientific TriPlus automatic liquid sampler. The analytical column used was a Thermo Scientific TR-Pesticide, 30 m x 0.25 mm i.d., 0.25 µm film thickness.

The GC-MS/MS conditions used are shown in Table 1, the list of selected reaction monitoring transitions applied is summarized in Table 2.

Results and Discussion

In this application note we present a simple and rapid method based on QuEChERS extraction and GC-tandem quadrupole mass spectrometry (GC/MS/MS) determination of 96 priority pesticides plus transformation products in jam samples. The samples of fruit jam were extracted with acetonitrile followed by dispersive SPE clean-up with PSA prior to detection by GC/MS/MS. The high sensitivity and selectivity of the TSQ Quantum XLS GC-MS/MS system has enabled direct splitless analysis using low volume (1.0 µL) aliquots of acetonitrile extracts. This has significantly simplified the sample preparation procedure, while meeting method performance criteria specified by EU method validation and quality control procedures for pesticide residues in food and feed.[4] To overcome matrix effects, calibration of the GC-MS/MS system was performed using matrix-matched standard calibration solutions.[5,6]

Validation of the methodology was carried out using samples spiked with known amounts of selected pesticides at concentrations between 0.01 and 0.05 mg/kg for 89 analytes. A further 7 analytes were spiked at higher levels (see Table 3), as reporting limits for these pesticides are correspondingly higher in UK monitoring. The recovery and precision data are summarized in Table 3. Except for chlorothalonil all of the pesticides met the EU DG SANCO method validation criteria.[4] Pesticides such as captan, dichlofluanid and iprodione, which are frequently difficult to analyse, showed good recovery and precision data. The study on the improvement of chlorothalonil recovery from the matrix is now under further investigation.

The calibration curves were linear over wide concentration ranges with correlation coefficients (r^2) > 0.98 for all analytes, except for dicofol. Also, the SRM chromatograms demonstrated high selectivity with no significant interferences observed and an excellent signal/noise ratio (> 5:1) for all analytes, even at the lowest calibrated level (5 ng/mL equivalent to 0.005 mg/kg). All analytes, except folpet and diphenylamine, could be confirmed with the second transition in the low level spiked samples. Examples of extracted ion chromatograms for dichlofluanid and deltamethrin at 0.01 mg/kg and captan at 0.02 mg/kg are illustrated in Figure 1. In all cases the detection limits comply with required MRLs.

Conclusion

The QuEChERS-GC/MS/MS multi-residue method described here is a simple, rapid and accurate approach suitable for the monitoring of GC amenable pesticides in accordance with EU requirements. Another advantage of the extraction method used is its applicability to pesticides amenable to LC analysis. During method validation it was found that recovery, % CV and linearity data were within EU DG SANCO criteria for all 96 pesticides, except chlorothalonil at 0.01 mg/kg and dicofol. However the recovery data for chlorothalonil spiked at 0.01 mg/kg (mean 63%, 11 % CV) and the consistent recovery and precision data obtained for dicofol showed that the methodology was suitable for screening purposes.

Extracted ion chromatograms of SRM traces of analytes demonstrated excellent selectivity with no interferences observed and excellent signal/noise ratios (> 5:1), for all analytes at the lowest calibrated level (typically 0.005 mg/kg).

The robustness of the system was further demonstrated during validation and analysis of pork ham samples for the UK Monitoring Programme, when similarly good quality analytical data was obtained for these analytes.

Thermo Scientific GC Trace Ultra Conditions		Thermo Scientific TSQ Quantum MS/MS Conditions	
Column	TR-Pesticide 30 m × 0.25 mm × 0.25 µm	Operating mode	Selected Reaction Monitoring (SRM)
Injector	Splitless	Ionization mode	EI
Injected volume	1 µL	Electron energy	70 eV
Injector temperature	225 °C	Emission current	50 µA
Carrier gas	Helium, 1 mL/min	Q1/Q3 resolution	0.7 u (FWHM)
Oven program	60 °C hold 1 min 15 °C/min to 160 °C hold 1 min 2.2 °C/min to 230 °C hold 1 min 5 °C/min to 290 °C hold 5 min Run Time 57.15 min	Collision gas Collision gas pressure	Argon 1 mTorr
	Transfer line temperature 280 °C	Polarity	Positive

Table 1: Instrumental conditions

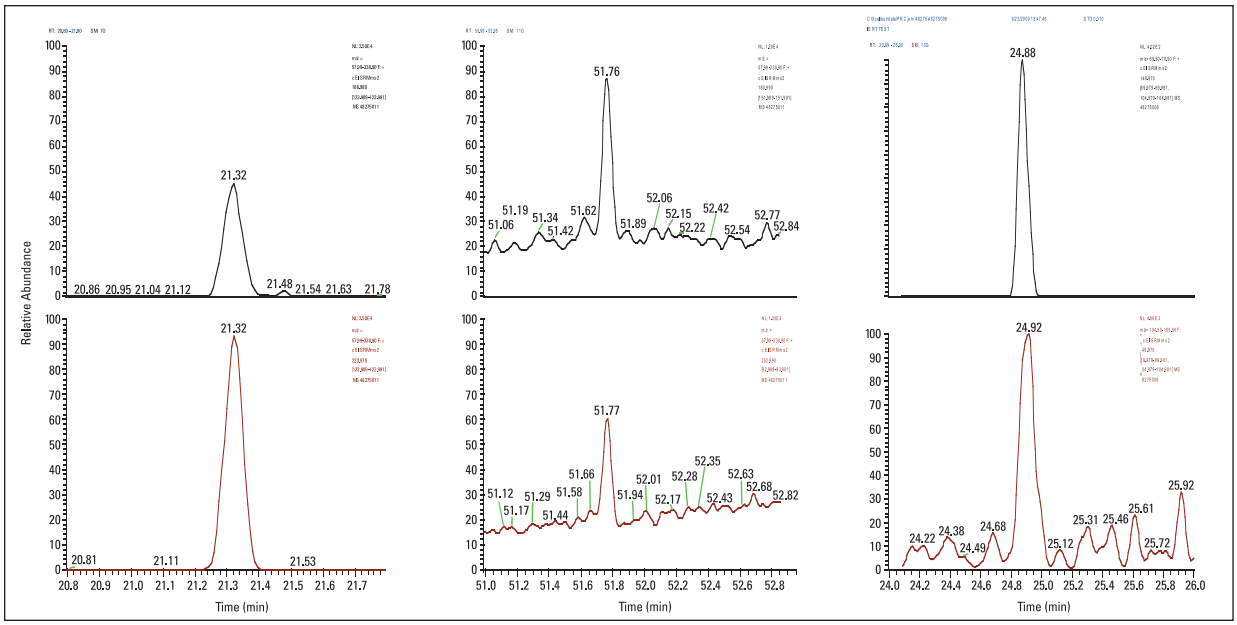

Figure 1: Extracted ion chromatogram of quantifier (upper trace) and qualifier ions (lower trace) for (A) dichlofluanid, (B) deltamethrin in a fruit preserve sample spiked at 0.01 mg kg^{-1} and (C) captan at 0.02 mg kg^{-1}. Transitions used are summarized in Table 2.

Name	Rt (min)	Transition	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Collision Energy (eV)	Timed SRMs Start (min)	Timed SRMs End (min)	Polarity
dichlorvos	6.44	Quant	184.95	92.98	17	5.44	7.44	Pos
dichlorvos	6.44	Qual	219.95	184.95	10	5.44	7.44	Pos
propham	8.90	Quant	179.09	137.07	10	7.90	9.90	Pos
propham	8.90	Qual	137.07	93.05	8	7.90	9.90	Pos
phthalimide (folpet bd*)	9.05	Quant	147.03	103.02	10	8.05	10.05	Pos
phthalimide (folpet bd*)	9.05	Qual	147.03	76.02	10	8.05	10.05	Pos
tetrahydrophthalimide (captan bd*)	9.35	Quant	151.06	122.05	10	8.35	10.35	Pos
tetrahydrophthalimide (captan bd*)	9.35	Qual	151.06	79.03	10	8.35	10.35	Pos
methacrifos	9.62	Quant	208.02	180.02	10	8.62	10.62	Pos
methacrifos	9.62	Qual	240.02	208.02	10	8.62	10.62	Pos
tecnazene	11.69	Quant	260.88	202.90	15	10.69	12.69	Pos
tecnazene	11.69	Qual	258.88	200.90	15	10.69	12.69	Pos
propachlor	11.80	Qual	176.06	120.04	10	10.80	12.80	Pos
propachlor	11.80	Qual	196.07	120.04	10	10.80	12.80	Pos
diphenylamine	12.02	Quant	167.09	139.07	25	11.02	13.02	Pos
ethoprophos	12.19	Quant	200.05	158.04	10	11.19	13.19	Pos
ethoprophos	12.19	Qual	158.04	114.03	10	11.19	13.19	Pos
chlorpropham	12.59	Quant	213.06	171.04	10	11.59	13.59	Pos
chlorpropham	12.59	Qual	213.06	127.03	15	11.59	13.59	Pos
dicrotophos	13.02	Quant	127.04	109.04	10	12.02	14.02	Pos
dicrotophos	13.02	Qual	193.06	127.04	10	12.02	14.02	Pos
trifluralin	13.14	Quant	306.10	264.09	15	12.14	14.14	Pos
trifluralin	13.14	Qual	264.09	160.05	15	12.14	14.14	Pos
cadusafos	13.41	Quant	159.05	131.04	10	12.41	14.41	Pos
cadusafos	13.41	Qual	159.05	97.03	20	12.41	14.41	Pos
hexachlorobenzene	14.27	Quant	283.81	248.84	20	13.27	15.27	Pos
hexachlorobenzene	14.27	Qual	285.81	250.83	20	13.27	15.27	Pos
dicloran	14.48	Quant	205.97	175.97	10	13.48	15.48	Pos
dicloran	14.48	Qual	207.96	177.97	10	13.48	15.48	Pos
simazine	14.80	Quant	201.08	173.07	10	13.80	15.80	Pos
simazine	14.80	Qual	201.08	138.05	10	13.80	15.80	Pos

Table 2: Selected Reaction Monitoring transitions used

Name	Rt (min)	Transition	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Collision Energy (eV)	Timed SRMs Start (min)	Timed SRMs End (min)	Polarity
carbofuran	14.88	Quant	164.08	149.07	10	13.88	15.88	Pos
carbofuran	14.88	Qual	221.11	164.08	5	13.88	15.88	Pos
HCH-gamma	15.53	Quant	218.89	182.91	15	14.53	16.53	Pos
HCH-gamma	15.53	Qual	180.91	108.95	25	14.53	16.53	Pos
quintozene	15.80	Quant	248.86	213.88	10	14.80	16.80	Pos
quintozene	15.80	Qual	294.84	236.87	20	14.80	16.80	Pos
fonofos	15.97	Quant	246.03	137.02	10	14.97	16.97	Pos
fonofos	15.97	Qual	137.02	109.01	10	14.97	16.97	Pos
diazinon	16.48	Quant	152.00	137.00	10	15.48	17.48	Pos
diazinon	16.48	Qual	304.10	179.06	15	15.48	17.48	Pos
tefluthrin	17.12	Quant	177.02	127.02	20	16.12	18.12	Pos
tefluthrin	17.12	Qual	197.03	141.02	15	16.12	18.12	Pos
chlorothalonil	17.14	Quant	265.88	169.92	20	16.14	18.14	Pos
chlorothalonil	17.14	Qual	265.88	132.94	20	16.14	18.14	Pos
etrimfos	17.35	Quant	292.06	181.04	10	16.35	18.35	Pos
etrimfos	17.35	Qual	292.06	153.03	10	16.35	18.35	Pos
formothion	17.94	Quant	126.00	93.00	8	16.94	18.94	Pos
formothion	17.94	Qual	224.00	125.00	15	16.94	18.94	Pos
chlorpyrifos-methyl	19.23	Quant	285.91	92.97	15	18.23	20.23	Pos
chlorpyrifos-methyl	19.23	Qual	124.96	78.97	10	18.23	20.23	Pos
parathion-methyl	19.23	Quant	263.00	109.00	15	18.23	20.23	Pos
parathion-methyl	19.23	Qual	263.00	127.00	20	18.23	20.23	Pos
vinclozolin	19.25	Quant	285.00	212.00	15	18.25	20.25	Pos
vinclozolin	19.25	Qual	212.00	172.00	15	18.25	20.25	Pos
tolclofos-methyl	19.48	Quant	264.96	249.96	18	18.48	20.48	Pos
tolclofos-methyl	19.48	Qual	264.96	219.97	20	18.48	20.48	Pos
fenitrothion	21.01	Quant	277.02	260.02	10	20.01	22.01	Pos
fenitrothion	21.01	Qual	277.02	109.01	20	20.01	22.01	Pos
pirimiphos-methyl	21.21	Quant	290.09	233.07	10	20.21	22.21	Pos
pirimiphos-methyl	21.21	Qual	305.10	290.09	15	20.21	22.21	Pos
ethofumesate	21.25	Quant	286.11	207.08	12	20.25	22.25	Pos
ethofumesate	21.25	Qual	207.08	161.06	10	20.25	22.25	Pos
dichlofluanid	21.45	Quant	223.97	122.99	15	20.45	22.45	Pos
dichlofluanid	21.45	Qual	166.98	123.99	12	20.45	22.45	Pos
aldrin	21.71	Quant	262.91	192.93	32	20.71	22.71	Pos
aldrin	21.71	Qual	292.90	257.91	20	20.71	22.71	Pos
chlorpyrifos	22.40	Quant	196.96	168.96	15	21.40	23.40	Pos
chlorpyrifos	22.40	Qual	313.93	257.95	15	21.40	23.40	Pos
dichlorobenzophenone, 4,4'- (dicofol bd*)	22.47	Quant	139.00	111.00	15	21.47	23.47	Pos
dichlorobenzophenone, 4,4'- (dicofol bd*)	22.47	Qual	250.00	139.00	10	21.47	23.47	Pos
parathion-ethyl	22.47	Quant	291.03	109.01	15	21.47	23.47	Pos
parathion-ethyl	22.47	Qual	125.01	97.01	8	21.47	23.47	Pos
chlorthal-dimethyl	22.71	Quant	331.90	300.91	15	21.71	23.71	Pos
chlorthal-dimethyl	22.71	Qual	300.91	222.93	25	21.71	23.71	Pos
isocarbofos	22.89	Quant	119.98	92.30	10	21.89	23.89	Pos
isocarbofos	22.89	Qual	135.96	108.34	10	21.89	23.89	Pos
nitrothal-isopropyl	23.10	Quant	236.08	194.07	10	22.10	24.10	Pos
nitrothal-isopropyl	23.10	Qual	236.08	148.05	20	22.10	24.10	Pos
pirimiphos-ethyl	24.08	Quant	304.12	168.06	15	23.08	25.08	Pos
pirimiphos-ethyl	24.08	Qual	318.12	166.06	13	23.08	25.08	Pos
isofenphos-methyl	24.22	Quant	199.06	121.04	15	23.22	25.22	Pos
isofenphos-methyl	24.22	Qual	241.07	199.06	10	23.22	25.22	Pos
oxychlordane	24.47	Quant	386.79	262.86	15	23.47	25.47	Pos
oxychlordane	24.47	Qual	386.79	322.83	15	23.47	25.47	Pos

Table 2 Continued: Selected Reaction Monitoring transitions used

Name	Rt (min)	Transition	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Collision Energy (eV)	Timed SRMs Start (min)	Timed SRMs End (min)	Polarity
pendimethalin	24.57	Quant	252.12	162.08	12	23.57	25.57	Pos
pendimethalin	24.57	Qual	252.12	191.09	12	23.57	25.57	Pos
pyrifenox-e (peak 1)	24.93	Quant	262.03	200.02	20	23.93	27.72	Pos
pyrifenox-e (peak 1)	24.93	Qual	262.03	192.02	20	23.93	27.72	Pos
tolylfluanid	24.98	Quant	238.09	137.05	15	23.98	25.98	Pos
tolylfluanid	24.98	Qual	137.05	91.03	20	23.98	25.98	Pos
chlozolinate	25.08	Quant	331.00	259.00	8	24.08	26.08	Pos
chlozolinate	25.08	Qual	259.00	188.00	8	24.08	26.08	Pos
captan	25.10	Quant	148.97	104.98	10	24.10	26.10	Pos
captan	25.10	Qual	148.97	69.98	15	24.10	26.10	Pos
isofenphos	25.31	Quant	213.07	121.04	17	24.31	26.31	Pos
isofenphos	25.31	Qual	213.07	185.06	10	24.31	26.31	Pos
quinalphos	25.44	Quant	146.03	118.02	15	24.44	26.44	Pos
quinalphos	25.44	Qual	157.03	129.02	13	24.44	26.44	Pos
folpet	25.54	Quant	259.91	129.96	16	24.54	26.54	Pos
folpet	25.54	Qual	146.95	102.97	10	24.54	26.54	Pos
furalaxyl	25.78	Quant	242.11	95.04	15	24.78	26.78	Pos
furalaxyl	25.78	Qual	301.13	225.10	10	24.78	26.78	Pos
procymidone	25.90	Quant	283.02	96.01	15	24.90	26.90	Pos
procymidone	25.90	Qual	283.02	255.02	10	24.90	26.90	Pos
chlordane (*cis/trans*)**	26.05	Quant	373.00	264.00	15	25.05	28.14	Pos
chlordane (*cis/trans*)**	26.05	Qual	372.81	265.87	15	25.05	28.14	Pos
methidathion	26.34	Quant	144.98	84.99	10	25.34	27.34	Pos
methidathion	26.34	Qual	144.98	57.99	15	25.34	27.34	Pos
bromophos-ethyl	26.56	Quant	358.89	302.91	20	25.56	27.56	Pos
bromophos-ethyl	26.56	Qual	358.89	330.90	10	25.56	27.56	Pos
endosulfan (I)	26.88	Quant	240.89	205.91	20	25.88	27.88	Pos
endosulfan (I)	26.88	Qual	271.88	236.89	10	25.88	27.88	Pos
tetrachlorvinphos	27.17	Quant	328.91	108.97	22	26.17	28.17	Pos
tetrachlorvinphos	27.17	Qual	330.91	315.91	22	26.17	28.17	Pos
hexaconazole	28.07	Quant	214.05	187.04	15	27.07	29.07	Pos
hexaconazole	28.07	Qual	214.05	159.04	15	27.07	29.07	Pos
prothiofos	28.41	Quant	308.97	238.97	5	27.41	29.41	Pos
prothiofos	28.41	Qual	266.97	238.97	10	27.41	29.41	Pos
dieldrin	28.74	Quant	276.91	240.92	10	27.74	29.74	Pos
dieldrin	28.74	Qual	262.91	192.93	26	27.74	29.74	Pos
DDE-pp	28.89	Quant	245.95	175.97	25	27.89	29.89	Pos
DDE-pp	28.89	Qual	317.94	247.95	20	27.89	29.89	Pos
fludioxonil	29.04	Quant	248.04	154.02	20	28.04	30.04	Pos
fludioxonil	29.04	Qual	248.04	182.03	15	28.04	30.04	Pos
buprofezin	29.74	Quant	249.13	193.10	10	28.74	30.74	Pos
buprofezin	29.74	Qual	305.16	172.09	10	28.74	30.74	Pos
flusilazole	29.84	Quant	233.07	152.05	20	28.84	30.84	Pos
flusilazole	29.84	Qual	233.07	165.05	20	28.84	30.84	Pos
bupirimate	30.16	Quant	273.14	193.10	10	29.16	31.16	Pos
bupirimate	30.16	Qual	316.16	208.10	10	29.16	31.16	Pos
endosulfan (II)	30.98	Quant	240.89	205.91	20	29.98	31.98	Pos
endosulfan (II)	30.98	Qual	271.88	236.89	18	29.98	31.98	Pos
chlorobenzilate	31.33	Quant	251.02	139.01	20	30.33	32.33	Pos
chlorobenzilate	31.33	Qual	251.02	111.01	20	30.33	32.33	Pos
DDD-pp/DDT-op	31.95	Quant	234.97	164.98	20	30.95	32.95	Pos
DDD-pp/DDT-op	31.95	Qual	234.97	198.97	18	30.95	32.95	Pos
ethion	32.45	Quant	230.99	174.99	15	31.45	33.45	Pos
ethion	32.45	Qual	230.99	129.00	20	31.45	33.45	Pos

Table 2 Continued: Selected Reaction Monitoring transitions used

Name	Rt (min)	Transition	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Collision Energy (eV)	Timed SRMs Start (min)	Timed SRMs End (min)	Polarity
benalaxyl	34.07	Quant	266.14	148.08	10	33.07	35.07	Pos
benalaxyl	34.07	Qual	234.12	174.09	10	33.07	35.07	Pos
endosulfan-sulphate	34.17	Quant	227.01	212.01	15	33.17	35.17	Pos
endosulfan-sulphate	34.17	Qual	271.88	236.89	15	33.17	35.17	Pos
methoxychlor bd	34.17	Quant	228.01	213.01	15	33.17	35.17	Pos
methoxychlor bd	34.17	Qual	273.88	238.89	15	33.17	35.17	Pos
DDT-pp	34.68	Quant	234.94	164.96	20	33.68	35.68	Pos
DDT-pp	34.68	Qual	234.94	198.95	15	33.68	35.68	Pos
TPP (IS)	36.19	Quant	326.07	325.00	10	35.19	37.19	Pos
TPP (IS)	36.19	Qual	326.07	215.00	25	35.19	37.19	Pos
propargite	36.26	Quant	135.06	107.05	15	35.26	37.26	Pos
propargite	36.26	Qual	173.08	105.05	12	35.26	37.26	Pos
diflufenican	36.55	Quant	394.07	266.05	10	35.55	37.55	Pos
diflufenican	36.55	Qual	266.05	246.05	10	35.55	37.55	Pos
iprodione	38.21	Quant	314.03	245.03	15	37.21	39.21	Pos
iprodione	38.21	Qual	314.03	271.03	10	37.21	39.21	Pos
phosmet	38.26	Quant	160.00	133.00	15	37.26	39.26	Pos
phosmet	38.26	Qual	160.00	77.00	20	37.26	39.26	Pos
pyridaphenthion	38.40	Quant	340.06	199.04	10	37.40	39.40	Pos
pyridaphenthion	38.40	Qual	340.06	203.04	25	37.40	39.40	Pos
bromopropylate	38.60	Quant	184.98	156.98	20	37.60	39.60	Pos
bromopropylate	38.60	Qual	342.96	184.98	20	37.60	39.60	Pos
EPN	38.70	Quant	169.02	141.02	10	37.70	39.70	Pos
EPN	38.70	Qual	157.02	110.01	15	37.70	39.70	Pos
dicofol	39.08	Quant	138.97	110.97	15	38.08	40.08	Pos
dicofol	39.08	Qual	164.09	107.06	17	38.08	40.08	Pos
tetramethrin	39.08	Quant	164.09	135.07	10	38.08	40.08	Pos
tetramethrin	39.08	Qual	250.94	138.97	15	38.08	40.08	Pos
methoxychlor	39.25	Quant	227.01	169.01	20	38.25	40.25	Pos
methoxychlor	39.25	Qual	227.01	212.01	15	38.25	40.25	Pos
TPE (alternative IS)	39.44	Quant	332.21	253.13	15	38.44	40.44	Pos
TPE (alternative IS)	39.44	Qual	332.21	254.14	15	38.44	40.44	Pos
fenpropathrin	39.60	Quant	181.09	152.07	23	38.60	40.60	Pos
fenpropathrin	39.60	Qual	265.13	210.10	15	38.60	40.60	Pos
fenazaquin	39.77	Quant	145.08	117.07	15	38.77	40.77	Pos
fenazaquin	39.77	Qual	160.09	117.07	20	38.77	40.77	Pos
tetradifon	40.61	Quant	355.88	228.93	10	39.61	41.61	Pos
tetradifon	40.61	Qual	226.93	198.94	18	39.61	41.61	Pos
phosalone	41.32	Quant	181.99	111.00	15	40.32	42.32	Pos
phosalone	41.32	Qual	366.99	181.99	10	40.32	42.32	Pos
fenarimol	43.10	Quant	139.01	111.01	15	42.10	44.10	Pos
fenarimol	43.10	Qual	251.02	139.01	15	42.10	44.10	Pos
pyrazophos	43.85	Quant	221.05	193.04	10	42.85	44.85	Pos
pyrazophos	43.85	Qual	232.05	204.05	10	42.85	44.85	Pos
bitertanol	45.06	Quant	170.09	141.07	20	44.06	46.06	Pos
bitertanol	45.06	Qual	170.09	115.06	25	44.06	46.06	Pos
permethrin *cis/trans***	45.36	Quant	183.04	168.03	15	44.36	46.36	Pos
permethrin *cis/trans***	45.36	Qual	183.04	165.03	15	44.36	46.36	Pos
pyridaben	45.58	Quant	147.06	117.04	20	44.58	46.58	Pos
pyridaben	45.58	Qual	309.12	147.06	15	44.58	46.58	Pos
cyfluthrin**	47.13	Quant	163.02	91.01	12	46.13	48.13	Pos
cyfluthrin**	47.13	Qual	226.03	206.03	5	46.13	48.13	Pos
cypermethrin**	47.94	Quant	181.03	152.03	25	46.94	48.94	Pos
cypermethrin**	47.94	Qual	163.03	127.02	10	46.94	48.94	Pos
fenvalerate-e/z**	50.14	Quant	167.05	125.04	10	49.14	51.14	Pos
fenvalerate-e/z**	50.14	Qual	419.13	225.07	10	49.14	51.14	Pos

Table 2 Continued: Selected Reaction Monitoring transitions used

Name	Rt (min)	Transition	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Collision Energy (eV)	Timed SRMs Start (min)	Timed SRMs End (min)	Polarity
deltamethrin	51.85	Quant	180.99	151.99	20	50.85	52.85	Pos
deltamethrin	51.85	Qual	252.99	93.00	18	50.85	52.85	Pos
famoxadone	52.52	Quant	330.11	224.08	10	51.52	53.52	Pos
famoxadone	52.52	Qual	330.11	237.08	15	51.52	53.52	Pos

** bd* *breakdown product not in standard mix*
*** rt cis-chlordane* *27.11*
*** Rt permethrin-trans* *45.77*
*** Rt cyfluthrin-II – IV* *47.51 – 47.72*
*** Rt cypermethrin-II – IV* *48.22 – 48.61*
*** Rt fenvalerate-z* *50.64*

Table 2 Continued: Selected Reaction Monitoring transitions used

Pesticide	0.01 mg/kg mean	0.01 mg/kg CV	0.05 mg/kg mean	0.05 mg/kg CV
aldrin	89	11	94	3
benalaxyl	101	6	108	3
bromophos-ethyl	93	10	101	4
bromopropylate	93	6	104	5
bupirimate	97	9	110	3
buprofezin	97	9	110	3
cadusafos	97	6	105	4
carbofuran	95	7	107	5
chlordane-cis	90	13	104	1
chlordane-trans	94	5	101	2
chlorobenzilate	103	7	108	2
chlorothalonil	63	11	84	5
chlorpyrifos	91	8	102	1
chlorpyrifos-methyl	95	4	103	3
chlorthal-dimethyl	87	9	102	3
chlozolinate	98	15	107	5
DDD-pp	94	6	103	3
DDE-pp	85	6	97	3
DDT-op	92	7	101	3
DDT-pp	94	5	102	2
deltamethrin	93	18	104	5
diazinon	97	7	107	5
dichlofluanid	80	9	94	6
dichlorvos	95	9	105	3
dicloran	92	5	105	5
dicrotophos	89	9	103	3
dieldrin	89	7	100	3
diflufenican	101	7	109	1
endosulfan-I	94	14	101	3
endosulfan-II	94	14	105	6
endosulfan-sulfate	102	8	109	3
EPN	95	9	105	3
ethion	103	9	105	3
ethofumesate	95	5	107	4
ethoprophos	102	9	108	4
etrimfos	94	4	106	4
famoxadone	92	8	100	6
fenarimol	95	7	105	3
fenazaquin	95	13	98	5
fenitrothion	93	8	106	7
fenpropathrin	95	13	107	4
fenvalerate	97	6	103	3
fludioxonil	103	7	104	5
flusilazole	93	11	111	6
folpet	93	9	97	8
fonofos	94	8	103	3
formothion	93	7	105	3
furalaxyl	96	6	108	5
HCB	72	10	86	4
HCH-gamma	94	5	103	3
hexaconazole	99	13	105	8
iprodione	101	6	110	3
isocarbofos	97	9	111	7
isofenphos	99	5	107	2
isofenphos-methyl	100	8	105	3
methacrifos	93	7	107	3
methidathion	100	8	104	3
methoxychlor	94	10	103	2
nitrothal-isopropyl	101	9	103	5
oxychlordane	99	21	99	6
parathion-ethyl	98	6	102	3
pendimethalin	97	9	103	4
parathion-methyl	100	8	105	5
permethrin	90	7	99	3
phosalone	103	7	105	3
phosmet	102	6	105	6
pirimiphos-ethyl	97	2	106	2
pirimiphos-methyl	95	7	104	3
procymidone	93	9	105	7
propachlor	92	11	107	2
propargite	98	11	107	4
propham	95	5	104	4
prothiofos	80	7	102	4
pyrazophos	97	7	106	3
pyridaben	100	6	104	3
pyridaphenthion	97	8	107	2
pyrifenox	97	6	107	1
quinalphos	102	9	102	3
quintozene	86	9	94	5
simazine	91	7	105	7
tecnazene	81	5	95	5
tefluthrin	90	8	103	3
tetrachlorvinphos	99	3	110	3
tetradifon	97	6	107	2
tetramethrin	97	8	106	2
tolclofos-methyl	95	8	101	3
tolylfluanid	98	8	100	3
trifluralin	97	8	104	2
vinclozolin	92	5	104	6

	0.02 mg/kg		0.10 mg/kg	
captan	108	8	96	9
cyfluthrin	110	5	105	4
	0.05 mg/kg		**0.25 mg/kg**	
chlorpropham	98	7	108	5
bitertanol	98	7	103	4
cypermethrin	103	6	102	3
dicofol	108	10	94	10
diphenylamine	94	6	99	4

Table 3: Validation data for fruit preserve (jam), n = 5

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Improved Screening for 250 Pesticides in Matrix using a LC-Triple Quadrupole Mass Spectrometer

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Overview

Purpose: Demonstration of the effect of a drag field in Q2 on signal intensity at short dwell times.

Methods: Pesticides were analyzed at various dwell times and in the presence of matrix on a Thermo Scientific™ TSQ Quantiva™ triple quadrupole mass spectrometer.

Results: We demonstrate effectiveness of a Q2 drag field in maintaining signal intensity at extremely low dwell times enabling data acquisition rates of 500 SRMs/sec which facilitate the monitoring of 250 pesticides simultaneously in an onion matrix.

Introduction

Both food safety and environmental regulatory requirements are demanding greater sensitivity on an increasing number of analytes. In addition, there are an increasing number of matrices to be evaluated. To meet these requirements, it is necessary to analyze a large number of analytes quickly at low levels. Triple quadrupole mass spectrometers are the industry standard for fast and reliable analysis achieved through the selected reaction monitoring experiment (SRM). This experiment is extremely efficient however, it too is limited by intrascan delays and dwell times required to get the maximum sensitivity and reproducibility. The experiments described here utilize a triple quadrupole MS equipped with a new Q2 collision cell enabling the rapid analysis of 250 pesticides in a screening application.

Methods

Liquid Chromatography

Separations were performed using a Thermo Scientific™ Dionex™ UltiMate™ 3000 XRS LC system with a Thermo Scientific™ Hypersil™ Gold aQ, 100x2.1mm ID, 1.9µm particle HPLC Column. The flow rate was 300 µL/min using the following chromatographic gradient:

Mobile Phase: A: 0.1% Formic Acid + 5 mM Ammonium Formate (aq)
B: Methanol + 0.1% Formic Acid + 5 mM Ammonium Formate

Gradient:

Time (min)	%B
0.00	2
0.25	30
35.00	100
40.00	100
40.01	2
45.00	2

Mass Spectrometer Method

Samples were analyzed using a TSQ Quantiva triple-quadrupole mass spectrometer in SRM mode. Ionization was performed using the new Ion Max NG ion source operated in heated electrospray ionization mode H(ESI), shown in Figure 1, with a vaporizer temperature of 425 °C and a capillary temperature of 350 °C. Sheath and auxiliary gas flows were 45 and 10 (arbitrary units) respectively with an ionization voltage of 3000 volts in both positive ion mode. Collision gas pressure was set at 1.5 mTorr throughout the experiments. Preliminary data was acquired for the mid-level standard (10 ppb) at dwell times of 1, 2, 5, 20, and 100 MS to assess the effect of dwell time on peak area; all subsequent data was acquired with a dwell time of 1 msecond with a total interscan delay of 1 msecond.

FIGURE 1. Ion Max NG source displaying drain extension for efficient removal of solvent and matrix reducing background, thereby improving sensitivity and overall system robustness in the presence of dirty matrices such as the onion matrix used in this study.

Results

Dwell Time Assessment

It is well known that in the absence of a drag field in Q2, decreasing dwell times result in a decrease in signal intensity, thus affecting both sensitivity and reproducibility, especially at low concentration levels of analytes. In the presence of the drag field, signal can be minimized even at dwell times as low as1 msec. The TSQ Quantiva MS creates a drag field by applying an axial DC potential to the 90° Q2 as shown in Figure 2. This is referred to as the "active collision cell". To assess the effect of the drag field, studies were performed 100, 20 ,5, 2, and 1 msec dwell times using the pesticide azoxystrobin. These studies assess the effect of the drag field and differing dwell times on signal intensity and reproducibility. It is clear from the results shown in Figure 3, that even at dwell times as low as 1 msec, there is no drop in signal and the reproducibility across all dwell times is only 3.14 % RSD.

The capability to reduce dwell times enables us to monitor more transitions per unit time thus, reducing HPLC run times, enabling the utilization of UPLC technology, or monitor numerous compounds at a single time.

FIGURE 2. Q2 from the TSQ Quantiva MS. Fragment ions are accelerated through the cell with the application of an axial DC potential, enabling data acquisition at dwell times as low as 1msec without signal loss. This facilitates the ability to acquire in excess of 500 SRMs/second.

FIGURE 3. Dwell time study on azoxystrobin. 10 ppb Azoxystrobin was analyzed at 100, 20, 5, 2, 1 msec dwell times. The %RSD for all dwell times combined was 3.14%.

	Sample Name	Area	ISTD Area	%RSD-RESP	RT	Exclude
1	Azoxy 1msec dwell	10459151	NA	3.14	2.51	
2	Azoxy 1msec dwell	10379082	NA	3.14	2.51	
3	Azoxy 1msec dwell	10494645	NA	3.14	2.50	
4	Azoxy 1msec dwell	10659557	NA	3.14	2.50	
5	Azoxy 1msec dwell	10648577	NA	3.14	2.51	
6	Azoxy 2msec dwell	11353046	NA	3.14	2.51	
7	Azoxy 2msec dwell	11636029	NA	3.14	2.50	
8	Azoxy 2msec dwell	11414801	NA	3.14	2.51	
9	Azoxy 2msec dwell	11415707	NA	3.14	2.51	
10	Azoxy 2msec dwell	11365536	NA	3.14	2.50	
11	Azoxy 5msec dwell	11204929	NA	3.14	2.51	
12	Azoxy 5msec dwell	11119550	NA	3.14	2.50	
13	Azoxy 5msec dwell	11124237	NA	3.14	2.50	
14	Azoxy 5msec dwell	11202701	NA	3.14	2.51	
15	Azoxy 20msec dwe	11032421	NA	3.14	2.50	
16	Azoxy 20msec dwe	10997641	NA	3.14	2.51	
17	Azoxy 20msec dwe	11506645	NA	3.14	2.50	
18	Azoxy 20msec dwe	11170524	NA	3.14	2.50	
19	Azoxy 20msec dwe	11294461	NA	3.14	2.51	
20	Azoxy 100msec dw	11141895	NA	3.14	2.51	
21	Azoxy 100msec dw	11127692	NA	3.14	2.51	
22	Azoxy 100msec dw	11138545	NA	3.14	2.51	
23	Azoxy 100msec dw	11423106	NA	3.14	2.51	

500 SRMs/second

After demonstrating no signal loss while monitoring a single transition at dwell times ranging from 1msecond to 100mseconds additional experiments were performed to assess the effect of monitoring 250 transitions per 0.5 seconds (rate equivalent to 500 SRMs/second. Two transitions (*m/z* 192→132,160) for pesticide carbendazim were acquired simultaneously with an additional 248 transitions for other common pesticide at a cycle time of 1.5 seconds (167 SRMs/second) and 0.5 seconds (500 SRM/sec.) . The results are shown in Figure 4 Panel A. The power of acquiring 500 SRM/sec. is clearly demonstrated in the increased number of data points across the peak shown in Panel A of Figure 4. Without the increase in data points it would not be possible to reproducibly integrate the peak and reproducibility would be impacted. However, as previously discussed, it is not typically possible to acquire data at these rates (corresponding to 1 msec. dwell times) without signal loss. Here we again demonstrate that even at 500 SRM/sec. (1 msec. dwell), in the presence of numerous additional transitions, we maintain the peak area, data shown in Figure 4, Panel B. Thus given the results we have demonstrated thus far we can monitor numerous compounds simultaneously with no signal loss and excellent reproducibility.

Our next experiment was to demonstrate the acquisition of 250 pesticides with two transitions each at a rate of 500 SRM/sec., this equates to a 0.5 second cycle time (1 msec. dwell), at 1-100 ppb.

FIGURE 4. Carbendazim acquired at the rate of 167 SRM/sec. and 500 SRM/sec. Panel A depicts the number data points across the peak at both acquisition rates. Panel B depicts the area response for the carbendazim transitions in the presence of 248 additional transitions at various dwell times.

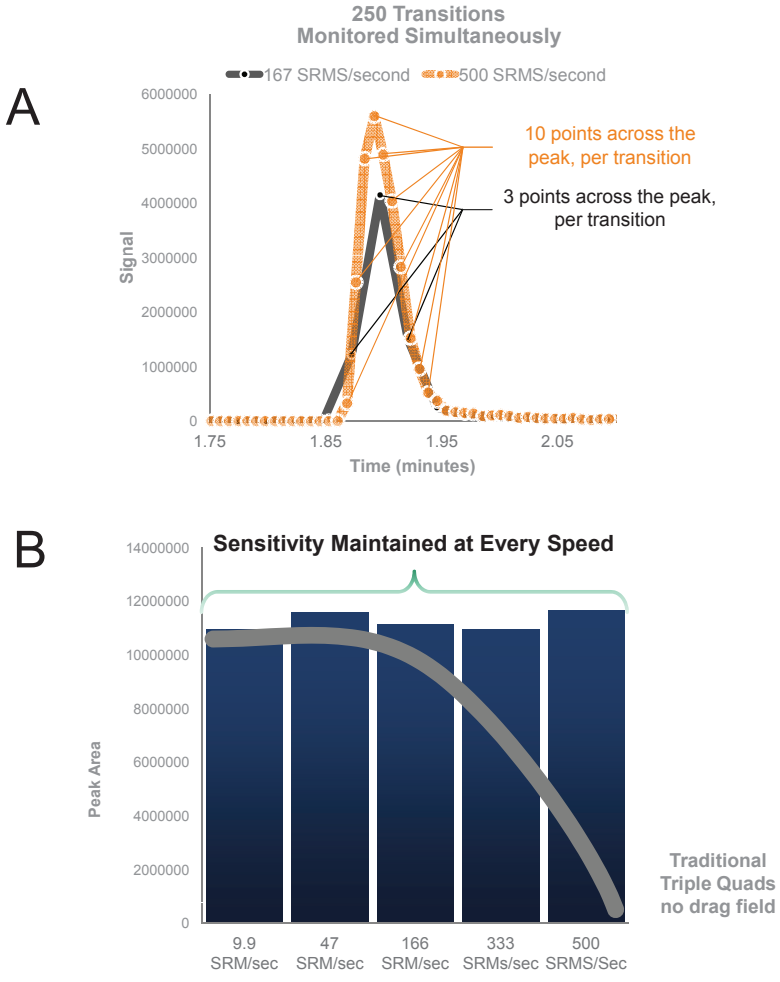


FIGURE 5. 1 ppb level of 250 pesticides (500 transitions) in onion matrix, acquired at a rate of 500SRM/sec. Panel A depicts the chromatograms for all 250 pesticides; Panel B depicts the peaks for 5 randomly chosen pesticides- displaying the number of data points across the peak and %RSD.

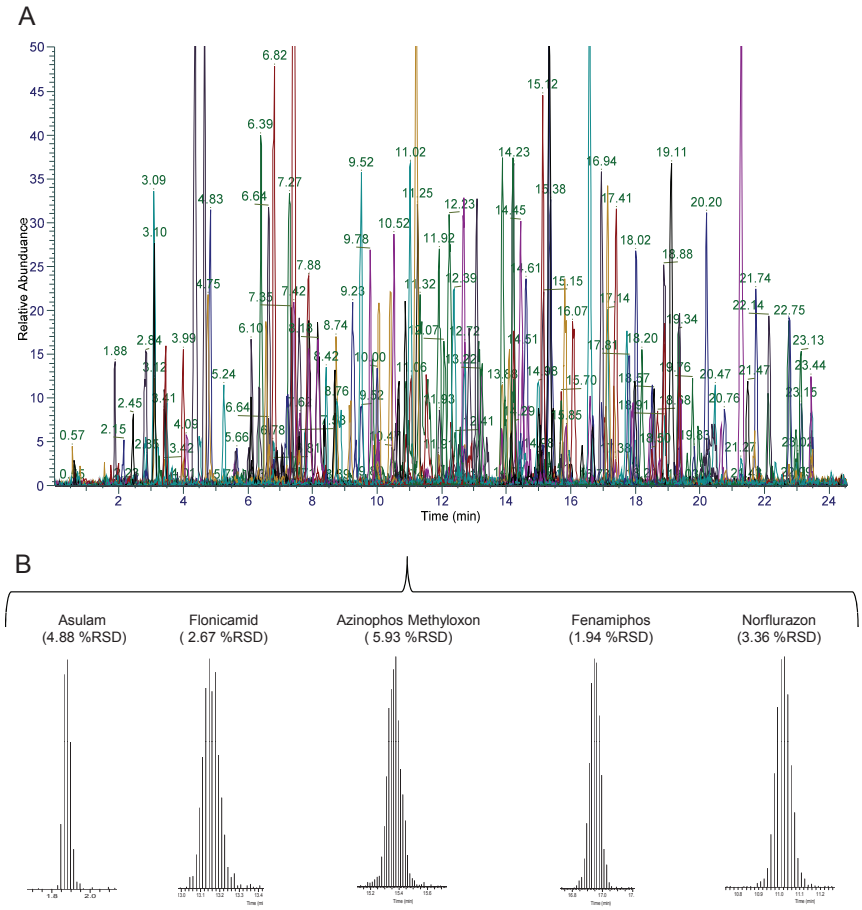
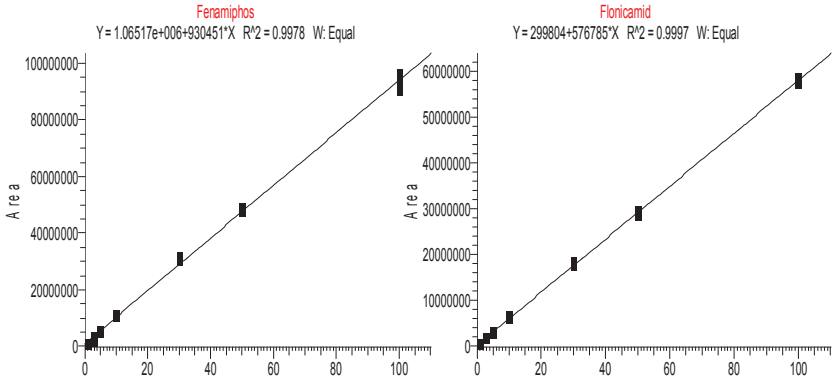


FIGURE 6. Calibration curves for fenamiphos and norflurazon, two of the compounds shown in Figure 5 above. Triplicate injections were made at each level; Note the excellent precision and linearity.



500 SRM/sec. in Onion Matrix

Having demonstrated the ability to, and the benefit of acquiring data at rates of 500 SRM/ sec., we now demonstrated the application of this method to 250 pesticides spiked into an onion matrix. Pesticides were spiked into an onion matrix at levels of 1 ppb through 100 ppb.

A representative chromatogram for a 1 ppb sample is shown in the Figure 5A. Figure 5B depicts the peaks for 5 randomly chosen pesticides, the number of data points acquired across the peak and the %RSD for triplicate injections.

Figure 6 contains the calibration curves for 2 of the 5 previously mentioned compounds. The data show that even in the presence of matrix, we are able to easily detect the compounds with excellent %RSDs, even at the 1 ppb level, while maintaining superb linearity.

The ability to acquire data for numerous compounds without specifying specific retention time is particularly helpful when running samples in matrix. This ability eliminates the need to adjust retention times that can change due to matrix effects on chromatographic conditions, thus simplifying the analysis of pesticides in food matrices.

Conclusion

- Q2 drag field enables 1 msec. SRM acquisitions without signal loss
- 1 msec. dwell times, with no signal loss, facilitates data acquisition rates of 500 SRM/sec.
- 500 SRM/sec. data acquisition rates allow us to
    - Reduce HPLC run times, enable the utilization of UPLC technology, or monitor numerous compounds in a single run.
    - Greatly simplify method setup by removing the need to set up specific time windows for compounds – run all the compounds, all the time.
- The analysis of pesticides in an onion matrix was simple, robust, sensitive, precise and linear.
- The new Ion Max NG source efficiently evacuates the source region therefore reducing the background and improving LODs and long term system robustness.

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Determination of 24 Pesticide Residues in Red Wine Using a QuEChERS Sample Preparation Approach and LC-MS/MS Detection

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Application Note 20830

Key Words
Pesticide residues, red wine, Accucore aQ, QuEChERS, dSPE, LC-MS/MS

Abstract
This application presents a fast, easy, and cost-effective method for the determination of 24 pesticide residues in red wine. Sample preparation involves the extraction of pesticides from red wine using the QuEChERS extraction method (AOAC version). The samples then undergo cleanup by dispersive solid-phase extraction (dSPE) using primary secondary amine (PSA) sorbent, which effectively retains organic acids, sugars, and phenolic pigments. A higher quantity of PSA than normally used in the dSPE step is required to sufficiently remove co-extracted phenolic compounds from red wine. The purified extract is subsequently separated using a solid core column prior to detection by a triple quadrupole mass spectrometer. The developed method was applied to commercially available red wine samples to test its applicability. Six out of the fourteen samples tested were found to contain pesticide residues at trace levels.

Introduction

Red wine is one of the most commonly consumed alcoholic beverages in the world with 241.9 million hectolitres consumed globally in 2011 [1]. Red wine is a rich source of phenolic antioxidants and is reported to reduce the risk of diabetes, cancer, Alzheimer's disease, and cardiovascular disease [2, 3]. To improve grape yields it is common practice in vineyards to use pesticides, such as fungicides and insecticides. However, if pesticide residues remain in the grapes prior to the winemaking process they can be transferred to the final product and, if present at significant levels, may be toxic to the consumer.

Due to the health risk that pesticides pose to humans it is important to monitor for their presence in food and beverages. No maximum residue levels (MRLs) have been established for pesticide residues in red wine; however, MRLs set for the raw commodity (e.g. wine grapes) can be applied to the processed product (e.g. wine) [4], thus the pesticide residues detected in the red wines tested in this study will be compared to the MRLs in wine grapes set by European Union (EU) [5].

The analysis of pesticide residues in red wine is challenging due to the complexity of the matrix, which contains alcohol, organic acids, sugars, and polyphenols (e.g. anthocyanins, flavonols, and tannins). Traditional sample preparation methods for red wine include liquid-liquid extraction (LLE) with different organic solvents, solid-phase extraction (SPE) with reversed-phase C18 or polymeric sorbents, solid-phase microextraction (SPME), and stir bar sorptive extraction (SBSE). However, these traditional methods have their own limitations, such as being labor intensive, costly (e.g. need for expensive glassware and solvents), using large quantities of organic solvent (environmental impact and disposal costs), requiring extensive method development and optimization, and possibly suffering from a lack of reproducibility or accuracy.

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The QuEChERS approach (acronym for Quick, Easy, Cheap, Effective, Rugged, and Safe) is a sample preparation technique that was first reported in 2003 by Anastassiades et al. for the analysis of pesticide residues in fruits and vegetables [6]. QuEChERS involves extracting pesticides (or other chemical residues) from a high aqueous sample into an organic solvent (most commonly acetonitrile) with the aid of salts, followed by dispersive solid-phase extraction (dSPE) to remove matrix co-extractives. This application note describes a modified QuEChERS extraction and dSPE cleanup method for the determination of pesticide residues in red wine. LC-MS/MS is used to accurately and quantitatively detect pesticides in red wine at low concentrations.

Thermo Scientific™ Accucore™ HPLC columns use Core Enhanced Technology™ to facilitate fast and high efficiency separations. The 2.6 µm diameter particles have a solid core and a porous outer layer. The optimized phase bonding creates a series of high-coverage, robust phases. The tightly controlled 2.6 µm diameter of Accucore particles results in much lower backpressures than typically seen with sub-2 µm materials. Accucore aQ columns are compatible with with 100% aqueous mobile phases and offer special selectivity for polar analytes.

Experimental Details

Consumables

A 5 mg/mL triphenyl phosphate stock solution in methyl tert-butyl ether was used as internal standard (IS).

Twenty-four neat pesticides (>96%) were obtained from a reputable supplier.

HPLC grade acetonitrile

HPLC grade methanol

Glacial acetic acid

Formic acid (>95%)

Ammonium formate (>99.995%)

Ultrapure water

Preparation of Pesticide Stock Solutions

A 1 mg/mL stock solution of each of the 24 pesticides was prepared by weighing 10 mg of the neat standard into a 10 mL volumetric flask and diluting to volume with acetonitrile.

Preparation of Pesticide Working Solutions

A 2 µg/mL pesticide working solution was prepared by mixing 100 µL of each of the 1 mg/mL stock solutions in a 50 mL volumetric flask, and diluting to volume with acetonitrile.

A 0.2 µg/mL pesticide solution was prepared by mixing 1 mL of the 2 µg/mL pesticide working solution with acetonitrile in a 10 mL volumetric flask, and diluting to volume with acetonitrile.

Preparation of Internal Standard Solution

A 30 µg/mL triphenyl phosphate working solution (IS) was made by mixing 60 µL of the 5000 µg/mL triphenyl phosphate solution with acetonitrile in a 10 mL volumetric flask, and diluting to volume with acetonitrile.

Standard Storage

All stock standards and working solutions were transferred to amber glass vials with Teflon®-lined caps and stored at -20 °C until needed.

Sample Preparation Supplies	Part Number
50 mL polypropylene centrifuge tube	
Thermo Scientific™ Mylar® pouch containing 6 g magnesium sulfate ($MgSO_4$) and 1.5 g sodium acetate	60105-335
Thermo Scientific 2 mL centrifuge tube containing 150 mg $MgSO_4$ and 150 mg PSA	60105-350
Thermo Scientific™ National™ Target™ 1 mL all-plastic disposable luer-slip syringes	S7510-1
Thermo Scientific™ Target2™ 0.2 µm, 22 nylon syringe filters	F2513-2
Thermo Scientific 2 mL screw-top autosampler vials	60180-508
Thermo Scientific™ Finntip™ pipet tips, 0.50–250 µL	14-245-150

Sample Preparation

The AOAC acetate buffered procedure was selected for sample extractions as it provides higher recovery for pymetrozine compared to the EN15662 citrate buffered or original non-buffered procedure.

AOAC QuEChERS extraction

1. Transfer 15 mL red wine sample into a 50 mL centrifuge tube.
2. Spike with 50 µL of the 30 µg/mL triphenyl phosphate solution (corresponding to 100 ng/mL).
3. Add 15 mL of acetonitrile containing 1% acetic acid and vortex for 1 min.
4. Add contents of the Mylar pouch containing 6 g $MgSO_4$ and 1.5 g sodium acetate, and shake vigorously on a horizontal shaker or vortex for 1 min.
5. Centrifuge at ≥3,750 rcf for 5 min.
6. The supernatant is now ready for dSPE cleanup.

dSPE cleanup

1. Transfer 1 mL of the supernatant into a 2 mL dSPE tube containing 150 mg $MgSO_4$ and 150 mg PSA and vortex for 30 s.
2. Centrifuge at ≥15,000 rcf for 5 min.
3. Transfer 0.3 mL of the purified extract into an autosampler vial, add 0.3 mL of reagent water, vortex, and filter with a 0.2 µm syringe filter.
4. The sample extract is now ready for LC-MS/MS analysis.

Preparation of Matrix-Matched Calibration Curve

A six-point matrix-matched calibration curve was prepared using sample extracts obtained from native wine samples prepared according to the procedure described above. The final extracts were spiked with appropriate volumes of pesticide working solution of 0.2 or 2 µg/mL to give final concentrations corresponding to 2, 10, 40, 100, 200, and 400 ng/mL of pesticides in red wine.

Separation Conditions		Part Number
Instrumentation:	Thermo Scientific™ Dionex™ UltiMate™ 3000 LC system	
Column:	Thermo Scientific Accucore 2.6 µm, 100 × 2.1 mm	17326-102130
Guard column:	Thermo Scientific™ Accucore™ aQ Defender™, 2.6 µm, 10 × 2.1 mm	17326-012105
Run time:	20 min (including re-equilibration time)	
Column temperature:	40 °C	
Injection volume:	10 µL	
Autosampler temperature:	10 °C	
Wash solvent:	Methanol / ultrapure water (1:1, v/v)	
Flow rate:	200 µL/min	
Mobile phase A:	0.3 % formic acid and 0.1 % ammonia formate in ultrapure water	
Mobile phase B:	0.1 % formic acid in methanol	
Preparation of mobile phase:	A: Dissolve 3 mL formic acid and 1 g ammonium formate in 1 L ultrapure water, and sonicate for 30 min.	
	B: Add 1 mL formic acid to 1 L methanol and sonicate for 30 min.	
Mobile phase gradient:	Time (min) B (%)	
	0.0 1	
	1.5 1	
	3.5 80	
	10.0 90	
	12.0 100	
	15.0 100	
	15.2 1	
	20.0 1	

The mobile phase was diverted to waste from 0 to 0.5 min and 15 to 20 min to prevent ion source contamination.

MS Conditions	
Instrumentation:	Thermo Scientific™ TSQ Vantage™ tandem mass spectrometer
Ionization mode:	ESI+
Spray voltage:	4000 V
Vaporizer temperature:	300 °C
Sheath gas pressure:	50 arbitrary units
Auxiliary gas pressure:	25 arbitrary units
Q1 and Q3 peak width:	0.2 and 0.7 Da
Collision gas:	Argon at 1.5 mTorr
Cycle time:	1 s
SRM parameters:	Table 1

SRM Transitions							
Pesticide	**t_R (min)**	**Precursor Ion**	**Product Ion 1**	**CE 1**	**Product Ion 2**	**CE 2**	**S-Lens (V)**
Methamidophos	1.28	142.0	124.6	14	111.6	5	60
Pymetrozine	1.31	218.0	104.9	18	176.0	16	70
Carbendazim	6.39	192.1	132.1	29	160.1	17	81
Dicrotophos	6.47	238.0	126.6	17	108.6	33	73
Acetachlor	6.48	269.4	111.9	15	71.7	33	72
Thiabendazole	6.61	202.1	131.1	31	175.1	24	103
DIMP	7.30	181.3	96.6	13	78.6	32	44
Tebuthiuron	7.32	228.9	115.6	26	171.6	17	72
Simazine	7.34	201.4	67.7	33	103.6	24	85
Carbaryl	7.41	202.0	126.6	30	144.6	7	40
Atrazine	7.69	216.0	67.7	35	173.6	16	79
DEET	7.72	191.9	118.6	15	90.7	28	92
Pyrimethanil	8.10	200.1	107.1	23	183.1	22	66
Malathion	8.08	331.0	98.6	23	126.9	12	60
Bifenazate	8.21	300.9	169.8	15	197.6	5	51
Tebuconazole	8.71	308.0	69.7	29	124.6	35	97
Cyprodinil	8.78	226.1	77.0	40	93.1	33	88
Triphenyl phosphate (IS)	8.80	327.1	77.02	37	152.1	33	98
Diazinone	8.85	305.1	153.1	15	169.1	14	89
Zoxamide	8.85	335.8	186.5	20	158.5	38	102
Pyrazophos	8.95	374.1	194.1	20	222.1	20	104
Profenofos	9.56	372.3	302.4	19	143.5	35	104
Chlorpyrifos	10.18	350.0	96.9	32	197.9	17	69
Abamectin	11.13	890.5	304.4	18	306.7	15	102
Bifenthrin	12.67	440.0	165.2	39	180.4	11	66

Table 1: Compound transition details

Data Processing	
Data processing:	Thermo Scientific™ TraceFinder™ software version 2.0

Results

Visual Appearance

The use of a high amount of PSA (150 mg) in dSPE cleanup was necessary for the efficient removal of organic acids, sugars, and polyphenolic pigments in red wine samples. The purified sample (Figure 1) is a clear colorless extract that is ready for LC-MS/MS analysis (extract can be filtered if desired).

Figure 1: Left: dSPE tubes with 150 mg $MgSO_4$ and 150 mg PSA before and after cleanup of 1 mL red wine extract; Right: Red wine extract before and after dSPE cleanup

Linearity and Limit of Quantitation (LOQ)

Matrix-matched calibration curves were prepared at concentrations of 2, 10, 40, 100, 200, and 400 ng/mL. An example of a calibration curve can be found in Figure 2. The responses were linear over the entire concentration range with correlation coefficient (R^2) ≥ 0.9963 (Table 2). The signal-to-noise ratio (S/N) at the lowest calibration level (2 ng/mL) was found to be ≥10 for all 24 pesticides. Therefore, the LOQ was estimated to be ≤2 ng/mL in this study.

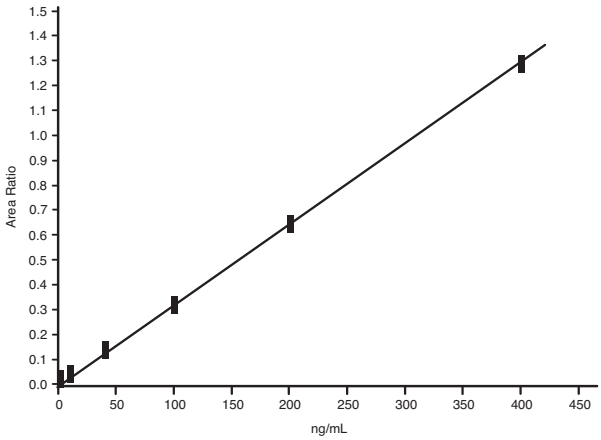


Figure 2: Simazine calibration curve

Pesticide	R^2
Methamidophos	0.9981
Pymetrozine	0.9979
Carbendazim	0.9989
Dicrotophos	0.9977
Acetachlor	0.9992
Thiabendazole	0.9966
DIMP	0.9998
Tebuthiuron	0.9996
Simazine	0.9998
Carbaryl	0.9986
Atrazine	0.9990
DEET	0.9996
Pyrimethanil	0.9983
Malathion	0.9997
Bifenazate	0.9987
Tebuconazole	0.9996
Cyprodinil	0.9995
Diazinone	0.9999
Zoxamide	0.9996
Pyrazophos	0.9997
Profenofos	0.9963
Chlorpyrifos	0.9965
Abamectin	0.9968
Bifenthrin	0.9991

Table 2: Linearity ranges and correlation coefficients (R^2)

Carryover

Blank acetonitrile was injected directly after the highest matrix-matched calibration standard (400 ng/mL) to check for sample carryover. No analyte carryover was observed.

Accuracy and Precision

Red wine made from organic grapes and determined to be free of pesticide residues was fortified with 10, 50, and 100 ng/mL pesticides (n=6) and prepared according the experimental procedure described above. As outlined in Table 3, the majority of results (≥95%) were found to be within an acceptable recovery range of 70–120% and RSD values ≤20%, demonstrating that this method is suitable for pesticide residue analysis of red wine samples.

Pesticide	10 ng/mL (n=6)		50 ng/mL (n=6)		100 ng/mL (n=6)	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Methamidophos	78.5	6.1	84.2	2.0	91.0	11.4
Pymetrozine	64.5	5.5	61.9	2.4	63.3	12.1
Carbendazim	66.3	4.1	66.2	4.1	53.4	19.6
Dicrotophos	82.0	2.4	80.2	1.0	81.4	13.6
Acetachlor	85.3	3.2	88.9	2.4	84.5	13.5
Thiabendazole	78.8	4.6	75.4	5.9	62.9	19.6
DIMP	95.8	2.9	94.0	4.3	91.4	13.2
Tebuthiuron	87.3	2.1	87.3	2.1	89.6	12.0
Simazine	97.7	2.5	99.3	2.5	92.2	11.4
Carbaryl	95.5	3.3	91.6	1.5	90.0	10.5
Atrazine	91.0	1.8	90.1	1.9	89.1	5.9
DEET	93.7	1.9	93.9	2.6	90.7	8.1
Pyrimethanil	94.2	3.1	91.0	2.1	82.7	13.7
Malathion	99.0	2.4	96.7	2.7	89.1	11.4
Bifenazate	103.3	3.4	97.5	3.0	84.5	11.3
Tebuconazole	95.0	3.0	94.1	3.1	83.6	8.4
Cyprodinil	98.7	2.3	96.6	2.3	90.4	5.2
Diazinone	98.5	2.5	100.1	3.5	80.2	17.6
Zoxamide	101.7	1.7	101.1	2.5	91.8	6.5
Pyrazophos	95.5	2.5	96.3	3.3	79.9	18.5
Profenofos	91.8	4.8	88.4	2.3	91.8	7.9
Chlorpyrifos	95.5	7.2	95.1	3.3	75.8	20.8
Abamectin	92.5	2.6	88.7	3.7	79.3	14.5
Bifenthrin	93.2	4.2	93.3	5.9	87.8	12.5
Overall average	**90.6**	**3.3**	**89.7**	**2.9**	**83.2**	**12.5**

Table 3: Accuracy and precision data of the 24 pesticides fortified into organic red wine at three concentrations

Application to Real Samples

Fourteen commercially available bottles of red wine from various geographical regions around the world were tested in duplicate using the developed method. Of the fourteen wines tested, six samples (#2, #9, #11–14) were found to contain one or more pesticides, namely carbendazim, pyrimethanil, bifenazate, tebuconazole, and cyprodinil (Table 4). The concentrations of pesticides detected ranged from 2.2 to 13 ng/mL (equal to 0.0022 to 0.013 mg/kg), which were approximately 100 to 1000 times lower than the MRLs set for wine grapes by the EU [5].

Pesticide Detected	Red Wine Sample	Concentration (ng/mL)
Carbendazim	#12	8.0
	#13	5.3
Pyrimethanil	#9	13
Bifenazate	#2	3.0
	#14	2.2
Tebuconazole	#11	2.8
	#14	7.4
Cyprodinil	#9	3.2
	#14	3.8

Table 4: Red wine samples and pesticides detected. For samples not listed, no pesticides were detected or the concentration was determined to be <LOQ (2 ng/mL).

Chromatograms

See Figure 3 for chromatograms of a red wine sample fortified with pesticides at 50 ng/mL.

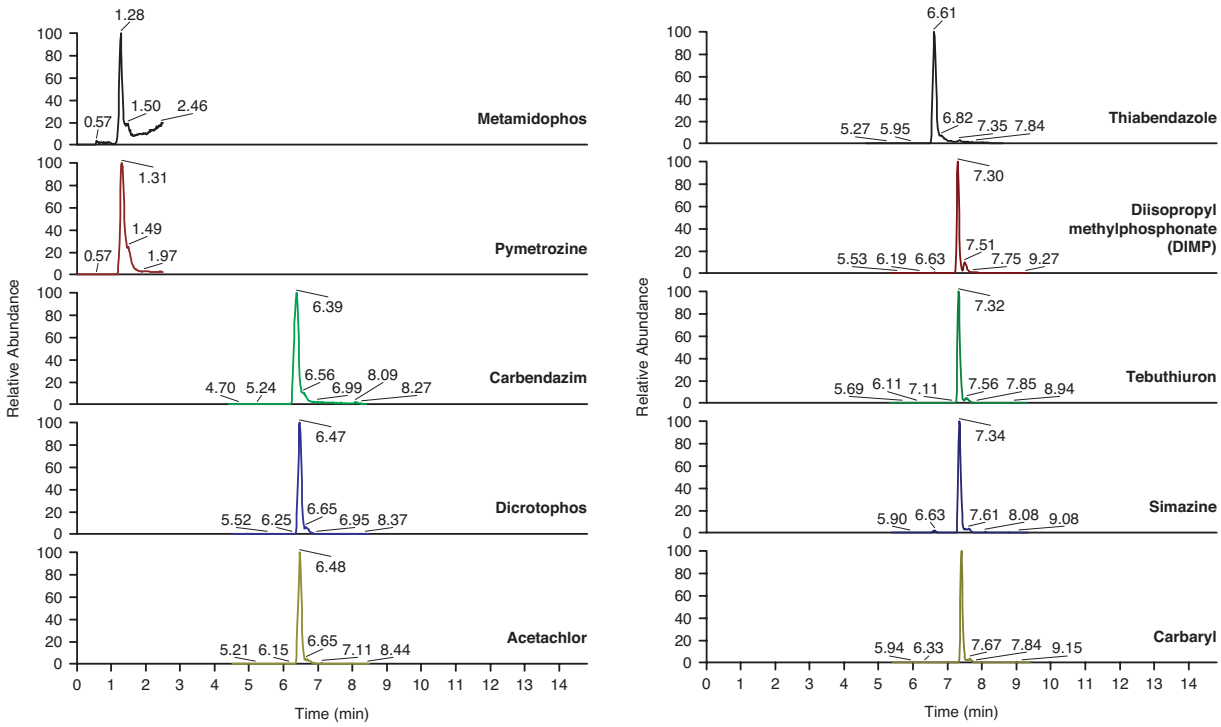
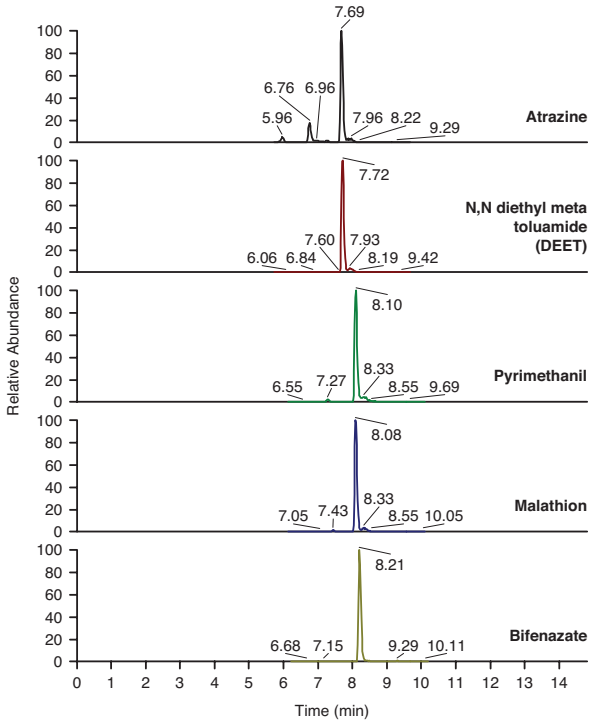
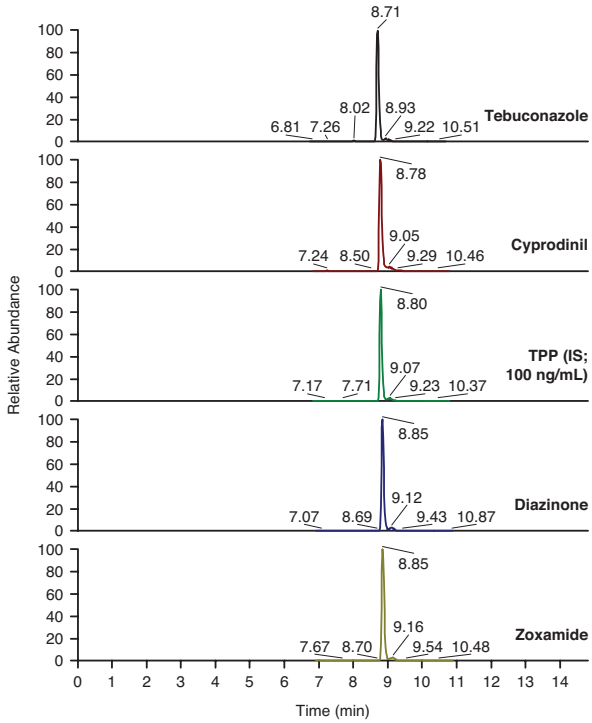


Figure 3: Chromatograms of a red wine sample spiked at 50 ng/mL





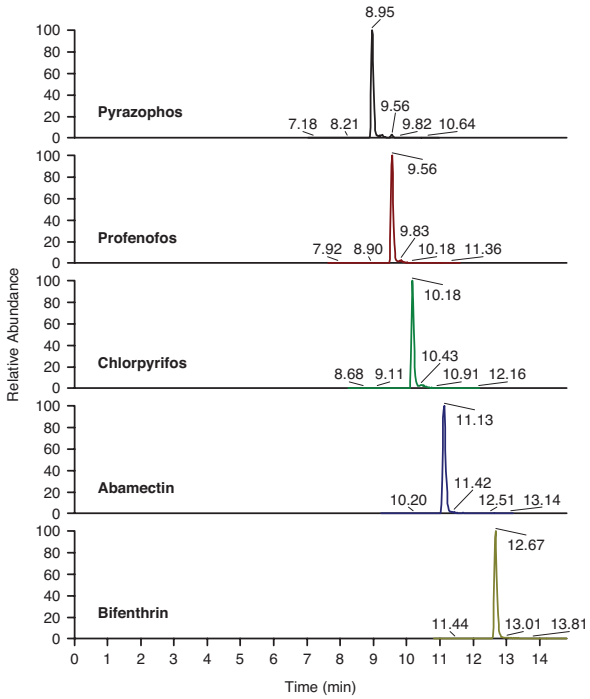


Figure 3 (continued): Chromatograms of a red wine sample spiked at 50 ng/mL

Conclusion

- A fast, easy and cost-effective method has been successfully developed using the QuEChERS-based approach.
- An increase in the amount of PSA (150 mg) in the dSPE cleanup was found to be necessary for the efficient removal of organic acids, sugars, and pigments that are present in wine, and produce a clean extract.
- LC-MS/MS was used for the quantitative analysis of 24 pesticides. The Accucore aQ HPLC columns gave good resolution and peak shapes for all of the pesticides.
- Good linearity, low LOQs, and satisfactory accuracy and precision data were obtained, indicating that this method is suitable for pesticide residue analysis in red wine.
- Fourteen commercially available red wine samples were analyzed to test the applicability of the method. Six samples were found to contain one or more pesticides but at concentrations (0.0022–0.013 mg/kg) far below the MRLs in wine grapes set by EU.

Reference

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Method: 52292

Determination of Carbendazim and Benomyl Residues in Oranges and Orange Juice by Automated Online Sample Preparation Using TLX-LC-MS/MS

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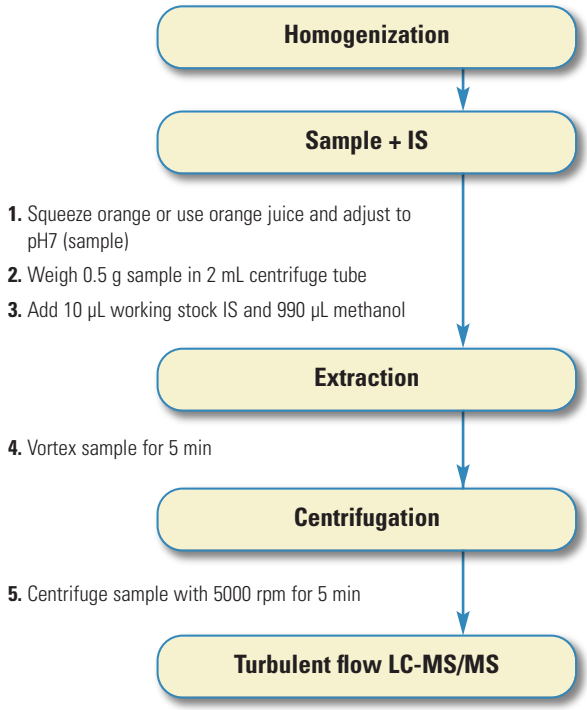
Key Words

- Transcend™ TLX
- TSQ Quantum Access MAX™
- Triple Quadrupole Mass Spec
- TurboFlow™ Technology
- Food Safety
- Fungicides

1. Schematic of Method



2. Introduction

Methyl 2-benzimidazole carbamate, most commonly known as carbendazim, is a widely used broad-spectrum benzimidazole fungicide and a decomposition product of benomyl. Carbendazim is used to control plant diseases in cereals and fruit, including citrus, bananas, strawberries, pineapples, and pome fruits. Although not permitted for use to treat citrus fruit in the USA and Australia, it is permitted in the EU and European Regulation 559/2011 sets a limit for carbendazim and benomyl (sum of carbendazim and benomyl expressed as carbendazim) of 0.2 mg/kg in oranges. Incidences of MRL exceedance have been common in the EU, with 23 Rapid Alert Notifications in 2011 for levels of carbendazim as high at 4 mg/kg in fruit, vegetables and herbs from Africa, S. America and Asia.[1] The most common occurrence was in yams and no instances of carbendazim in oranges or orange juice were reported. Orange juice from Brazil imported into the USA has been found to contain carbendazim and an action limit of 0.01 m/kg has been applied by the FDA.[2]

Many methods in widespread use for monitoring carbendazim have been developed for multi-residue determination of fungicides and employ a variety of sample preparation and cleanup techniques. In recent years the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method has become widely adopted for handling

Thermo Scientific Transcend TLX system coupled with the TSQ Quantum Access MAX triple quadrupole mass spectrometer

fruit such as oranges. However, despite its undoubted advantages, it requires many manual sample manipulation steps, making it labor-intensive, especially when large numbers of samples have to be analyzed. Therefore, it is beneficial to consider options for automation of multi-residue methods, which can be both cost-effective as well as offer a high degree of reliability in recovery and repeatability. While the preliminary stages of homogenization and solvent extraction of food matrices inevitably require manual intervention, once a crude extract has been obtained there is scope for a fully automated procedure thereafter. The method described in this document is an adaptation of an existing online, multi-residue pesticide method (Thermo Scientific Method 52213[3]) proven and verified specially for the actual carbendazim contamination issue of orange juices in the US.

3. Scope

This method can be applied to oranges and orange juice at a limit of quantification (LOQ) below 0.01 mg/kg, the action limit used by the FDA for monitoring purposes. The method has been validated for carbendazim and the sum of benomyl + carbendazim in oranges and orange juice, but can be readily extended to a larger number of residues.

4. Principle

This method is the adaptation of carbendazim and extension for benomyl of an online sample preparation technique based on an existing in-house validated method (Thermo Scientific Method 52213[3]) for the determination of 50 pesticides in grape, baby food and wheat flour matrices. The method uses TurboFlow technology as a possible alternative to the QuEChERS method since TurboFlow is more suitable for high-throughput fungicide analysis. Sample pre-concentration, cleanup and analytical separation is carried out in a single run, using an online coupled TurboFlow method (Thermo Scientific Transcend TLX). TurboFlow technology serves as a novel sample preparation technique due to its special flow profile, size exclusion, reversed phase column chemistry and very effective separation of matrix and target compounds, resulting in relatively clean sample extracts. Macromolecules such as sugars, fats and proteins are removed from the sample extract with high efficiency, while target analytes are retained on the column based on different chemical interactions. After application of a wash step, the trapped compounds are transferred onto the analytical LC column and separated conventionally. The complete method involves internal standardization, solvent extraction of the homogenized orange juice, solvent extraction, centrifugation and injection into an automated cleanup system. Cleanup using Transcend TLX system has been optimized for maximum recovery of carbendazim or benomyl and minimal injection of co-extractives into the MS/MS. Identification of carbendazim and benomyl is based on retention time, ion-ratios using selected reaction monitoring (SRM) of characteristic transition ions, and quantification using matrix-matched standards of one of the selected SRM ions.

5. Reagent List

		Part Number
5.1	Acetone, HPLC Grade	A/0606/17
5.2	Acetonitrile, LC-MS Grade	A/0638/17
5.3	Ammonium formate, for HPLC	A/5080/53
5.4	Methanol, Optima LC/MS grade	A456-212
5.5	Formic acid, extra pure for HPLC	F/1850/PB08
5.6	Isopropanol, HPLC grade	P/7507/17
5.7	Water, LC-MS grade	W/0112/17
5.8	Ammonia (35% solution)	10508610

6. Calibration Standards

6.1 Standards

6.1.1 Carbendazim (analytical standard) from Sigma-Aldrich®

6.1.2 Benomyl (analytical standard) from Sigma-Aldrich

6.2 Internal standards:

6.2.1 Imidaclorprid-4,4,5,5-d_4 (analytical standard) from Sigma-Aldrich

7. Standards and Reagent Preparation

7.1 **Stock solution:** Weigh 10.00 mg of the compounds (recalculate the amount regarding actual purity of the standard) into a volumetric flask, dissolve in methanol and dilute to 100 mL. The final concentration of the two fungicides is 100 µg/mL. The solution of carbendazim can be used for 3 months when stored refrigerated, however benomyl stock solution remains stable only for 0.5 days.

7.2 **Individual working mixture:** Transfer 50 µL of stock solution of either carbendazim or benomyl (100 µg/mL), respectively, to a 50 mL volumetric flasks and dilute to the mark with methanol. The solution should be prepared fresh every time before using. Final concentration of each standard is 0.1 µg/mL.

7.3 **Stock standard solution of internal standard:** Weigh 10.00 mg of Imidacloprid-d_4 (recalculate the amount regarding actual purity of the standard) into volumetric flask, dissolve in methanol and dilute to 100 mL. The solution can be stored at 4 °C for at least 3 months. Final concentration is 100 µg/mL.

7.4 **Working standard solution of internal standard:** Transfer 100 µL of stock solution of imidacloprid-d4 (100 µg/mL) to a 10 mL volumetric flask and dilute to marked volume with methanol. The solution should be prepared fresh every time before using. The final concentration of imidacloprid-d_4 is 1 µg/mL.

7.5 **5 M Ammonia solution:** Weigh 24.3 g of ammonia (35% solution) to 100 mL volumetric flask and dilute to marked volume with deionized water.

8. Apparatus

		Part Number
8.1	Sartorius analytical balance	ME235S
8.2	Thermo Scientific Barnstead EASYpure II water	3125753
8.3	Vortex shaker	3205025
8.4	Vortex universal cap	3205029
8.5	Accu-Jet pipettor	3140246
8.6	Orion™ 2 Star, pH meter	10539752
8.7	Thermo Scientific Heraeus Fresco 17 micro centrifuge	208590
8.8	Transcend TLX-1 system with TSQ Quantum Access MAX MS/MS	40500

9. Consumables

		Part Number
9.1	LC vials	3205111
9.2	LC caps	3151266
9.3	Thermo Scientific Pipette Finnpipette 100–1000 µL	321453
9.4	Pipette Finnpipette™ 10–100 µL	3166472
9.5	Pipette Finnpipette 500–5000 µL	3166473
9.6	Pipette holder	3651211
9.7	Pipette tips 0.5–250 µL, 500/box	3270399
9.8	Pipette tips 1–5 mL, 75/box	3270420
9.9	Pipette tips 100–1000 µL, 200/box	3270410
9.10	Spatula, 18/10 steel	3458179
9.11	Spatula, nylon	3047217
9.12	Tube holder	3204844
9.13	Wash bottle, PTFE	3149330
9.14	Vial rack (2 mL)	12211001
9.15	Centrifuge plastic tube (2 mL)	3150968
9.16	TurboFlow Cyclone MCX-2 (50 × 0.5 mm) column	CH-953457
9.17	Thermo Scientific Hypersil GOLD 150 × 4.6 mm, 5 µm column	25005-154630
9.18	UNIGUARD holder	850-00
9.19	Hypersil GOLD™ 10 × 4 mm, 5 µm guard column	25005-014001

10. Glassware

		Part Number
10.1	Volumetric flask, 10 mL	FB50143
10.2	Volumetric flask, 25 mL	FB50147
10.3	1 mL glass pipette	FB50211
10.4	1 L bottle	9653650
10.5	500 mL bottle	9653640
10.6	100 mL volumetric flask	FB50151

11. Procedure

11.1 Sample Preparation

11.1.1 **Orange samples:** Prepare orange samples prior to injection into TLX-MS/MS system: Collect at least 10 representative oranges (min 1 kg) and cut into two halves.[4] Squeeze them on a kitchen squeezer and collect the pressed juice. Adjust the pH of the juice to 7 by adding 5 M ammonia solution.

11.1.2 **Orange juice samples:** Orange juice can be used directly after vigorous shaking and adjusting the pH to 7 with 5 M ammonia solution.

11.2 Sample Extraction

11.2.1 Weigh 0.5 g sample on an analytical balance into a 2 mL centrifuge tube

11.2.2 Add 990 µL methanol and 10 µL working IS solution

11.2.3 Vortex the sample for 5 min

11.2.4 Centrifuge in the centrifuge at 5000 rpm for 5 min

11.2.5 Transfer the supernatant into the LC vial for TLX-LC-MS/MS clean up and determination

		TurboFlow								Analytical					
Step	Duration [s]	Flow mL/min	Grad	A%	B%	C%	D%	Tee	Loop	Flow mL/min	Grad	A%	B%	C%	D%
1	60	1.50	step		100			–	out	0.50	step		100		
2	60	1.50	step		95		5	–	out	0.50	step		100		
3	80	0.16	step		100			Tee	in	1.44	step		100		
4	60	1.00	step			100		–	in	1.60	ramp		55		45
5	60	1.00	step	10			90	–	in	1.60	ramp		40		60
6	220	0.20	step		100			–	out	1.60	ramp				100
7	60	0.20	step		100			–	out	1.60	step				100
8	180	0.20	step		100			–	out	1.00	step		100		
		Mobile phases for the TurboFlow: A: water pH=3 B: water C: 40% acetonitrile 40% isopropanol and 20% acetone D: 5 mM ammonium-formiate in methanol + 0.1% formic acid								Solvent channels for analytical: A: not in use B: 5 mM ammonium-formiate in water + 0.1% formic acid C: not in use D: 5 mM ammonium-formiate in methanol + 0.1% formic acid					

Table 1: Gradient program table for Thermo Scientific Aria control software

12. Analysis

12.1 LC Operating Conditions

The TLX system was optimized for both TurboFlow methods and analytical separation.

12.1.1 LC conditions for TurboFlow and analytical columns

Operation was carried out in focus mode setup (Figure 1) with 1:0.75 splitting before MS/MS entrance using a divert valve connection. A TurboFlow Cyclone MCX-2 column was installed (9.17) and a Hypersil Gold column equipped with guard column was used (9.18–9.20). Installed loop volume was 200 µL.

Table 1 gives details of the method program. Sample load (Step 1) was applied with 1.5 mL/min flow rate in turbulent flow, whereby matrix components were eluted in the waste and target fungicides were trapped on the TurboFlow column. After washing the TurboFlow column with a 5% organic/aqueous mixture (Step 2), the trapped fungicides were eluted and transferred (Step 3) after 2 minutes from the TurboFlow to the analytical column with simultaneous dilution of the eluate enabling pre-concentration of fungicides at the beginning of the analytical column. The analytical column was equilibrated and conditioned during loading and washing steps. After transfer of the fungicides, the analytical separation started with gradient elution (Step 4–7), while the TurboFlow column was washed and conditioned and the loop was filled with the TurboFlow eluent. After the gradient run, analytical column was washed in acetonitrile and conditioned for the next run. The total run time of the method with TurboFlow sample preparation and analytical separation, with preparation for the next run, is 13 minutes to keep method capable for multi-fungicide residue analysis. In order to minimize sample carry-over and cross-contamination, the injection needle and valve were washed with both strong and weak wash solvents 4 times (conditions in 12.1.2).

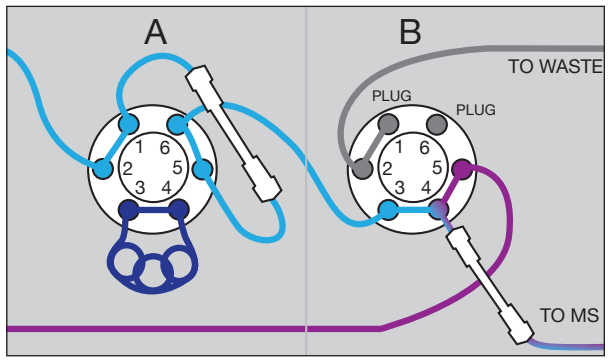


Figure 1: Focus mode system set up and method setting in Aria control software on the Transcend TLX system

12.1.2 Injector set up

Injector: Thermo Scientific Pal injector with 100 µL injection syringe volume

Sample holder temperature: 10 °C

Cleaning solvents: Solvent channel 1 – 80:20 methanol/acetone
Solvent channel 2 – acetonitrile

Injector settings:

- Pre clean with solvent 1 [steps]: 2
- Pre clean with solvent 2 [steps]: 2
- Pre clean with sample [steps]: 1
- Filling speed [µL/s]: 50
- Filling strokes [steps]: 2
- Injection port: LC Vlv1 (TX channel)
- Pre inject delay [ms]: 500
- Post inject delay [ms]: 500
- Post clean with solvent 1 [steps]: 4
- Post clean with solvent 2 [steps]: 4
- Valve clean with solvent 1 [steps]: 4
- Valve clean with solvent 2 [steps]: 4
- Injection volume: 20 µL

12.2 Mass Spectrometric Conditions

Mass spectrometric detection was carried out using a TSQ Quantum Access MAX triple quadrupole mass spectrometer in SRM mode. All SRM traces were individually tuned for the target fungicides (Table 2). MS software programming was set in Thermo Scientific Xcalibur Eazy mode set up.

MS settings:

- Scan type: SRM (details in Table 2)
- Cycle time [s]: 0.3
- Peak width: 0.7 Da FWHM
- Collision gas pressure [mTorr]: 1.5
- Capillary temperature [°C]: 290 °C
- Vaporizer temperature [°C]: 290 °C
- Sheath gas pressure [arb]: 40
- Aux gas pressure [arb]: 10
- Ion sweep pressure [arb]: 0
- Spray voltage [V]: 3200
- Skimmer offset [V]: 2
- Polarity: positive for all compounds
- Trigger: 1.00e5

13. Calculation of Results

Calibration by internal standardization is applied for the determination of carbendazim and benomyl. This quantification method requires determination of response factors R_f defined by the equation below. Calculation of final result is performed using the following equations.

Calculation of the response factor:

$$R_f = \frac{A_{St} \times c_{[IS]}}{A_{[IS]} \times c_{St}}$$

$\mathbf{R_f}$ – the response factor

$\mathbf{A_{St}}$ – the area of the fungicide peak in the calibration standard

$\mathbf{A_{[IS]}}$ – the area of the internal standard peak of the calibration standard

$\mathbf{c_{St}}$ – fungicide concentration of the calibration standard solution

$\mathbf{c_{[IS]}}$ – the internal standard concentration of the calibration standard solution

Calculations for each sample of the absolute amount of fungicide that was extracted from the sample:

$$X_{analyte} = \frac{A_{analyte} \times X_{[IS]}}{A_{[IS]} \times R_f}$$

$\mathbf{X_{analyte}}$ – the absolute amount of fungicide that was extracted from the sample

$\mathbf{A_{analyte}}$ – the area of fungicide peak in the sample

$\mathbf{A_{[IS]}}$ – the area of the internal standard peak in the sample

$\mathbf{X_{[IS]}}$ – the absolute amount of internal standard added to the sample

The concentration of fungicide in the sample [ng/g]:

$$c = \frac{X_{analyte}}{m}$$

m – the weight of sample [g]

$\mathbf{X_{analyte}}$ – absolute analyte amount [ng]

14. Method Performance Characteristics

In-house validation of the method was carried out according to IUPAC and AOAC guidelines for single laboratory validation and it was also demonstrated that method performance characteristics fulfilled the legislative criteria set for pesticide residue methods.[5-8]

Samples used for the determination of method performance characteristic parameters were prepared by spiking of appropriate amount of working standard solution and work solution of internal standard into the 0.5 g sample and total volume was adjusted to 1 mL with methanol (equivalent total volume according to 10.2.).

With reference to the low stability and fast transformation of benomyl into carbendazim, the validation study was carried out with samples spiked only with carbendazim to establish the method performance parameters.[6] After establishing validation parameters, samples were run additionally with spiked carbendazim and benomyl, in order to check degradation and contribution of benomyl to the carbendazim peak area (Figure 2). In order to keep control on benomyl degradation, all these samples were analyzed within 2 hours after preparation.

14.1 Selectivity

Method (SRM) selectivity was confirmed based on presence of specific ion transitions at the corresponding retention time (Table 2), as well as the observed ion ratio values corresponding to those of the standards. Acceptance criteria for retention time and ion ratios were set according to Reference 4.

14.2 Linearity, Response Factor

The linearity of calibration curves was assessed by internal standardization over the range from 0–0.1 mg/kg. The matrix-matched calibration curves were created at seven levels (and blank) and injected in duplicate. Calibration levels were 0, 0.005, 0.010, 0.015, 0.025, 0.035, 0.050 and 0.100 mg/kg. R_f values for internal standardization were determined from the calibration curves by calculating cumulative average response factor over the whole calibration range and resulted R_f = 3.2, which was used for quantitative analysis. The details on calibration are shown in Table 3.

14.3 Accuracy

Method accuracy and precision was assessed by recovery studies using blank matrices spiked at three concentration levels injected in six individually prepared replicates. Samples were spiked at 0.005, 0.010 and 0.050 mg/kg concentration levels. Found concentrations, recovery and relative standard deviation (% RSD) were calculated (Table 3). Recovery values were in the range 96–115% and were deemed to be acceptable (criteria 70–120%).

14.4 Repeatability and Intermediate Precision

Method within-day (repeatability) and between-day precision (intermediate precision) values ranged from 6.8–9.8% (Table 4) and were deemed acceptable (below 20%).

14.5 Limits of Detection (LODs) and Quantification (LOQs)

Limits of detection and quantification were estimated following the IUPAC approach which consisted of analyzing the blank sample to establish noise levels and then testing experimentally estimated LODs and LOQs for signal/noise, 3 and 10 respectively. The method LOD and LOQ values resulted as 0.00015 mg/kg and 0.0005 mg/kg (Figures 3 and 4). The expectation of the method was to meet the US rejection limit for orange juices set by the FDA at 0.010 mg/kg as well as the European MRL value (0.2 mg/kg) at LOQ level. Method LOQ fulfilled both legislation criteria.

14.6 Matrix Effect

Matrix effect was investigated by comparison of calibration results in solvent and in matrix. Youden plot of both calibration series was applied. Slope of fitted linear resulted y=0.8497× which represents less than 20 % deviation from the idealistic y=× value indicating no matrix effect for the investigated matrix (Figure 5).

14.7 Survey Samples

The method was applied to 6 different orange juice samples (n=3) and oranges (n=3) purchased from local stores. Survey samples were of organic origin from Spain and Germany. No carbendazim was found above 0.01 mg/kg in any of survey samples (Table 5).

15. Conclusion

This method enables convenient, fast and cost-effective automated determination of carbendazim and benomyl in oranges and orange juice. Based on the short total run time and a simple online sample preparation technique, 100 samples per day can be analyzed at a level of 0.01 mg/kg, with faster and more precise analysis compared to the QuEChERS technique. Method performance characteristics were established by in-house validation for oranges and orange juice. Based on its method performance parameters, the developed TLX system is suitable for routine use for regulatory purposes and possesses potential as alternative to the widely used QuEChERS method. The TLX system can readily be extended to a larger and wider range of fungicide residues, and has previously been demonstrated as being applicable to other matrices such as cereals, grapes and baby food.[1]

16. References

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17. Annex

17.1 Tables, Chromatograms and Matrix Study

Analyte	Retention time [min]	Precursor Ion	Product Ion (EcoII)	Product Ion2 (EcoII)	Ion Ratio	Tube Lens
Carbendazim	6.01	191.8	160.1 (18)	132.1 (29)	0.25	100
d4-Imidacloprid	6.21	259.9	213.1 (17)	179.1 (20)	0.82	97
Benomyl	9.03	291.1	192.1 (12)	160.1 (27)	0.85	101

Table 2: Ion transitions of target compounds for SRM setting

	Linearity			Recovery [%] (RSD%)		
Compound	**Slope**	**Intercept**	**R^2**	**0.005 mg/kg**	**0.010 mg/kg**	**0.050 mg/kg**
Carbendazim	0.1501	0.1787	0.9981	99 (5.5)	101 (6.8)	108 (4.0)
Carbendazim + Benomyl	0.3377	0.1840	0.9891	115 (14.6)	96 (9.4)	104 (3.6)

Table 3: Linearity (n=2) and recovery (n=6) of target compounds

	Precision [%]			
	Identification (tr)		Quantification (Peak Area)	
Compound	**Repeatability**	**Intermediate Precision**	**Repeatability**	**Intermediate Precision**
Carbendazim	0.1	0.1	6.8	9.5
Carbendazim + Benomyl	–	–	7.5	9.8

Table 4: Repeatability and intermediate precision of target compounds

Sample #	Type of Sample	Carbendazim [mg/kg]
1	juice	0.001
2	juice	0.002
3	juice	0.005
4	orange	0.001
5	orange	<LOD
6	orange	<LOD

Table 5: Survey sample results

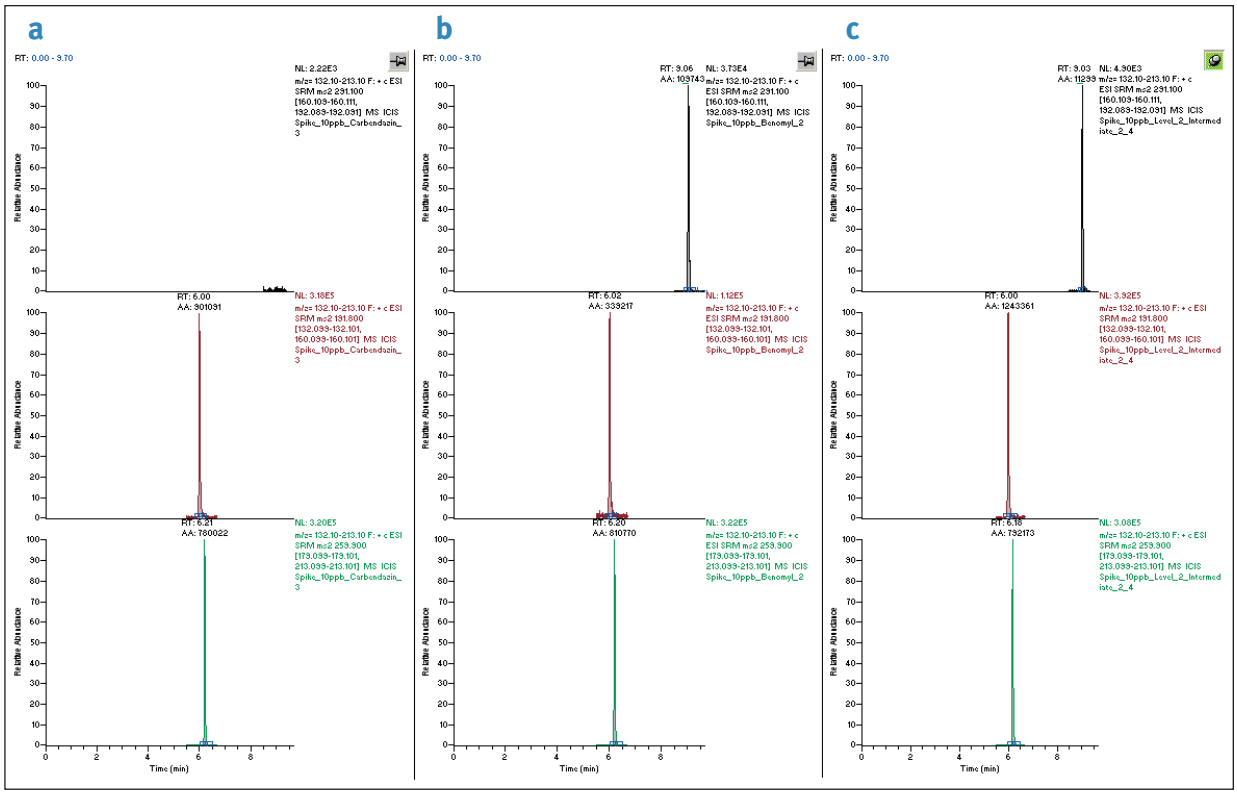

Figure 2: Demonstration of transformation of benomyl into carbendazim. Traces from top: benomyl, carbendazim and d4-imidacloprid (IS). Chromatograms showing a) 10 ng/mL carbendazim solution, b) 10 ng/mL benomyl solution after 2 hrs of preparation, c) chromatogram of solution containing 10 ng/mL carbendazim and benomyl after 2 hrs of preparation. Significant amount of benomyl transforms into carbendazim.

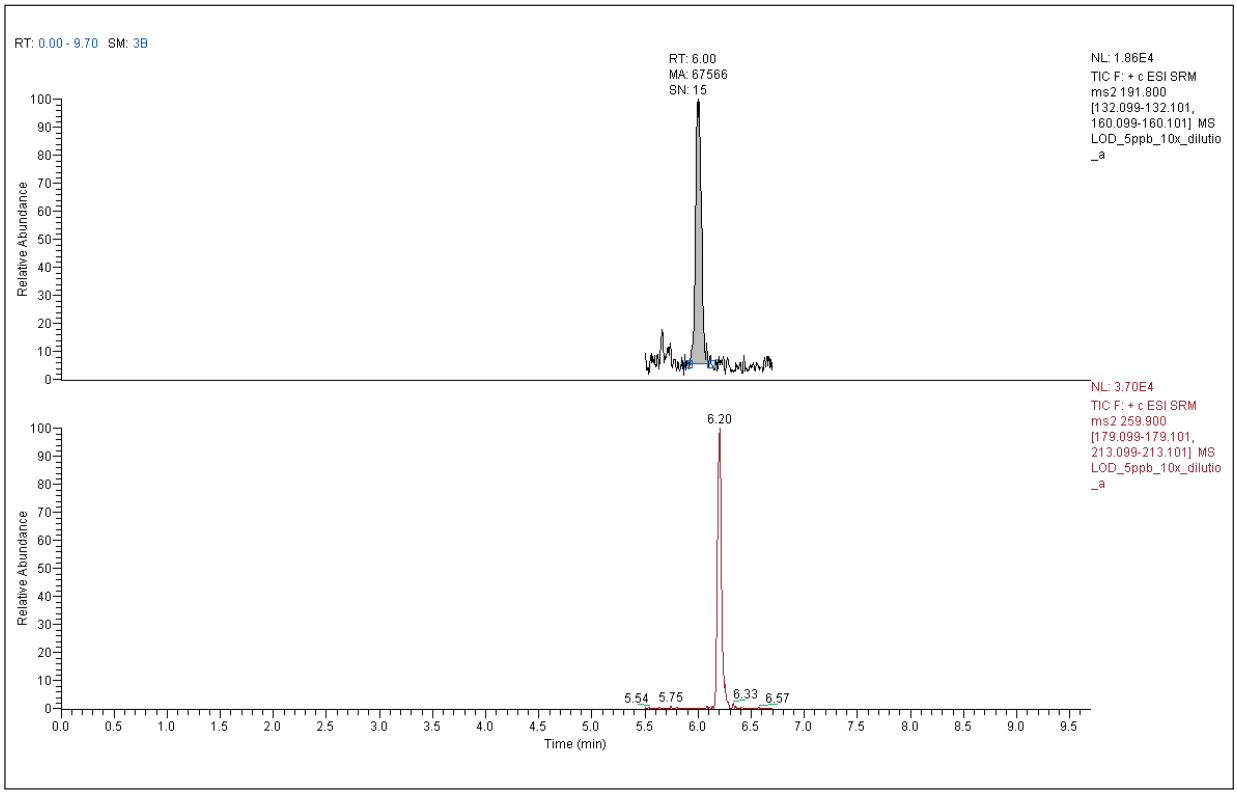

Figure 3: Chromatogram of 0.0005 mg/kg carbendazim in orange juice representing signal intensity at LOQ level.
On top: carbendazim, below: d4-imidacloprid (IS).

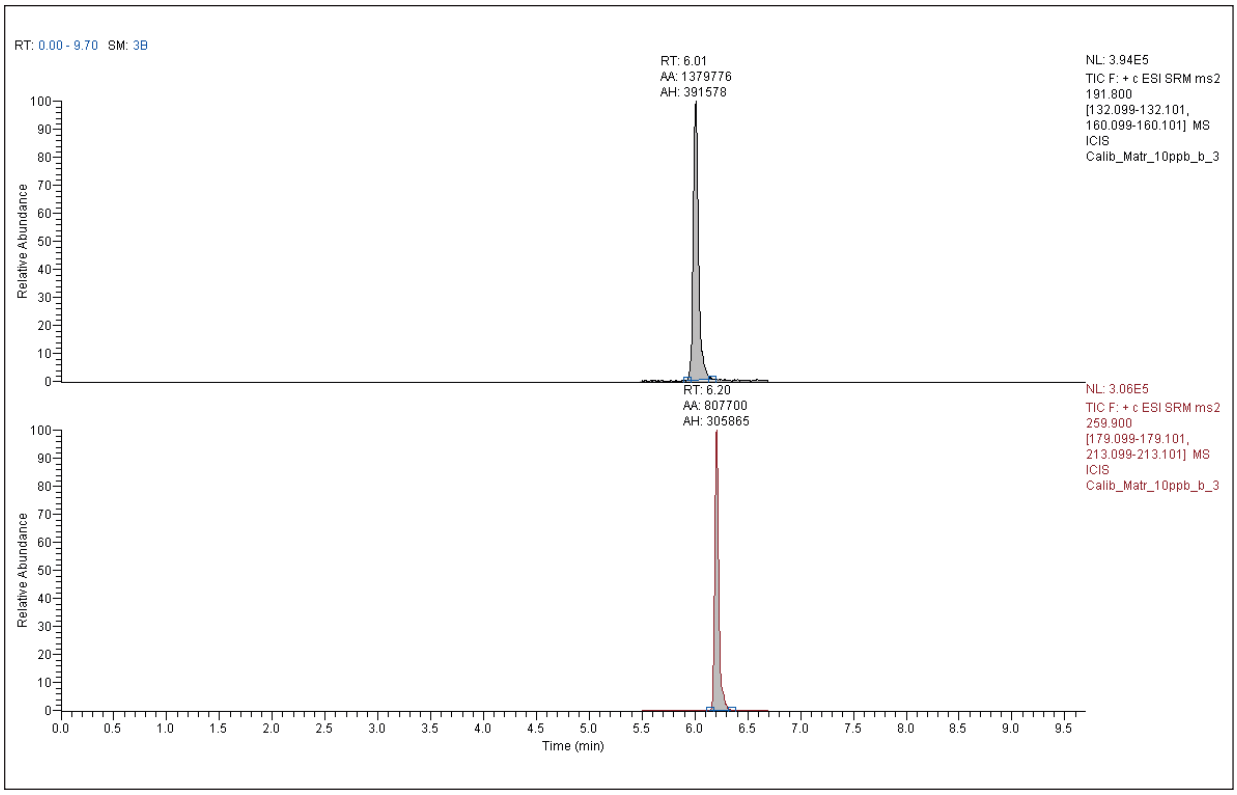

Figure 4: Chromatogram of 0.01 mg/kg carbendazim matrix (orange juice) matched calibration standard representing peak intensity at current US (FDA) rejection level. On top: carbendazim, below: d4-imidacloprid (IS).

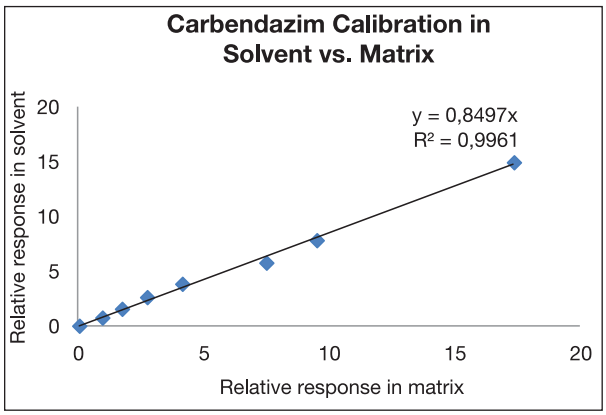

Figure 5: Matrix effect study. Plot of relative responses of calibration levels in solvent vs in orange juice.

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Simplifying Complex Multi-Residue Pesticide Methodology in GC-MS/MS

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Technical Note 10319

Key Words
Pesticide analysis, triple quadrupole GC/MS, AutoSRM, SRM, MRM

Overview

Easing Implementation of Multi-Residue Pesticide Methodology

The task of setting up a triple quadrupole GC/MS pesticide analysis can be daunting, regardless of your starting point. Perhaps you are brand new to GC/MS pesticide analysis, and you need all the help you can get. Maybe you analyze a small set of pesticides and want to expand your target list, or you analyze a large pesticide set in multiple runs on a single quadrupole and want to combine these into a single MRM analysis. Perhaps you already have a comprehensive MRM method, but want to move this to a Thermo Scientific™ TSQ™ 8000 triple quadrupole GC-MS/MS system to take advantage of its robustness, removable ion source under vacuum, and its ease in adding new target pesticides through AutoSRM. Whatever your starting point, when adopting new technology to address complex analytical challenges, you need tools that enable you to be productive, quickly.

With your needs and requirements in mind, the Thermo Scientific TSQ 8000 Pesticide Analyzer (Figure 1) has been developed. Provided within this comprehensive package are all the tools you need to set up a complex pesticide method, regardless of your starting point.

Everyone who is new to pesticide analysis on the TSQ 8000 GC-MS/MS system will appreciate the provided list of optimized pesticide transitions. Also, with an easy to follow step-by-step description of how to develop new transitions using AutoSRM, you'll find the ease of adding new pesticides to your MRM method is now a competitive advantage for your laboratory. And for those who need more assistance, the TSQ 8000 Pesticide Analyzer contains a complete instrument method developed on an included column with provided compound retention times and MRM parameters—eliminating days, if not weeks, of method development.

Figure 1. The TSQ 8000 Pesticide Analyzer. Details of its contents can be found in the *TSQ 8000 Pesticide Analyzer Brochure (BR10318)*.

In addition to simplified method startup, another advantage of using the analyzer is that it utilizes Timed-SRM methodology, allowing for easy-to-use, high-analyte-capacity methodology. The usability and scanning efficiency of Timed-SRM are complemented by the fast-scanning capability of the TSQ 8000 instrument, making the analysis of hundreds of pesticides, with a total of over one thousand transitions, not just possible, but easy.

Finally, the TSQ 8000 Pesticide Analyzer has the ability to analyze full scan data at the same time as your targeted MRM analysis. This allows you to harness the power of existing EI full scan libraries to, for example, find potential high-level contaminants you would otherwise miss in a targeted analysis, or monitor the matrix background for possible interference.

Thermo SCIENTIFIC

Using the Startup Kit

Starting Point 1: Starting from Scratch

When creating your method within Thermo Scientific™ TraceFinder™ EFS software, the instrument control and data processing software included with the TSQ 8000 Pesticide Analyzer, the use of the TraceFinder Pesticide Compound Database (CDB) will greatly simplify the method development process. Multiple transitions for each compound in the database have been optimized on the TSQ 8000 instrument with AutoSRM to within ± 1 eV of the optimum collision energy.

Simply select the compounds of interest in the CDB (Figure 2). This will create not only the TraceFinder software processing method, but also the TSQ 8000 mass spectrometer acquisition list. Since the instrument employs Timed-SRM, SRM windows for data acquisition will be centered on your retention times, so that all peaks elute far from acquisition-window breaks. The complete step-by-step procedure, including software screen captures, is detailed in the *TSQ 8000 Pesticide Analyzer Installation Guide*, which is also included with the TSQ 8000 Pesticide Analyzer.

After selecting your compounds of interest, you are now ready to acquire samples in MRM with your TSQ 8000 instrument.

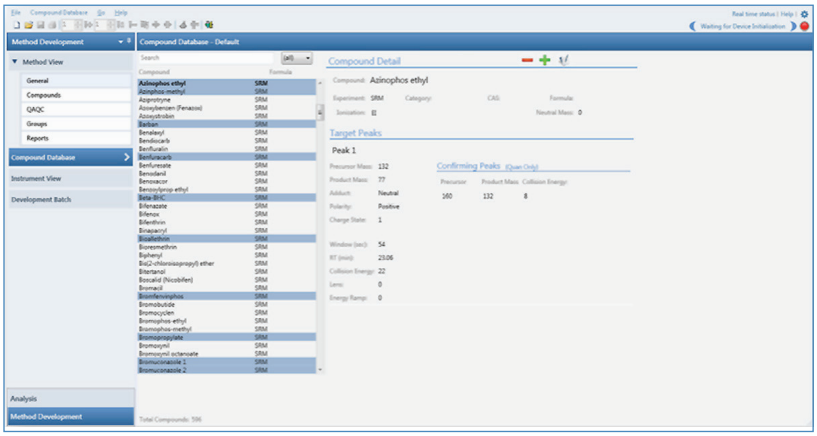

Figure 2. Selecting compounds from the TraceFinder EFS Compound Data Base. This will populate both your TraceFinder Processing Method and your acquisition list. For more information on creating TSQ 8000 methods with the TraceFinder CDB, see *AB52300: Thermo Scientific TSQ 8000 GC-MS/MS Method Sync.*

Starting Point 2: Starting from an Established GC Method

If you already have a preferred GC method, and know the retention times of your target compounds, you can update the pesticides in the CDB with the known retention times. Next, simply select the compounds you are interested in analyzing from the updated CDB, as shown in Figure 2. Again, this will create both the TraceFinder EFS processing method and the TSQ 8000 system Timed-SRM acquisition list, with acquisition windows centered on the retention times of the target peaks.

If you do not know exact retention times, you can easily widen acquisition windows while in TraceFinder EFS software for all compounds (Figure 3) to ensure your peaks fall within their acquisition window. Now update your TraceFinder EFS software method with the new retention times as you would in a normal data review, and your acquisition windows will be centered on each compound. After updating the retention times, follow the same step to reduce acquisition windows back to defaults in order to maximize dwell time for the analysis.

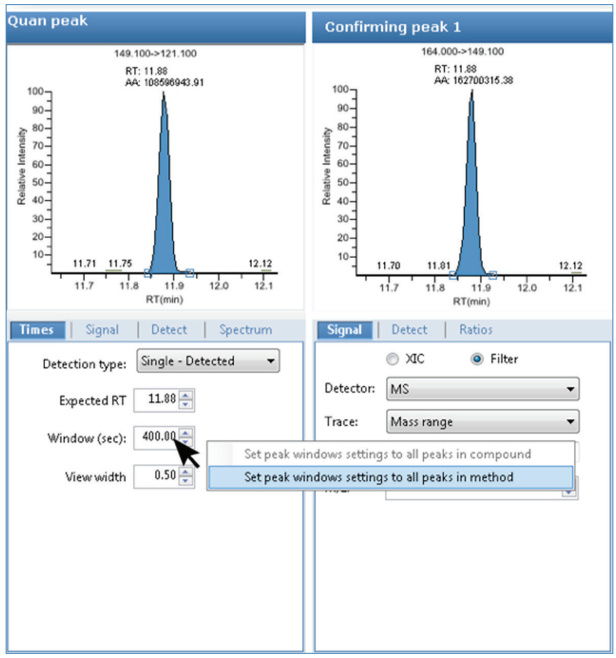

Figure 3. Widening acquisition windows in TraceFinder EFS software to find peaks with unknown retention times.

Tools to Get You Productive

The software features of the TSQ 8000 system have been designed with complex pesticide analysis in mind. These features include AutoSRM, a tool that makes the instrument the easiest for developing and adding new compounds to an existing pesticide method. Another useful feature is Timed-SRM, which enables accurate pesticide identification and quantitation, even for very dense pesticide methodologies. Finally, the ability of the TSQ 8000 instrument to perform simultaneous full scan/MRM provides the capability to identify general unknowns in conjunction with your target pesticides, filling a classic gap in targeted MRM analysis.

Addition of New Compounds

For those compounds provided in the TSQ 8000 Pesticide Analyzer CDB, the addition of new compounds to your methodology is extremely simple. If you are using the method and GC column provided with the TSQ 8000 Pesticide Analyzer, simply select additional compounds to your method from the CDB. The instrument software now adds the selected compounds to both the method acquisition list and the TraceFinder EFS software processing list with the correct retention times.

For those pesticides not yet in the TSQ 8000 Pesticide Analyzer CDB, AutoSRM can be used to quickly develop these new transitions (Figure 4). Once fully developed, the new compounds are easily imported into the CDB and added to your TraceFinder software method. A step-by-step walkthrough of this is described in detail in the *TSQ 8000 Pesticide Analyzer Installation Guide*, which is provided as part of the TSQ 8000 Pesticide Analyzer package. For more details on how AutoSRM works, see *AB52298: Introducing AutoSRM*.

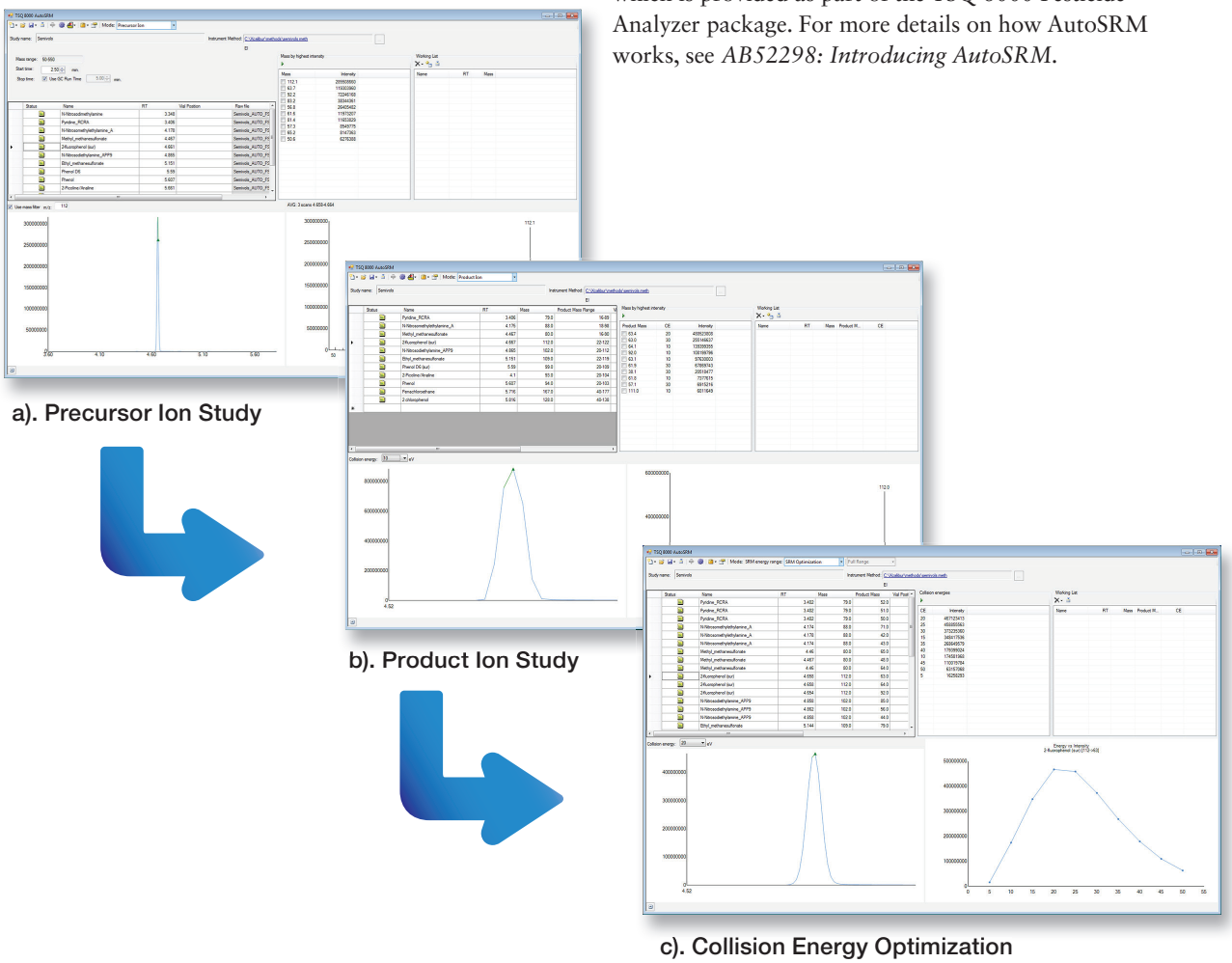

Figure 4. Screen shots showing the three-step process of AutoSRM. a.) In the first step, AutoSRM acquires full scan data for selecting precursor ions. b.) In the second step, product ions are selected from product ion scan data. c.) In the final step, collision energies are varied for each of the selected SRM's to determine the optimal collision energy.

High Compound Capacity Methods

One of the primary challenges of modern pesticide analysis is the sheer number of pesticides that need monitoring in order to meet international standards. This is particularly true in food analysis where products are transported across country borders, requiring exporters to meet the regulatory demands of many countries. Triple quadrupole instruments help meet this demand due to the high selectivity of MRM analysis, which allows for spectral separation of coeluting peaks due to unique reactions in the collision cell. This enables monitoring of more compounds in a single chromatographic run without prohibitive interference. However, due to the targeted nature of the MRM process, individual scan events must be created for each pesticide to be monitored, placing a strain on the amount of time devoted to the monitoring of each compound, and thus the sensitivity of the analysis of each compound.

With a traditional style analysis, this issue can be partially resolved by slicing up the acquisition list into discreet time segments, so that all transitions are not being monitored at the same time. However, this can quickly lead to problems when analyzing more than 50 pesticides in one run. This is because, due to the density of the peaks in the heart of the method, it is difficult to find a time for a segment break when no target peaks are eluting.

This then forces a compromise between adding many compounds per segment, reducing individual SRM dwell times and sensitivity, and adding segment breaks between closely eluting peaks, which causes the risk of false negatives due to shifts in peak retention times outside of acquisition windows because, for example, a large bit of matrix coelutes with a peak.

The TSQ 8000 system takes an approach called Timed-SRM that eliminates this compromise. Timed-SRM removes the limitations of segmented SRM by centering acquisition windows on the retention time of each peak and allowing for acquisition window overlap, so that acquisition windows for all nearby eluting compounds are not forced to start and stop at the same time (Figure 5). The user simply needs to enter the retention time of each compound, and the instrument method takes care of the rest, eliminating the need for creating segments.

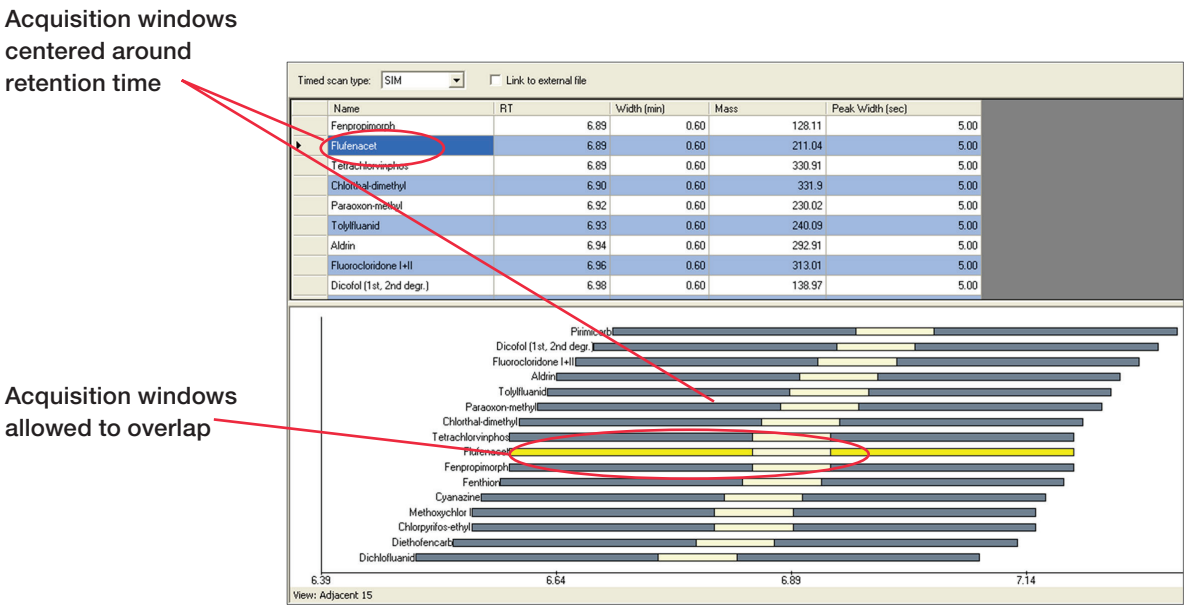

Figure 5. The TSQ 8000 system Timed-SRM Acquisition list, showing SRM acquisition windows centered on retention times and overlapping nearby transitions.

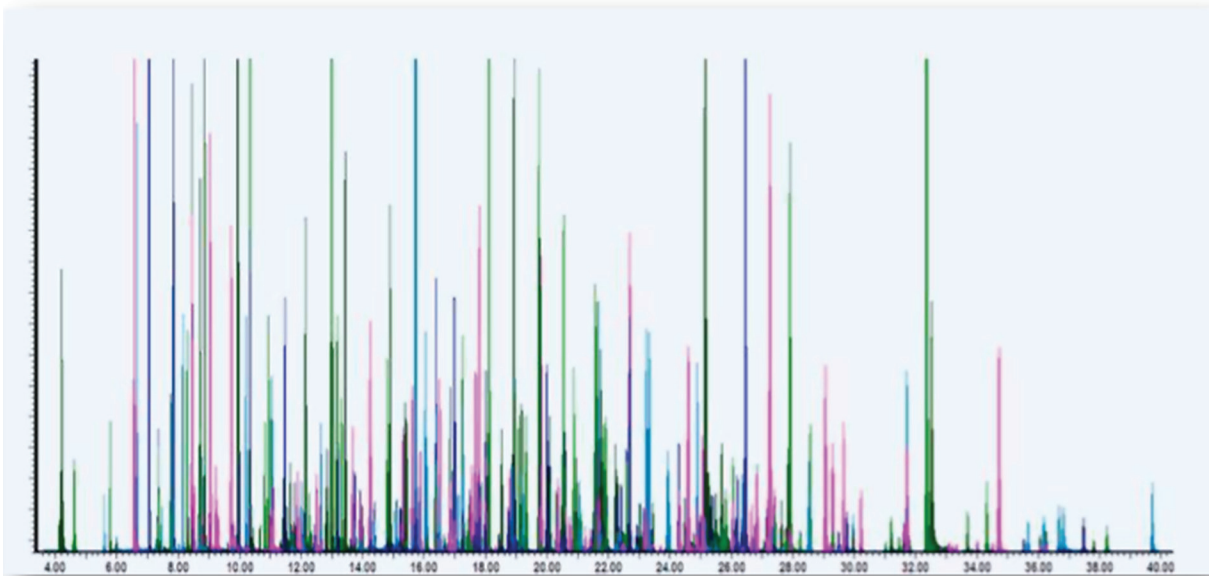

Figure 6. Real-world acquisition of over 300 pesticides in a single chromatographic run using Timed-SRM.

Figure 6 shows a real-world example of a pesticide analysis of over 300 compounds using Timed-SRM. As shown in the Table 1 comparison with Segmented-SRM, Timed-SRM increases both the sensitivity of the analysis by reducing the number of transitions being acquired simultaneously and the time between when target peaks elute and when their acquisition window begins or ends.

Table 1. Comparison of Segmented-SRM vs. Timed-SRM for method of over 300 pesticides. Timed-SRM can dramatically reduce the average number of transitions occurring simultaneously, while increasing the minimum time between an eluting peak and an acquisition window break.

	Segmented-SRM	Timed-SRM
Average number of simultaneous transtions during run	55 Transitions	15 Transitions
Shortest time interval between a compound retnetion time and an acquisiton window break	5 Seconds	15 Seconds

General Unknown Screening

Another limitation of the classic MRM approach to pesticide analysis is that, due to its targeted nature, if a compound is not part of your target list, you are not going to find it, even if it is present in large quantities in your sample. This limitation is removed with capability of the TSQ 8000 system to perform simultaneous full scan/MRM.

The TSQ 8000 system allows you to set up a full scan acquisition throughout the duration of your MRM analysis. Each acquisition will then have full scan data to identify non-target compounds, in addition to MRM data to confirm and quantitate the target list. This mode of analysis is facilitated with the TraceFinder EFS software qualitative processing view within its standard quantitative batch analysis, which automatically detects, identifies, and reports non-target compounds (Figure 7).

Figure 7. Qualitative view of TraceFinder EFS software for analyzing fruit juice with simultaneous full scan/Timed-SRM on the TSQ 8000 system. In addition to quantitating and confirming the 158 target compounds by MRM (top), TraceFinder EFS software has identified three high-level unknowns by full scan analysis (bottom): 2,4-bis(1,1-dimethylethyl)-phenol, triethyl citrate, and Vitamin E.

Conclusion

For the lab just starting up a complex pesticide analysis by triple quadrupole GC-MS, the TSQ 8000 Pesticide Analyzer offers the easiest and quickest path to success. The included methodology, consumables, and SRM transition list with accurate retention times enable the creation of your pesticide method to be as simple as selecting the compounds you want to analyze. With multiple SRM transitions per compound optimized to within ± 1 eV, the pesticide analyzer is useful for anyone who wants to take advantage of the unique features of the TSQ 8000 system designed to make complex pesticide analysis simple.

The TSQ 8000 Pesticide Analyzer fully takes advantage of these features, including the ability to do Timed-SRM, which significantly increases low-level sensitivity through a more efficient SRM scheduling. Also, the full scan/MRM capability of the TSQ 8000 mass spectrometer combines the elite quantitation capabilities of MRM analysis with classic general unknown identification through full scan quadrupole library searching. Finally, the ability to easily develop and add new pesticides to an existing pesticide method through AutoSRM makes the TSQ 8000 Pesticide Analyzer the most flexible system for expanding your pesticide target list to meet future regulatory or client demands.

Certified System ISO 9001 QMI-SAI Global

Thermo Fisher Scientific, Austin, TX USA is ISO 9001:2008 Certified.

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Multi-Residue Pesticide Analysis in Herbal Products Using Accelerated Solvent Extraction with a Triple Quadrupole GC-MS/MS System

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Application Note 52291

Key Words
Pesticides, Tea, Herbal products, ASE, SRM, MRM, Multi-residue analysis, TSQ 8000 GC-MS/MS

Introduction

The residue analysis of pesticides has developed in recent years into a comprehensive methodology for the detection of many hundreds of potential contaminating compounds. A multi-residue method for herbal products and teas is faced with additional challenges from the worldwide origin of the products and the complex matrix of the dried materials. In the due quality control of raw materials, the unknown or undeclared local plant protection treatments must be taken into account with a wide variety of potential pesticide contaminations.

Dried leaves, fruits or seeds and other herbal products of medical use deliver highly complex extracts from the sample preparation due to the rich content of active ingredients, essential oils and the typical high boiling natural polymer compounds from broken cells, leaves or fruit skins. A thorough clean up of the extracted sample can lead to losses of critical analytes of interest. A complete characterization of pesticide, and other residue, contamination is done by both LC and GC-MS/MS to cover the complete range of functional groups.

This application report describes the methodology used for the multi-residue pesticide analysis of herbal products using accelerated solvent extraction (ASE) and gel permeation chromatography (GPC) sample preparation with detection and quantitation by the Thermo Scientific TSQ 8000 GC-MS/MS system.

A routine screening method for more than 200 pesticide compounds was applied to a wide variety of different sample types, ranging from regular black tea or sage leaves, to seeds like fennel and herbs of medical and fragrance use like thyme and chamomile. The data processing and reporting was achieved by using the Thermo Scientific TraceFinder quantitation software suite.

The sensitivity requirement for this analysis was determined by the regulatory background. The analysis of pesticide residues in tea and herbal products follows the regulations of the European Directorate General for Health and Consumer Affairs (SANCO) for “Method Validation and Quality Control Procedures for Pesticide Residue Analysis in Food and Feed” [1]. The sensitivity requirements for these products as referenced in the Codex Alimentarius [2] result in maximum residue levels of 0.01 mg/kg for most of the pesticide compounds.

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Sample Preparation

Herbal and tea samples were extracted with an accelerated solvent extraction method using the Thermo Scientific Dionex ASE 350 Accelerated Solvent Extractor. The ASE method used is described in an official pesticide standard method [3]. The collected extracts were concentrated using a rotary evaporator (Rotavap) and further cleaned up via gel permeation chromatography (GPC). The GPC step used a polystyrene gel (Bio-Beads® S-X3) with an ethylacetate/cyclohexane mobile phase. After additional concentration by the Rotavap, the extracts were ready for GC injection using ethylacetate as the main solvent.

Method Setup

The analytical method comprised sample handling and injection using the Thermo Scientific TriPlus RSH liquid autosampler, TRACE GC 1310 gas chromatograph equipped with an instant connect, temperature programmable PTV injection system, and the TSQ™ 8000 triple quadrupole GC-MS/MS detection system. The MRM detection method was taken from a routinely employed Thermo Scientific TSQ Quantum XLS GC-MS/MS method without any further optimization on the TSQ 8000 GC-MS/MS system [4]. The TSQ 8000 system automatically optimized acquisition windows and optimized instrument duty cycle using timed-SRM (t-SRM) for maximum sensitivity. This enabled the avoidance of lengthy manual set-ups usually required when adopting new instrumentation (Figure 1).

ASE™ 350 Accelerated Solvent Extraction

Sample weight	10 g
Extraction solvent	Ethylacetate/cyclo-Hexane 1:1, same as GPC solvent
Temperature	120 °C
Pressure	100 bar
Extraction time	5 min, 1 cycle
Flushing with solvent	60% of cell volume
Flushing with nitrogen	100 s

TriPlus™ RSH Autosampler

Syringe	10 µL
Injection volume	1 µL
Injection type	Fast liquid band injection, 100 ms injection time
Washing cycles	3 x 10 µL, solvent ethylacetate

TRACE™ 1310 Gas Chromatograph

Injector PTV	Splitless mode
Base temperature	50 °C
Transfer	10 °C/s to 250 °C, until end of run
Flow	Constant flow, 1.2 mL/min, helium
Analytical column	40 m, ID 0.18 mm, 0.18 µm film, 5%-phenyl phase (5MS type)
Pre-column	5 m, ID 0.18 mm, empty deactivated, no backflush
Column oven	Temperature programmed
Start	70 °C, for 1.50 min
Ramp 1	15 °C/min to 190 °C
Ramp 2	7 °C/min to 290 °C, 12 min
Transfer line	250 °C

TSQ 8000 Mass Spectrometer

Ion source temperature	220 °C
MRM Detection	Timed SRM mode (see Appendix)

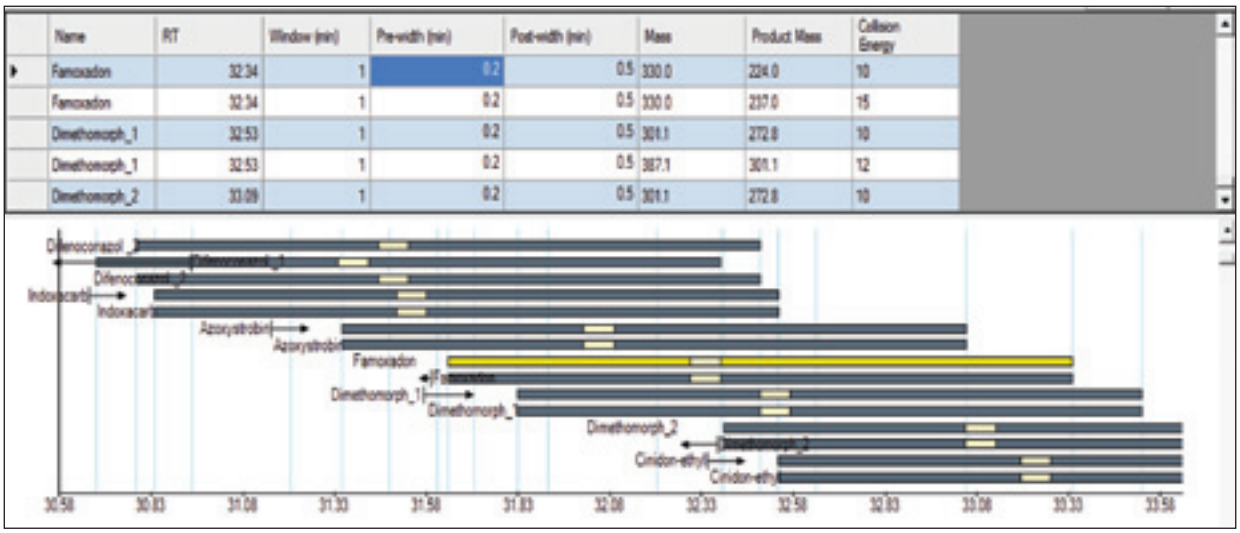

Figure 1. Screenshot of a section of the analytical run showing the "acquisition map" automatically created by the TSQ 8000 system using t-SRM. This mode ensures the instrument only monitors for compounds when they elute to optimize sensitivity.

Calibration and Linearity

The quantitative calibration and linearity check for the method was performed by using six calibration points in the range of 0.004 µg/mL to 1.0 µg/mL. This range represents an analyte concentration of 0.01 to 2.5 mg/kg in the samples (10 – 2500 ppb).

For setting up the calibration solutions, a stock solution containing target pesticide compounds in herbal products was used. The calibration solution was prepared in a standard matrix with a matrix load equivalent to the typical herbal extracts used. The standard matrix blank consisted of lemon peel extracted using the standard procedure. The pesticide blank level was tested before applying as a blank standard matrix. Standard solutions were prepared containing lemon peel extract dissolved 1:1 with ethyl acetate. The correlation coefficients, R^2, achieved during method calibration exceeded 0.99 for all compounds (Figure 2).

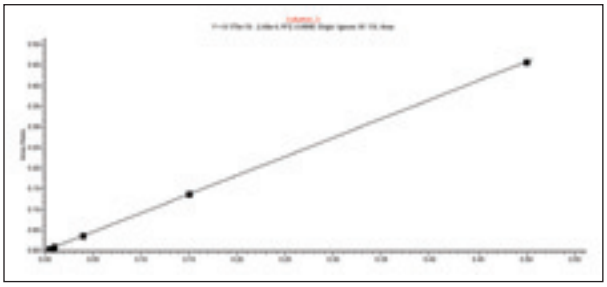

Figure 2. Calibration curve for Cyfluthrin, $R^2 = 0.9996$

Results and Discussion

Sensitivity (LOD)

Using the standard pool of pesticides, the method detection limits in the standard lemon peel were estimated. Using the 4 ppb (pg/µL) matrix standard level, S/N values were used to estimate the limits of detection (LOD). The S/N values in matrix are given in Table 1 for a selection of critical compounds taken at retention times that are affected most from the eluting matrix. Although the compounds are eluting in heavily impacted matrix regions of the chromatogram, the high selectivity of the TSQ 8000 GC-MS/MS for the target pesticides at low level against an intense matrix load is demonstrated in Figure 3 and Figure 4.

Table 1. Detection limit S/N for selected pesticide compounds in matrix

Pesticide	RT [min]	S/N @ 4 ppb
Terbacil	13:83	24
Alachlor	14:78	12
Tolylfluanid	16:75	44
Pyridaben	24:17	83

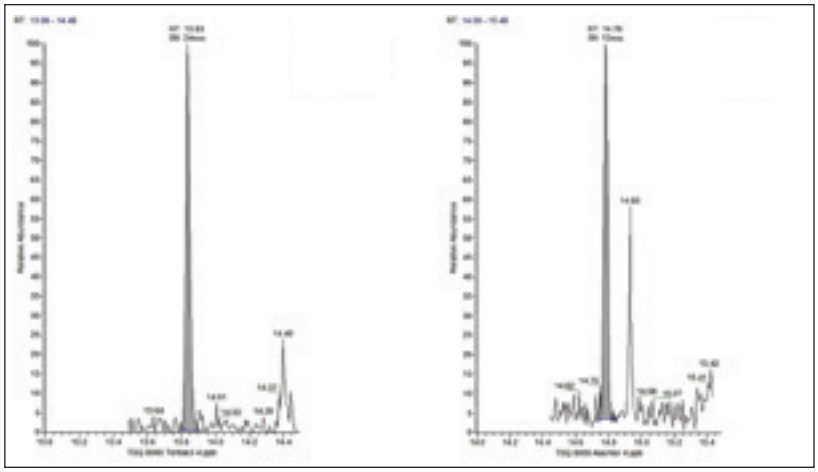

Figure 3. SRM peaks at 4 ppb from Terbacil (left, 161.1 › 88.0, CE 15 V) and Alachlor (right, 188.1 › 130.1, CE 25 V). SRM transitions were taken from the Pesticide Method Reference, 2nd ed. 2011. [4]

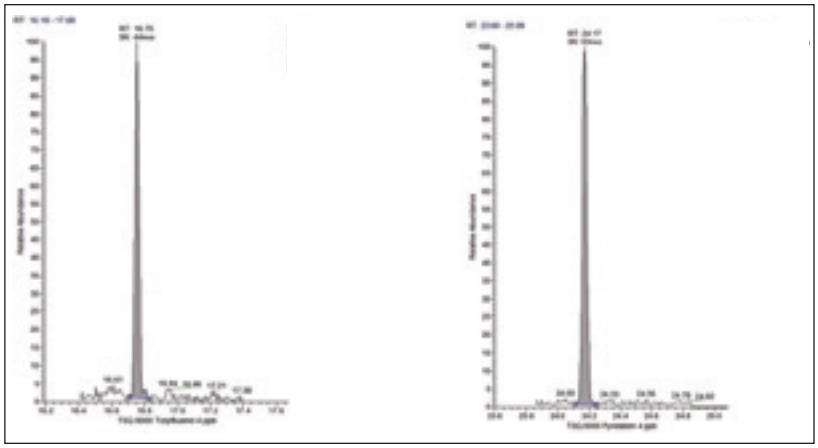

Figure 4. SRM peaks at 4 ppb from Tolylfluanid (left, 238.1 › 137.1, CE 15 V) and Pyridaben (right, 309.1 › 147.1, CE 15 V). SRM transitions were taken from the Pesticide Method Reference, 2nd ed. 2011. [4]

Robustness and Maintenance

Routine preventative maintenance on the GC was performed using routine standard operating procedures. The calibration chromatograms seen in Figures 3 and 4 have been acquired after a persistent matrix load to the system through routine analysis of more than 500 matrix samples.

This level of robustness meant that even with persistent and very high matrix load, it was not necessary to clean the removable ion source short term.

The innovative instant connect modularity of the injectors and detectors of the TRACE 1310 GC, used here as the front-end to the mass spectrometer, allows the user quick accessibility to any injector part for rapid cleaning. Furthermore the unique ability to replace the entire injector module within minutes represents an excellent way of postponing routine maintenance to when the laboratory schedule allows while keeping the GC-MS/MS system operational.

Analytical Precision

Within a routine series of 50 commercial samples, the quality control samples were measured with replicate injections. The results for a range of compounds is given in Table 2. The relative effects on known problematic pesticide compounds can be seen, while coefficients of variation (CV%) for unaffected compounds all stay well below 10% even within this long series of matrix injections.

Table 2. Coefficients of variation for lemon peel matrix spiked QC samples for a set of 60 pesticides under investigation (avg. 7.4%, 24 injections)

Diflubenzofuron	10.0%	Penconazol	7.5%	Diniconazol	2.9%
Biphenyl-d10	7.5%	Allethrin	8.4%	Aclonifen	9.0%
Biphenly	9.5%	Pyrifenox	5.5%	Trifloxystrobin	6.0%
o-Phenylphenol	8.2%	Procymidon	5.7%	Propiconazol	3.1%
Fenobucarb	6.0%	Triadimenol	11.5%	Propargit	6.0%
Diphenylamin	5.7%	Picoxystrobin	7.0%	Tebuconazol	4.3%
Terbutylazin	4.4%	Flutriafol	6.3%	Nitralin	9.2%
Propyzamid	3.1%	Hexaconazol	9.2%	Piperonyl butoxid	8.3%
Terbazil	5.8%	Isoprothiolan	9.7%	Brompropylat	5.8%
Fipronil-desulfinyl	6.9%	Uniconazol	7.0%	Fenoxycarb	9.1%
Alachlor	6.7%	Kresoxim-methyl	9.9%	Etoxazol	8.8%
Prometryn	8.3%	Myclobutanil	9.2%	Fenazaquin	3.3%
Ethofumesat	7.4%	Flusilazol	4.4%	Metconazol	5.3%
Bromacil	8.3%	Cinerin 1	8.1%	Pyriproxyfen	8.5%
Chlorpyrifos	6.9%	Buprofezin	7.4%	Fenamirol	8.5%
Tetraconazol	6.2%	Diclobutrazol	2.6%	Fluquinconazol	4.9%
Triadimefon	11.7%	Cyproconazol	2.6%	Pyridaben	5.2%
Dicapton	10.7%	Chlorbenzilat	3.3%	Etofenprox	10.2%
Butralin	6.6%	Etoconazol	4.4%	Silafluofen	10.2%
Fipronil	5.5%	Iprodion	11.1%	Indoxacarb	8.5%

Results from Real Life Samples

The above method was used for the analysis of a wide variety of herbs, teas and dried fruit known as one of the most challenging analytical task for controlling the pesticide maximum residue levels due to the heavy matrix impact. Table 3 gives a representative overview of positive results from different samples with the indication of the pesticide compound and concentration found. All compounds were detected by using at least two SRM traces and were subsequently confirmed by checking the calibrated ion ratios. The concentration ranges covered were from close to the MRL level of 10 mg/kg to high levels of up to 50 times above the regulated maximum. Figure 5 provides an example of confirmed residue detection in a thyme sample.

Table 3. Positive results above MRL level found in samples of various matrices

Sample Matrix	Pesticide Residues Found	Concentration (mg/kg)
Dried Herbs	o-Phenylphenol	0.017
Dried Herbs	Tebuconazol	0.023
Dried Fruit	Diflubenzuron	0.049
Dried Fruit	Myclobutanil	0.023
Dried Fruit	Propargit	0.479
Dried Fruit	Tebuconazol	0.081
Dried Fruit	Difenconazol	0.013
Dried Herbs	Picoxystrobin	0.228
Dried Herbs	Picoxystrobin	0.233
Dried Herbs	o-Phenylphenol	0.011
Herbal Tea	o-Phenylphenol	0.014
Herbal Tea	o-Phenylphenol	0.011
Herbal Tea	Terbutylazin	0.016

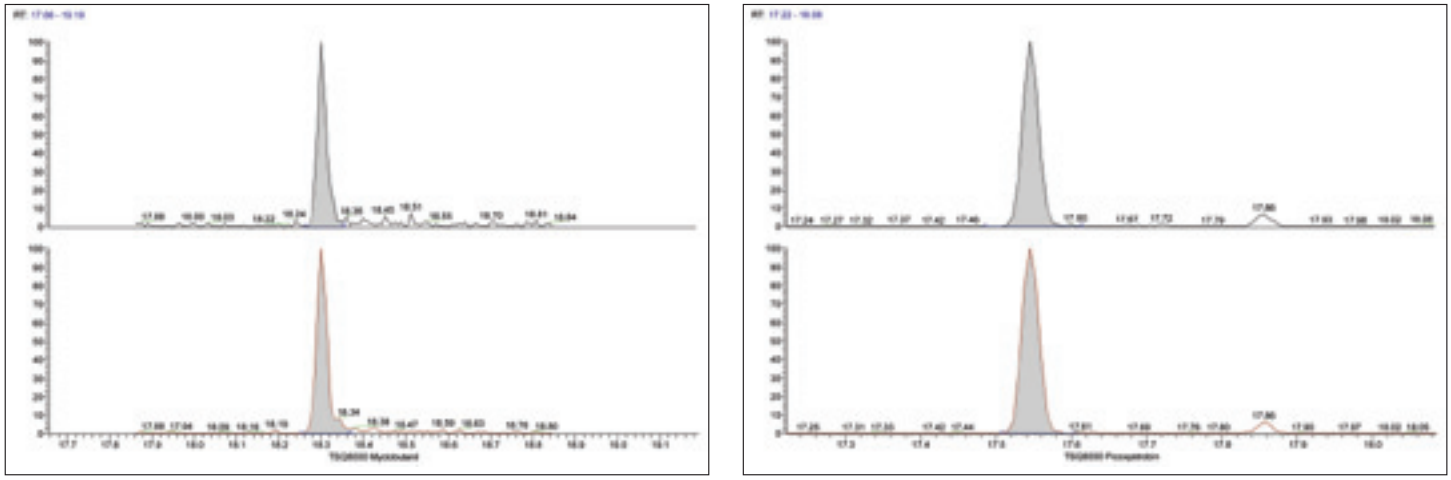

Figure 5. Positive results for Myclobutanil in green apple (0.023 mg/kg, left) and Picoxystrobin in thyme (0.228 mg/kg, right), both detected on two SRM traces

Data Analysis and Reporting

The data processing was performed using TraceFinder™ quantitation software. TraceFinder software contains a compound data store containing a large number of pesticide compound entries from which required compounds for the method had been selected. For each pesticide, the necessary parameters for MRM acquisition and compound identification, such as SRM transition, retention time, and ion ratios, as well as quantitation details like quantitation mass and recovery requirement, are stored.

The analytical sequence setup, data acquisition and result processing was done from one software platform integrating the complete analytical process. In Figure 6, the analytical sequence is shown in the upper part of the screen, with the compounds included in the method to the right. The actual chromatograms for the selected pesticide compounds are displayed in the bottom part for review by the operator.

Figure 6. TraceFinder software analysis view:

A. Acquisition sequence table for calibration, QC and sample runs

B. Compound list with status flags

C. Compound chromatogram windows with integrated quantitation and confirmation peaks

Expanded Productivity

The total cycle time of the analytical runs was 30 minutes, which allowed the throughput of two samples per hour and resulted in a load of up to 48 samples, including QC checks during the day for the control of more than 200 pesticide compounds in each run.

This expanded productivity was a combined result of the TSQ 8000 triple quadrupole GC-MS/MS system with its enhanced analyte selectivity in matrix samples, the high method and system robustness, and the advanced data processing using TraceFinder software. Pesticide peaks were typically baseline-separated with a high signal-to-noise ratio allowing for an accurate automated area integration with significantly reduced manual control required. A number of quality control parameters within TraceFinder software immediately provided visible flagging for compounds that may need manual attention. Automatic ion ratio checks provided a fast and solid confirmation in the case of positive findings. The high processing speed of TraceFinder software provided for multi-residue analysis and quick and comprehensive reporting for each sample.

Conclusion

The TSQ 8000 GC-MS/MS delivered high sensitivity and matrix selectivity for routine pesticide analysis even in difficult matrix samples. The data acquisition using the unique timed-SRM allowed for the detection of a virtually unlimited number of pesticide compounds in one run without sacrificing the high sensitivity for individual compounds. Quantitative calibrations were performed in a standard matrix and showed excellent linearity and precision over the relevant concentration range to control the regulated MRL levels.

The high matrix selectivity of the TSQ 8000 system allowed for reduced sample preparation, providing high recoveries for a wide range of chemically diverse pesticide compounds. The very high matrix selectivity delivered low chemical matrix background with well-defined pesticide peaks that were safe and easy to integrate, thus eliminating the need for time-consuming manual baseline corrections.

Positive pesticide compound signals were confirmed by TraceFinder software checking the calibrated ion ration of the two monitored SRM transitions.

The TSQ 8000 GC-MS/MS system is well prepared for routine analysis and provides high robustness of the chromatographic system and ion source, thus reducing the need for frequent maintenance and avoiding system downtime for high sample throughput and productivity. The system is easy to use, durable, and robust even with the most challenging sample types and is fully automated in sampling capabilities to found and not-found report generation.

References

1. SANCO Document N° SANCO/12495/2011, Method Validation and Quality Control Procedures for Pesticide Residue Analysis in Food and Feed, Implemented by 01/01/2012.
2. Codex Alimentarius (www.codexalimentarius.net/mrls/pesticides/jsp/pest-q-e.jsp)
3. Pesticide determination according to § 64 LFGB L 00.00-34 (German legislation) Modul E9 (ASE); GPC
4. Pesticide Method Reference, 2nd Edition, 2011 Thermo Fisher Scientific, p/n 120390.

Appendix: List of pesticides with MRM transitions used (from [4])

Pesticide Name	RT (min)	Precursor Mass (*m/z*)	Product Mass (*m/z*)	Collision Energy (V)
Difluorobenzamid Degradation (Isocyanat)	6.93	152.93	90.01	20
Difluorobenzamid Degradation (Isocyanat)	6.93	152.93	125.01	20
Carbofuran 1	8.80	149.06	121.05	10
Carbofuran 1	8.80	164.08	149.07	10
Difluorobenzamid Degradation	8.62	141.00	63.11	25
Difluorobenzamid Degradation	8.62	141.00	113.09	15
Biphenyl-d10_ISTD	9.24	160.00	160.16	10
Biphenyl	9.28	154.08	153.08	15
Biphenyl	9.28	153.08	152.08	15
Carbofuran-3-hydroxy 1	10.43	137.05	81.01	18
Carbofuran-3-hydroxy 1	10.43	180.05	137.01	15
Tetrahydrophthalimid	10.84	151.04	79.01	25
Tetrahydrophthalimid	10.84	151.04	122.09	10
O-Phenylphenol	11.00	170.07	141.06	20
O-Phenylphenol	11.00	170.07	115.05	20
Molinate	11.10	187.10	126.07	10
Molinate	11.10	126.07	98.05	5
Chlorfenprop methyl	11.59	196.00	165.00	10
Chlorfenprop methyl	11.59	165.00	137.00	10
Fenobucarb	11.20	121.07	77.05	15
Fenobucarb	11.20	150.09	121.07	10
Propachlor	11.76	176.06	120.04	10
Propachlor	11.76	120.04	92.03	10
Propachlor	11.76	169.06	120.04	10
Propachlor	11.76	196.07	120.04	10
Cycloate	11.98	154.10	83.05	10
Cycloate	11.98	215.13	154.10	5
Diphenylamin	11.49	169.01	168.09	20
Diphenylamin	11.49	169.01	167.09	20
Chloropropham	12.26	213.06	127.03	15
Chloropropham	12.26	213.06	171.04	10
Phosmet-oxon	12.09	160.00	132.96	15
Phosmet-oxon	12.09	104.00	75.88	10
Phosmet-oxon	12.09	160.00	76.96	20
Prometon	13.10	225.16	183.13	10
Prometon	13.10	225.16	210.15	10
Carbofuran 2	13.13	149.06	121.05	10
Carbofuran 2	13.13	164.08	149.07	10
Profluralin	13.22	318.10	199.06	15
Profluralin	13.22	330.23	252.45	25
Swep	13.46	187.05	123.95	18
Swep	13.46	219.11	174.02	15
Trietazine	13.48	229.14	200.14	15
Trietazine	13.48	214.14	186.10	15
Dimethipin	13.53	117.98	57.97	10
Dimethipin	13.53	210.10	76.02	10
Terbutylazin	12.97	214.10	132.06	10
Terbutylazin	12.97	214.10	104.05	10
Propyzamid	13.04	173.01	145.01	15
Propyzamid	13.04	173.01	109.01	18
Propyzamid	13.04	175.02	147.01	15
Propyzamid	13.04	254.02	226.02	15
Isocarbamide	13.67	142.03	70.01	15
Isocarbamide	13.67	142.03	113.01	10
Dinoseb	13.92	211.13	116.99	15
Dinoseb	13.92	211.13	163.11	10
Terbazil	13.42	161.05	88.03	15
Terbazil	13.42	160.05	76.02	15
Bromocylen	14.37	358.79	242.85	15
Bromocylen	14.37	356.93	241.24	15
Dimethenamid	14.60	230.06	154.04	10
Dimethenamid	14.60	232.06	154.04	10
Dimethachlor	14.61	197.08	148.06	10
Dimethachlor	14.61	199.08	148.06	10
Acetochlor	14.65	174.11	146.15	15
Acetochlor	14.65	223.19	147.17	10
Desmetryn	14.68	213.11	171.08	10
Desmetryn	14.68	213.11	198.10	10
Flurprimidol	14.77	269.12	106.98	20
Flurprimidol	14.77	270.18	107.04	20
Alachlor	14.26	188.10	160.07	10
Alachlor	14.26	188.10	130.12	25
Alachlor	14.26	237.14	160.15	10
Metribuzin	14.14	198.08	82.03	20
Metribuzin	14.14	198.08	89.04	16
Propanil	15.00	217.01	161.00	10
Propanil	15.00	219.01	163.00	10
Fipronildesulfinyl	14.15	333.00	231.20	20
Fipronildesulfinyl	14.15	333.00	281.30	20
Carbofuran-3-hydroxy 2	15.02	137.05	81.01	18
Carbofuran-3-hydroxy 2	15.02	180.05	137.01	15
Prometryn	14.49	241.14	184.10	15
Prometryn	14.49	226.13	184.10	12
Tridiphan	15.18	186.94	158.94	15
Tridiphan	15.18	219.09	184.09	20
Ethofumesat	14.80	206.82	160.86	10
Ethofumesat	14.80	285.75	206.82	12
Pentanochlor	15.73	141.05	106.05	15
Pentanochlor	15.73	239.05	141.05	15
Chlorpyrifos	15.78	257.97	165.98	20
Chlorpyrifos	15.78	314.05	258.18	15
Bromacil	15.03	205.01	188.01	15
Bromacil	15.03	207.01	190.01	15

Pesticide Name	RT (min)	Precursor Mass (*m/z*)	Product Mass (*m/z*)	Collision Energy (V)
Anthrachinon	15.44	207.97	151.99	20
Anthrachinon	15.44	180.04	152.05	15
Anthrachinon	15.44	207.97	180.10	10
Nithrothal isopropyl	16.09	236.08	194.07	10
Nithrothal isopropyl	16.09	236.08	148.05	20
Triadimefon	15.41	208.07	181.06	10
Triadimefon	15.41	210.07	183.06	10
Tiocarbazil	16.15	156.08	100.05	8
Tiocarbazil	16.15	279.10	156.07	6
Tetraconazol	15.39	336.02	218.01	20
Tetraconazol	15.39	338.02	220.01	20
Butralin	15.54	266.14	220.11	15
Butralin	15.54	266.14	190.10	15
Dicapthon	15.44	262.00	262.00	9
Dicapthon	15.44	262.00	216.00	13
Crufomat	16.30	256.20	226.15	25
Crufomat	16.30	276.20	182.09	10
Allethrin	16.17	123.07	80.98	10
Allethrin	16.17	136.04	92.98	10
Dinobuton	16.89	163.06	116.04	15
Dinobuton	16.89	211.07	117.04	18
Penconazol	16.89	248.06	157.04	25
Penconazol	16.89	248.06	192.04	15
Pyrifenox 1	16.17	262.03	192.02	20
Pyrifenox 1	16.17	262.03	200.02	20
Pyrifenox 2	16.81	262.03	192.02	20
Pyrifenox 2	16.81	262.03	200.02	20
Tolylfluanid	16.92	238.09	137.05	15
Tolylfluanid	16.92	240.09	137.05	15
Fipronil	17.01	368.95	214.97	30
Fipronil	17.01	366.95	254.96	25
Triflumizol	17.20	206.05	179.04	15
Triflumizol	17.20	179.04	144.04	15
Procymidon	17.22	283.05	95.93	10
Procymidon	17.22	285.05	95.97	10
Procymidon	17.22	285.05	257.30	10
Triadimenol 1	16.45	168.11	69.99	15
Triadimenol 1	16.45	128.05	100.04	10
Triadimenol 2	16.64	168.11	69.99	15
Triadimenol 2	16.64	128.05	100.04	10
Butachlor	17.54	237.13	160.09	10
Butachlor	17.54	176.09	146.08	10
Chlorbenside	17.57	124.97	88.98	20
Chlorbenside	17.57	124.97	63.02	30
Fenothiocarb	17.68	160.07	72.01	15
Fenothiocarb	17.68	160.07	106.00	10
Picoxystrobin	17.69	335.09	303.09	10
Picoxystrobin	17.69	303.09	157.04	20
Paclobutrazole	17.75	236.10	125.06	15

Pesticide Name	RT (min)	Precursor Mass (*m/z*)	Product Mass (*m/z*)	Collision Energy (V)
Paclobutrazole	17.75	238.11	127.06	15
Chinomethionat	17.78	206.06	147.98	15
Chinomethionat	17.78	234.08	206.06	10
Napropamid	18.07	271.16	128.07	5
Napropamid	18.07	128.07	72.04	10
Flutriafol	18.11	219.07	123.04	15
Flutriafol	18.11	123.04	75.03	15
Flurodifen	18.14	190.02	126.01	10
Flurodifen	18.14	190.02	146.01	5
Bisphenol A	18.17	213.14	119.06	15
Bisphenol A	18.17	213.14	164.99	20
Bisphenol A	18.17	228.15	213.07	10
Chlorfenson_ISTD	18.20	302.00	110.90	20
Hexaconazol	18.22	214.08	159.07	20
Hexaconazol	18.22	214.08	151.98	25
Imazalil	18.24	172.96	144.96	15
Imazalil	18.24	172.96	108.95	25
Isoprothiolan	18.24	203.99	117.95	7
Isoprothiolan	18.24	203.99	84.90	25
Isoprothiolan	18.24	290.06	118.03	15
Flamprop-methyl	18.39	230.05	170.04	10
Flamprop-methyl	18.39	276.06	105.02	10
Kresoximmethyl	18.48	206.10	131.09	15
Kresoximmethyl	18.48	206.10	116.01	10
Buprofezin	18.51	175.08	116.96	20
Buprofezin	18.51	175.08	131.99	15
Buprofezin	18.51	249.16	105.93	20
Buprofezin	18.51	249.16	193.20	10
Uniconazol	18.57	234.12	136.99	15
Uniconazol	18.57	234.12	101.95	25
Uniconazol	18.57	234.12	165.08	10
Cinerin 1	18.60	123.08	95.06	10
Cinerin 1	18.60	123.08	81.05	10
Cinerin 1	18.60	150.10	108.09	10
Flusilazol	18.60	233.16	165.13	25
Flusilazol	18.60	233.16	152.06	20
Myclobutanil	18.65	179.00	125.00	15
Myclobutanil	18.65	179.00	89.95	25
Methoprotryne	18.66	256.14	212.11	15
Methoprotryne	18.66	256.14	200.11	15
Diclobutrazol	18.75	270.07	159.04	15
Diclobutrazol	18.75	272.08	161.04	15
Azaconazole	18.78	217.02	173.01	15
Azaconazole	18.78	219.02	175.01	15
Perthane	18.95	223.15	179.10	18
Perthane	18.95	223.15	167.06	18
Cyproconazol	19.14	222.09	125.05	20
Cyproconazol	19.14	224.09	127.05	20
Flamprop-isopropyl	19.14	276.08	105.03	15

Pesticide Name	RT (min)	Precursor Mass (*m/z*)	Product Mass (*m/z*)	Collision Energy (V)
Flamprop-isopropyl	19.14	278.17	104.99	20
Chloropropylat	19.16	251.02	139.01	20
Chloropropylat	19.16	251.02	111.01	20
Ancymidol	19.18	228.15	121.02	15
Ancymidol	19.18	215.15	107.02	15
Chlorbenzilat	19.22	251.02	139.01	20
Chlorbenzilat	19.22	251.02	111.01	20
Cyprofuram	19.36	211.12	132.02	10
Cyprofuram	19.36	211.12	166.05	10
Etaconazol 1	19.38	245.04	173.03	15
Etaconazol 1	19.38	245.04	191.03	10
Etaconazol 2	19.38	245.04	173.03	15
Etaconazol 2	19.38	245.04	191.03	10
Diniconazol	19.47	268.06	232.05	15
Diniconazol	19.47	270.06	234.05	15
Jasmolin 1	19.58	123.08	81.05	10
Jasmolin 1	19.58	123.08	95.06	10
Jasmolin 1	19.58	164.16	109.15	10
Aclonifen	19.70	212.02	182.02	10
Aclonifen	19.70	264.03	194.02	15
Tetrasul	19.85	251.92	216.93	20
Tetrasul	19.85	253.92	218.93	20
Carfentrazone ethyl	19.95	340.03	312.03	10
Carfentrazone ethyl	19.95	312.15	150.99	20
Benodanil	19.99	322.98	230.99	15
Benodanil	19.99	322.98	195.99	5
Trifloxystrobin	20.02	222.13	162.14	10
Trifloxystrobin	20.02	115.99	88.95	15
Trifloxystrobin	20.02	222.13	130.02	15
Chlordecone	20.06	271.91	237.16	15
Chlordecone	20.06	273.91	239.15	20
Famophos (Famphur)	20.16	218.07	108.94	15
Famophos (Famphur)	20.16	218.07	126.95	20
Iprodion Degradation	18.63	186.87	123.99	20
Iprodion Degradation	18.63	186.87	159.02	15
Iprodion Degradation	18.63	243.94	187.02	10
Iprodion	20.57	314.06	245.25	15
Iprodion	20.57	186.99	123.87	20
Iprodion	20.57	316.00	247.35	15
Iprodion	20.57	316.00	273.11	10
Propiconazol 1	19.38	259.02	173.02	20
Propiconazol 1	19.38	172.94	144.91	15
Propiconazol 2	19.54	259.02	173.02	20
Propiconazol 2	19.54	172.94	144.91	15
Pyraflufen-ethyl	20.30	412.02	349.02	15
Pyraflufen-ethyl	20.30	349.02	307.02	15
Clodinafop-propargyl	20.36	349.05	266.04	15
Clodinafop-propargyl	20.36	349.05	238.04	15
Lenacil	20.70	153.05	136.06	15

Pesticide Name	RT (min)	Precursor Mass (*m/z*)	Product Mass (*m/z*)	Collision Energy (V)
Lenacil	20.70	153.05	135.15	15
Diclofop methyl	20.77	253.02	162.01	15
Diclofop methyl	20.77	340.04	253.02	15
Propargit	20.79	173.08	135.04	15
Propargit	20.79	173.08	106.93	20
Propargit	20.79	350.21	173.10	15
Diflufenican	20.83	394.07	266.05	10
Diflufenican	20.83	266.05	246.05	10
Piperonylbutoxid	20.87	176.11	131.08	15
Piperonylbutoxid	20.87	176.11	103.06	10
Piperonylbutoxid	20.87	176.11	145.09	15
Tebuconazol	20.97	250.12	125.06	20
Tebuconazol	20.97	252.12	127.06	20
Nitralin	21.09	316.02	274.15	10
Nitralin	21.09	273.99	216.07	10
Benzoylpropethyl	21.22	292.05	105.02	15
Benzoylpropethyl	21.22	172.03	145.02	14
Captafol	21.22	311.06	78.94	20
Captafol	21.22	311.06	276.21	10
Epoxyconazol	21.29	192.04	138.03	10
Epoxyconazol	21.29	192.04	111.02	10
Bromuconazol 1	21.73	294.96	174.98	15
Bromuconazol 1	21.73	292.96	172.98	15
Brompropylat	21.76	340.93	183.05	20
Brompropylat	21.76	340.93	185.04	20
Etoxazol	21.83	300.14	270.38	20
Etoxazol	21.83	330.17	300.44	25
Fenoxycarb	21.85	186.08	109.05	15
Fenoxycarb	21.85	255.11	186.08	10
Phosmet	20.79	160.00	133.00	15
Phosmet	20.78	160.00	104.00	20
Phosmet	20.78	316.99	160.00	5
Fenpiclonil	21.94	235.99	200.99	15
Fenpiclonil	21.94	237.99	200.99	15
Fenazaquin	22.22	160.09	145.08	10
Fenazaquin	22.22	145.05	116.99	15
Fenazaquin	22.22	160.09	117.08	20
Phenothrin 1	22.27	183.10	153.08	18
Phenothrin 1	22.27	183.10	165.09	10
Phenothrin 2	22.42	183.10	153.08	18
Phenothrin 2	22.42	183.10	165.09	10
Bromuconazol 2	22.35	294.97	174.97	15
Bromuconazol 2	22.35	292.97	172.97	15
Metconazol	22.41	125.00	88.93	20
Metconazol	22.41	250.20	124.88	25
Triticonazole	22.80	235.10	217.09	10
Triticonazole	22.80	235.10	182.07	10
Pyriproxyfen	22.82	226.15	186.22	15
Pyriproxyfen	22.82	136.00	95.95	15

Pesticide Name	RT (min)	Precursor Mass (*m/z*)	Product Mass (*m/z*)	Collision Energy (V)
Azinphosmethyl	22.95	160.00	132.00	10
Azinphosmethyl	22.95	160.00	104.64	10
Pyriproxyfen	23.06	136.00	77.92	20
Fenamirol	23.55	251.02	139.01	15
Fenamirol	23.55	330.03	139.01	10
Pyridaben	24.50	364.14	309.12	5
Pyridaben	24.50	309.12	147.06	15
Fluquinconazol	24.59	340.01	298.01	22
Fluquinconazol	24.59	342.01	300.01	22
Etofenprox	26.05	163.09	107.06	16
Etofenprox	26.05	163.09	135.07	10
Etofenprox	26.05	376.14	135.02	30
Etofenprox	26.05	376.14	163.09	10
Silafluofen	26.25	179.00	151.00	7
Silafluofen	26.25	286.13	258.12	15
Difenconazol 1	26.91	323.05	265.04	15
Difenconazol 1	26.91	325.05	267.04	20
Difenconazol 2	27.05	323.05	265.04	15
Difenconazol 2	27.05	325.05	267.04	20
Indoxacarb	28.55	264.02	176.14	10
Indoxacarb	28.55	264.02	148.03	20
Indoxacarb	28.55	321.05	289.34	10

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Application Note 52279

High Precision Pesticide Analysis in Produce using GC Triple Quadrupole and U-SRM Mode

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Key Words
1 ppb Levels, Challenging Compounds, Pesticide Analysis, PTV Backflush, U-SRM Mode, Selected Reaction Monitoring, Selectivity, Timed-SRM

Introduction

Pesticides are widely used in agriculture to protect crops and to improve efficiency of production. Consequently, governments, food producers and food retailers have the duty to ensure that any residues occurring in foods for human consumption are at or below Statutory Maximum Residue Levels (MRLs). Regulation EC 396/2005 adopted in the European Union sets MRLs for more than 500 different pesticides in over 300 different food commodities.[1]

Many of these MRLs are set at a default value of 0.01 mg/kg, the typical limit of determination of routine analytical methods. Thus, there is a requirement for food safety laboratories to test a wide array of foods for a large number of pesticide residues at concentrations at or below 0.01 mg/kg, with low costs and fast turnaround times (often <48 hours). For the efficient control of the regulated MRL levels, the overall method sensitivity in matrix is required to be a factor of 10 lower. This is most often achieved using multi-residue methods based on the use of a combination of LC-MS/MS and GC-MS techniques to determine pesticide residues in a single generic solvent extract of the sample. One such example is the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) procedure, which is based on acetonitrile extraction and dispersive solid phase extraction.[2] After the QuEChERS extraction, a solvent exchange was made to facilitate the GC injection.

The productivity benefit of using the QuEChERS extraction technique is the fast turnaround time for a large number of samples with small sample volumes in the range of 10 g. Limitations of this approach are typically arising from the heavy matrix load of QuEChERS extract requiring increased robustness of the GC inlet system and increased selectivity offered by using a MS/MS analyzer. This application note describes the high quality and low level analysis of pesticides in produce samples using the Thermo Scientific TSQ Quantum XLS Ultra GC-MS/MS system.

For most of the pesticide compounds included in the method, the complete list of the compounds with their respective SRM transitions have been downloaded from the Pesticides Method Reference CD (provided with the manual p/n 120390) into the instrument acquisition method. Each transition has been determined for optimal sensitivity and selectivity, with the complete list documented for TSQ Quantum XLS users.

Over 400 pesticides have been monitored in several matrices such as wheat, blackcurrants and cucumber; the results of the most challenging pesticides in terms of activity and response are highlighted, showing calibration curves, repeatability and ion ratio stabilities.

The TSQ Quantum XLS Ultra™ is able to perform SRM with a higher mass resolution (0.1 Da) setting thus allowing for better selectivity. Not all pesticides in all matrices benefit from a higher mass resolution setting, but depending on the matrix and the compound analyzed, there can be a significant improvement on the signal to noise ratio. Some examples are shown in the 'Advanced GC-MS/MS Experiment' section of this application note.

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Experimental Conditions

All samples were prepared using the QuEChERS technique, and calibration was performed using a blank QuEChERS extract from cucumber. All target compounds were measured using at least two SRM transitions for each compound to a level of 0.001 mg/kg, which is ten times lower than the current maximum concentration limit.

All sample analyses were carried out using the TSQ Quantum XLS Ultra GC-MS/MS system, equipped with a Thermo Scientific TRACE GC Ultra gas chromatograph.

The TRACE GC Ultra™ was configured with a B.E.S.T. PTV injector equipped with a backflush device. Sample introduction was performed using the Thermo Scientific TriPlus RSH autosampler. The capillary column was a Thermo Scientific TraceGOLD TG-5MS column (5% phenyl film) of 30 m length, 0.25 mm inner diameter and 0.25 µm film thickness (Table 1).

The pre-column used was a 1.2 m TG-5HT, 0.15 µm film thickness and 0.53 mm inner diameter (see Table 1).

TRACE GC Ultra	
Injection Volume	2 µL injection
Liner	Siltec® baffled liner (part number 453T2120)
Carrier Gas	He, constant flow, 1.3 mL/min
Column Type	TraceGOLD™ TG-5MS column (5% phenyl film) of 30 m length, 0.25 mm inner diameter and 0.25 µm film thickness (part number 26098-1420)
Precolumn	1.2 m of TraceGOLD TG-5HT column of 30 m length, 0.53 mm inner diameter and 0.15 µm film thickness (part number 26095-0620)
GC Method	Initial 65 °C, Hold 1.5 min, Ramp 30.0 °C/min–150 °C, Ramp 5.0 °C/min–290 °C, Ramp 30.0 °C/min–320 °C, Hold 5.0 min
Transfer Line	300 °C
TRACE GC Ultra PTV Program	
Injector Temperature	70 °C, splitless injection 1.5 min
PTV Inject	70 °C, 0.2 min, 8 °C/sec to transfer step
PTV Transfer	280 °C, 21 min, 10 °C/sec to clean step
PTV Clean	350 °C, 33 min, clean flow 30 mL/min
Transfer Time	21 min
TSQ Quantum XLS Ultra Mass Spectrometer	
Source Temperature	240 °C, CEI volume
Ionization	EI, 70 eV
Emission Current	50 µA
Resolution	0.7 Da Q1, Q3; 0.1 Da on Q1, 0.7 on Q3 for the wheat examples
Collision Gas	Argon, 1.5 mTorr

Table 1: Selected instrument conditions for the employed TRACE GC Ultra and TSQ Quantum XLS Ultra mass spectrometer

Maximizing Robustness

High boiling compounds in sample matrix have a negative effect on the analytical column's quality and lifetime, requiring a bake out process at high temperatures, thus limiting sample throughput. A backflush process was used to protect the column, allowing more samples to be injected before the phase attachment on the surface of the column becomes weak. Being able to inject more samples before necessary column replacement improves throughput and reduces costs per analyses.

During backflushing of the pre-column, the injector was set to a higher temperature and increased flow. This also allowed the injector liner to be swept of residual matrix contaminants during analysis time. This concurrent backflush operation results in the complete system staying clean and inert for a high number of injections, resulting in less maintenance frequencies.[3]

Method Setup

The method parameters for the PTV concurrent backflush operation, GC separation and TSQ Quantum XLS Ultra mass spectrometer setup are given in Table 1.

Each compound SRM transition was only monitored for a narrow time window around the established retention time (timed SRM). This led to a fully optimized instrument duty cycle for maximum analytical performance being handled automatically by the system. The complete list can be copied into the instrument method, thus saving time and avoiding entry errors.[4]

For data acquisition, the two most selective transitions were chosen after reviewing data from spiked matrix samples. Selection criteria were based on the absence of interferences from the matrix, along with signal generation of the transition.

Results and Discussion

Advanced GC-MS/MS Experiments – U-SRM

The patented Thermo Scientific HyperQuad technology in the TSQ Quantum XLS Ultra system offered high sensitivity by high ion transmission already found at the standard nominal mass resolution settings (0.7 Da FWHM). In addition, the HyperQuad™ technology allows the possibility to enhance the applied mass resolution for increased selectivity during analysis. The significantly increased selectivity further reduces the background caused by matrix components, thus giving a cleaner peak detection and high signal-to-noise results.

Some compound transitions are more susceptible to matrix interference than others. Standard SRM resolution (0.7 Da) can often provide enough selectivity to overcome most matrix interference challenges. In complex matrices, however, even with the structure-selective SRM acquisitions, removal of the isobaric matrix interference is insufficient.

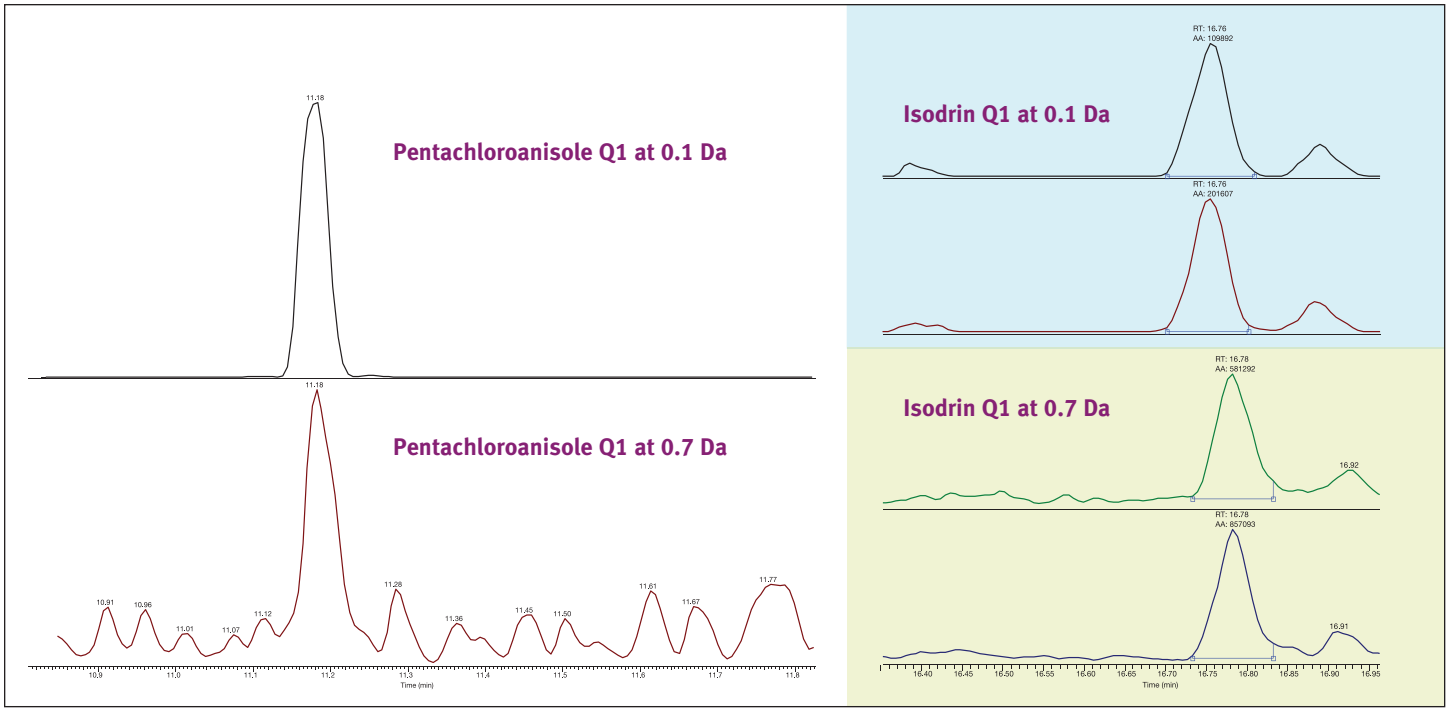


Figure 1: Comparison of U-SRM and standard SRM for pentachloroanisole and isodrin in wheat at 10 ppb levels;
Top: The chromatogram in U-SRM SRM (Q1 FWHM at 0.1 Da); Bottom: The same sample in standard mode (Q1 FWHM at 0.7 Da).

By increasing the mass resolution (down to 0.1 Da) of the first quadrupole during SRM acquisitions, a more selective isolation of the compound pre-cursor ion is achieved. This acquisition mode is known as Ultra-Selective Reaction Monitoring (U-SRM).

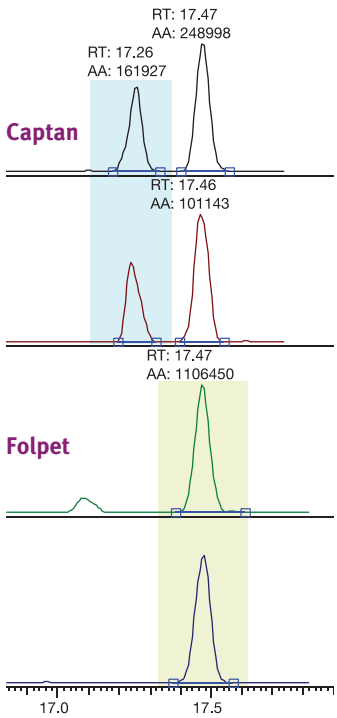
Figure 1 gives examples of U-SRM acquisition of pentachloroanisole and isodrin at 10 ppb in wheat matrix.

Analytical Performance

The complete method validation was performed using standard mass resolution settings at 0.7 Da.

A very comfortable detection of virtually all pesticides was achieved at the 1 ppb level. Excellent linearity was also observed with correlation values exceeding 0.995 for the linear calibration. In addition to this, the residual errors for each calibration point were less than 10% for all compounds (RSD). This included a calibration point at the 1 ppb level.

Also, more difficult compounds such as Captan and Folpet showed excellent peak signal and repeatability when using this method.



Injections (10 ppb blackcurrant)	Captan (area)	Folpet (area)
1	221347	247021
2	180365	229513
3	196336	273992
4	189745	277547
5	199317	273831
6	176386	270323
7	174745	296082
8	203117	231265
9	210897	248086
10	231017	234245
11	193543	264083
12	208722	292804
13	184633	246250
14	175942	285853
15	204764	295590
Average	196725.1	264432.3
Standard Deviation	16988.97	23534.93
RSD	**8.64%**	**8.90%**

Figure 2 and Table 2: Captan (RT 17.26) and Folpet (RT 17.47) in blackcurrant extract spiked at 10 ppb level, showing both transitions

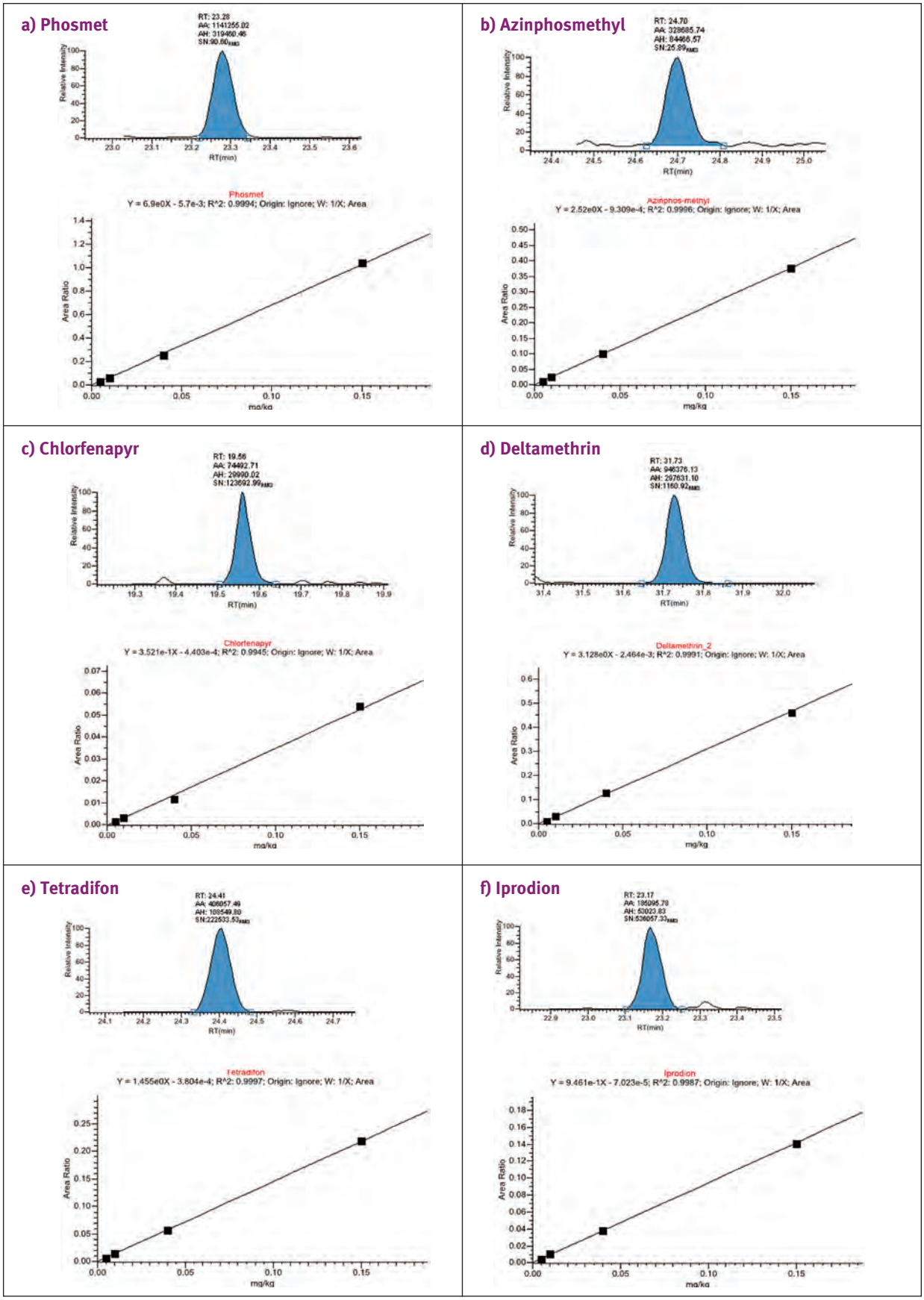


Figure 3: Calibration curves and peaks at 1 ppb level with 2 µL injection

As an additional test, the ion ratio at different levels has been monitored and the deviation of the transitions has been calculated.

Compound	Ion Ratio Deviation RSD in % (n=5)
Phosmet	0.79
Azinphosmethyl	3.65
Chlorfenapyr	15.08
Deltamethrin	0.88
Iprodion	5.34
Alfa Endosulfan	3.63
Methidathion	0.84
Carbaryl	3.64
Cyfluthrin	3.55
Pyrimifos	3.83

Table 3: Ion ratio deviation of some challenging pesticides in cucumber matrix at several levels of concentration

Figure 3 (a) through (f) show a 1 ppb matrix spike and calibration data obtained for select targeted pesticides in cucumber matrix.

Conclusions

- Advances in HyperQuad technology offers increased analytical performance for routine applications such as pesticide analysis.
- A true multi-compound method was developed for over 400 pesticides using timed SRM; easily transferable from a spreadsheet.
- A high level of accuracy and precision was reached during data evaluation, on several cornerstones of analysis, such as repeatability, linearity and ion ratio stability.
- Furthermore, all examples shown are the more challenging pesticides faced analytically in terms of stability, activity and response.
- This resolution technology development allows for advanced GC-MS/MS operations to be performed, such as U-SRM to further increase selectivity in complex matrices. This not only improves quantitative measurements, but it is also amenable when using a reduced sample clean-up which is typical for QuEChERS methodologies.

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4. Pesticides Method Reference, 2nd ed. 2011, Thermo Fisher Scientific, Austin, TX, USA, (part number 120390).

For reference on Thermo Scientific QuEChERS products, please see our catalog, Thermo Scientific HyperSep Dispersive SPE Products (part number BRGSCQUECHERS 1109).

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Method: 52213

Determination of Pesticides in Grapes, Baby Food and Wheat Flour by Automated Online Sample Preparation LC-MS/MS

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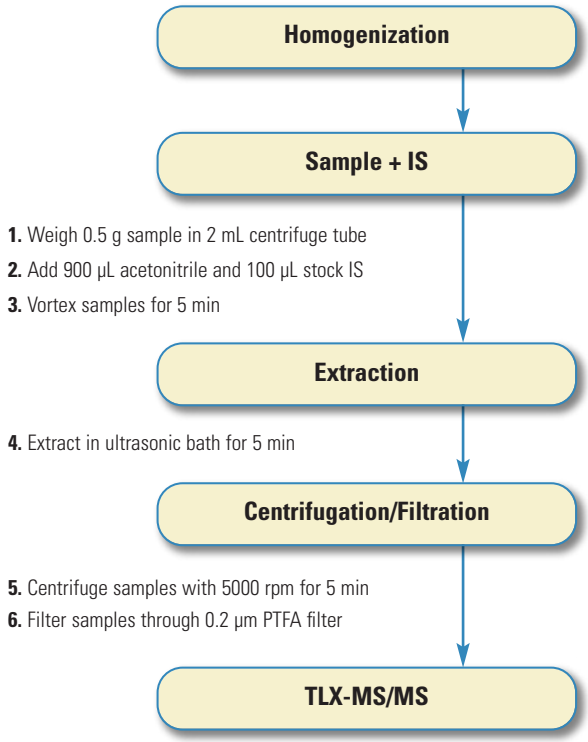
Key Words

- Transcend TLX
- TSQ Quantum Access MAX
- TurboFlow Technology
- Food Safety

1. Schematic of Method



2. Introduction

European Regulation 396/2005 sets maximum residue levels of pesticides in different products of plant and animal origin. These regulations present a significant analytical challenge with respect to the low limits of quantification which are required for some specified food matrices such as baby food. Many gas chromatography (GC) and high pressure liquid chromatography (HPLC) methods have been developed for multi-residue determination of pesticides and are in widespread use – employing a variety of sample preparation and cleanup techniques. In recent years the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method has become widely adopted for handling fruit and vegetables. However, QuEChERS requires many manual sample manipulation steps, making it labor-intensive when large numbers of samples have to be analyzed. It is therefore beneficial to consider options for automation of multi-residue methods, which can be cost-effective and can offer a high degree of reliability in recovery and repeatability. While the preliminary stages of homogenization and solvent extraction of food matrices inevitably require manual intervention, once a crude extract has been obtained, the procedure is fully automated thereafter. This automated procedure is included in the method, which utilizes turbulent flow chromatography with online liquid chromatography-mass spectrometry (LC-MS/MS).

Thermo Scientific Transcend TLX system coupled with the TSQ Quantum Access Max triple quadrupole mass spectrometer

3. Scope

This multi-residue pesticide method can be applied to fruits, cereals and composite baby foods at limits of detection (LODs) in the range of 0.8–10.3 µg/kg which are below respective EU maximum residue limits (MRLs). The method has been validated for 48 pesticides from different classes, but can be readily extended to a larger number of residues.

4. Principle

This method describes a novel sample preparation technique as a possible alternative to the QuEChERS method for high throughput pesticide analysis. Sample concentration, cleanup and analytical separation are carried out in a single run using an online coupled turbulent flow chromatography – reversed phase chromatography system (Thermo Scientific Transcend TLX system powered by Thermo Scientific TurboFlow technology). TurboFlow™ technology enables very effective separation of matrix and target compounds – resulting in relatively clean sample extracts. Macromolecules are removed from the sample extract with high efficiency, while target analytes are retained on the column based on different chemical interactions. After application of a wash step, the trapped compounds are transferred onto the analytical LC column and separated conventionally. The complete method involves internal standardization, solvent extraction of the homogenized food sample, centrifugation and injection into an automated cleanup system. Cleanup using TurboFlow technology has been optimized for maximum recovery of pesticide residues and minimal injection of co-extractives into the MS/MS. Identification of residues is based on ion-ratios using multiple reaction monitoring (MRM) of characteristic transition ions, and quantification using matrix-matched standards of one of the selected MRM ions.

5. Reagent List

		Part Number
5.1	Acetone, HPLC grade	A/0606/17
5.2	Acetonitrile, LC/MS grade	A/0638/17
5.3	Ammonium formate, for HPLC	A/5080/53
5.4	Methanol, Optima LC/MS grade	A456-212
5.5	Formic acid, extra pure for HPLC	F/1850/PB08
5.6	Isopropanol, HPLC grade	P/7507/17
5.7	Water, LC/MS grade	W/0112/17

6. Standard List

6.1 Pesticides: all standards from Sigma-Aldrich

Abamectin, ametryn, azinphos-me, azoxystrobin, bifenazate, carbaryl, carbendazim, carfentrazone-ethyl, chlormequate, clofentezin, cymoxanil, cypermethrin, dazomet, diazinon, dimethoate, dimethomorph A, dimethomorph B, ediphenfos, fenazaquin, fluazifop P, fluzilazol, hexithaizox, imazalil, imidacloprid, isoproturon, isoxaben, lactofen, malathion, metalaxyl, methomyl, metribuzin, myclobutanyl, omethoate, oxadyxil, oxamyl, pethoxamid, profenofos, promecarb, propoxur, pymetrozin, pyperonil-butoxide, pyrimethanyl, quinoxifen, spirodiclofen, tebuconazol, thiacloprid, triadimefon, trifloxistrobin.

6.2 Internal Standards

d_4-imidacloprid-, d_6-isoproturon, d_6-primicarb, d_{10}-parathion-ethyl (Sigma)

6.3 Quality Control Materials

FAPAS #963 (pasta matrix), FAPAS #966 (maize flour matrix), FAPAS #19110 (lettuce puree matrix)
(Note: FAPAS samples were selected primarily on content of target pesticides, however, matrices are different from the validated matrices with the exception of flour.)

7. Standards and Reagent Preparation

7.1 Concentration of mixed **pesticide working stock solution** (2 µg/mL and 1 µg/mL) in methanol. Prepare 2 µg/mL working stock standard solution by 10× dilution of intermediate stock standard solution in a 10 mL volumetric flask with methanol. Prepare 1 µg/mL working stock standard mix, by diluting intermediate stock standard solution by 20× in a 10 mL volumetric flask.

7.2 To prepare **individual stock standard solutions**, weigh 10 mg from each analyte into a 20 mL amber screw cap vial on the five digit analytical balance. Add 10 mL methanol from a calibrated pipette and note the weight of both analyte and solvent. If undissolved crystals are seen, put the vial in an ultrasonic bath until complete dissolution.

7.3 To prepare **intermediate stock standard solution**, pipette 200 µL from each individual stock standard into a 10 mL volumetric flask and fill up to the mark with methanol.

7.4 Concentration of **stock internal standard** (for sample spiking for internal standardization) is 100–100 ng/mL for d_4-imidacloprid and d_6-isoproturon, 10000 ng/mL for d_6-primicarb and 700 µg/mL d_{10}-parathion-ethyl in methanol. Prepare stock internal standard mixture by pipetting 7 mL of d_{10}-parathion-ethyl individual stock solution and 1 mL of intermediate stock internal standard mixture into a 10 mL volumetric flask and fill up to the mark with methanol.

7.5 To prepare **individual stock internal standard** solutions, weigh 10 mg of each analyte into a 20 mL amber screw cap vial on the five digit analytical balance. Add 10 mL methanol from a calibrated pipette and note the weight of both analyte and solvent.

7.6 To prepare **intermediate stock internal standard** mixture, pipette 1000 µL d_6-primicarb individual solution and 100–100 µL d_4-imidacloprid and d_6-isoproturon individual solutions into a 10 mL volumetric flask and fill to the mark with methanol.

8. Apparatus

		Part Number
8.1	Fisher precision balance	XP-1500FR
8.2	Sartorius analytical balance	ME235S
8.3	Thermo Scientific Barnstead EASYpure II water	3125753
8.4	Ultrasonic bath Elmasonic S40H	1002006
8.5	ULTRA-TURRAX® – G25 dispergation tool	1713300
8.6	ULTRA-TURRAX	3565000
8.7	Vortex shaker	3205025
8.8	Vortex universal cap	3205029
8.9	Accu-Jet pipettor	3140246
8.10	Thermo Scientific Heraeus Fresco 17 micro centrifuge	3208590
8.11	Transcend™ TLX-1 system	
8.12	Thermo Scientific TSQ Quantum Access MAX triple stage quadrupole mass spectrometer	

9. Consumables

		Part Number
9.1	LC vials	24014019
9.2	Pipette Finnpipette 100–1000 µL	3214535
9.3	Pipette Finnpipette 10–100 µL	3166472
9.4	Pipette Finnpipette 500–5000 µL	3166473
9.5	Pipette holder	3651211
9.6	Pipette tips 0.5–250 µL, 500/box	3270399
9.7	Pipette tips 1–5 mL, 75/box	3270420
9.8	Pipette tips 100–1000 µL, 200/box	3270410
9.9	Spatula, 18/10 steel	3458179
9.10	Spatula, nylon	3047217
9.11	Tube holder	3204844
9.12	Wash bottle, PTFE	3149330
9.13	2 mL vial rack	12211001
9.14	0.2 µm PTFA syringe filter	F2513-4
9.15	1 mL disposable plastic syringe	S7510-1
9.16	1.7 mL centrifuge plastic tube	3150968
9.17	TurboFlow Cyclone MCX-2 (50 × 0.5 mm) column	CH-953457
9.18	Thermo Scientific Hypersil GOLD 150 × 4.6 mm, 5 µm column	25005-154630
9.19	UNIGUARD holder	850-00
9.20	Hypersil GOLD™ 10 × 4 mm, 5 µm guard column	25005-014001

10. Glassware

		Part Number
10.1	Volumetric flask, 10 mL	FB50143
10.2	Volumetric flask, 25 mL	FB50147
10.3	1 mL glass pipette	FB50211
10.4	1 L bottle	9653650
10.5	500 mL bottle	9653640

11. Procedure

11.1 Sample Preparation

Solid Samples

Extract solid samples prior to injection into the Transcend system coupled to the TSQ Quantum Access MAX™ mass spectrometer. If samples are table grapes, these are treated as semisolid samples and need to be homogenized prior to extraction. Baby food and flour samples are treated as fine and homogenous solid matrices, so intensive manual mixing with a spatula is satisfactory.

11.2 Homogenization of Semisolid Samples

11.2.1 Select approximately 10–15 individual grapes randomly from the bunch and put into an appropriate size (depending on grape type and size ~100 mL) beaker and label it.

11.2.2 Attach the G25 dispergation tool to the ULTRA-TURRAX homogenizer

11.2.3 Start homogenization at middle rotation speed (speed level 2-3) and continue it to form a smooth puree

11.3 Extraction

11.3.1 Weigh 0.5 g sample into a 1.7 ml centrifuge tube

11.3.2 Add 900 µL acetonitrile stock IS

11.3.3 Vortex the sample for 5 min (to wet all the solid samples throughout)

11.3.4 Put the well-mixed samples into the Ultrasonic bath for 5 min.

11.3.5 Centrifuge in the micro centrifuge at 5000 rpm for 5 min.

11.3.6 Remove supernatant and filter it through 0.2 µm PTFE syringe filter directly into the LC vial

12. Analysis

Sample concentration, cleanup and analytical separation are carried out in a single run using an automated online sample preparation system, which includes the Transcend system and Thermo Scientific Aria operating software. TurboFlow technology with the Transcend system enables very effective separation of matrix and target compounds due to its special size exclusion and reversed phase chemistry. Macromolecules are removed from the sample extract with high efficiency, while target analytes are retained on the column based on different chemical interactions. After application of a wash step, the trapped compounds are transferred onto the analytical LC column and separated conventionally. Consequently the method was optimized for both TurboFlow technology and analytical chromatography.

		TurboFlow Technology								LC					
Step	**Duration [s]**	**Flow**	**Grad**	**A%**	**B%**	**C%**	**D%**	**Tee**	**Loop**	**Flow**	**Grad**	**A%**	**B%**	**C%**	**D%**
1	60	1.50	step		100			–	out	0.50	step		100		
2	60	1.50	step		95		5	–	out	0.50	step		100		
3	80	0.16	step		100			Tee	in	1.44	step		100		
4	60	1.00	step			100		–	in	1.60	ramp		55		45
5	60	1.00	step	10			90	–	in	1.60	ramp		40		60
6	220	0.20	step		100			–	out	1.60	ramp				100
7	60	0.20	step		100			–	out	1.60	step				100
8	180	0.20	step		100			–	out	1.00	step		100		
		Mobile phases for the TurboFlow method: A: water pH=3 B: water C: 40% acetonitrile 40% isopropanol and 20% acetone D: 5 mM ammonium-formiate in methanol + 0.1% formic acid								Solvent channels for LC: A: acetonitrile B: 5 mM ammonium-formiate in water + 0.1% formic acid C: water D: 5 mM ammonium-formiate in methanol + 0.1% formic acid *Note: LC channel C can be used for column wash purposes*					

Table 1: Gradient program table for Aria™ control software

12.1 LC Conditions for Transcend TLX System

Operation was carried out in focus mode setup (Figure 1) with 1:1 splitting before the TSQ Quantum Access MAX mass spectrometer entrance using a divert valve connection. The TurboFlow Cyclone MCX-2 column was installed as the TurboFlow column (9.17). The Hypersil GOLD column equipped with a guard column was used as the analytical LC column (9.18–9.20). Installed loop volume was 200 µL.

Sample load (Step 1) was applied with 1.5 mL/min flow rate, whereby matrix components were eluted in the waste, and target pesticides were trapped on the TurboFlow column. After washing the TurboFlow column with 5% organic/aqueous mixture (Step 2), the trapped pesticides were eluted and transferred (Step 3) after 2 min from the TurboFlow column to the analytical LC column. Simultaneous dilution of the eluate occurs enabling pre-concentration of pesticides at the beginning of the analytical column. The analytical LC column was equilibrated and conditioned during loading and washing steps. After transfer of the pesticides, the analytical separation started with gradient elution (Steps 4–7), while the TurboFlow column was washed and conditioned, and the loop was filled with the eluent. After the gradient run, the Hypersil GOLD column was washed in acetonitrile and conditioned for the next run. The total run time of the method with automated online sample preparation and analytical separation was 13 min. Table 1 gives details of the method program. In order to minimize sample carry-over and cross-contamination, the injection needle as well as the injection valve was washed 4 times with both cleaning solvents.

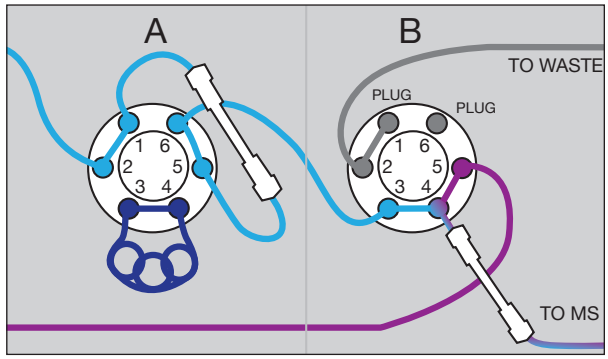


Figure 1: Focus mode system set up and method setting in Aria control software on the Transcend TLX system

12.1.1 Injector settings

Injector: Transcend TLX autosampler with 100 µL injection syringe volume

Sample holder temperature: 10 °C

Cleaning solvents: Solvent channel 1–80%MeOH/acetone
Solvent channel 2–50%MeOH/H_2O

Injector settings:

- Pre Clean with solvent 1 [steps]: 2
- Pre Clean with solvent 2 [steps]: 2
- Pre Clean with sample [steps]: 1
- Filling speed [µL/s]: 50
- Filling strokes [steps]: 2
- Injection port: LC Vlv1 (TurboFlow method channel)
- Pre inject delay [ms]: 500
- Post inject delay [ms]: 500
- Post clean with solvent 1 [steps]: 4
- Post clean with solvent 2 [steps]: 4
- Valve clean with solvent 1 [steps]: 4
- Valve clean with solvent 2 [steps]: 4
- Injection volume: 10 µL

12.1.2 Mass Spec conditions

Mass spectrometric detection was carried out by TSQ Quantum Access MAX triple stage quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) in selected reaction monitoring (SRM) mode. All SRM traces were individually tuned for each target pesticide. MS programming was set in Thermo Scientific Xcalibur software in Eazy set up mode.

Settings were:

- Scan type: SRM (details in table below)
- Cycle time [s]: 0.3
- Peak width: 0.7 Da FWHM
- Collision gas pressure [mTorr]: 1.0
- Capillary Temperature [°C]: 290
- Vaporizer Temperature [°C]: 290
- Sheath gas pressure [arb]: 40
- Aux gas pressure [arb]: 10
- Ion sweep pressure [arb]: 0
- Spray voltage [V]: 3200
- Polarity: positive for all compounds
- Trigger: 1.00e5

12.2 Calculation of Results

Calibration by the internal standardization is applied for the determination of pesticides. This quantification method requires determination of response factors R_f defined by the equation below. Calculation of final results is performed using the following equations.

Calculation of the response factor:

$$R_f = \frac{A_{St} \times c_{[IS]}}{A_{[IS]} \times c_{St}}$$

$\mathbf{R_f}$ – the response factor

$\mathbf{A_{St}}$ – the area of the pesticide peak in the calibration standard

$\mathbf{A_{[IS]}}$ – the area of the internal standard peak of the calibration standard

$\mathbf{c_{St}}$ – pesticide concentration of the calibration standard solution

$\mathbf{c_{[IS]}}$ – the internal standard concentration of the calibration standard solution

Calculations for each sample of the absolute amount of pesticide that was extracted from the sample:

$$X_{analyte} = \frac{A_{analyte} \times X_{[IS]}}{A_{[IS]} \times R_f}$$

$\mathbf{X_{analyte}}$ – the absolute amount of pesticide that was extracted from the sample

$\mathbf{A_{analyte}}$ – the area of pesticide peak in the sample

$\mathbf{A_{[IS]}}$ – the area of the internal standard peak in the sample

$\mathbf{X_{[IS]}}$ – the absolute amount of internal standard added to the sample

The concentration of pesticide in the sample [ng/g]:

$$c = \frac{X_{analyte}}{m}$$

m – the weight of sample [g]

$\mathbf{X_{analyte}}$ – absolute analyte amount [ng]

13. Method Performance Characteristics

In-house validation of the method was carried out on all matrices and target pesticides. International Union of Pure and Applied Chemistry (IUPAC)/Association of Official Analytical Chemists (AOAC) guideline for single laboratory validation[1,2] was used as a template and it was also demonstrated that method performance characteristics fulfilled the legislative criteria set for pesticide residue methods.[3]

13.1 Selectivity

Method (SRM) selectivity was confirmed based on the presence of specific ion transitions at the corresponding retention time (Table 2), as well as the observed ion ratio values corresponding to those of the standards. Acceptance criteria for retention time and ion ratios were set according to Reference 1.

13.2 Linearity, Response Factor

The linearity of calibration curves was assessed over the range from 10–500 ng/g. In all cases, the correlation coefficients of linear functions were better than 0.985. The calibration curves were created at five levels (matrix-matched) and injected in duplicate. R_f values for internal standardization were determined from the calibration curves for each matrix, and internal standards by calculating cumulative average response factors over the whole calibration range.

13.3 Accuracy

Method accuracy and precision was assessed by recovery studies using blank matrices spiked at three concentration levels injected in six individually prepared replicates. Samples were spiked at 10, 100 and 250 ng/g concentration levels. Found concentrations, recovery and relative standard deviation (%RSD) were calculated (Table 3). Recovery values are deemed acceptable if between 70–125%. Additional accuracy was established for selected target analytes by analysing FAPAS #963, 966 and 19110 proficiency test materials. All measured concentrations of the relevant compounds (diazinon, tebuconazole, trifloxistrobin, malathion, azoxystrobin and dimethomorph) were within the acceptable satisfactory ranges.

13.4 Precision

Method within-day and between-day precision values were determined for each matrix at middle spiking level (100 ng/g) each in 6 replicates and expressed as %RSD over 3 days with individually prepared samples. Mean within-day precision values were determined as average of the 3 individual days' mean precision, while between-day precision was expressed as mean of the overall precision data. Measured values are shown in Table 4.

13.5 Limits of Detection (LODs) and Quantification (LOQs)

LODs and LOQs were estimated following the IUPAC approach which consisted of analyzing the blank sample to establish noise levels and then testing experimentally estimated LODs and LOQs for signal/noise, 3 and 10 respectively. The method LOD values are listed in Table 5. The expectation of the method was to meet MRL values at least at LOD level. The lowest MRL values were defined for baby food matrices (10 ng/g), which were achieved in all cases.

13.6 Robustness

A robustness study was performed by varying parameters like extraction time, centrifugation speed, time by 20%, shaker (horizontal shaker, vortex) and extraction mode (ultrasonic bath, vortex shaking). Results were compared to the original method and significant differences were sought based on ANOVA analysis. None of the parameters which were varied led to significant differences in measured values, consequently indicating that the method was robust.

14. Conclusion

The method described here enables convenient, fast and cost-effective automated determination of selected pesticides, from polar to non-polar compound chemistry, in different matrix types. Based on the short total run time and Transcend system with TurboFlow technology, 100 samples per day can be analyzed under controlled sample preparation conditions. Method performance characteristics were established by in-house validation for baby food, grapes and wheat flour matrices. The method performance indicates it is suitable for routine use for regulatory purposes and can be readily extended to a larger and wider range of pesticide residues.

15. References

1. http://www.aoac.org/Official_Methods/slv_guidelines.pdf
2. http://www.scribd.com/doc/4922271/Harmonized-Guidelines-for-Single-Laboratory-Validation-of-Methods-Of
3. http://ec.europa.eu/sanco_pesticides/public/index.cfm

16. Annex

16.1 Tables and Chromatograms

Analyte	Precursor Ion	Product Ion (CE)	Product Ion2 (CE)	Retention Time [min]
Abamectin	890.2	305.1 (25)	567.4 (12)	10.1
Ametryn	228.1	96.1 (25)	116.1 (26)	7.76
Azinphos methyl	339.8	132.1 (19)	160.2 (12)	7.87
Azoxystrobin	404.1	344.1 (25)	372.1 (14)	7.99
Bifenazate	301.1	198.1 (7)	170.1 (19)	8.36
Carbaryl	219.1	202.1 (5)	127.1 (32)	7.31
Carbendazim	191.8	160.1 (18)	132.1 (29)	5.96
Carfentrazone-ethyl	429.1	412.2 (12)	384.2 (18)	8.71
Chlormequate	122.1	58.5 (31)	63.3 (21)	4.06
Clofentezin	304.7	138.1 (26)	102.1 (38)	9.07
Cymoxanil	199.3	83.9 (20)	111.1 (20)	6.71
Cypermethrin	433.1	416.3 (5)	191.2 (15)	8.72
Dazomet	163.1	120.1 (11)	90.2 (9)	5.83
Diazinon	304.9	169.1 (21)	153.1 (21)	8.90
Dimethoate	230.2	125.3 (21)	170.7 (13)	6.43
Dimethomorph A&B	388.1	300.9 (21)	164.9 (31)	8.12/8.34
Ediphenfos	310.8	283.1 (11)	111.2 (19)	8.80
Fenazaquin	307.2	161.2 (16)	57.2 (21)	10.18
Fluazifop P	384.3	282.1 (18)	254.2 (27)	9.29
Fluzilazol	316.1	165.1 (27)	247.1 (18)	8.66
Hexithaizox	353.1	228.1 (14)	167.8 (24)	9.66
Imazalil	296.9	159.1 (23)	176.2 (20)	7.50
Imidacloprid	256.1	209.2 (15)	175.2 (17)	6.16
d4-Imidacloprid	259.9	213.1 (17)	179.1 (20)	6.24
Isoproturon	207.1	72.1 (18)	165.3 (14)	7.73
d6-Isoproturon	213.2	78.3 (19)	171.1 (14)	7.71
Isoxaben	333.1	165.1 (20)	149.9 (38)	8.15
Lactofen	479.1	462.1 (5)	344.2 (15)	9.35
Malathion	347.9	330.7 (5)	99.4 (29)	8.22
Metalaxyl	279.9	220.2 (13)	192.1 (18)	7.63
Methomyl	163.1	106.1 (10)	88.1 (8)	5.95
Metribuzin	215.2	187.1 (16)	74.1 (34)	7.21
Myclobutanyl	289.1	70.3 (18)	124.9 (30)	8.38
Omethoate	214.2	125.1 (22)	155.2 (15)	5.58
Oxadyxil	296.2	279.2 (5)	219.3 (15)	6.80
Oxamyl	236.9	72.2 (14)	90.3 (5)	5.75
d10-Parathion-ethyl	302.1	238.1 (17)	270.1 (11)	8.83
Pethoxamid	296.1	131.1 (20)	250.2 (12)	8.48
d6-Primicarb	245.2	185.1 (16)	78.3 (28)	6.86
Profenofos	374.8	304.9 (17)	222.8 (31)	9.37
Promecarb	225.2	207.9 (7)	151.2 (6)	8.29
Propoxur	210.1	111.1 (14)	168.2 (7)	7.12
Pymetrozin	218.0	105.2 (23)	78.3 (37)	5.53
Pyperonil-butoxide	356.0	177.1 (13)	147.1 (29)	9.49
Pyrimethanyl	200.1	181.2 (35)	168.1 (28)	8.00
Quinoxifen	307.9	196.8 (31)	214.1 (33)	9.68
Spirodiclofen	410.9	313.1 (9)	71.1 (12)	9.83
Tebuconazol	308.2	70.2 (22)	124.9 (33)	8.88
Thiacloprid	253.1	126.1 (19)	90.1 (33)	6.55
Triadimefon	294.1	197.1 (15)	69.4 (20)	8.32
Trifloxistrobin	409.5	186.3 (17)	206.4 (13)	9.24

Table 2: Ion transitions for SRM setting

	Grape [Rec %] (%RSD)			Baby Food [Rec %] (%RSD)			Wheat Flour [Rec %] (%RSD)		
Analyte	**10 ng/g**	**100 ng/g**	**250 ng/g**	**10 ng/g**	**100 ng/g**	**250 ng/g**	**10 ng/g**	**100 ng/g**	**250 ng/g**
Abamectin	66 (17)	64 (18)	71 (11)	68 (19)	76 (5)	76 (4)	89 (17)	99 (5)	101 (7)
Ametryn	111 (16)	99 (18)	118 (9)	111 (8)	115 (5)	125 (5)	108 (16)	111 (4)	109 (7)
Azinphos-me	111 (9)	121 (19)	110 (11)	105 (5)	100 (4)	112 (5)	85 (13)	92 (6)	124 (4)
Azoxystrobin	105 (15)	69 (8)	104 (9)	86 (4)	90 (5)	88 (2)	87 (5)	118 (3)	117 (2)
Bifenazate	90 (14)	88 (5)	96 (9)	101 (5)	106 (5)	113 (4)	121 (5)	112 (4)	108 (3)
Carbaryl	69 (8)	86 (8)	90 (8)	98 (5)	111 (6)	120 (4)	110 (4)	110 (3)	107 (3)
Carbendazim	93 (14)	108 (5)	104 (8)	122 (7)	89 (5)	97 (3)	73 (14)	123 (6)	116 (3)
Carfentrazone-ethyl	85 (14)	74 (11)	84 (11)	92 (6)	102 (5)	104 (3)	112 (7)	119 (4)	114 (2)
Chlormequate	LOD	90 (12)	77 (17)	74 (16)*	90 (10)	89 (10)	LOD	106 (7)	100 (7)
Clofentezin	78 (18)*	71 (9)	84 (6)	71 (18)	73 (12)	82 (10)	123 (10)*	110 (7)	94 (13)
Cymoxanil	110 (13)	93 (14)	114 (13)	96 (19)	80 (17)	78 (7)	89 (19)	101 (15)	83 (12)
Cypermethrin	121(13)*	84 (17)	74 (11)	122 (12)	79 (12)	87 (9)	123 (13)*	115 (9)	114 (11)
Dazomet	106 (19)	107 (18)	117 (9)	80 (17)	114 (5)	118 (5)	84 (7)	102 (5)	99 (5)
Diazinon	80 (15)	75 (5)	87 (10)	87 (9)	99 (6)	103 (4)	122 (3)	108 (2)	105 (3)
Dimethoate	90 (4)	88 (10)	95 (4)	106 (3)	114 (4)	117 (3)	73 (7)	118 (4)	112 (4)
Dimethomorph A	70 (15)	84 (8)	74 (8)	81 (5)	85 (4)	86 (4)	112 (4)	98 (3)	98 (2)
Dimethomorph B	89 (11)	71 (4)	77 (4)	86 (4)	91 (4)	89 (4)	110 (8)	114 (5)	118 (4)
Ediphenfos	94 (14)	72 (7)	90 (8)	109 (6)	110 (5)	114 (4)	105 (11)	111 (8)	110 (6)
Fenazaquin	101 (6)	88 (12)	78 (4)	78 (4)	83 (7)	85 (8)	104 (10)	81 (12)	73 (16)
Fluazifop P	101 (17)	72 (16)	86 (13)	101 (8)	100 (7)	103 (6)	116 (5)	107 (4)	106 (4)
Fluzilazol	87 (12)	69 (9)	89 (9)	91 (9)	102 (6)	107 (5)	122 (5)	110 (3)	106 (5)
Hexithaizox	75 (17)	82 (15)	93 (15)	93 (15)	119 (8)	120 (12)	102 (5)*	94 (11)	91 (14)
Imazalil	79 (8)	82 (11)	85 (8)	88 (5)	95 (8)	102 (6)	85 (19)	81 (5)	77 (12)
Imidacloprid	86 (8)	93 (6)	97 (5)	111 (4)	117 (3)	124 (2)	107 (3)	112 (3)	110 (3)
Isoproturon	95 (8)	74 (10)	86 (7)	104 (5)	109 (4)	101 (4)	123 (18)	109 (4)	114 (3)
Isoxaben	84 (14)	74 (5)	87 (7)	95 (4)	103 (4)	103 (3)	115 (5)	121 (3)	114 (2)
Lactofen	91 (17)	70 (15)	81 (12)	104 (7)	108 (5)	116 (9)	131 (7)	111 (6)	109 (7)
Malathion	117 (9)	83 (13)	75 (10)	103 (6)	91 (4)	88 (5)	104 (9)	94 (5)	112 (4)
Metalaxyl	79 (9)	76 (9)	80 (5)	88 (5)	98 (5)	97 (5)	74 (8)	123 (4)	115 (3)
Methomyl	75 (9)	68 (8)	81 (10)	73 (12)	81 (4)	87 (5)	99 (10)	96 (10)	89 (10)
Metribuzin	89 (11)	73 (6)	87 (4)	106 (10)	112 (5)	113 (7)	103 (13)	112 (4)	107 (3)
Myclobutanyl	90 (17)	75 (11)	90 (10)	102 (8)	104 (5)	110 (4)	105 (3)	119 (4)	117 (3)
Omethoate	70 (20)*	72 (8)	76 (9)	76 (18)	78 (7)	81 (11)	71 (16)*	75 (14)	70 (6)
Oxadyxil	71 (9)	72 (7)	87 (5)	84 (4)	101 (4)	100 (4)	87 (6)	123 (4)	117 (2)
Oxamyl	69 (9)	71 (9)	69 (7)	74 (8)	78 (5)	79 (6)	96 (11)	95 (10)	88 (7)
Pethoxamid	74 (10)*	70 (6)	77 (8)	89 (5)	88 (8)	91 (6)	123 (3)	115 (3)	108 (2)
Profenofos	112 (17)	72 (12)	95 (11)	109 (6)	115 (4)	120 (4)	115 (8)	106 (3)	105 (2)
Promecarb	90 (10)	86 (5)	94 (5)	104 (6)	114 (3)	115 (4)	128 (4)	122 (3)	112 (2)
Propoxur	84 (6)	87 (6)	84 (7)	98 (6)	106 (4)	108 (4)	91 (6)	115 (4)	110 (4)
Pymetrozin	101 (8)	94 (4)	121 (14)	101 (4)	112 (5)	113 (3)	89 (3)	117 (3)	110 (2)
Pyperonil-butoxide	78 (17)	93 (9)	86 (9)	95 (4)	102 (4)	109 (4)	115 (10)	113 (6)	111 (3)
Pyrimethanyl	120 (13)	121 (7)	108 (13)	80 (14)	114 (5)	101 (4)	94 (10)	106 (5)	110 (6)
Quinoxifen	90 (19)	78 (20)	104 (6)	87 (10)	99 (8)	105 (7)	98 (12)	90 (7)	86 (9)
Spirodiclofen	83 (11)	79 (17)	78 (17)	89 (16)	102 (6)	103 (7)	83 (4)	98 (5)	96 (5)
Tebuconazol	83 (15)	79 (8)	83 (6)	94 (4)	93 (6)	98 (4)	121 (7)	115 (4)	117 (3)
Thiacloprid	95 (8)	80 (10)	89 (8)	109 (5)	113 (5)	109 (3)	69 (8)	124 (6)	116 (4)
Triadimefon	69 (12)	68 (8)	83 (5)	96 (8)	104 (6)	109 (4)	118 (8)	115 (3)	114 (3)
Trifloxistrobin	82 (5)	76 (8)	81 (11)	99 (6)	97 (6)	104 (4)	109 (4)	98 (5)	92 (4)

Table 3: Average method recovery [%] and %RSD [%] values at 3 different spike levels in the investigated matrices (n=6)

*LOD: spike level at or below LOD, * spike level at or below LOQ*

		Grape		Baby Food		Wheat Flour	
Analyte	**Spike Level [ng/g]**	**Mean within day precision [%RSD]**	**Between day precision [%RSD]**	**Mean within day precision [%RSD]**	**Between day precision [%RSD]**	**Mean within day precision [%RSD]**	**Between day precision [%RSD]**
Abamectin	100	11	14	6	11	10	11
Ametryn	100	11	19	9	12	8	16
Azinphos-me	100	12	15	5	6	9	11
Azoxystrobin	100	14	22	7	10	6	6
Bifenazate	100	10	17	7	9	6	9
Carbaryl	100	16	19	7	16	8	17
Carbendazim	100	8	11	7	12	7	9
Carfentrazone-ethyl	100	9	17	10	15	8	10
Chlormequate	100	12	15	10	15	8	10
Clofentezin	100	14	21	11	15	9	11
Cymoxanil	100	16	19	14	21	11	15
Cypermethrin	100	12	16	12	16	10	12
Dazomet	100	15	20	13	20	15	21
Diazinon	100	9	17	6	16	8	12
Dimethoate	100	12	17	9	17	10	13
Dimethomorph A	100	11	17	7	16	8	10
Dimethomorph B	100	6	10	7	11	7	14
Ediphenfos	100	10	11	7	7	6	6
Fenazaquin	100	12	21	9	13	13	13
Fluazifop P	100	9	14	8	8	11	10
Fluzilazol	100	9	19	6	10	5	8
Hexithaizox	100	8	19	9	18	15	19
Imazalil	100	10	18	10	15	10	17
Imidacloprid	100	7	8	5	6	14	16
Isoproturon	100	15	21	6	12	7	12
Isoxaben	100	12	17	7	9	7	7
Lactofen	100	12	17	7	20	12	15
Malathion	100	7	19	8	17	5	17
Metalaxyl	100	12	19	6	11	8	8
Methomyl	100	12	18	7	14	10	20
Metribuzin	100	8	16	7	8	8	9
Myclobutanyl	100	10	14	8	10	8	14
Omethoate	100	18	19	14	16	13	14
Oxadyxil	100	12	18	4	10	6	13
Oxamyl	100	10	19	7	15	9	15
Pethoxamid	100	8	19	8	16	5	10
Profenofos	100	8	19	5	19	11	11
Promecarb	100	10	20	4	5	12	14
Propoxur	100	7	19	6	8	8	9
Pymetrozin	100	11	16	6	10	9	10
Pyperonil-butoxide	100	6	19	6	15	6	15
Pyrimethanyl	100	14	20	6	8	9	11
Quinoxifen	100	9	18	9	10	10	13
Spirodiclofen	100	9	18	8	18	10	13
Tebuconazol	100	8	13	9	10	6	6
Thiacloprid	100	16	17	9	13	9	13
Triadimefon	100	9	19	8	11	7	8
Trifloxistrobin	100	13	18	8	11	10	13

Table 4: Method (intermediate) precision values for all matrices

	Baby Food		Grape		Wheat Flour	
Compound	**LOD [ng/g]**	**LOQ [ng/g]**	**LOD [ng/g]**	**LOQ [ng/g]**	**LOD [ng/g]**	**LOQ [ng/g]**
Abamectin	2.4	7.2	2.0	6.0	3.1	9.3
Ametryn	2.5	7.5	2.5	7.5	1.4	4.2
Azinphos-Me	1.1	3.3	1.1	3.3	1.2	3.6
Azoxystrobin	0.9	2.7	0.9	2.7	0.9	2.7
Bifenazate	2.8	8.4	2.7	8.1	2.9	8.7
Carbaryl	1.5	4.5	1.6	4.8	1.2	3.6
Carbendazim	1.3	3.9	1.4	4.2	2.6	7.8
Carfentrazone-ethyl	1.5	4.5	1.5	4.5	2.1	6.4
Chlormequate	6.0	18.0	10.3	31.0	9.2	27.7
Clofentezin	3.2	9.6	4.1	12.3	4.5	13.5
Cymoxanil	3.3	9.9	3.1	9.3	3.2	9.6
Cypermethrin	3.0	9.0	5.0	15.0	4.5	13.5
Dazomet	1.4	4.3	1.3	4.0	1.2	3.6
Diazinon	1.1	3.3	1.0	3.0	1.3	3.9
Dimethoate	1.2	3.6	1.2	3.6	1.2	3.6
Dimethomorph	1.0	3.0	1.0	3.0	2.0	6.0
Edifenphos	1.2	3.6	1.1	3.3	1.2	3.6
Fenazaquin	2.0	6.0	2.5	7.5	2.2	6.6
Fluazifop P	1.0	3.0	1.2	3.6	1.8	5.4
Fluzilazol	1.0	3.0	1.0	3.0	1.5	4.5
Hexithiazox	3.0	9.1	3.4	10.2	4.0	12.0
Imazalil	1.2	3.6	1.4	4.2	1.5	4.5
Imidacloprid	1.1	3.3	1.2	3.6	1.2	3.6
Isoproturon	1.7	5.1	1.8	5.4	1.3	4.0
Isoxaben	1.0	3.0	1.0	3.0	1.1	3.3
Lactofen	1.4	4.2	1.9	5.7	2.5	7.5
Malathion	3.0	9.0	1.8	5.4	1.6	4.8
Metalaxyl	0.9	2.7	0.9	2.7	2.1	6.3
Methamyl	1.6	4.8	1.4	4.2	1.7	5.1
Metribuzin	1.5	4.5	1.6	4.8	1.9	5.7
Myclobutanyl	2.0	6.0	1.4	4.2	1.5	4.5
Omethoate	3.0	9.0	3.5	10.5	3.6	10.8
Oxadyxil	1.8	5.4	1.7	5.1	2.5	7.5
Oxamyl	2.5	7.5	3.3	9.9	2.9	8.7
Pethoxamid	2.7	8.1	3.5	10.5	2.9	8.7
Profenofos	1.9	5.7	1.9	5.7	2.5	7.5
Promecarb	1.8	5.4	1.7	5.1	1.9	5.7
Propoxur	1.6	4.8	1.5	4.5	1.2	3.6
Pymetrozin	1.1	3.3	1.4	4.2	1.1	3.3
Pyperonil-butoxide	0.8	2.4	0.8	2.4	0.8	2.4
Pyrimethanil	1.9	5.7	2.3	6.9	3.1	9.2
Quinoxifen	1.5	4.5	1.8	5.4	2.0	6.0
Spirodiclofen	2.5	7.5	2.6	7.8	3.2	9.6
Tebuconazol	1.3	3.9	1.8	5.4	2.2	6.6
Thiacloprid	1.0	3.0	1.0	3.0	1.4	4.2
Triadimefon	1.4	4.2	1.5	4.5	2.9	8.7
Trifloxistrobin	1.2	3.6	1.6	4.8	1.6	4.8

Table 5: Limits of detection and limits of quantification (LODs and LOQs) of the method for different matrices

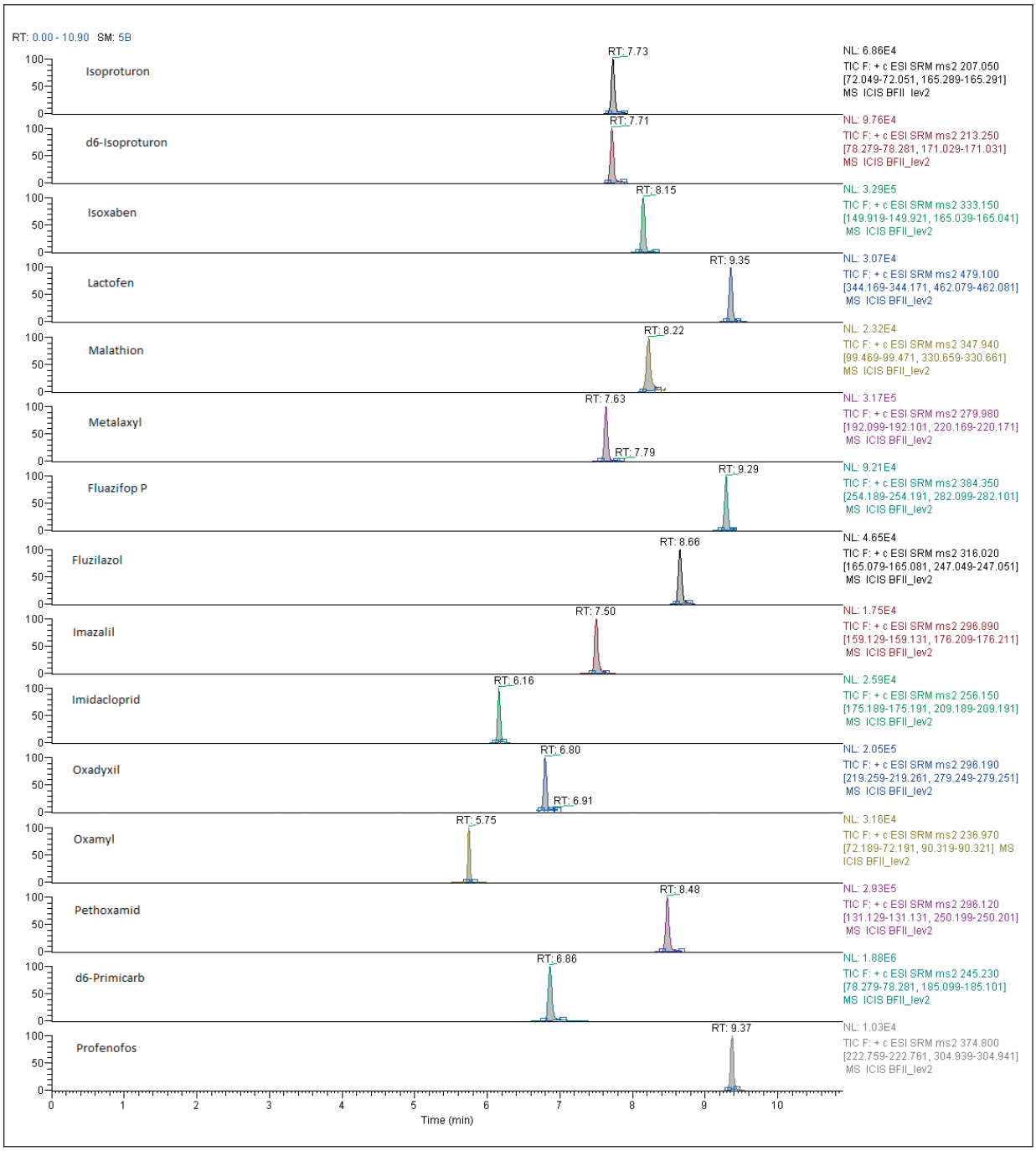


Figure 2: Illustration of selected target substance peaks and internal standards in baby food matrix spiked at legislation limit 10 ng/g

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Comparing LC and GC Triple Quadrupole MS for the Screening of 500 Pesticides in Matrix

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Overview

Purpose: The goal of this project is to compare the screening of more than 500 pesticides in matrix by LC and GC triple quadrupole, and determine the value of a comprehensive LC and GC screening approach.

Methods: The methodology included the vegetable extraction by QuEChERS followed by GC-MS/MS and LC-MS/MS analysis of over 500 pesticides in matrix.

Results: The majority of compounds could be detected to levels acceptable by EU standards by either GC/MS or LC/MS. All but eight pesticides could be determined to acceptable levels by the combined GC/LC methodology.

Introduction

Modern pesticide analysis is extremely challenging due to the diversity of compounds required to be reported, especially in the area of food safety control. Furthermore, the pressure to report large numbers of pesticides quickly makes it attractive to use large single injection methods. Triple quadrupole mass spectrometry has emerged as a primary technique for screening large target lists of pesticides due to its high sensitivity and selectivity against matrix. However, because of the chemical diversity of pesticides, LC or GC introduction alone may not be ideal, or even sufficient for a comprehensive analysis. Presented is a comparison of both LC and GC sample introduction techniques coupled to triple quadrupole mass spectrometer for the screening of more than 500 pesticides at ppb levels.

Methods

Sample Preparation

Pesticide standards were obtained from the U.S. Food and Drug Administration (FDA). In order to determine detection limits of such a wide range of pesticides, standards were prepared at multiple levels, enabling the selection of an appropriate level to determine the detection limit of each compound.

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Vegetable matrices were prepared for analysis by using a modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method, which is a sample preparation procedure used to extract pesticides from food[1]. The QuEChERS extracts were obtained from California Department of Food and Agriculture. For the QuEChERS extraction, 15 g of homogenized sample and 15 mL of acetonitrile were used.

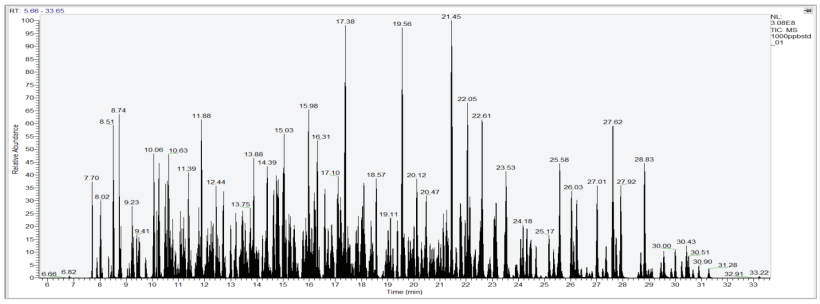
GC/MS Instrument Methodology

Gas Chromatograph Method Conditions

A method was developed for the Thermo Scientific™ TRACE™ 1310 Gas Chromatograph and Thermo Scientific™ TSQ™ 8000 Mass Spectrometer. A Programmable Temperature Vaporization (PTV) injector was used on the TRACE 1310. The ability to program a temperature ramp with this injector was utilized so that thermally labile pesticides would be transferred to the analytical column at as low a temperature possible.

Similarly, the oven on the TRACE 1310 gas chromatograph was ramped, volatilizing pesticides on the column as their boiling points were reached. A slow ramp of 5 ºC/min was employed between an oven temperature of 180 ºC and 280 ºC, which is the range in which the majority of these pesticides are volatilized, to achieve optimal separation during this most dense part of the chromatogram. Figure 1 shows the total ion chromatogram resulting from the GC/MS method, and Figure 2 lists the GC method parameters.

FIGURE 1. GC/MS Total Ion Chromatogram.



The analytical column used was a Thermo Scientific™ TraceGOLD™ TG-5SILMS, with dimensions 30 m x 0.25 mm x 0.25 µm. The liner employed was a baffled, Siltek™ deactivated inlet liner.

FIGURE 2. Gas Chromatograph Parameters.

Injection Volume	
Injection Volume (µL):	1.0
Trace 1310 GC PTV Inlet	
PTV mode:	Splitless
Inlet (°C):	75
Split flow(ml/min):	50
Splitess time (min)	1
PTV inject:	75 °C , 0.1 min to transfer step
PTV transfer:	300 °C, 2.5 °C/sec for 3 min to clean step
PTV Clean:	330 °C, 14.5 °C/sec for 20 min
Carrier Flow He (mL/min):	1.2
Oven Temperature Program	
Temperature 1 (°C):	40
Hold Time (min):	1.5
Rate (°C/min)	25
Temperature 2 (°C):	90
Hold Time (min):	1.5
Rate (°C/min)	25
Temperature 3 (°C):	180
Hold Time (min):	0
Rate (°C/min)	5
Temperature 4 (°C):	280
Hold Time (min):	0
Rate (°C/min)	10
Temperature 5 (°C):	300
Hold Time (min):	5

GC-Triple Quadrupole Method Conditions

Transitions for all pesticides were taken from the Thermo Scientific™ TSQ 8000 Pesticide Analyzer. These transitions were originally developed with the use of AutoSRM software, which provided automated SRM development with collision energies optimized to ± 1 eV. Thermo Scientific TraceFinder™ software was used for acquisition and processing of the extracted samples. Selecting the appropriate compounds from the pesticide analyzer automatically populated the SRM acquisition list in the instrument method and the compound processing parameters in the Thermo Scientific™ TraceFinder™ software processing method. One ion per compound was used for quantitation and two additional ions were used for ion ratio confirmation. Figure 3 lists additional MS parameters used.

FIGURE 3. GC-Mass Spectrometer Parameters

Mass Spec Parameters	
Transfer line (°C):	**250**
Source temperature (°C):	**300**
Mode:	**SRM**
Ionization:	**EI, 70 eV**
Collision Gas:	**Argon**
Resolution:	**Q1 normal**

LC/MS Instrument Methodology

U-HPLC Method Conditions

Chromatographic analysis was performed using the Thermo Scientific™ Accela™ 1250 UHPLC system. The autosampler was an HTC-PAL™ Autosampler (CTC Analytics, Zwingen, Switzerland). The column used was a Thermo Scientific™ Hypersil™ GOLD aQ column (100 x 2.1 mm, 1.9 μm particle size). Displayed in Figure 4 is the total ion chromatogram. The UHPLC conditions are listed in Figure 5.

FIGURE 4. LC/MS Total Ion Chromatogram

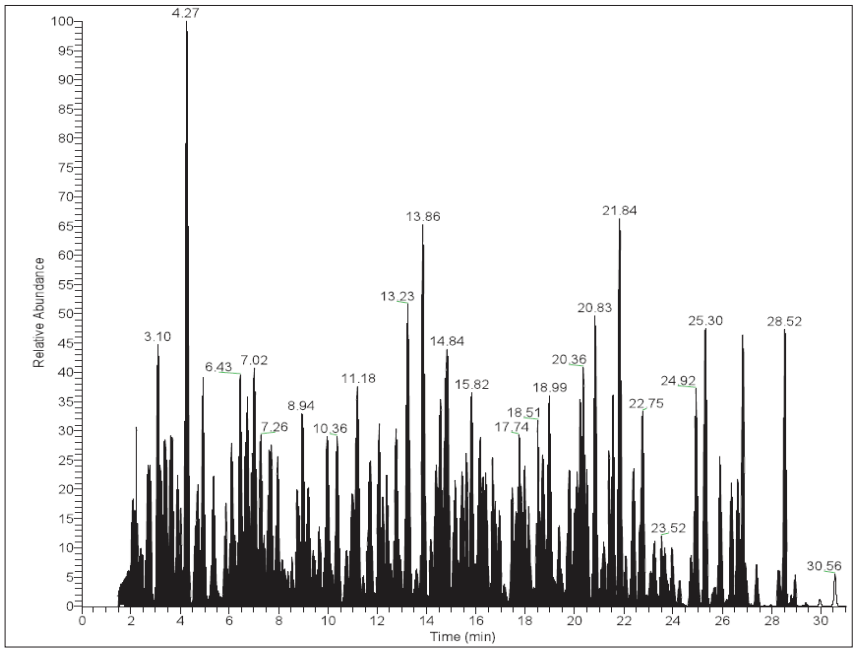


FIGURE 5. HPLC Parameters

HPLC Parameters			
Mobile Phase A:	Water with 0.1% formic acid and 4 mM ammonium formate		
Mobile Phase B:	Methanol with 0.1% formic acid and 4 mM ammonium formate		
Flow Rate:	300 µL/min		
Column Temperature:	40 °C		
Sample Injection Volume:	10 µL		
Gradient:	Gradient Time (min)	%A	%B
	0.00	98	2
	0.25	70	30
	35.00	0	100
	40.00	0	100
	40.01	98	2
	45.00	98	2

TSQ Quantum Access MAX LC-Triple Quadrupole Method Conditions

All samples were analyzed on the Thermo Scientific™ TSQ Quantum Access MAX™ triple stage quadrupole mass spectrometer with a heated electrospray ionization (HESI) source. To maximize the performance of the mass spectrometer, time-specific SRM windows were employed at the retention times of the target compounds. In addition, Quantitation-Enhanced Data-Dependent scanning, which delivers SRM-triggered MS/MS data, was used for structural confirmation. Alternating positive and negative polarity switching was utilized in the method. The MS conditions are listed in Figure 6 below.

FIGURE 6. LC-Mass Spectrometer Parameters.

Mass Spec Parameters	
Sheath Gas Flow Rate:	55 units
Aux Gas Flow Rate:	15 units
Spray Voltage:	3500 V
Capillary Temp:	280 °C
Heater Temp:	295 °C
Cycle Time:	0.2 s

Results and Discussion

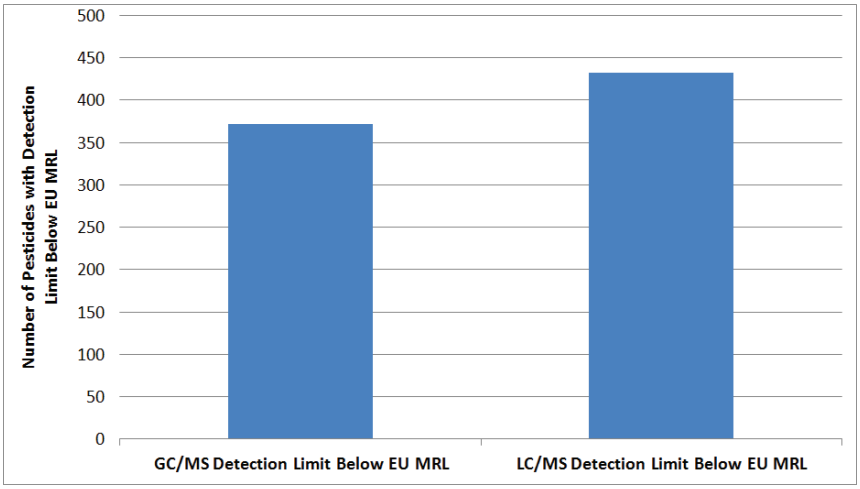
Determination of Method Detection Limit

For both GC/MS and LC/MS methods, spiked matrix samples were analyzed at several concentrations close to or below the European Union Method Reporting Limit (EU MRL). Each concentration level was injected several times and a statistical determination[2] of the method detection limit was calculated for comparison to the EU MRL for an onion matrix for each pesticide. When a required MRL was not available for the pesticide in onion, a 10 parts per billion MRL was used as stated in EU regulations.

Comparison of GC/MS to LC/MS

The majority of compounds were detected below EU MRLs by either the GC/MS or LC/MS method used (Figure 7). Out of the total 524 compounds analyzed, 372 pesticides had MDLs less than EU MRLs for the GC/MS methodology, compared with 432 pesticides with MDLs below the EU MRLs for the LC/MS methodology. Note that a10 µL injection was used in the LC/MS methodology compared with a 1 µL injection employed in the GC/MS methodology.

FIGURE 7. Number of compounds with method detection limits lower than EU MRLs for GC/MS and LC/MS methods

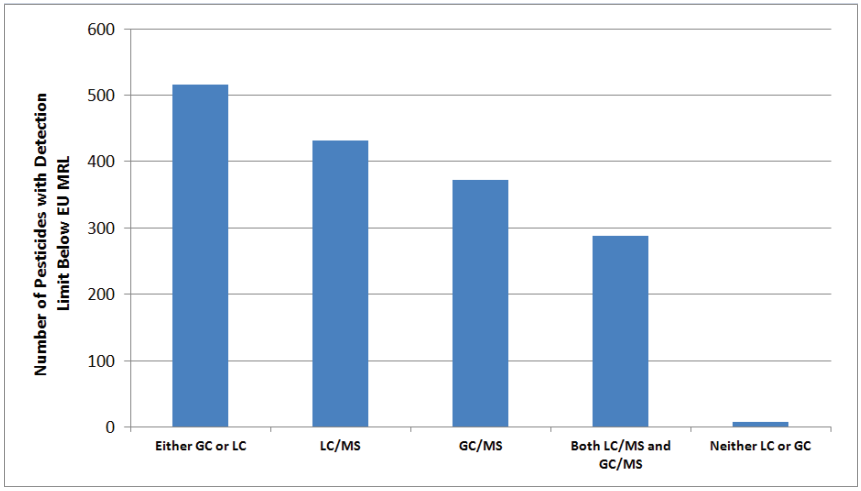


Benefits of Comprehensive GC/LC Methodology

By combining both GC and LC methodologies in a comprehensive screening methodology, 516 pesticides were detected below their MRLs for an onion matrix. This is 144 more than were detected below their MRLs for GC/MS methodology alone, and 84 more than by LC/MS alone. Only 8 pesticides had calculated detection limits for both GC/MS and LC/MS greater than their EU MRLs. On average, these 8 compounds" detection limits were four times their EU MRLs for the technique that gave them their lowest detection limit.

Furthermore, 288 compounds were able to be detected at concentrations below the EU MRL by both GC/MS and LC/MS methodology. This indicates that for a majority of these pesticides the two orthogonal techniques can be used together to increase confidence in the identification and quantitation. Figure 8 displayed below details these results.

FIGURE 8. Number of pesticides with detection limits below the EU MRL for GC/LC combined methodology compared with LC and GC methodology separately. Also displayed are numbers of pesticides detected below the MRL for both GC and LC methodology, and by neither methodology.



Conclusion

Methodology for both GC and LC/MS was developed and employed to analyze over 500 pesticides in a food matrix extracted with QuEChERS methodology. A summary of results, conclusions and possible future investigations for this project are as follow:

- 372 of 524 total pesticides were detected at levels under EU MRLs for onion samples by GC/MS
- 432 of 524 were detected at levels under EU MRLs for onion samples by LC/MS
- 516 of 524 were detected by either GC/MS, LC/MS, or by both GC/MS and LC/MS, demonstrating the power of combining these two techniques.
- For future work, a 10 µL large volume GC injection could be employed for the GC/MS methodology to better compare with the LC/MS methodology, and to try to lower the eight problematic pesticides detection limits under the EU MRL.
- Also, future work could explore techniques to selectively increase sensitivity for the eight problematic compounds, such as weighting SRM dwell time more heavily for these compounds, or decreasing resolution for these compounds, trading selectivity for sensitivity.

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Application Note: 425

Determination of Different Classes of Pesticide Residues in Processed Fruits and Vegetables by LC-MS Using the TSQ Quantum Ultra According to EU Directive 91/414 EEC

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Key Words

- TSQ Quantum Ultra™
- Surveyor™ HPLC System
- H-SRM
- Food Safety
- Pesticide Residues
- Sensitivity

Introduction

A diet rich in fruits and vegetables is thought to reduce the risk of some types of cancer, atherosclerosis, and heart disease. However, commercially grown produce often contains high levels of pesticide residues that can lead to serious health problems when consumed. Due in large part to growing public concern over the amount of pesticide residues in foods, the European Union (EU) has enacted several directives to fix Maximum Residue Limits (MRLs) for different pesticide residues in food of plant origin. MRLs represent the maximum amount of pesticide residues that might be expected in a commodity produced under conditions of good agricultural practice and typically range between 0.01 mg/kg and 10 mg/kg[1]. Although MRLs are not maximum toxicological limits, care is taken to ensure that these maximum levels do not generate toxicological concerns. Thus far, MRLs have been set for approximately 250 active substances. To cover the full variety of agricultural raw commodities (approximately 260 products of plant and animal origin), MRLs must be established for more than 260,000 pesticide/commodity combinations[1,2].

In the EU, pesticides are regulated principally by Directive 91/414/EEC concerning the placing of plant-protection products on the market[3]. According to this legislation, chemical substances or micro-organisms in pesticides are approved for use only if they have undergone a peer-reviewed safety assessment. All foodstuffs intended for human consumption or animal feed in the EU are now subject to a maximum residue limit for pesticides to protect human and animal health. Regulation (EC) 396/2005[4] consolidates in a single act all the limits applicable to various types of food and feed. It establishes MRLs for products of plant and animal origin at the Community level, taking into account good agricultural practices. It was based on several substantial amendments in the Council Directives:

- 76/895/EEC[5], which relates to the fixing of maximum levels for pesticide residues in and on specific fruits and vegetables
- 86/362/EEC[6] for cereals and cereal products
- 86/363/EEC[7] for products of animal origin
- 90/642/EEC[8] for plant products

Additionally, more stringent legislation has been established concerning pesticides in baby food. Since 1999, the EU has introduced the Commission Directives 1999/39/EC[9] and 1999/50/EC[10], which limit all pesticide residues to an MRL value of 0.010 mg/kg in processed cereal-based foods and in fruit and vegetables intended for the production of baby foods. MRLs below 0.010 mg/kg have been established for a few pesticides of higher toxicity, while the use of certain very toxic pesticides has been completely prohibited in the production of baby foods, as underlined in Commission Directives 2003/13/EC[11] and 2006/125/EC[12].

New "active" ingredients entering the market to replace compounds banned by Directive 91/414/ EEC possess considerably different physicochemical properties, and thus demand the development of multi-residue analytical methods. Analytical methodologies used to determine pesticide residues in foods must be capable of quantifying very low levels of residues as well as confirming their identity. This task becomes more difficult as MRLs are decreased and the number of target pesticides and metabolites increases. Therefore, the challenge is to develop a sensitive, cost-effective, multi-residue analytical method that can quickly identify and confirm pesticide residues belonging to various chemical classes in food products. At the same time, the method must accurately quantify these residues at low levels, thus fulfilling the performance criteria described in "Method Validation and Quality Control Procedures for Pesticide Residues Analysis in Food and Feed," European Commission Document SANCO 2007/3131[13].

Goal

To develop a multi-residue liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) method for the detection and quantification of 45 pesticides, including parent compounds and their transformation products from different chemical classes, in various food matrices.

Experimental Conditions

LC-ESI-MS/MS is the analytical technique of choice to assay environmental and food matrices with high sensitivity and selectivity. The technique is especially well-suited for the identification and quantification of polar and thermally labile pesticides and metabolites down to mg/kg levels.

The pesticides included in this study are listed in Table 1.

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Compound	t_R (min)[a]	Parent ion (*m/z*)	Quantifier ion (*m/z*)	Quantifier ion (*m/z*)
Acephate	2.5	184	143 (10V)	125 (10V)
Aldicarb[b] (Na+)	19.7	213	89 (22 V)	116 (22 V)
Aldicarb-sulfoxide (Na^+)	2.7	229	166 (9V)	109 (15V)
Aldicarb-sulfone (Na^+)	4.1	245	109 (25V)	166 (25V)
Acetamiprid	17.3	223.0	126 (21 V)	90 (35 V)
Azoxystrobin	28.8	404.2	372 (16 V)	344 (26 V)
Carbaryl	24.0	202.1	145 (25 V)	127 (25 V)
Carbofuran	23.1	222.1	123 (24 V)	165 (24 V)
3-hydroxy-carbofuran (-H_2O)	15.8	220	135 (15V)	163 (15V)
Chlorpropham	29.6	214.0	172 (12 V)	154 (19 V)
Carbendazim + benomyl[b]	2.5	192.0	160 (22 V)	132 (31 V)
Cyprodinil	25.3	226.1	93 (40 V)	77 (46 V)
Demeton-S	26.0	259.0	89 (22 V)	116 (22 V)
Demeton-S-methyl	22.8	253.0	61 (40 V)	89 (20 V)
Demeton-S-methyl-sulfone	7.0	263.0	109 (30 V)	169 (20 V)
Demeton-S-methyl-sulfoxide	3.3	247.0	169 (17 V)	109 (29 V)
Dimethomorph A[c] / B[c]	26.8 27.3	388.1	301 (23 V)	165 (35 V)
Disulfoton	33.8	275.0	89 (15 V)	61 (30 V)
Disulfoton-sulfone	27.1	307.0	125 (20 V)	153 (20 V)
Disulfoton-sulfoxide	24.2	291.0	185 (15 V)	157 (25 V)
Ethoprofos	29.2	243.0	131 (21 V)	173 (21 V)
Fenhexamid[d]	29.0	302.0 304.0	97 (22 V)	 97 (26 V)
Flusilazole	29.8	316.1	247 (21 V)	165 (31 V)
Imazalil	21.4	297.0	159 (24 V)	255 (25 V)
Imidachloprid	15.3	256.1	209 (22 V)	175 (22 V)
Kresoxim-methyl	31.8	314.0	222 (14 V)	116 (19 V)
Metalaxyl	24.8	280.1	220 (15 V)	192 (25 V)
Methiocarb	27.6	226.0	169 (11 V)	121 (19 V)
Methiocarb sulfoxide (Na^+)	6.6	185.0	122 (23 V)	170 (23 V)
Methomyl	5.0	163.0	106 (12 V)	88 (12 V)
Myclobutanil	28.7	289.0	125 (35 V)	70 (25 V)
Oxamyl (Na^+)	4.2	242	70 (20V)	121 (20V)
Penconazole	30.0	284.0	159 (35 V)	70 (35 V)
Pirimicarb	7.3	239.1	182 (15 V)	72 (30 V)
Propiconazole	30.8	342.0	159 (31 V)	69 (31 V)
Propoxur	22.7	210.1	111 (17 V)	168 (10 V)
Pyrimethanil	21.2	200.0	182 (35 V)	168 (35 V)
Tetraconazole[d]	29.4	372.0 374.0	159 (38 V)	 161 (31 V)
Thiabendazole	2.5	202.0	131 (36 V)	175 (36 V)
Thiachloprid	21.0	253.0	99 (45 V)	126 (25 V)
Thiodicarb	23.3	355.0	88 (20 v)	108 (20 V)
Thiophanate-methyl	22.5	343.0	151 (23 V)	311 (15 V)
Triadimefon	28.9	294.1	197 (19 V)	225 (19 V)
Triadimenol A[c]/ B[c]	27.1/27.5	296.1	70 (16 V)	99 (16 V)
Triazophos	30.4	314.1	162 (19 V)	119 (33 V)

[a] Retention time

[b] Benomyl was measured as carbendazim[14]

[c] Dimethomorph and triadimenol exist as two isomers with different retention times

[d] For fenhexamid and tetraconazole, the isotopic parent ions were selected due to the lack of a second sound transition

Table 1: Retention times and compound-specific ESI(+)-MS/MS parameters

Sample Preparation

A stock mix solution of all the pesticides was prepared at a concentration of 1 mg/L. Calibration solutions in the concentration range 0.5-100 µg/L were prepared by serial dilution of the stock solution.

Samples were prepared for analysis using extraction with ethyl acetate. Individual samples of fruits and vegetables were first homogenized. After homogenization, a 10.0 g sample was extracted using ethyl acetate and anhydrous sodium sulfate. The mixture was ultra-sonicated for 20 minutes. The mixture was filtrated through a thin layer of anhydrous sodium sulfate and the filtrate was evaporated. The extracts were then reconstituted in 5 mL of methanol. The solution was diluted with water and then filtered through a 0.45 µm syringe filter[14].

HPLC

HPLC analysis was performed using the Surveyor HPLC System (Thermo Fisher Scientific, San Jose, CA). Each 20 µL sample was injected onto a 150×2.1 mm, 3.5 µm, C18 HPLC column equipped with a 10×2.1 mm, 3.5 µm, C18 HPLC guard column. A gradient LC method used mobile phases A (0.1% formic acid) and B (0.1% formic acid in acetonitrile) at a flow rate of 0.2 mL/min. The gradient was: 0–3 min A:B = 90:10 (v/v), 3–31 min A:B = 90:10 (v/v) to A:B = 10:90 (v/v), 31–36 min A:B = 10:90 (v/v), 36–36.5 min A:B = 10:90 (v/v) to A:B = 90:10 (v/v), 36.5–45 min A:B = 90:10 (v/v).

MS

MS analysis was carried out on a TSQ Quantum Ultra triple stage quadrupole mass spectrometer with an electrospray ionization source (Thermo Fisher Scientific, San Jose, CA).

The MS conditions were as follows:
Ion source polarity: Positive
Spray voltage: 4000 V
Sheath gas pressure (N_2): 40 units
Auxiliary gas pressure (N_2): 10 units
Ion transfer tube temperature: 350 °C
Collision gas pressure (Ar): 1.0 mTorr
Q1 resolution: 0.2 FWHM (H-SRM)
Q3 resolution: 0.7 FWHM
Scan Type: H-SRM
Dwell time: 20–50 ms

The LC-MS/MS method was developed according to the scheme shown in Figure 1. The run was divided into four time segments based on the retention times of the target compounds. Multiple scan events were included in each time segment. For each target compound, the protonated molecule $[M+H]^+$ was usually investigated, except in the cases of compounds where the adduct $[M+Na]^+$ was the base peak in the ESI(+) spectra. Two transitions were selected per compound in order to perform quantification and identification simultaneously.

The SRM transitions that were monitored are summarized in Table 1. Identification criteria for the target compounds were based on the LC retention time (t_R) and on the ratio of the two monitored transitions for each compound.[13,14]

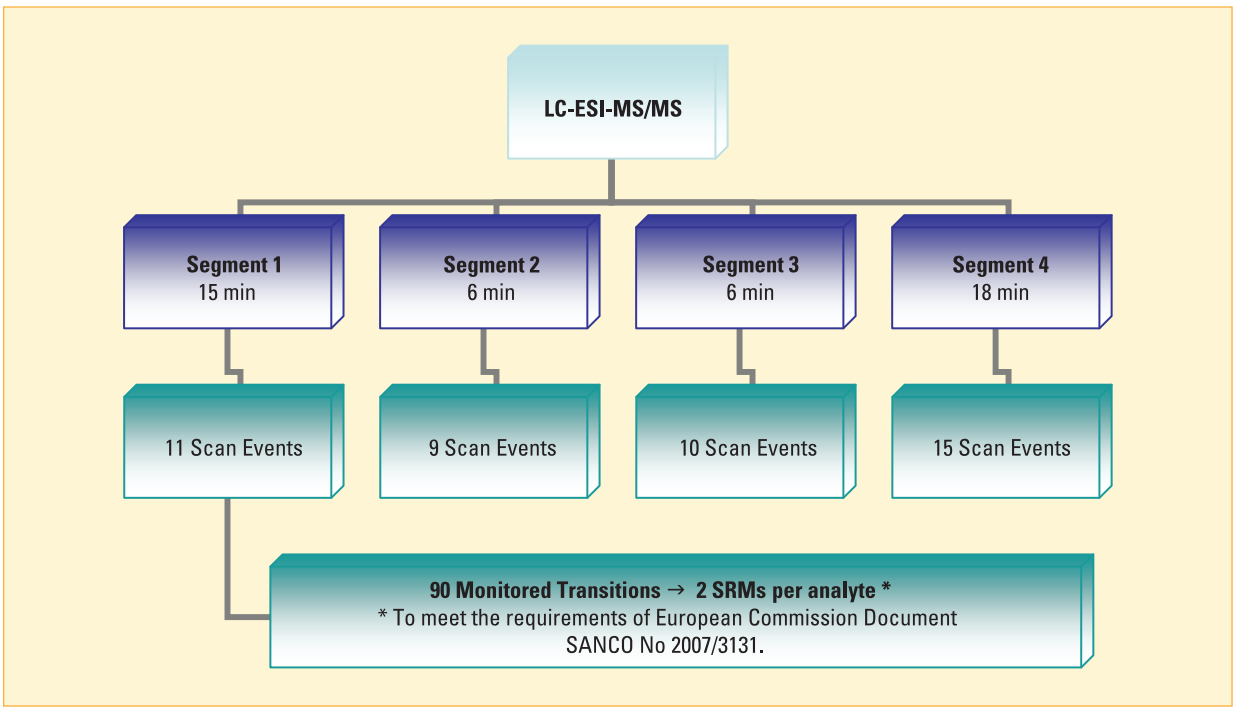

Figure 1: LC-ESI-MS/MS method

Results and Discussion

Although LC-MS/MS is a selective technique, interferences due to isobaric compounds can appear in chromatograms. These isobaric interferences increase the chemical background and can make it difficult to integrate the desired analyte peak reproducibly. Among the compounds included in this study were three sets of isobaric compounds and one set of compounds that share the same fragment ions, which increases the likelihood of cross-talk. Therefore, to eliminate the noise and lower the detection limits, all of the assays in this study were run in the Highly Selective Reaction Monitoring (H-SRM) mode with the Q1 FWHM peak width set at 0.2[14].

The H-SRM chromatograms of a mix solution of certain pesticides at a concentration of 1 µg/L are shown in Figure 2. Linearity of the method was proven for all cases because the R^2 values were usually greater than 0.99 for the linear regression equations (1/x weighted) in the concentration ranges tested. The instrumental detection limits (IDLs) were, in most cases, below 0.5 µg/L. Figure 3 displays the linearity plots of selected compounds. Linearity data for certain compounds are summarized in Table 2.

Using the H-SRM mode reduced the matrix effects by minimizing the chemical noise caused by co-eluting isobaric compounds. Consequently, the signal-to-noise ratio was enhanced in the complicated food matrices. This effect can be observed in the chromatograms in Figure 4, which show the analysis of a peas sample in the SRM and H-SRM modes. The top two SRM chromatograms illustrate the background in a blank peas extract whereas the bottom two SRM chromatograms show the peaks for methomyl in a peas extract spiked with 1 ppb of methomyl. The narrower window of the Q1 set at 0.2 FWHM in the H-SRM mode improves the selectivity of the analysis and increases the signal-to-noise ratio.

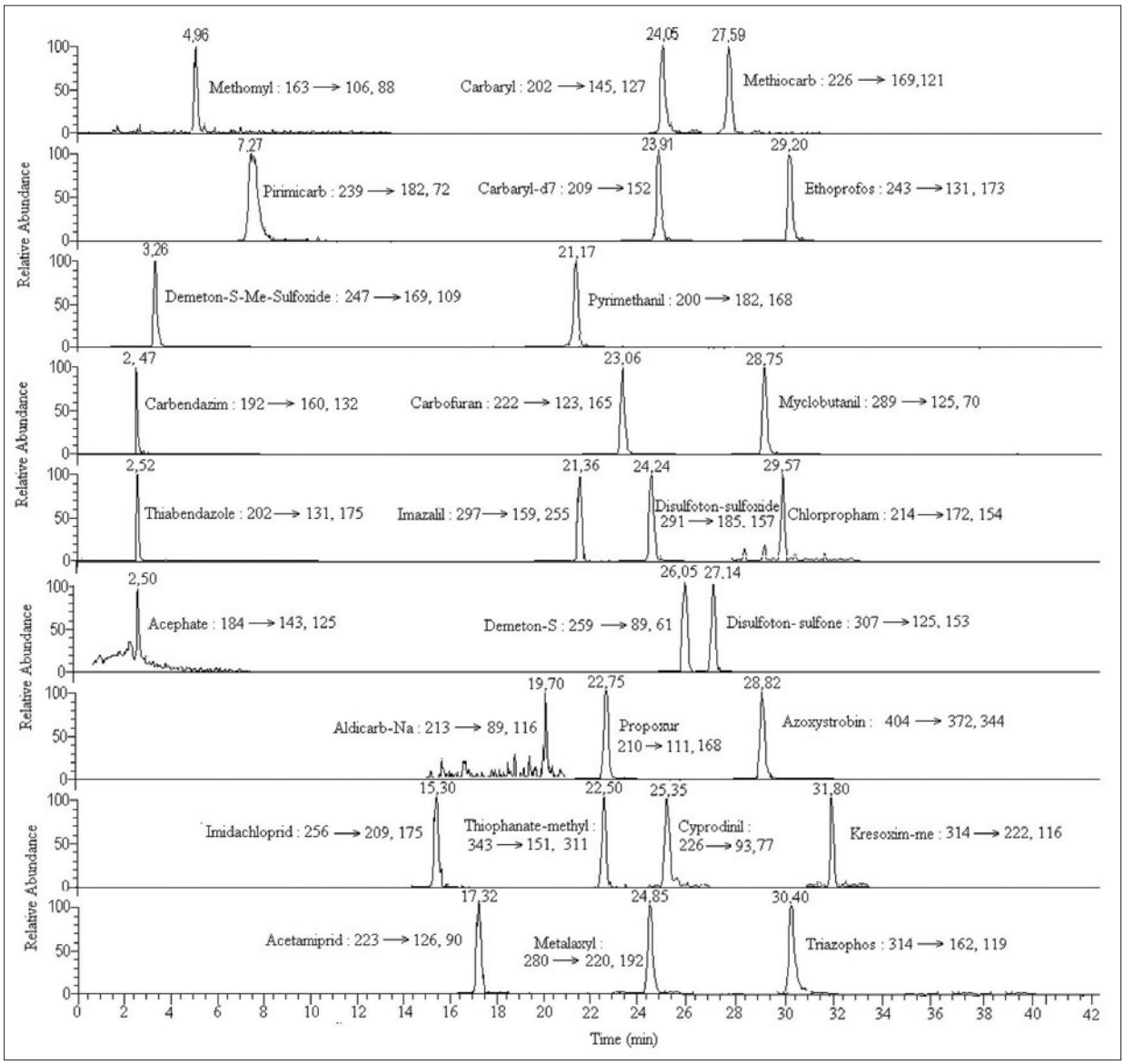

Figure 2: SRM chromatograms for certain pesticides of the standard mix solution

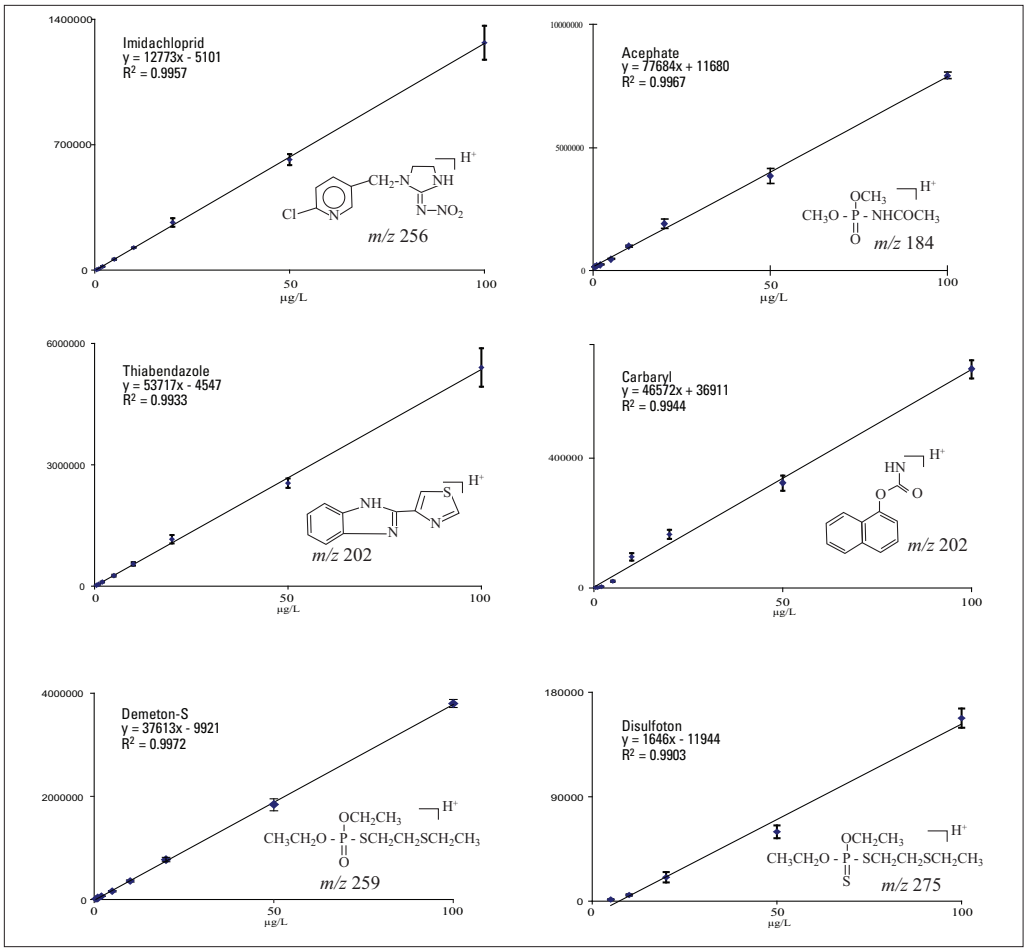

Figure 3: Linearity plots for certain compounds

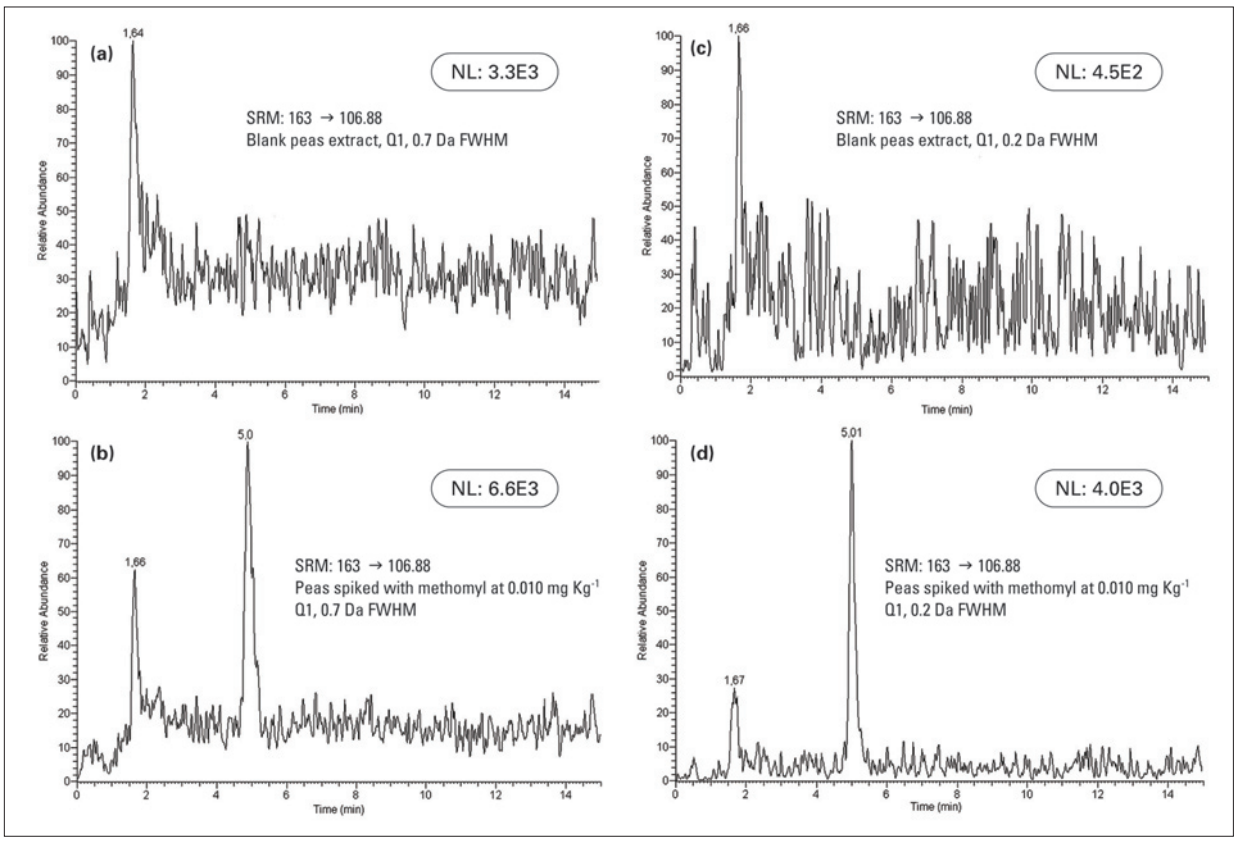

Figure 4: H-SRM and SRM chromatograms of methomyl in pea sample matrix

The matrix-matched calibration curves of methomyl at different Q1 settings are shown in Figure 5 and data of the calibration curves are listed in Table 3. The signal itself is reduced by a factor of two when the Q1 FWHM peak width is changed from 0.7 to 0.2, yet the linearity and accuracy are improved (as demonstrated by the correlation coefficients and back-calculated values of the matrix-matched standards at the low concentration levels, in Table 3).

Some samples were found to contain pesticide residues. Figure 6 displays SRM chromatograms of a sample of frozen peas that contained residues of triazophos and myclobutanil. The confirmation of identity was based on the ion ratio of monitored transitions in the sample and in the standard solution according to the EU Guidelines for pesticide residues monitoring.[13] The concentrations of the residues found in the sample were below the Maximum Residue Limits (MRLs)[1,2].

Compound	Linear regression equations	Concentration range (µg/L)	R^2	IDLs (µg/L)
Acephate	Y=116806+77683.7 X	(1-100)	0.9967	0.5
Aldicarb	Y=-590.6 +1115.4 X	(1-100)	0.9900	0.7
Azoxystrobin	Y=-363884 + 213698 X	(0.5-100)	0.9912	0.2
Carbaryl	Y=36911 + 46572 X	(0.5-100)	0.9903	0.3
Carbendazim	Y=10192 + 211684 X	(0.5-100)	0.9964	0.1
Carbofuran	Y=11107 + 161251 X	(0.5-100)	0.9920	0.2
Chlorpropham	Y=-5289 + 6893.5 X	(1-100)	0.9954	0.6
Cyprodinil	Y=-57425 + 30565.4 X	(0.5-50)	0.9931	0.3
Demeton-S	Y=-9921 + 37615 X	(0.5-100)	0.9972	0.3
Disulfoton	Y=-11944 + 1646.2 X	(5-100)	0.9903	1.5
Disulfoton Sulfoxide	Y=40274 + 141033 X	(0.5-100)	0.9961	0.4
Disulfoton Sulfone	Y=-1633.2 + 8994 X	(0.5-100)	0.9904	0.4
Ethoprofos	Y=-10106 + 40922 X	(0.5-100)	0.9940	0.3
Imidachloprid	Y=-5101.1 +12773.2 X	(0.5-100)	0.9957	0.3
Kresoxim-methyl	Y=-7877.4 + 3056.8 X	(2-100)	0.9900	1.0
Metalaxyl	Y=28427.5 +117245 X	(0.5-100)	0.9964	0.3
Methiocarb	Y=4861 + 48380.4 X	(0.5-100)	0.9921	0.3
Methomyl	Y=-2440.7 +13847.8 X	(0.5-100)	0.9990	0.4
Myclobutanil	Y=-16905.7 +10101.5 X	(0.5-100)	0.9953	0.4
Pirimicarb	Y=23403 +168260 X	(0.5-100)	0.9953	0.2
Propoxur	Y=9181 + 151300 X	(0.5-100)	0.9947	0.2
Pyrimethanil	Y=-4723.7 + 9197.2 X	(0.5-100)	0.9900	0.4
Thiabendazole	Y=-4546.8 + 53716.7 X	(0.5-100)	0.9933	0.3
Triazophos	Y=-18350 +134057 X	(0.5-100)	0.9954	0.3

Table 2: Linearity data and instrumental detection limits (IDLs) for certain pesticides

	Peas Matrix 0.1 g/mL Q1: 0.2 FWHM	Peas Matrix 0.1 g/mL Q1: 0.7 FWHM	Peas Matrix 0.2 g/mL Q1: 0.2 FWHM	Peas Matrix 0.2 g/mL Q1: 0.7 FWHM
1/x	Y = -2469.2 + 10863 X	Y = 4631.3 + 18381.3 X	Y = -3845.1 + 8212 X	Y = 10244 + 19142 X
R^2	0.9966	0.9851	0.9945	0.9861
Accuracy of Matrix-Matched Calibration Curves (1/x)				
1 µg/L	0.91 µg/L (91%)	0.68 µg/L (68%)	0.87 µg/L (87%)	1.24 µg/L (124%)
5 µg/L	4.89 µg/L (97%)	4.74 µg/L (94%)	5.26 µg/L (105%)	4.56 µg/L (91%)
10 µg/L	9.78 µg/L (97%)	11.5 µg/L (85%)	10.5 µg/L (95%)	9.05 µg/L (90%)

Table 3: Linearity and accuracy data for methomyl in pea matrix

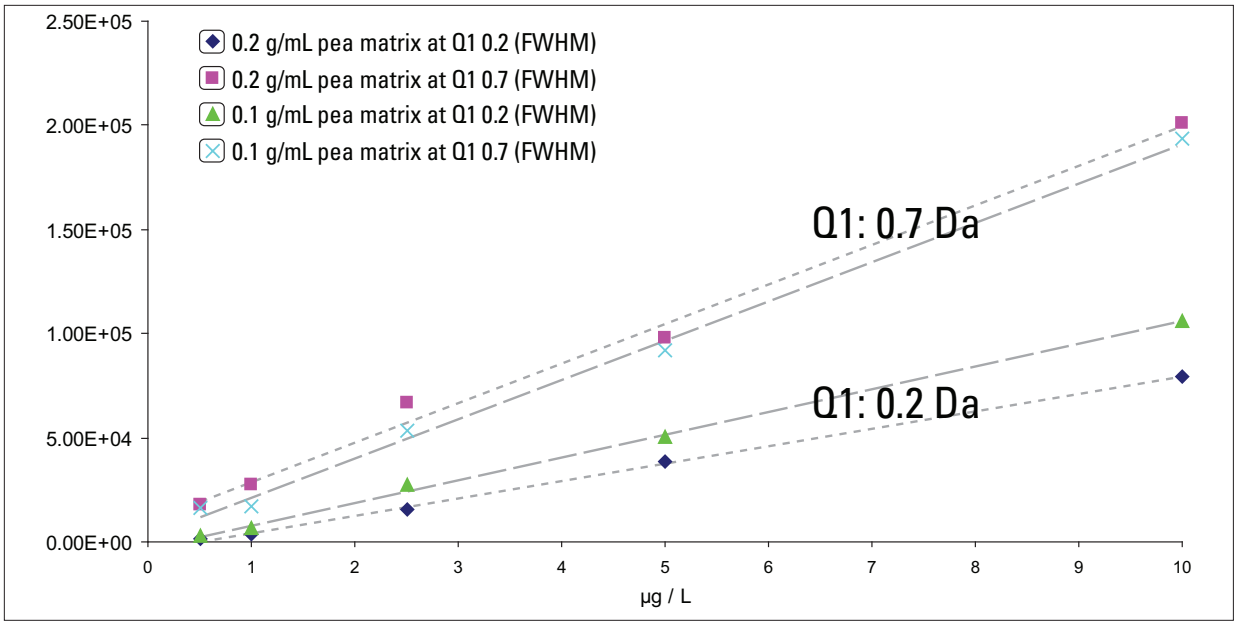

Figure 5: Matrix-matched calibration curves of methomyl in pea extract at Q1: 0.2 (FWHM) and 0.7 (FWHM)

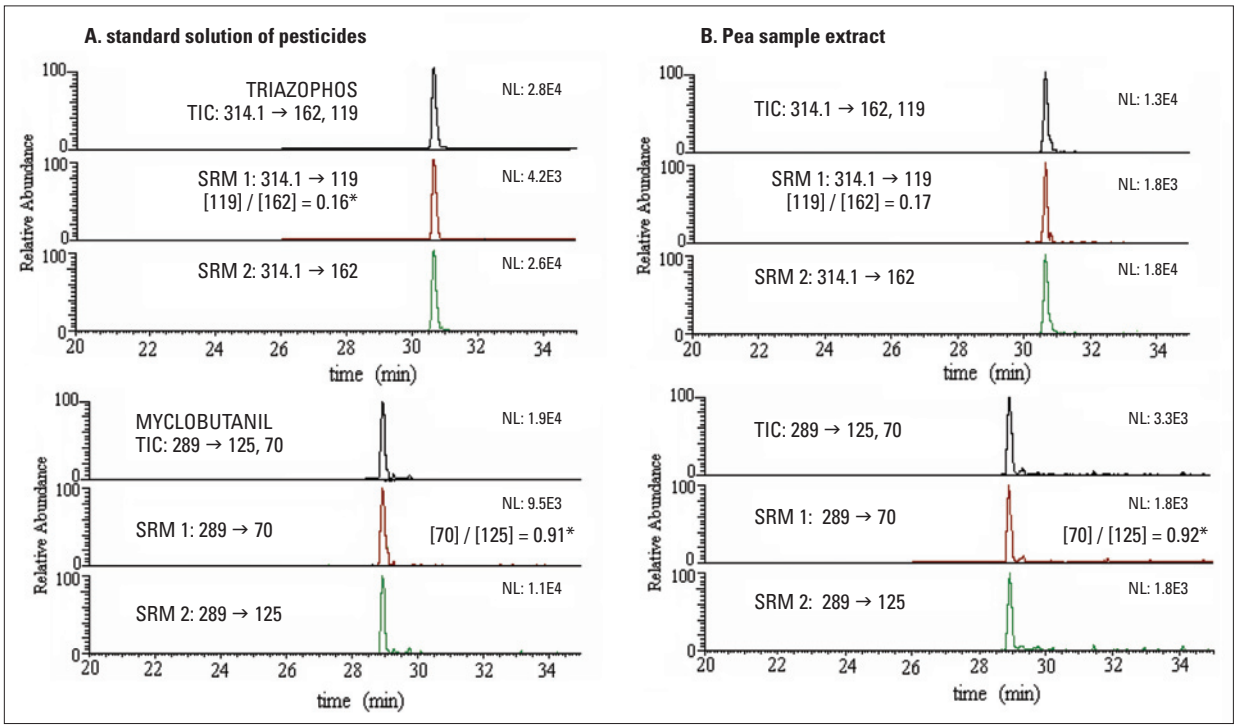

Figure 6: LC-ESI-SRM chromatograms of frozen pea sample extract, with residues of triazophos and myclobutanil

Conclusion

A multi-residue LC-ESI-MS/MS method was developed for the reliable confirmation and quantification of pesticides from different chemical classes at low ppb levels in food matrices. The method uses the Highly Selective Reaction Monitoring (H-SRM) mode of the TSQ Quantum Ultra triple quadrupole mass spectrometer to effectively reduce the background interference and improve the signal-to-noise ratios. For the pesticides investigated, satisfactory precision and accuracy were achieved and Limit of Quantitation (LOQ) values of 0.010 mg/kg were established. The method can be expanded to include more pesticides and their metabolites to improve the range of pesticide residues monitored in food commodities.

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Application Note: 387

Multi-residue Analysis of Pesticides in Food using GC/MS/MS with the TSQ Quantum GC

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Key Words

- TSQ Quantum GC
- H-SRM
- Pesticide Residues in Food
- Positive List System
- QED
- SRM

Introduction

Food safety concerns are on the rise amongst consumers worldwide. In 2006, sweeping changes were made to the Food Hygiene Law in Japan regarding residues of agricultural chemicals, including pesticides, in foods. As a result, standard residue values were established for approximately 800 pesticides. All food items produced in or imported into Japan are required to meet the standards established by this law. If pesticide residues in any food items exceed these standards, then the distribution and sale of the food is prohibited. This Positive List System has had a significant effect not only on the Japanese domestic production, but also on much of the food exported to Japan from various foreign countries such as China, the United States, and Taiwan.

There are numerous types of pesticides regularly used in the agricultural industry, including insecticides, fungicides, herbicides, and growth regulators. Because each type has different physicochemical properties, there are limitations on simultaneous analysis. Among the pesticides for which standard values are currently set, GC/MS/MS can analyze approximately 300 compounds. The superior selectivity of this technique allows interference-free quantification, even with peak coelution, and provides positive confirmation of various pesticides in a single analytical run.

To accurately monitor pesticide residues, a high throughput multi-residue screening method that can quantitate a large number of pesticide residues during a single analytical run is needed.

Goal

To simultaneously analyze 103 pesticides using the TSQ Quantum™ GC system, using SRM and H-SRM. Additionally, to show the utility of QED MS/MS for structural confirmation of the analytes undergoing quantification.

Experimental Conditions

Sample Preparation

Green pepper, carrot, grapefruit and banana samples were prepared for analysis using a method based on the simple and quick QuEChERS approach.[1] A 10 g sample of food was homogenized in a food processor and placed in a polypropylene centrifuge tube. The sample was extracted with 20 mL of acetonitrile in a homogenizer. Then, 4 g of anhydrous magnesium sulfate and 1 g of sodium chloride were added and the resulting mixture was centrifuged. After centrifugation, the supernatant was loaded onto a graphite carbon/PSA dual layer solid phase extraction column and eluted with 50 mL of acetonitrile/toluene (3:1). After the eluate was concentrated under reduced pressure, it was dissolved (1 g/mL) in 10 mL of acetone/n-hexane to give the test solution.

GC

GC analysis was performed using the TRACE GC Ultra™ System (Thermo Fisher Scientific, Milan, Italy). The GC conditions were as follows:

Column: Rxi-5MS 30 m x 0.25 mm I.D., 0.25 m df (Restek Corp., Bellefonte, PA)
Injection mode: Splitless with surge injection (200 kPa, 1 min)
Injection temperature: 240 °C
Oven temperature: 80 °C (1 min) – 20 °C/min – 180 °C – 5 °C/min – 280 °C (10 min)
Flow rate: Constant flow 1.2 mL/min
Transfer line temperature: 280 °C

AS

The samples were injected through the TriPlus™ autosampler (Thermo Fisher Scientific, Milan, Italy). The autosampler conditions were as follows:

Injection volume: 1 µL
Injection mode: Hot needle
Syringe: 80 mm

MS

MS analysis was carried out on a TSQ Quantum GC triple stage quadrupole mass spectrometer. (Thermo Fisher Scientific, San Jose, CA). The MS conditions were as follows:

Ionization mode: EI positive ion
Ion volume: Closed EI
Emission current: 25 µA
Ion source temperature: 220 °C
Scan type: SRM and H-SRM
Scan width: 0.002 a.m.u.
Scan time: 0.01 s
Peak width: Q1, 0.7 Da; Q3, 0.7 Da FWHM
Peak width for H-SRM: Q1, 0.4 Da; Q3, 0.7 Da FWHM
Collision gas (Ar) pressure: 1.2 mTorr

A total of 103 pesticides were analyzed to determine the product ion to be used for quantitation. Table 1 lists the SRM transitions and the optimum collision energy for each of the compounds and a summary of the calibration range, linearity, and the reproducibility of each individual compound at 5 ppb (ng/mL).

	R.T.	Precursor Ion (m/z)	Product Ion (m/z)	Collision Energy	R^2	Range	CV(%) n=5
Mevinphos	6.44	192	127	10	0.9999	0.1-100	4.03
XMC	7.52	122	107	10	0.9999	0.1-100	2.55
Tecnazene	8.03	261	203	15	0.9996	0.1-100	5.41
Ethoprpphos	8.22	200	114	10	0.9981	0.1-100	7.91
Ethalfluralin	8.42	316	276	10	0.9997	0.1-100	4.14
Benfluralin	8.62	292	264	10	0.9989	0.1-100	1.86
Monocrotophos	8.62	192	127	10	0.9754	5-100	19.47
α-BHC	9.03	219	183	15	0.9999	0.1-100	4.51
Dicloran	9.25	206	176	10	0.9994	0.1-100	2.30
Simazine	9.30	201	172	10	0.9999	0.1-100	4.33
Propazine	9.50	214	172	10	0.9998	0.1-100	1.99
β-BHC	9.57	219	183	15	1.0000	0.1-100	3.51
γ-BHC	9.73	219	183	15	0.9998	0.1-100	6.57
Cyanophos	9.78	243	109	10	0.9996	0.1-100	3.56
Pyroquilon	9.90	173	130	20	0.9996	0.1-100	2.95
Diazinon	4.94	304	179	15	0.9995	0.1-100	4.40
Phosphamidon-1	10.06	264	127	10	0.9989	0.1-100	10.31
Prohydrojasmon-1	10.12	184	83	20	0.9992	0.1-100	7.39
δ-BHC	10.26	219	183	15	0.9994	0.1-100	5.17
Prohydrojasmon-2	10.66	264	127	10	0.9972	0.1-100	17.11
Benoxacor	10.7	259	120	15	0.9999	0.1-100	3.30
Propanil	10.95	262	202	10	0.9993	0.1-100	3.65
Phosphamidon-2	10.97	264	127	10	0.9970	0.1-100	8.77
Dichlofenthion	10.99	279	223	15	0.9994	0.1-100	2.21
Dimethenamid	11.06	230	154	10	0.9996	0.1-100	2.51
Bromobutide	11.09	232	176	10	0.9990	0.1-100	5.91
Paration-methyl	11.24	263	109	10	0.9982	0.1-100	3.74
Tolclofos-methyl	11.38	265	250	15	0.9998	0.1-100	2.52
Ametryn	11.43	227	170	10	0.9999	0.1-100	0.90
Mefenoxam	11.57	249	190	10	0.9995	0.1-100	5.81
Bromacil	11.98	205	188	15	0.9988	0.1-100	3.87
Pirimiphos-methyl	12.00	305	276	10	0.9995	0.1-100	4.08
Quinoclamine	12.18	207	172	10	0.9989	0.1-100	4.24
Diethofencarb	12.34	225	125	15	0.9985	0.1-100	4.64
Cyanazine	12.52	225	189	10	0.9994	0.1-100	3.41
Chlorpyrifos	12.57	314	258	15	0.9991	0.1-100	3.37
Parathion	12.59	291	109	15	0.9962	0.1-100	9.76
Triadimefon	12.67	208	111	25	0.9986	0.1-100	6.10
Chlorthal-dimethyl	12.73	301	223	20	1.0000	0.1-100	1.23
Nitrothal-isopropyl	12.78	236	148	15	0.9974	0.1-100	5.53
Fthalide	13.04	272	243	10	0.9993	0.1-100	4.32
Fosthiazate	13.05 13.12	195	103	10	0.9956	5-100	6.29
Diphenamid	13.1	239	167	10	0.9997	0.1-100	4.67
Pyrifenox-Z	13.64	262	200	15	0.9979	0.2-100	4.54
Fipronil	13.79	367	213	25	0.9991	0.1-100	3.49
Allethrin	13.67	123	81	10	0.9991	5-100	3.79
Dimepiperate	13.87	145	112	10	0.9987	0.1-100	3.74
Phenthoate	13.87	274	121	10	0.9987	0.1-100	1.82
Quinalphos	13.88	146	118	10	0.9984	0.1-100	1.96
Paclobutrazol	14.45	236	125	15	0.9961	0.1-100	7.41
Endosulfan-α	14.67	241	206	15	0.9996	0.1-100	4.54
Butachlor	14.73	237	160	10	0.9998	0.1-100	5.26
Imazamethabenz-methyl	14.81	256	144	20	0.9932	2-100	12.09
Butamifos	15.00	286	202	15	0.9958	0.1-100	4.66
Napropamide	15.01	271	128	5	0.9989	0.1-100	8.96

	R.T.	Precursor Ion (m/z)	Product Ion (m/z)	Collision Energy	R^2	Range	CV(%) n=5
Flutlanil	15.06	173	145	15	0.9986	0.1-100	1.93
Hexaconazole	15.06	214	172	15	0.9924	0.1-100	8.98
Profenofos	15.28	337	267	15	0.9968	0.1-100	6.61
Uniconazole-P	15.38	234	137	15	0.9966	0.1-100	11.37
Pretilachlor	15.37	162	132	15	0.9982	0.1-100	6.72
Flamprop-methyl	15.66	276	105	10	0.9986	0.1-100	3.93
Oxyfluorfen	15.69	361	300	10	0.9980	0.5-100	6.07
Azaconazole	15.79	217	173	15	0.9981	0.1-100	7.07
Bupirimate	15.82	316	208	10	0.9982	0.1-100	4.65
Thifluzamide	15.84	449	429	10	0.9972	0.1-100	2.75
Fenoxanil	16.25	293	155	20	0.9989	0.1-100	3.73
Chlorbenzilate	16.43	251	139	15	0.9976	0.1-100	0.81
Pyriminobac-methyl-Z	16.76	302	256	15	0.9986	0.1-100	2.70
Oxadixyl	16.86	163	132	10	0.9998	0.1-100	3.72
Triazophos	17.30	257	162	10	0.9941	0.2-100	6.72
Fluacrypyrim	17.38	189	129	10	0.9988	0.1-100	2.15
Edifenphos	17.72	310	173	10	0.9927	0.1-100	7.95
Quinoxyfen	17.74	272	237	10	0.9993	0.1-100	4.50
Lenacil	17.78	153	136	15	0.9979	0.1-100	5.19
Trifloxystrobin	18.01	222	162	10	0.9966	0.1-100	8.47
Pyriminobac-methyl-E	18.19	302	256	15	0.9982	0.1-100	2.12
Tebuconazole	18.39	250	125	20	0.9907	0.2-100	13.03
Diclofop-methyl	18.51	253	162	15	0.9991	0.1-100	2.14
Mefenpyr-diethyl	19.15	253	189	20	0.9992	0.1-100	3.35
Pyributicarb	19.24	165	108	10	0.9973	0.1-100	2.00
Pyridafenthion	19.46	340	199	10	0.9940	0.2-100	4.71
Acetamiprid	19.39	152	116	20	1.0000	50-100	–
Bromopropylate	19.64	341	185	15	0.9956	0.1-100	3.72
Piperophos	19.84	320	122	10	0.9939	0.2-100	7.51
Fenpropathrin	19.98	265	210	10	0.9973	0.1-100	6.87
Etoxazole	20.06	300	270	20	0.9969	0.1-100	8.84
Tebufenpyrad	20.10	333	171	20	0.9978	0.5-100	13.35
Anilofos	20.31	226	157	15	0.9948	0.2-100	5.56
Phenothrin-1	20.49	183	165	10	0.9967	5-100	16.13
Tetradifon	20.54	356	229	10	0.9998	0.2-100	4.17
Phenothrin-2	20.66	183	165	10	0.9968	0.1-100	3.79
Mefenacet	21.22	192	136	15	0.9955	0.1-100	4.90
Cyhalofop-buthyl	21.23	357	229	10	0.9967	0.1-100	5.52
Cyhalothrin-1	21.30	181	152	20	0.9975	0.2-100	3.21
Cyhalothrin-2	21.66	181	152	20	0.9984	0.2-100	6.67
Pyrazophos	22.06	373	232	10	0.9963	0.1-100	10.46
Bitertanol	22.80 22.97	170	141	20	0.9873	0.1-100	6.76
Pyridaben	23.18	147	117	20	0.9958	0.1-100	1.29
Cafenstrole	24.03	100	72	5	0.9958	0.1-100	9.77
Cypermethrin-1	24.72	181	152	20	0.9983	2-100	9.29
Halfenprox	24.79	263	235	15	0.9979	0.1-100	10.25
Cypermethrin-2	24.92	181	152	20	0.9982	2-100	6.91
Cypermethrin-3	25.06	181	152	20	0.9985	2-100	16.27
Cypermethrin-4	25.13	181	152	20	0.9948	2-100	13.79
Fenvalerate-1	26.47	167	125	10	0.9977	0.1-100	3.11
Flumioxazin	26.50	354	176	20	0.9937	0.1-100	9.66
Fenvarelate-2	26.91	167	125	10	0.9979	0.1-100	3.26
Deltamethrin+ Tralomethrin	28.15	181	152	20	0.9967	0.2-100	8.20
Tolfenpyrad	29.11	383	171	20	0.9968	2-100	4.84
Imibenconazole	30.35	375	260	15	1.0000	50-100	–

Table 1: Retention times, SRM conditions, calibration range, linearity, and the reproducibility of each individual pesticide residue compound

Results and Discussion

Figure 1 shows an example calibration curve for Propazine at 0.1-100 ppb with a corresponding chromatogram at 1 ppb, showing excellent reproducibility (r^2 = 0.9998).

Figure 2 shows examples of GC/MS/MS chromatograms of various pesticides in which 1 ppb of each pesticide was added to green pepper. Even at this extremely low concentration (1/10 of the uniform standard value for pesticides), it was possible to make measurements with remarkably high sensitivity with the TSQ Quantum GC.

Figure 3 shows the chromatograms for cypermethrin, fenvalerate and deltamethrin (+ tralomethrin). Cypermethrin is a synthetic pyrethroid compound with a high detection ratio in agricultural produce. In addition to having a slow elution time in the GC, it has 4 peaks that are due to different isomers that must be resolved. As the chromatograms show, measurements with good sensitivity were obtained even at the low concentration of 5 ppb.

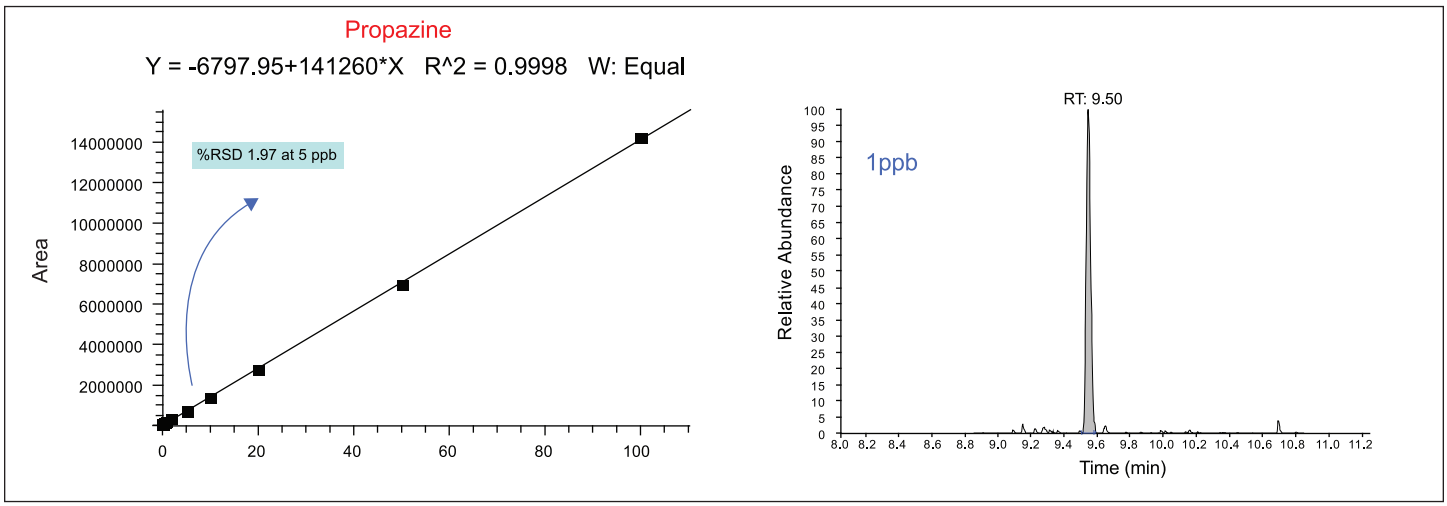

Figure 1: Calibration curve (0.1-100 ppb) and SRM chromatogram (1 ppb) for Propazine

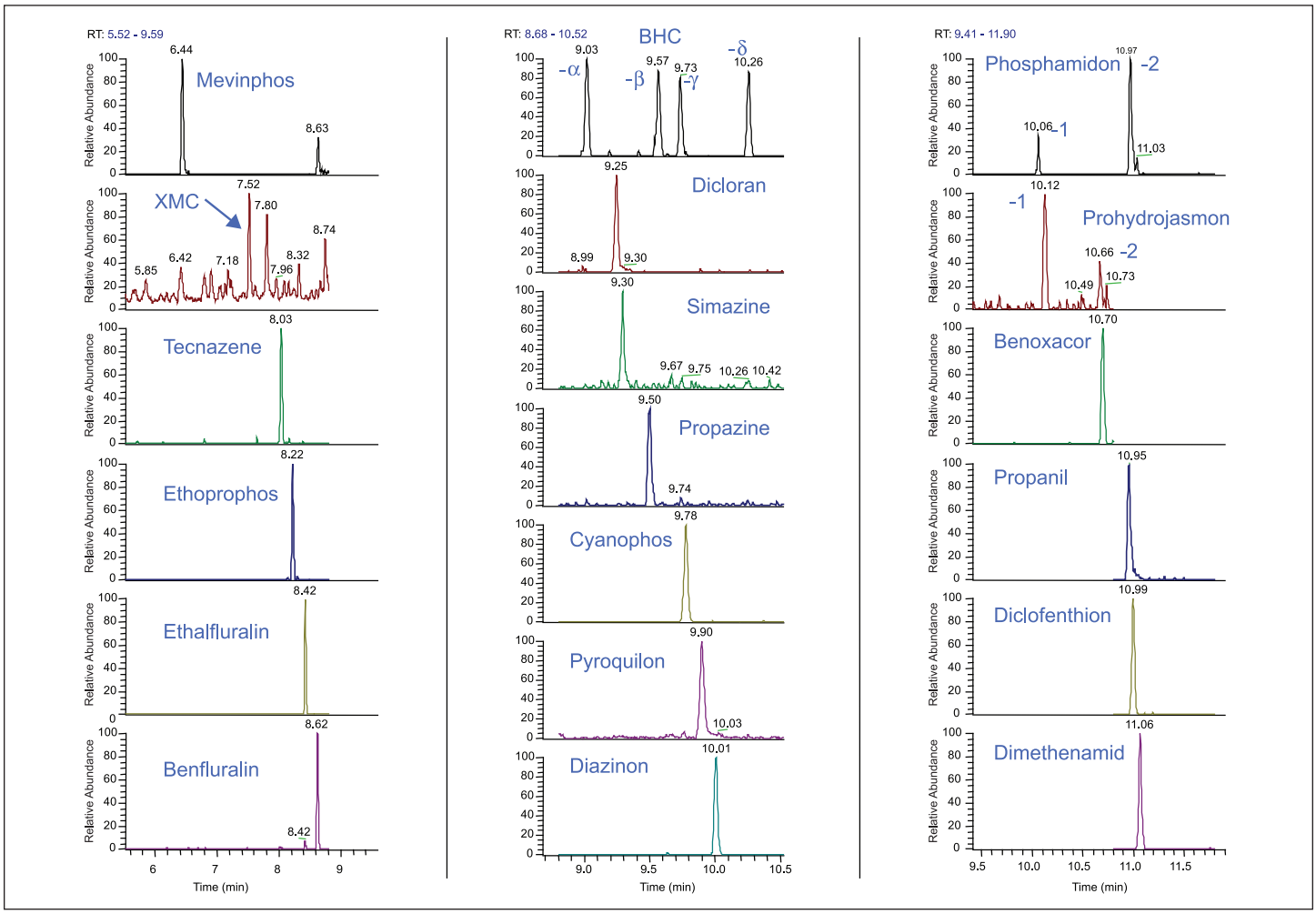

Figure 2: GC/MS/MS chromatograms of various pesticides at 1 ppb in green pepper samples

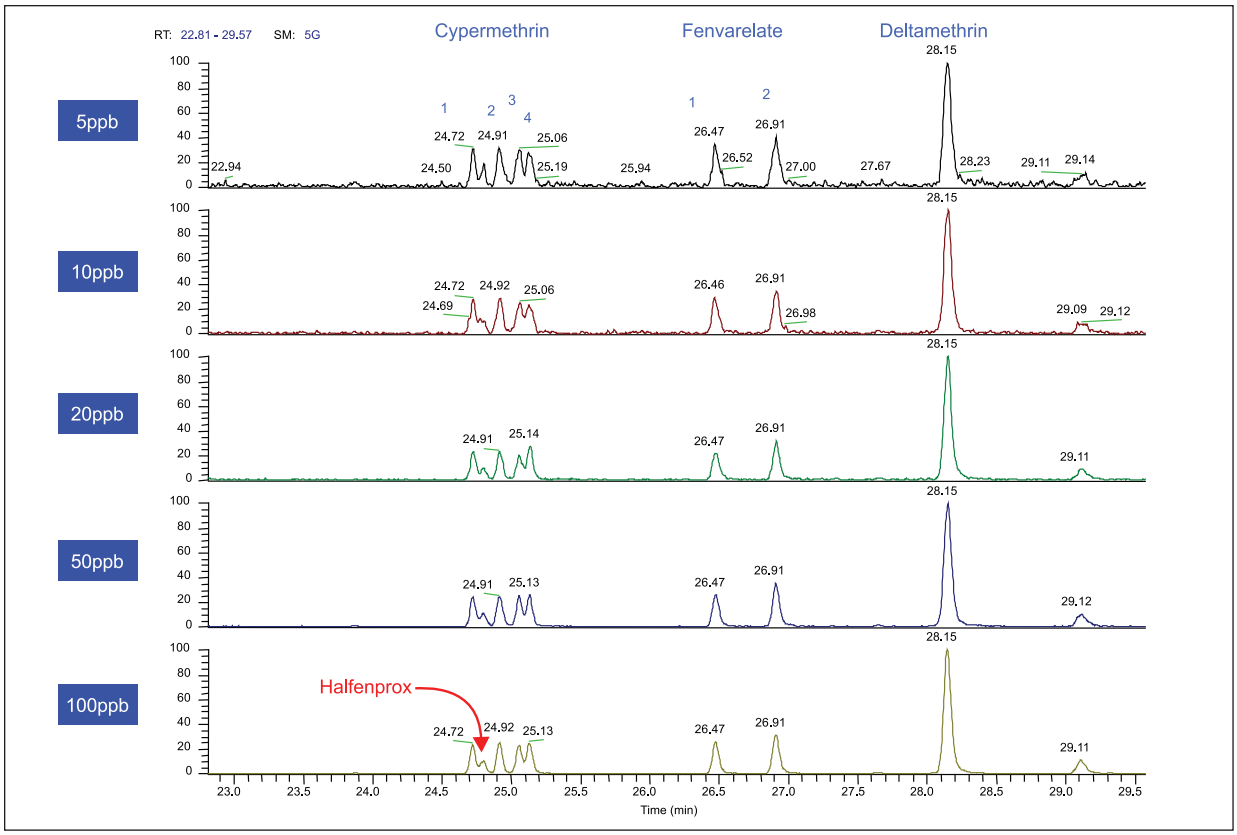

Figure 3: Chromatograms for cypermethrin, fenvalerate and deltamethrin

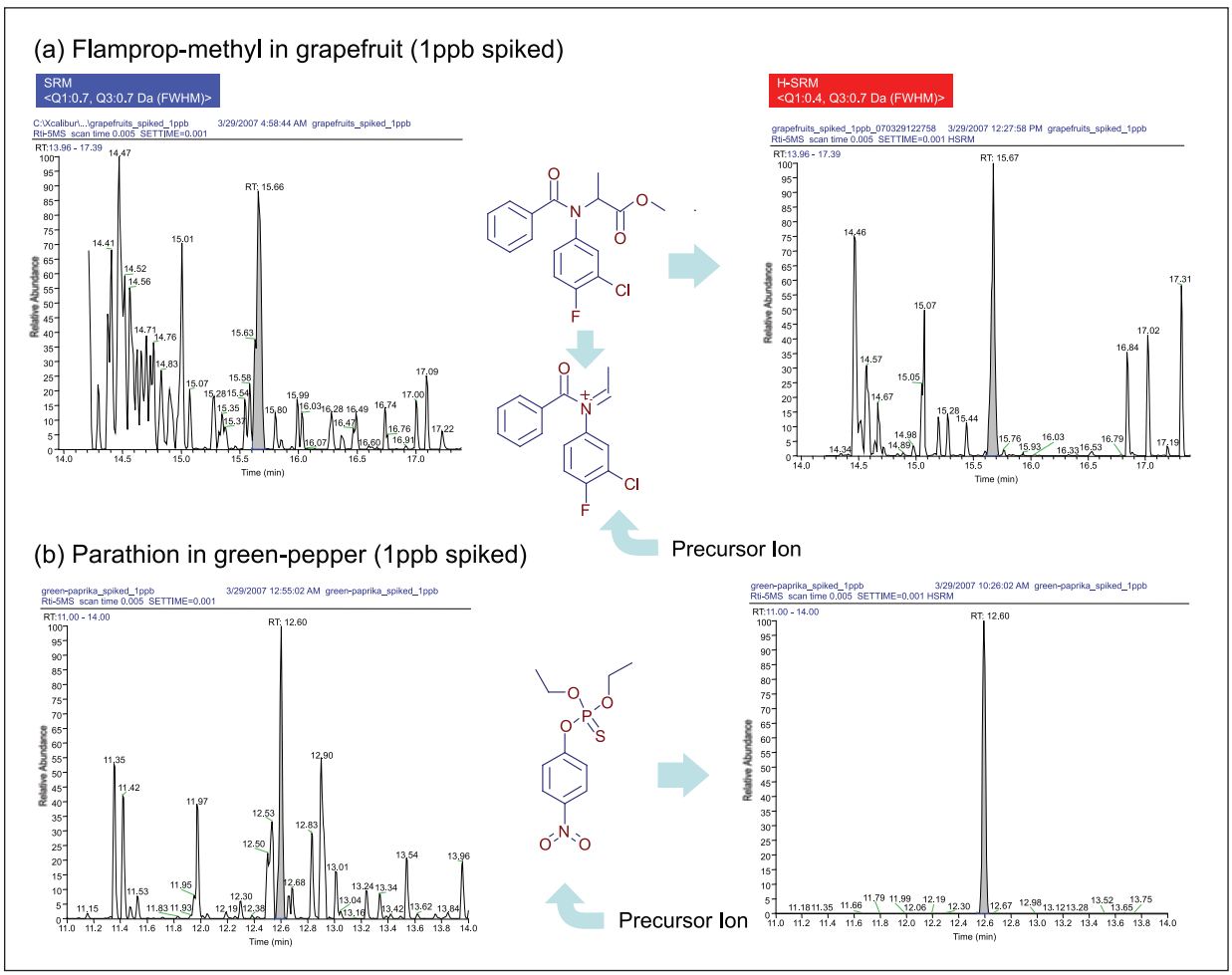

Figure 4: Comparison of SRM Mode with H-SRM Mode. (a) Flamprop-methyl in grapefruit (1 ppb). (b) Parathion in green-pepper (1 ppb).

Advantages of H-SRM

H-SRM is an acronym for Highly-Selective Reaction Monitoring, which is a more advanced form of Selective Reaction Monitoring (SRM). H-SRM can eliminate chemical noise, lower detection limits, and reduce the likelihood of generating false positives. For many pesticides that are subject to matrix-dependent interference, the measurements can be successfully carried out using the H-SRM mode. With H-SRM, the precursor ion is selected with a smaller peak width. The more stringent tolerance accounts for the higher selectivity, which can lower LOQs and increase precision and accuracy at the limits of detection. The effects of H-SRM over SRM are illustrated for flamprop-methyl in grapefruit and parathion in green-pepper in Figure 4.

Structural Confirmation with QED

QED MS/MS stands for Quantification Enhanced by Data Dependant™ MS/MS. A QED scan on a triple quadrupole instrument delivers an information rich mass spectrum that can be used for structural confirmation of analytes while undergoing quantification by SRM (or H-SRM). The specificity provided by H-SRM followed by QED MS/MS provides uncompromised quantitation performance at low levels followed by a fast, highly-specific full MS/MS scan for confirmation. Figure 5 shows the QED scan results obtained from a carrot test sample spiked with 10 ppb diazinon.

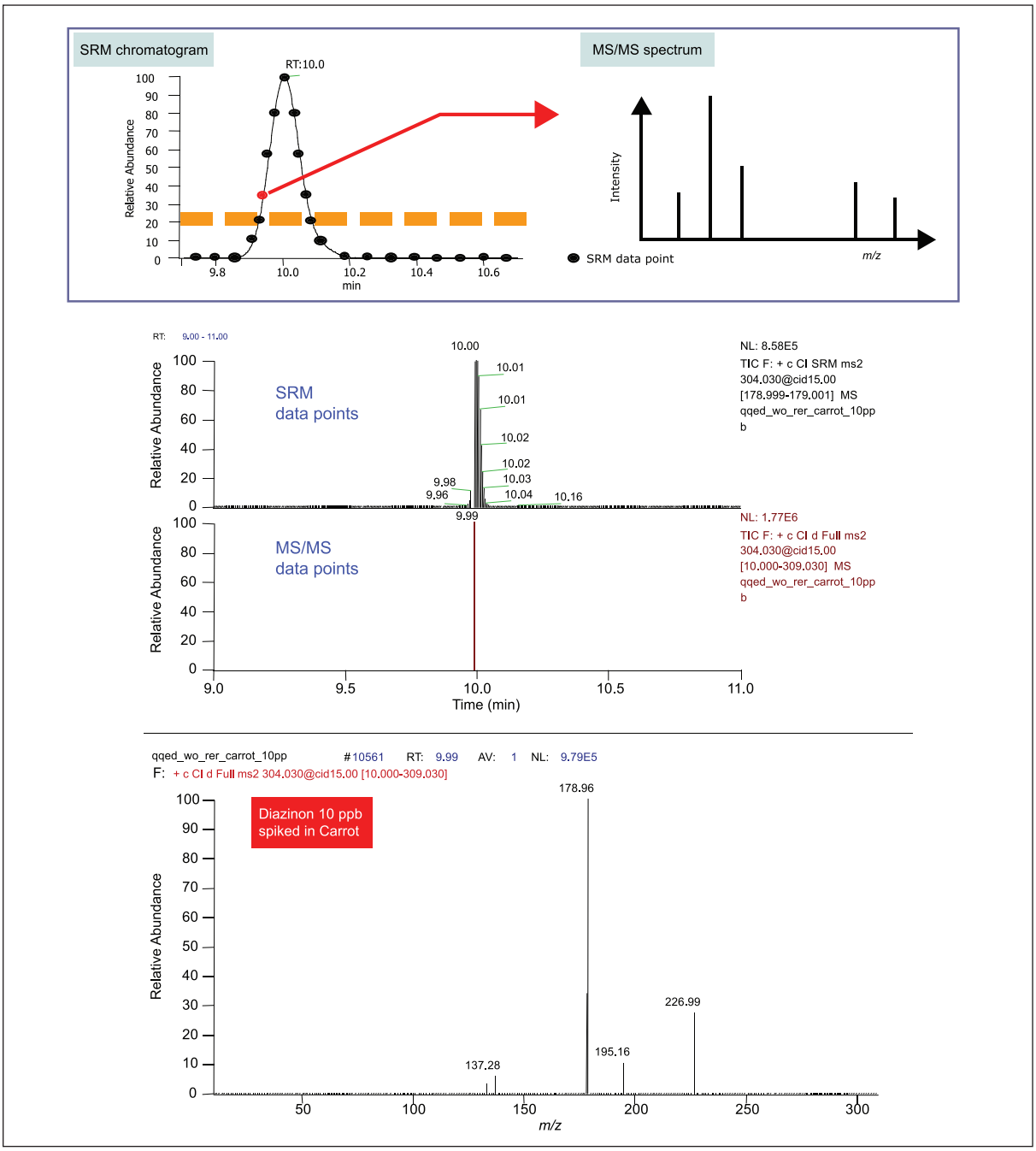

Figure 5: Chromatogram from a carrot test sample (upper row) and the MS/MS spectrum obtained with QED (lower row)

Zero Cross-talk

Cross-talk can potentially occur when fragment ions from one SRM transition remain in the collision cell while a second SRM transition takes place. This can cause signal artifacts in the second SRM transition's chromatogram. It can be especially problematic when different SRM events have the same product ions formed from different precursor ions. However, the orthogonal design of the collision cell in the TSQ Quantum eliminates cross-talk. Figure 6 shows the absence of cross-talk between two different SRM transitions of paclobutrazol and thifluzamide. Both yield a product ion of *m/z* 125, but no artifacts are seen in either chromatogram with a scan time of 10 ms. Similarly, the SRM transitions of triszophos and diclofop-methyl 5 also show no evidence of cross talk, even though they both yield product ions at *m/z* 162.

Conclusion

Simultaneous analysis was carried out on multi-component pesticide residues in food products using a quadrupole GC/MS/MS system, the TSQ Quantum GC. Results obtained indicated excellent sensitivity (0.1 ppb), reproducibility (10% at 5 ppb) and linearity (R2 > 0.995) in the range of 0.1-100 ppb. No cross-talk was observed for the analysis of closely eluting multi-component mixtures. Using H-SRM, interferences from the sample matrix background were substantially reduced, leading to improved LOQs. In addition, QED provided MS/MS structural confirmation of the analytes undergoing quantification.

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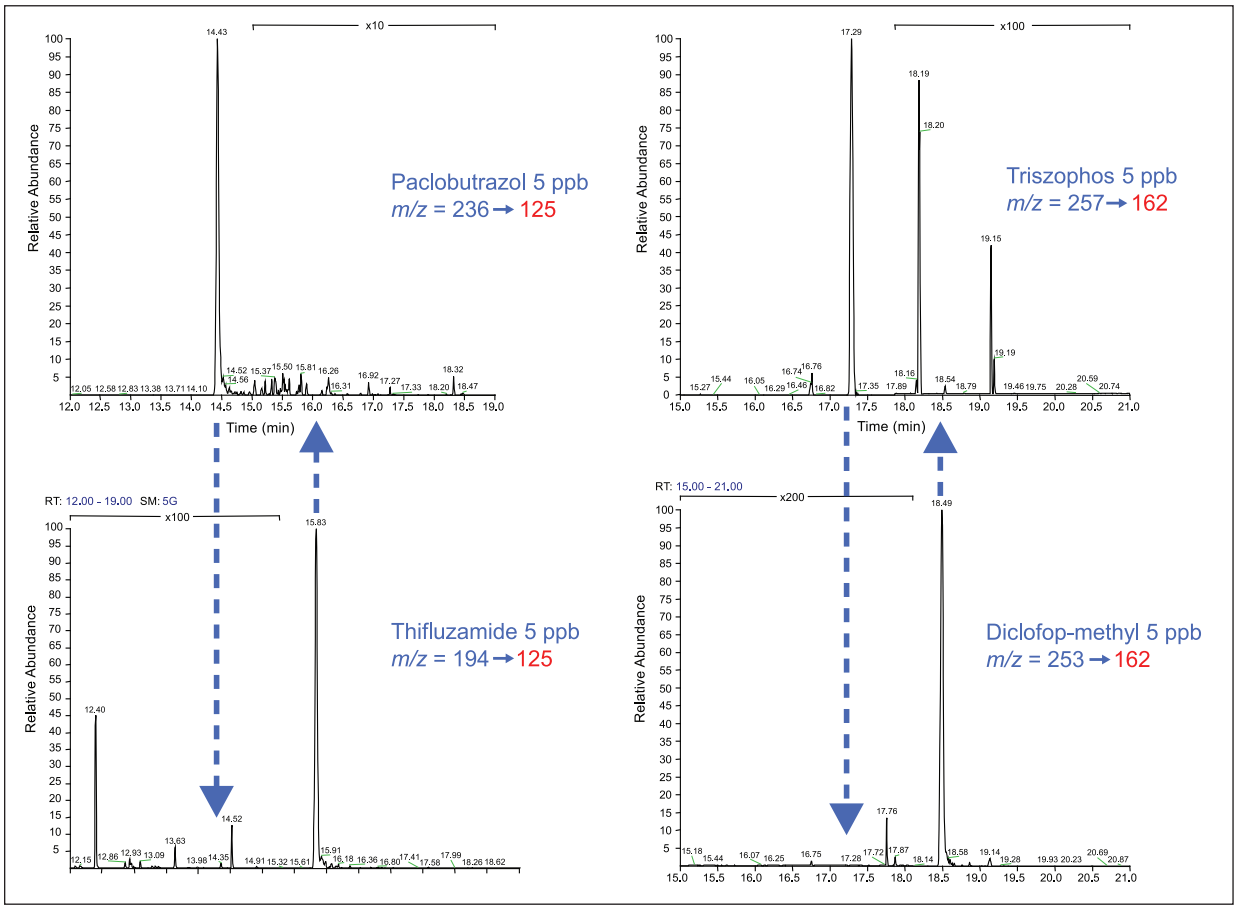

Figure 6: No cross-talk was observed in the SRM transitions of paclobutrazol and thifluzamide or in the SRM transitions of triszophos and diclofop-methyl.

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Application Note: 51878

Non-targeted Screening and Accurate Mass Confirmation of 510 Pesticides on the High Resolution Exactive Benchtop LC/MS Orbitrap Mass Spectrometer

Allen Zhang, James S. Chang, Christine Gu, Mark Sanders, Thermo Fisher Scientific, San Jose, CA, USA

Key Words

- Exactive
- High Mass Accuracy
- High Resolution
- Orbitrap Technology
- Pesticide Analysis

Overview

As agricultural trade grows and food safety concerns mount, stricter pesticide regulations are being enforced around the world. Increased pesticide testing and reductions in maximum permissible residue levels have driven demand for fast, sensitive and cost-effective analytical methods for high-throughput screening of multi-class pesticides in food. Detection of 510 pesticides at low ppb levels was achieved within 12 minutes using the Thermo Scientific Exactive benchtop LC/MS system powered by Orbitrap technology. The high resolving power of the Thermo Scientific Orbitrap platform enables accurate mass confirmation of all compounds, including isobaric pesticides. Accurate, robust, easy to use and cost-efficient, the Exactive™ LC/MS is ideally suited for routine, comprehensive screening of targeted and non-targeted pesticides at or below the 0.01 mg/kg (10 ppb) default limit set by EU and Japanese legislation.

Introduction

In 2007, the United States Environmental Protection Agency (EPA) completed a ten-year reassessment of 9,721 pesticide tolerances to meet more stringent safety standards and recommended the revocation or modification of thousands of uses of pesticides in food.[1] China published national standard GB 2763-2005 in 2005, which established 478 maximum residue levels (MRLs) for 136 pesticides.[2] Japan's Positive List System, introduced in 2006, established MRLs for hundreds of agricultural chemicals, including approximately 400 pesticides, in food and set a uniform limit of 10 ppb to chemicals for which MRLs have not been determined.[3] Regulation (EC) No. 396/2005 of the European Parliament, implemented in 2008, harmonized all pesticide MRLs for European Union (EU) member states and set default limits of 0.01 mg/kg for all pesticide/commodity combinations for which no MRLs have been set.[4] A pesticide safety review of about 1,000 active substances on the market was mandated by EU Directive 91/414/EEC and, upon completion in 2009, led to the approval of only about 250 substances, effectively setting the permissible levels of over 700 de-listed pesticides to the default limit.[5] The EU and Japanese regulations are among the most stringent in the world and have fueled the need for faster and more sensitive analytical methods for cost-efficient, high-throughput screening of multi-class pesticide residues.

Pesticides in food were traditionally monitored and quantified using gas chromatography (GC) coupled with either selective detectors (e.g. electron capture) or mass spectrometry (MS). GC/MS continues to be widely used in pesticide analysis because it is highly selective, provides confirmation of multiple classes of pesticides in a single analytical run, and is relatively inexpensive and easy to operate. However, GC/MS cannot detect polar, thermally unstable or low volatility compounds without derivatization. Recent improvements in liquid chromatography (LC) throughput and MS detection capabilities have led to a surge in the use of LC/MS-based techniques for screening, confirmation and quantitation of ultra-trace levels of multi-class pesticide residues, including those that are not GC-amenable. LC-triple quadrupole tandem MS (LC/MS/MS) enables highly selective and sensitive quantification and confirmation of hundreds of target pesticides in a single run, but this approach requires extensive compound-dependent parameter optimization and cannot be used to screen for untargeted pesticides. Full scan approaches using high performance time-of-flight (TOF) or Orbitrap™ mass spectrometers coupled to ultra-high pressure LC (U-HPLC) facilitate rapid and sensitive screening and detection of LC-amenable pesticide residues present in a sample. The superior resolving power of the Orbitrap mass spectrometer (up to 100,000 FWHM) compared to TOF instruments (10,000–20,000) ensures the high mass accuracy required for complex sample analysis.[6] High resolution LC/MS instrumentation, however, can be cost-prohibitive for many routine monitoring laboratories.

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The Thermo Scientific Exactive benchtop LC/MS Orbitrap mass spectrometer was designed for accurate and reliable screening of complex samples in a wide range of demanding high-throughput applications. Built on Orbitrap mass analyzer technology, the Exactive delivers exceptional mass resolution (up to 100,000) to ensure highly accurate mass measurements and to enable confident discrimination of co-eluting, isobaric compounds in complex samples.[6,7] A wide in-scan dynamic range (3-4 orders of magnitude) facilitates the detection of trace levels of compounds in the presence of highly abundant matrix interferences. High scan speeds and polarity switching ensure full compatibility with U-HPLC and high-throughput methods. Cost-effective and easy to operate, the Exactive is an ideal tool for compliance monitoring in regulatory labs. In this note, we demonstrate rapid screening and accurate mass confirmation of 510 pesticides at low ppb levels using U-HPLC coupled to a high resolution Exactive benchtop Orbitrap mass spectrometer. Full scan U-HPLC-single stage Orbitrap MS can be used to screen a virtually limitless number of pesticides and, unlike MS/MS methods, does not require compound-dependent parameter optimization.

Materials and Methods

Sample Preparation

Pesticide standards were obtained from the U.S. Food and Drug Administration (FDA). A stock solution of a mixture of 510 pesticides was prepared at a concentration of 3 mg/L. Calibration solutions, with concentrations of 1-250 ppb, were prepared by serial dilution of the stock solution in 50:50 (v/v) acetonitrile/water.

Spiked spinach samples were prepared for analysis using a modified QuEChERS method (Figure 1). QuEChERS, an acronym for Quick, Easy, Cheap, Effective, Rugged, and Safe, is a sample preparation procedure used to extract pesticides from food.[8] Malathion D6 was used as an internal standard for calibration.

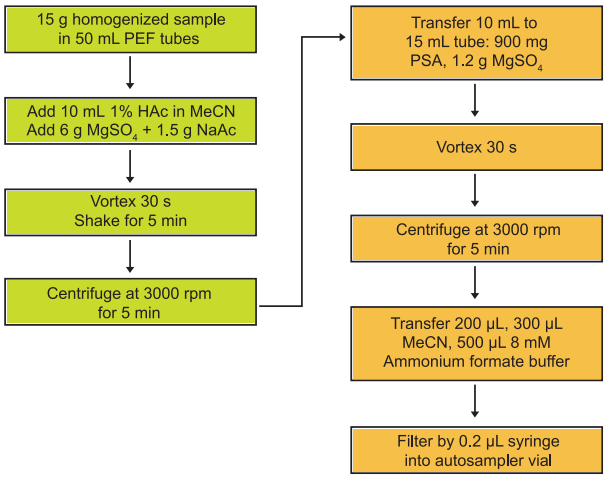


Figure 1: Schematic of the modified QuEChERS workflow used to extract pesticides from spinach matrices

Experimental Conditions

Instrumentation

LC/MS analysis was performed using a Thermo Scientific Accela U-HPLC system. With a CTC Analytics PAL autosampler coupled to an Exactive benchtop Orbitrap mass spectrometer (Figure2). Data acquisition was performed using Thermo Scientific Xcaliber software. Thermo Scientific Pathfinder software was used for data processing.

Figure 2: LC/MS analysis was performed using an Accela™ U-HPLC system coupled to an Exactive benchtop Orbitrap mass spectrometer

LC Parameters

Column:	Thermo Scientific Hypersil GOLD aQ C18 column (100 x 2.1 mm, 1.9 µm particle size)		
Mobile Phase:	A: Water with 0.1% formic acid and 4 mM ammonium formate B: Methanol with 0.1% formic acid and 4 mM ammonium formate		
Flow Rate:	300 µL/min		
Column Temperature:	ambient		
Sample Injection Volume:	10 µL		
Gradient:	Time (min)	%A	%B
	0	100	0
	1	100	0
	8	0	100
	12	0	100
	12.5	100	0
	14	100	0

MS Parameters

Full mass scan positive/negative ion mode (mass range = 100 to 1500)	
Resolution:	50,000
Automatic Gain Control (AGC) Target Value:	10e6
Heated Electrospray Ionization Source Conditions:	
Spray Voltage:	2200 V
Capillary Temperature:	280 °C
Sheath Gas:	32 au
Auxiliary Gas:	7 au
Vaporizer Temperature:	200 °C

Results and Discussion

U-HPLC improves chromatographic resolution, speed and sensitivity, and when coupled to MS, facilitates rapid, high-throughput analysis of challenging samples. Using U-HPLC-single stage Orbitrap MS, a mixture of 510 pesticides representing a broad spectrum of chemical classes was separated and detected within 12 minutes (Table 1). High resolution (50,000) and high mass accuracy (< 5 ppm without internal calibration for most compounds) enabled identification of all analytes (Table 1). Separation of isobaric pesticides was achieved only at the high resolving powers provided by Orbitrap MS, as demonstrated in Figure 3. Excellent linearity in detector response was observed over the range of 1-250 ppb, with correlation coefficients greater than 0.99 for the majority of pesticides (Table 1). Chromatograms and calibration curves for eight representative pesticides are shown in Figure 4. For the concentration range studied (1-250 ppb), limits of quantitation (LOQs) were estimated from triplicate injections (CV < 15%) of standard solutions at concentration levels corresponding to a signal-to-noise ratio of 10. As shown in Table 1, LOQs ranged from 1-50 ppb, and for 499 pesticides, LOQs were at or below 10 ppb, the MRL imposed by EU and Japanese regulations.

To evaluate the applicability of this technique to complex food samples, U-HPLC-single stage Orbitrap MS was used to screen for pesticides extracted from a spiked spinach matrix. An extraction procedure based on fast and efficient QuEChERS methodology was used to facilitate rapid high-throughput multiresidue analysis. Table 2 summarizes this and mass spectral data obtained for a representative set of extracted pesticides. Extracted ion chromatograms and calibration curves for six pesticides extracted from the spiked spinach matrix are depicted in Figure 5. The detection and quantitation capabilities of this method were assessed using the EPA method detection limit (MDL) procedure.[9] For all pesticides, limits of detection (LODs) and LOQs were lower than 1 ppb (Table 2).

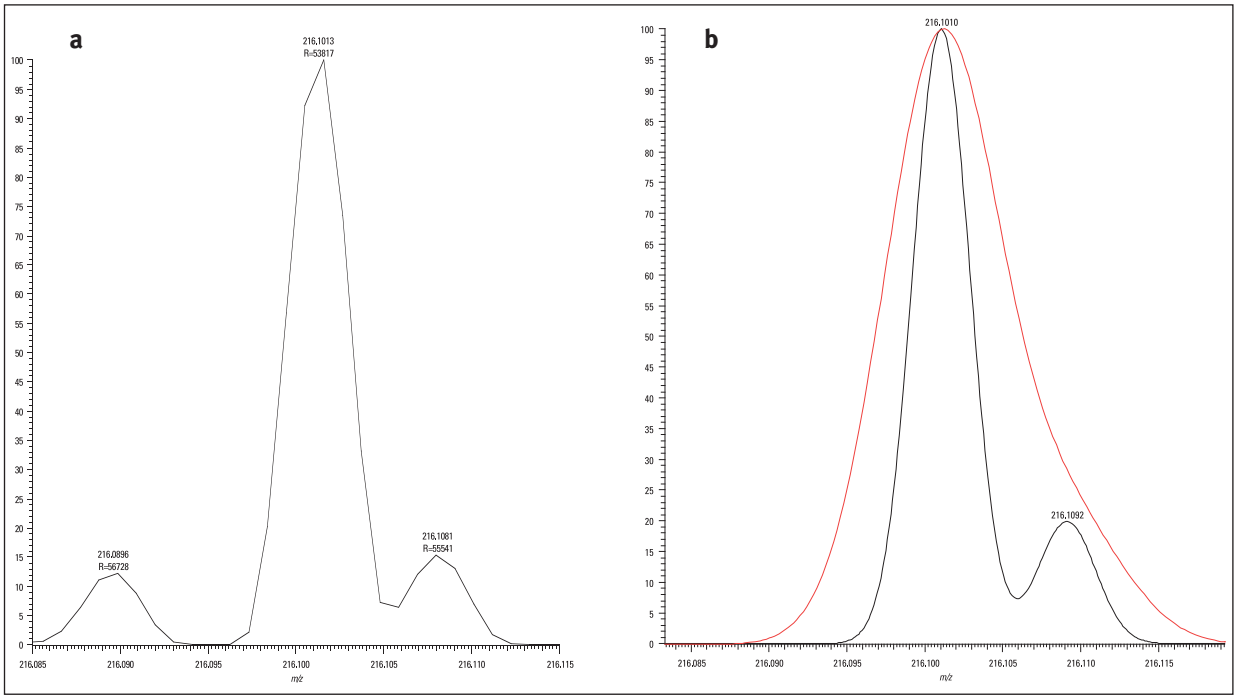


Figure 3: The high resolving power of the Exactive benchtop Orbitrap mass spectrometer enabled separation of the $[M+H]^+$ ion of atrazine (m/z = 216.1012) from the $[M+NH_4]^+$ ion of cymoxanil (m/z = 216.1088). (a) Mass spectra of the two isobaric pesticides at a resolution of 50,000. (b) Simulated mass spectra of the isobaric pesticides at resolutions of 25,000 (red line) and 50,000 (black line).

LC/MS data for Pesticide Standards *(Table 1)*

Compound	Formula	Polarity	Theoretical Mass (*m/z*)	Experimental Mass (*m/z*)	Mass Deviation (ppm)	LOQ (ppb)	R^2
Abamectin B1a	C48H72O14	+	890.526	890.5261	0	10	0.9898
Abamectin B1b	C47H70O14	+	876.5104	876.5138	3.8	10	0.9315
Acephate	C4H10NO3PS	+	184.0192	184.0193	0.8	1	0.9994
Acequinocyl	C24H32O4	+	402.2639	402.2638	0.2	1	0.9886
Acetamiprid	C10H11C N4	+	223.0745	223.0747	0.7	1	0.9989
Acibenzolar S-methyl	C8H6N2OS2	+	210.9994	211.0004	4.4	10	0.9936
Acifluorfen	C14H7ClF3NO5	-	359.9892	359.9896	1.1	1	0.9961
Aclonifen	C12H9ClN2O3	+	282.064	282.065	3.6	25	0.9812
Acrinathrin	C26H21F6NO5	+	559.1662	559.1664	0.3	1	0.9931
Akton	C12H14Cl3O3PS	+	374.954	374.9536	1	25	0.9859
Alachlor	C14H20C NO2	+	270.1255	270.1255	0.3	1	0.9890
Alanycarb	C17H25N3O4S2	+	400.1359	400.1369	2.5	1	0.9049
Aldicarb	C7H14N2O2S	+	208.1114	208.1116	0.6	1	0.9989
Aldicarb sulfone	C7H14N2O4S	+	223.0747	223.0747	0.3	1	0.9987
Aldicarb sulfoxide	C7H14N2O3S	+	207.0798	207.0798	0.1	1	0.9998
Allethrin	C19H26O3	+	303.1955	303.1957	0.6	1	0.9983
Allidochlor	C8H12ClNO	+	174.068	174.068	0	1	0.9936
Ametryn	C9H17N5S	+	228.1277	228.1278	0.4	1	0.9979
Amicarbazone	C10H19N5O2	+	242.1612	242.1612	0.1	1	0.9986
Aminocarb	C11H16N2O2	+	209.1285	209.1285	0.3	1	0.9997
Aminopyralid	C6H4Cl2N2O2	-	204.9577	204.9571	2.9	1	0.9629
Amitraz	C19H23N3	+	294.1965	294.1965	0.1	1	0.9725
Ancymidol	C15H16N2O2	+	257.1285	257.1284	0.4	1	0.9954
Anilazine	C9H5Cl3N4	-	272.9507	272.9572	2.4	1	0.9653
Anilofos	C13H19C NO3PS2	+	368.0305	368.0304	0.3	1	0.9986
Anilofos	C13H19C NO3PS2	+	368.0305	368.0304	0.3	1	0.9971
Antimycin A	C28H40N2O9	-	547.2661	547.2668	1.2	1	0.9928
Aramite	C15H23ClO4S	+	352.1344	352.1345	0.4	1	0.9946
Aspon	C12H28O5P2S2	+	379.0926	379.0927	0.1	1	0.9853
Asulam	C8H10N2O4S	+	248.07	248.07	0	1	0.9986
Atrazine	C8H14ClN5	+	216.1011	216.1012	0.8	1	0.9991
Azaconazole	C12H11Cl2N3O2	+	300.0301	300.0302	0.1	1	0.9940
Azadirachtin	C35H44O16	+	738.2968	738.2968	0	1	0.9904
Azafenidrin	C15H13Cl2N3O2	+	338.0458	338.0458	0	1	0.9932
Azamethiphos	C9H10ClN2O5PS	+	324.9809	324.981	0.2	1	0.9991
Azinphos methyl oxon	C10H12N3O4PS	+	302.0359	302.0359	0.1	1	0.9969
Azinphos-ethyl	C12H16N3O3PS2	+	346.0444	346.0443	0.1	1	0.9906
Azinphos-methyl	C10H12N3O3PS2	+	318.0131	318.0129	0.3	1	0.9957
Azoxystrobin	C22H17N3O5	+	404.1241	404.124	0.2	1	0.9948
Barban	C11H9Cl2NO2	+	275.0349	275.0355	2.4	10	0.9953
Benalaxyl	C20H23NO3	+	326.1751	326.175	0.4	1	0.9986
Benazolin	C9H6C NO3S	+	243.983	243.9827	1.2	1	0.9863
Bendiocarb	C11H13NO4	+	224.0917	224.0919	0.8	1	0.9993
Benfluralin	C13H16F3N3O4	+	353.1431	353.143	0.2	1	0.9883
Benfuracarb	C20H30N2O5S	+	428.2214	428.2211	0.6	1	0.9956
Benodanil	C13H10INO	+	323.988	323.9879	0.4	1	0.9925
Benoxacor	C11H11Cl2NO2	+	260.024	260.024	0.1	10	0.9989
Bensulide	C14H24NO4PS3	+	415.0943	415.0944	0.1	1	0.9872
Bentazone	C10H12N2O3S	+	241.0641	241.0643	0.5	1	0.9982
Benthiavalicarb	C15H18FN3O3S	+	340.1126	340.114	4.2	1	0.9047
Benzoximate	C18H18C NO5	+	364.0946	364.0944	0.7	1	0.9965
Bifenazate	C17H20N2O3	+	301.1547	301.1546	0.1	1	0.9892
Bifenox	C14H9Cl2NO5	+	359.0196	359.0193	0.8	10	0.9668
Bifenthrin	C23H22C F3O2	+	423.1333	423.1322	2.6	10	0.9729
Binapacryl	C15H18N2O6	+	340.1503	340.1496	2.2	10	0.9688
Bispyribac-sodium	C19H17N4NaO8	+	453.1017	453.1018	0.1	10	0.9843
Bitertanol	C20H23N3O2	+	338.1863	338.1861	0.5	1	0.9916
Boscalid	C18H12Cl2N2O	+	343.04	343.0399	0.1	1	0.9797
Brodifacoum	C31H23BrO3	-	521.0758	521.0755	0.5	1	0.9905

Compound	Formula	Polarity	Theoretical Mass (*m/z*)	Experimental Mass (*m/z*)	Mass Deviation (ppm)	LOQ (ppb)	R^2
Bromadiolone	C30H23BrO4	-	525.0707	525.0706	0.1	1	0.9879
Bromoxynil	C7H3Br2NO	-	273.8509	273.8506	1	1	0.9990
Bromuconazole(cis-)	C13H12BrCl2N3O	+	375.9614	375.9613	0	1	0.9961
Bromuconazole(trans-)	C13H12BrCl2N3O	+	375.9614	375.9613	0	1	0.9912
Bufencarb	C13H19NO2	+	222.1489	222.149	0.6	1	0.9965
Bupirimate	C13H24N4O3S	+	317.1642	317.1641	0.2	1	0.9978
Buprofezin	C16H23N3OS	+	306.1635	306.1632	0.7	1	0.9974
Butachlor	C17H26ClNO2	+	329.199	329.1989	0.3	10	0.9928
Butafenacil	C20H18ClF3N2O6	+	492.1144	492.1144	0.1	1	0.9981
Butocarboxim	C7H14N2O2S	+	208.1114	208.1116	0.6	1	0.9971
Butoxycarboxim	C7H14N2O4S	+	223.0747	223.0747	0.3	1	0.9990
Butralin	C14H21N3O4	+	296.1605	296.1604	0.4	1	0.9983
Butylate	C11H23NOS	+	218.1573	218.1575	0.9	1	0.9993
Cadusafos	C10H23O2PS2	+	271.095	271.0948	0.8	1	0.9874
Carbaryl	C12H11NO2	+	202.0863	202.0855	3.9	1	0.9923
Carbendazim	C9H9N3O2	+	192.0768	192.0767	0.4	1	0.9986
Carbetamide	C12H16N2O3	+	237.1234	237.1235	0.6	1	0.9982
Carbofuran	C12H15NO3	+	222.1125	222.1126	0.4	1	0.9980
Carbofuran, 3OH-	C12H15NO4	+	255.1339	255.1338	0.5	1	0.9986
Carboxin	C12H13NO2S	+	236.074	236.074	0.3	1	0.9972
Carfentrazone-ethyl	C15H14Cl2F3N3O3	+	429.0703	429.0702	0.1	1	0.9957
Carpropamid	C15H18Cl3NO	+	334.0527	334.0526	0.1	1	0.9982
Chinomethionate	C10H6N2OS2	+	252.026	252.0267	2.7	10	0.9963
Chlorantraniliprole	C18H14BrCl2N5O2	+	481.9781	481.978	0.1	1	0.9718
Chlorbromuron	C9H10BrClN2O2	+	292.9687	292.9688	0.2	1	0.9958
Chlorbufam	C11H10ClNO2	+	241.0738	241.073	3.5	1	0.9864
Chlordimeform	C10H13ClN2	+	197.084	197.084	0.1	10	0.9973
Chlorfenvinphos	C12H14Cl3O4P	+	358.9768	358.9767	0.2	1	0.9976
Chlorfluazuron	C20H9Cl3F5N3O3	+	556.9968	556.9968	0.1	1	0.9963
Chloroxuron	C15H15ClN2O2	+	291.0895	291.0893	0.8	1	0.9978
Chlorpropham	C10H12ClNO2	+	214.0629	214.0632	1.3	10	0.9910
Chlorpyrifos	C9H11Cl3NO3PS	+	349.9336	349.9336	0	1	0.9951
Chlorpyrifos oxon	C9H11Cl3NO4P	+	333.9564	333.9564	0.1	1	0.9903
Chlorpyrifos-methyl	C7H7Cl3NO3PS	+	321.9023	321.9022	0.1	25	0.9763
Chlorthiamid	C7H5Cl2NS	+	222.9858	222.9852	2.8	10	0.9857
Chlorthion	C8H9C NO5PS	+	314.9966	314.9971	1.6	25	0.9812
Chlorthiophos	C11H15Cl2O3PS2	+	360.965	360.9643	1.9	25	0.9632
Chlortoluron	C10H13ClN2O	+	213.0789	213.079	0.6	1	0.9976
Clethodim	C17H26ClNO3S	+	360.1395	360.1395	0.2	1	0.9923
Clofentezine	C14H8Cl2N4	+	320.0464	320.045	4.5	10	0.9935
Clothianidin	C6H8C N5O2S	+	250.016	250.016	0.2	1	0.9916
Coumaphos	C14H16ClO5PS	+	363.0217	363.0217	0.1	1	0.9983
Coumaphos oxon	C14H16ClO6P	+	347.0446	347.0446	0	1	0.9951
Crotoxyphos	C14H19O6P	+	332.1258	332.1255	0.7	1	0.9982
Crufomate	C12H19ClNO3P	+	309.1129	309.112	3.1	1	0.9914
Cumyluron	C17H19ClN2O	+	303.1259	303.1258	0.2	1	0.9989
Cyanazine	C9H13ClN6	+	241.0963	241.0963	0.2	1	0.9951
Cyazofamid	C13H13ClN4O2S	+	342.0786	342.077	4.6	1	0.9895
Cyclanilide	C11H9Cl2NO3	-	271.9887	271.9891	1.8	1	0.9991
Cycloate	C11H21NOS	+	216.1417	216.1418	0.4	1	0.9913
Cyclohexamide	C15H23NO4	+	299.1965	299.1966	0.3	1	0.9977
Cycluron	C11H22N2O	+	199.1805	199.1805	0.1	1	0.9922
Cyflufenamid	C20H17F5N2O2	+	413.1283	413.1282	0.2	1	0.9977
Cyfluthrin	C22H18Cl2FNO3	+	451.0986	451.098	1.3	10	0.7124
Cyhalothrin	C23H19ClF3NO3	+	467.1344	467.1339	1	1	0.9859
Cymoxanil	C7H10N4O3	+	216.1091	216.1088	1.3	1	0.9885
Cypermethin	C22H19Cl2NO3	+	433.108	433.108	0	10	0.9859
Cyphenothrin	C24H25NO3	+	393.2173	393.2173	0	1	0.9959
Cyproconazole	C15H18ClN3O	+	292.1211	292.1211	0.2	1	0.9978

LC/MS data for Pesticide Standards *(Table 1 continued)*

Compound	Formula	Polarity	Theoretical Mass (*m/z*)	Experimental Mass (*m/z*)	Mass Deviation (ppm)	LOQ (ppb)	R^2
Cyprodinil	C14H15N3	+	226.1339	226.1339	0.3	1	0.9967
Cyprosulfamide	C18H18N2O5S	+	375.1009	375.1009	0.1	1	0.9977
Cyromazine	C6H10N6	+	167.104	167.1039	0.2	1	0.9445
Daimuron	C17H20N2O	+	269.1648	269.1646	0.7	1	0.9992
Dazomet	C5H10N2S2	+	163.0358	163.0358	0.1	1	0.9451
DEF (Tribufos)	C12H27OPS3	+	315.1034	315.1033	0.3	1	0.9840
Deltamethrin	C22H19Br2NO3	+	521.007	521.0073	0.5	1	0.9986
Demeton S-methyl	C6H15O3PS2	+	231.0273	231.0275	0.9	1	0.9966
Demeton S-sulfone	C6H15O5PS2	+	263.0171	263.0173	0.8	10	0.9914
Demeton-O	C8H19O3PS2	+	259.0586	259.0586	0.1	1	0.9960
Demeton-S (Disulfoton oxon)	C8H19O3PS2	+	259.0586	259.0586	0.1	1	0.9960
Desmedipham	C16H16N2O4	+	318.1448	318.1448	0	1	0.9975
Desmetryn	C8H15N5S	+	214.1121	214.1122	0.6	1	0.9986
Dialifor	C14H17C NO4PS2	+	411.0363	411.0363	0.1	1	0.9984
Diallate	C10H17Cl2NOS	+	270.0481	270.0482	0.5	1	0.9636
Diamidafos (Nellite)	C8H13N2O2P	+	201.0787	201.0787	0	1	0.9986
Diazinon	C12H21N2O3PS	+	305.1083	305.1081	0.9	1	0.9983
Diazinon hydroxy	C12H21N2O4PS	+	321.1032	321.1031	0.6	1	0.9985
Diazinon oxon	C12H21N2O4P	+	289.1312	289.1311	0.2	1	0.9385
Dicapthon	C8H9C NO5PS	+	314.9966	314.9971	1.6	25	0.9812
Dichlofluanid	C9H11Cl2FN2O2S2	+	349.9961	349.9961	0.2	1	0.9930
Dichlorfenthion	C10H13Cl2O3PS	+	314.9773	314.9768	1.5	10	0.9966
Dichlormid	C8H11Cl2NO	+	208.0291	208.0292	0.6	1	0.9923
Dichlorvos	C4H7Cl2O4P	+	220.9532	220.9533	0.4	10	0.9920
Diclobutrazol	C15H19Cl2N3O	+	328.0978	328.0978	0.1	1	0.9949
Dicrotophos	C8H16NO5P	+	238.0839	238.0839	0.2	1	0.9991
Diethofencarb	C14H21NO4	+	268.1543	268.1543	0.1	1	0.9994
Difenacoum	C31H24O3	+	445.1798	445.1798	0.1	1	0.9972
Difenoconazole	C19H17Cl2N3O3	+	406.072	406.0719	0.3	1	0.9914
Diflenoxuron	C16H18N2O3	+	287.139	287.1389	0.6	1	0.9938
Diflubenzuron	C14H9ClF2N2O2	-	309.0248	309.0246	0.6	1	0.9985
Dimepiperate	C15H21NOS	+	264.1417	264.1429	4.9	1	0.9994
Dimethachlor	C13H18C NO2	+	256.1099	256.1098	0.3	1	0.9921
Dimethametryn	C11H21N5S	+	256.159	256.1588	0.8	1	0.9983
Dimethenamid	C12H18C NO2S	+	276.082	276.0818	0.5	1	0.9977
Dimethoate	C5H12NO3PS2	+	230.0069	230.007	0.3	1	0.9993
Dimethomorph	C21H22C NO4	+	388.131	388.131	0	1	0.9970
Dimethylvinphos. Z-	C10H10Cl3O4P	+	330.9455	330.9455	0.1	1	0.9950
Dimetilan	C10H16N4O3	+	241.1295	241.1295	0.1	1	0.9990
Dimoxystrobin	C19H22N2O3	+	327.1703	327.1702	0.4	1	0.9905
Diniconazole	C15H17Cl2N3O	+	326.0821	326.0821	0.2	1	0.9899
Dinotefuran	C7H14N4O3	+	203.1139	203.1139	0.1	1	0.9957
Dioxacarb	C11H13NO4	+	224.0917	224.0919	0.8	1	0.9978
Dioxathion	C12H26O6P2S4	+	474.0426	474.0426	0	1	0.9900
Diphenamid	C16H17NO	+	240.1383	240.1383	0.1	1	0.9992
Diphenylamine	C12H11N	+	170.0964	170.0965	0.3	1	0.9952
Dipropetryn	C11H21N5S	+	256.159	256.1588	0.8	1	0.9983
Disulfoton	C8H19O2PS3	+	275.0358	275.0355	0.9	1	0.9935
Ditalimfos	C12H14NO4PS	+	300.0454	300.0452	0.6	1	0.9967
Dithianon	C14H4N2O2S2	+	314.0052	314.0064	3.6	10	0.9235
Dithiopyr	C15H16F5NO2S2	+	402.0615	402.0617	0.3	10	0.9866
Diuron	C9H10Cl2N2O	+	233.0243	233.0244	0.6	1	0.9947
DNOC	C7H6N2O5	-	197.0204	197.0205	1.5	1	0.9948
Dodemorph	C18H35NO	+	282.2791	282.279	0.6	1	0.9946
Doramectin	C50H74O14	+	916.5417	916.5418	0.1	10	0.9888
Edifenphos	C14H15O2PS2	+	311.0324	311.0322	0.6	1	0.9952
EPN	C14H14NO4PS	+	341.0719	341.0721	0.3	1	0.9983
Epoxiconazole	C17H13C FN3O	+	330.0804	330.0803	0.2	1	0.9953
Eprinomectin B1a	C50H75NO14	+	914.526	914.526	0	1	0.9852

Compound	Formula	Polarity	Theoretical Mass (*m/z*)	Experimental Mass (*m/z*)	Mass Deviation (ppm)	LOQ (ppb)	R^2
Eprinomectin B1b	C49H73NO14	+	900.5104	900.5131	3	10	0.9738
EPTC (eptam)	C9H19NOS	+	190.126	190.1261	0.2	1	0.9938
Esprocarb	C15H23NOS	+	266.1573	266.1572	0.4	1	0.9981
Etaconazol	C14H15Cl2N3O2	+	328.0614	328.0613	0.3	1	0.9980
Ethaboxam	C14H16N4OS2	+	321.0838	321.0839	0.3	1	0.9907
Ethalfluralin	C13H14F3N3O4	+	334.1009	334.0994	4.6	1	0.9845
Ethidimuron	C7H12N4O3S2	+	265.0424	265.0422	0.6	1	0.9805
Ethiofencarb	C11H15NO2S	+	226.0896	226.0898	0.8	1	0.9987
Ethiolate	C7H15NOS	+	162.0947	162.0947	0.2	1	0.9960
Ethion	C9H22O4P2S4	+	384.9949	384.9948	0.1	1	0.9914
Ethion monoxon	C9H22O5P2S3	+	369.0177	369.0177	0	1	0.9975
Ethiprole	C13H9Cl2F3N4OS	+	414.0165	414.0164	0.1	1	0.9817
Ethirimol	C11H19N3O	+	210.1601	210.1602	0.4	1	0.9984
Ethofumesate	C13H18O5S	+	304.1213	304.1213	0.1	1	0.9986
Ethoprop	C8H19O2PS2	+	243.0637	243.0637	0	1	0.9865
Ethoxyquin	C14H19NO	+	218.1539	218.1541	0.7	1	0.9967
Etobenzanid	C16H15Cl2NO3	+	340.0502	340.0502	0.1	1	0.9969
Etofenprox	C25H28O3	+	394.2377	394.2379	0.6	1	0.9928
Etoxazole	C21H23F2NO2	+	360.177	360.1769	0.1	1	0.9976
Etrimfos	C10H17N2O4PS	+	293.0719	293.0718	0.6	1	0.9982
Famoxadone	C22H18N2O4	+	392.1605	392.1603	0.4	1	0.9937
Famphur	C10H16NO5PS2	+	343.0546	343.0531	4.4	1	0.9973
Famphur oxon	C10H16NO6PS	+	327.0774	327.0775	0.2	1	0.9955
Fenamidone	C17H17N3OS	+	312.1165	312.1163	0.6	1	0.9986
Fenamiphos	C13H22NO3PS	+	304.1131	304.113	0.3	1	0.9944
Fenamiphos sulfone	C13H22NO5PS	+	336.1029	336.1029	0.1	1	0.9924
Fenamiphos sulfoxide	C13H22NO4PS	+	320.108	320.1079	0.2	1	0.9936
Fenarimol	C17H12Cl2N2O	+	331.04	331.0399	0.3	1	0.9825
Fenazaquin	C20H22N2O	+	307.1805	307.1805	0.1	1	0.9881
Fenbuconazole	C19H17ClN4	+	337.1215	337.1214	0.1	1	0.9970
Fenhexamid	C14H17Cl2NO2	+	302.0709	302.0709	0.2	1	0.9965
Fenitrothion	C9H12NO5PS	+	295.0512	295.0517	1.6	10	0.9971
Fenoxanil	C15H18Cl2N2O2	+	346.1084	346.1083	0.1	1	0.9914
Fenoxycarb	C17H19NO4	+	302.1387	302.1386	0.5	1	0.9943
Fenpiclonil	C11H6Cl2N2	+	254.0246	254.0246	0.3	1	0.9817
Fenpropathrin	C22H23NO3	+	350.1751	350.1759	2.4	1	0.9954
Fenpropimorph	C20H33NO	+	304.2635	304.2633	0.5	1	0.9919
Fenpyroximate	C24H27N3O4	+	422.2074	422.2074	0.2	1	0.9966
Fensulfothion	C11H17O4PS2	+	309.0379	309.0378	0.3	1	0.9969
Fenthion	C10H15O3PS2	+	279.0273	279.0286	4.5	1	0.9941
Fenthion oxon	C10H15O4PS	+	263.0501	263.0501	0.1	1	0.9975
Fenthion sulfone	C10H15O5PS2	+	328.0437	328.0439	0.6	1	0.9993
Fenthion sulfoxide	C10H15O4PS2	+	295.0222	295.022	0.6	1	0.9957
Fenuron	C9H12N2O	+	165.1022	165.1022	0.4	1	0.9998
Fenvalerate	C25H22ClNO3	+	437.1627	437.1629	0.7	10	0.9919
Fipronil	C12H4Cl2F6N4OS	-	434.9314	434.9316	0.4	1	0.9968
Flonicamid	C9H6F3N3O	-	228.039	228.0384	2.6	1	0.9989
Florasulam	C12H8F3N5O3S	+	360.0373	360.0374	0.2	1	0.9956
Fluazinam	C13H4Cl2F6N4O4	-	462.9441	462.945	1.9	1	0.9946
Flubendiamide	C23H22F7IN2O4S	-	681.016	681.0154	0.9	1	0.9917
Flucarbazone	C12H11F3N4O6S	+	414.069	414.069	0	1	0.9924
Fluchloralin	C12H13ClF3N3O4	+	373.0885	373.0894	2.4	10	0.9605
Flucythrinate	C26H23F2NO4	+	469.1933	469.1933	0.2	1	0.9932
Fludioxonil	C12H6F2N2O2	+	266.0736	266.0736	0.1	1	0.9749
Flufenacet	C14H13F4N3O2S	+	364.0737	364.0736	0.4	1	0.9980
Flufenoxuron	C21H11ClF6N2O3	+	489.0435	489.0436	0.1	1	0.9929
Flumetralin	C16H12ClF4N3O4	+	422.0525	422.0537	2.8	25	0.9917
Flumetsulam	C12H9F2N5O2S	+	326.0518	326.0516	0.6	1	0.9988
Flumioxazin	C19H15FN2O4	+	355.1089	355.1089	0	10	0.9677

LC/MS data for Pesticide Standards *(Table 1 continued)*

Compound	Formula	Polarity	Theoretical Mass (*m/z*)	Experimental Mass (*m/z*)	Mass Deviation (ppm)	LOQ (ppb)	R^2
Fluometuron	C10H11F3N2O	+	233.0896	233.0897	0.4	1	0.9983
Fluopicolide	C14H8Cl3F3N2O	+	382.9727	382.9728	0.2	1	0.9911
Fluorochloridone	C12H10Cl2F3NO	+	329.043	329.0431	0.4	1	0.9837
Fluorodifen	C13H7F3N2O5	+	346.0645	346.0652	2	10	0.9963
Fluoxastrobin	C21H16C FN4O5	+	459.0866	459.0865	0.3	1	0.9983
Fluquinconazole	C16H8Cl2FN5O	+	376.0163	376.0163	0	10	0.9939
Fluroxypyr	C7H5Cl2FN2O3	-	252.9588	252.9581	2.7	10	0.9928
Flusilazole	C16H15F2N3Si	+	316.1076	316.1076	0.1	1	0.9932
Flutolanil	C17H16F3NO2	+	341.1471	341.1471	0	1	0.9948
Flutriafol	C16H13F2N3O	+	302.11	302.11	0	1	0.9942
Fluvalinate ?	C26H22C F3N2O3	+	520.1609	520.1613	0.7	10	0.9968
Fonophos	C10H15OPS2	+	247.0375	247.0375	0.2	1	0.9165
Fonophos O-analog	C10H15O2PS	+	231.0603	231.0601	0.8	10	0.9526
Forchlorfenuron	C12H10C N3O	+	248.0585	248.0585	0.1	1	0.9967
Formasafen	C15H10C F3N2O6S	-	436.9827	436.9817	2.2	1	0.9972
Formetanate	C11H15N3O2	+	239.1503	239.1503	0.1	1	0.9981
Fosthiazate	C9H18NO3PS2	+	284.0539	284.0538	0.2	1	0.9958
Fuberidazole	C11H8N2O	+	185.0709	185.0708	0.9	1	0.9972
Furalaxyl	C17H19NO4	+	302.1387	302.1386	0.5	1	0.9943
Furathiocarb	C18H26N2O5S	+	383.1635	383.1635	0.1	1	0.9980
Griseofulvin	C17H17ClO6	+	353.0786	353.0787	0.2	1	0.9968
Halofenozide	C18H19C N2O2	-	329.1062	329.1063	0.3	1	0.9984
Haloxyfop-methyl	C16H13C F3NO4	+	376.0558	376.0556	0.4	1	0.9965
Heptenophos	C9H12ClO4P	+	251.0235	251.0235	0.2	10	0.9983
Hexaconazole	C14H17Cl2N3O	+	314.0821	314.082	0.4	1	0.9947
Hexaflumuron	C16H8Cl2F6N2O3	-	458.9743	458.9745	0.4	1	0.9834
Hexazinone	C12H20N4O2	+	253.1659	253.1658	0.5	1	0.9975
Hexythiazox	C17H21C N2O2S	+	353.1085	353.1084	0.4	1	0.9807
Hydramethylnon	C25H24F6N4	+	495.1978	495.1976	0.3	1	0.9965
Imazalil	C14H14Cl2N2O	+	297.0556	297.0555	0.4	1	0.9960
Imazamox	C15H19N3O4	+	306.1448	306.1447	0.5	1	0.9962
Imazapyr	C13H15N3O3	+	262.1186	262.1185	0.3	1	0.9972
Imazaquin	C17H17N3O3	+	312.1343	312.1341	0.5	1	0.9970
Imibenconazole	C17H13Cl3N4S	+	410.9999	411	0.2	1	0.9909
Imidacloprid	C9H10ClN5O2	+	256.0596	256.0595	0.5	1	0.9983
Imiprothrin	C17H22N2O4	+	319.1652	319.1651	0.4	1	0.9663
Inabenifide	C19H15C N2O2	+	339.0895	339.0895	0	1	0.9974
Indanofan	C20H17ClO3	+	341.0939	341.0938	0.4	1	0.9824
Indoxacarb	C22H17C F3N3O7	+	528.078	528.0779	0.2	1	0.9922
Ioxynil	C7H3I2NO	-	369.8231	369.8237	0.2	1	0.9955
Ipconazole	C18H24C N3O	+	334.1681	334.1679	0.4	1	0.9968
Iprobenfos	C13H21O3PS	+	289.1022	289.1021	0.1	1	0.9977
Iprovalicarb	C18H28N2O3	+	321.2173	321.2171	0.4	1	0.9993
Isazophos	C9H17ClN3O3PS	+	314.049	314.0489	0.3	1	0.9988
Isocarbamid	C8H15N3O2	+	186.1237	186.1237	0	1	0.9967
Isocarbophos	C11H16NO4PS	+	307.0876	307.0876	0.1	1	0.9941
Isofenfos	C15H24NO4PS	+	346.1236	346.1236	0.2	1	0.9911
Isofenfos O-analog	C15H24NO5P	+	330.1465	330.1473	2.6	10	0.9344
Isoprocarb	C11H15NO2	+	194.1176	194.1177	0.8	1	0.9978
Isopropalin	C15H23N3O4	+	310.1761	310.1761	0.2	1	0.9932
Isoprothiolane	C12H18O4S2	+	291.0719	291.0718	0.6	1	0.9961
Isoproturon	C12H18N2O	+	207.1492	207.1492	0.2	1	0.9939
Isoxaben	C18H24N2O4	+	333.1809	333.1809	0.1	1	0.9982
Isoxadifen-ethyl	C18H17NO3	+	296.1281	296.1281	0	1	0.9968
Isoxaflutole	C15H12F3NO4S	+	377.0777	377.0779	0.4	1	0.9919
Isoxathion	C13H16NO4PS	+	314.061	314.0608	0.7	1	0.9895
Ivermectin B1a	C48H74O14	+	892.5417	892.5415	0.2	10	0.9915
Ivermectin B1b	C47H72O14	+	883.4814	883.4818	0.4	50	0.9695
Kresoxim-methyl	C18H19NO4	+	314.1387	314.1386	0.2	1	0.9969

Compound	Formula	Polarity	Theoretical Mass (*m/z*)	Experimental Mass (*m/z*)	Mass Deviation (ppm)	LOQ (ppb)	R^2
Lactofen	C19H15ClF3NO7	+	479.0827	479.0828	0.1	1	0.9883
Linuron	C9H10Cl2N2O2	+	249.0192	249.0191	0.3	1	0.9977
Lufenuron	C17H8Cl2F8N2O3	+	510.9857	510.9833	4.7	1	0.9808
Malathion	C10H19O6PS2	+	348.0699	348.07	0.4	1	0.9950
Malathion O-analog	C10H19O7PS	+	315.0662	315.0661	0.2	1	0.9948
Mandipropamid	C23H22ClNO4	+	412.131	412.131	0.1	1	0.9978
Mefenacet	C16H14N2O2S	+	299.0849	299.0848	0.4	1	0.9985
Mefluidide	C11H13F3N2O3S	+	328.0937	328.0937	0.1	1	0.9987
Mepanipyrim	C14H13N3	+	224.1182	224.1184	0.6	1	0.9887
Mephospholan	C8H16NO3PS2	+	270.0382	270.038	0.6	1	0.9915
Mepronil	C17H19NO2	+	270.1489	270.1487	0.4	1	0.9938
Mesotrione	C14H13NO7S	+	340.0486	340.0502	4.9	1	0.9952
Metaflumizone	C24H16F6N4O2	-	505.1105	505.1106	0.1	1	0.9745
Metalaxyl	C15H21NO4	+	280.1543	280.1542	0.6	1	0.9988
Metazachlor	C14H16ClN3O	+	278.1055	278.1054	0.3	1	0.9984
Metconazole	C17H22ClN3O	+	320.1524	320.1523	0.4	1	0.9881
Methabenzthiazuron	C10H11N3OS	+	222.0696	222.0698	0.9	1	0.9982
Methacrifos	C7H13O5PS	+	258.056	258.0559	0.1	1	0.9958
Methamidophos	C2H8NO2PS	+	142.0086	142.0087	0.4	1	0.9990
Methidathion	C6H11N2O4PS3	+	319.9957	319.9956	0.2	1	0.9971
Methiocarb	C11H15NO2S	+	226.0896	226.0898	0.8	1	0.9987
Methomyl	C5H10N2O2S	+	163.0536	163.0534	0.9	1	0.9991
Methoprotryne	C11H21N5OS	+	272.154	272.1537	1	1	0.9978
Methoxyfenozide	C22H28N2O3	+	369.2173	369.2172	0.2	1	0.9935
Metobromuron	C9H11BrN2O2	+	259.0077	259.0077	0.2	1	0.9948
Metofluthrin	C18H20F4O3	-	359.1276	359.1277	0.2	1	0.9887
Metolachlor	C15H22ClNO2	+	284.1412	284.1411	0.1	1	0.9981
Metominostrobin(E-)	C16H16N2O3	+	285.1234	285.1232	0.7	1	0.9957
Metosulam	C14H13Cl2N5O4S	+	418.0138	418.0137	0.3	1	0.9924
Metoxuron	C10H13ClN2O2	+	229.0738	229.074	0.6	1	0.9995
Metrafenone	C19H21BrO5	+	409.0645	409.0643	0.4	1	0.9963
Metribuzin	C8H14N4OS	+	215.0961	215.0963	0.7	1	0.9969
Mevinphos	C7H13O6P	+	242.0788	242.0788	0.1	1	0.9977
Mexacarbate	C12H18N2O2	+	223.1441	223.1443	0.7	1	0.9991
Milbemectin A3	C31H44O7	+	546.3425	546.3421	0.8	10	0.9819
Milbemectin A4	C32H46O7	+	560.3582	560.3584	0.4	1	0.9905
Molinate	C9H17NOS	+	188.1104	188.1104	0.2	1	0.9881
Monocrotophos	C7H14NO5P	+	224.0682	224.0685	1	1	0.9989
Monolinuron	C9H11ClN2O2	+	215.0582	215.0583	0.7	1	0.9977
Moxidectin	C37H53NO8	+	640.3844	640.3847	0.5	1	0.9966
Myclobutanil	C15H17ClN4	+	289.1215	289.1214	0.1	1	0.9940
Naled	C4H7Br2Cl2O4P	+	395.8164	395.8164	0.1	10	0.9908
Naphthol	C10H8O	+	145.0648	145.0648	0.2	1	0.9939
Napropamide	C17H21NO2	+	272.1645	272.1644	0.5	1	0.9933
Naptalam sodium	C18H12NNaO3	+	331.1053	331.1067	4.2	1	0.9931
Neburon	C12H16Cl2N2O	+	275.0713	275.0711	0.5	1	0.9941
Nitenpyram	C11H15ClN4O2	+	271.0956	271.0948	3.2	1	0.9876
Nitralin	C13H19N3O6S	+	346.1067	346.1083	4.6	1	0.9824
Nitrothal-isopropyl	C14H17NO6	+	313.1394	313.1385	3.5	10	0.8345
Norflurazon	C12H9ClF3N3O	+	304.0459	304.0458	0.3	1	0.9858
Novaluron	C17H9ClF8N2O4	-	491.005	491.0053	0.6	1	0.9902
Noviflumuron	C17H7Cl2F9N2O3	-	526.9617	526.9613	0.7	1	0.9759
Nuarimol	C17H12ClFN2O	+	315.0695	315.0693	0.5	1	0.9907
Octhilinone (2-Octyl-4-isothiazoline-3-one)	C11H19NOS	+	214.126	214.1262	0.8	1	0.9977
Ofurace	C14H16ClNO3	+	299.1157	299.1156	0.2	1	0.9974
Omethoate (Dimethoate oxon)	C5H12NO4PS	+	214.0297	214.0298	0.4	1	0.9997
Orbencarb	C12H16ClNOS	+	258.0714	258.0712	0.6	1	0.9969

LC/MS data for Pesticide Standards *(Table 1 continued)*

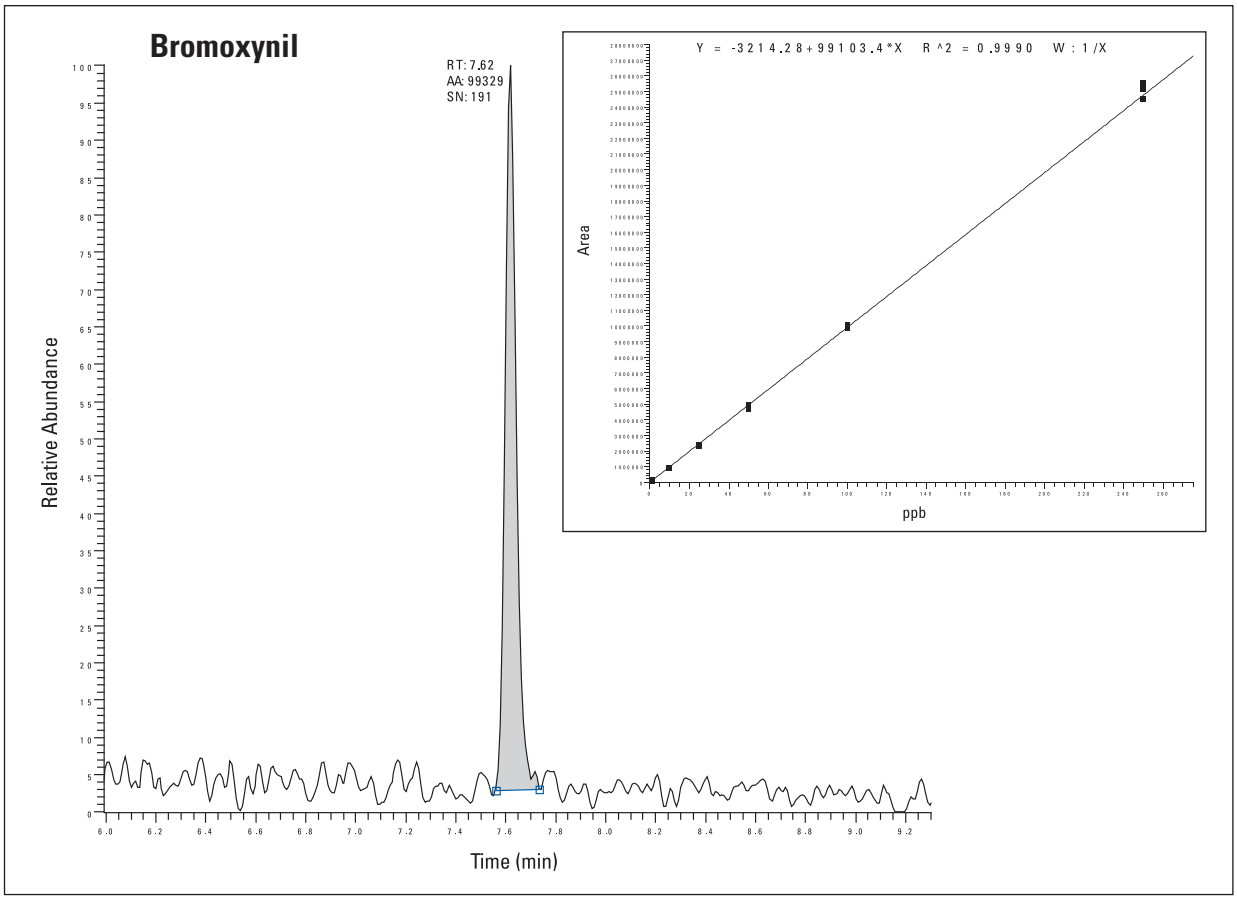
Compound	Formula	Polarity	Theoretical Mass (*m/z*)	Experimental Mass (*m/z*)	Mass Deviation (ppm)	LOQ (ppb)	R^2
Oryzalin	C12H18N4O6S	-	345.0874	345.0876	0.5	1	0.9895
Oxadiazon	C15H18Cl2N2O3	+	362.1033	362.1032	0.1	1	0.9969
Oxadixyl	C14H18N2O4	+	279.1339	279.1339	0	1	0.9994
Oxamyl	C7H13N3O3S	+	237.1016	237.1017	0.5	1	0.9997
Paclobutrazol	C15H20C N3O	+	294.1368	294.1367	0.3	1	0.9955
Parathion	C10H14NO5PS	+	309.0669	309.0679	3.2	10	0.9645
Parathion methyl oxon	C8H10NO6P	+	265.0584	265.0585	0.5	10	0.9903
Parathion oxon	C10H14NO6P	+	293.0897	293.0896	0.3	1	0.9928
Pebulate	C10H21NOS	+	204.1417	204.1417	0.1	1	0.9929
Penconazole	C13H15Cl2N3	+	284.0716	284.0715	0.4	1	0.9931
Pencycuron	C19H21C N2O	+	329.1415	329.1414	0.5	1	0.9986
Pendimethalin	C13H19N3O4	+	282.1448	282.1448	0.2	10	0.9949
Penoxsulam	C16H14F5N5O5S	+	484.0709	484.071	0.3	1	0.9928
Penthiopyrad	C16H20F3N3OS	+	360.1352	360.1352	0.1	1	0.9935
Permethrin(cis-)	C21H20Cl2O3	+	408.1128	408.1129	0.2	1	0.9935
Permethrin(trans-)	C21H20Cl2O3	+	408.1128	408.1129	0.2	1	0.9935
Phenmedipham	C16H16N2O4	+	318.1448	318.1448	0	1	0.9975
Phenothrin	C23H26O3	+	368.222	368.2222	0.6	1	0.9944
Phenthoate	C12H17O4PS2	+	321.0379	321.0378	0.4	1	0.9929
Phenylphenol(o-)	C12H10O	+	188.107	188.107	0.2	1	0.9854
Phorate	C7H17O2PS3	+	261.0201	261.02	0.3	10	0.9812
Phorate oxon	C7H17O3PS	+	230.0974	230.0982	3.5	1	0.9973
Phorate oxon sulfone	C7H17O5PS2	+	277.0328	277.0327	0.5	1	0.9979
Phorate oxon sulfoxide	C7H17O4PS2	+	261.0379	261.0377	0.8	1	0.9995
Phorate sulfone	C7H17O4PS3	+	310.0365	310.0363	0.6	1	0.9951
Phorate sulfoxide	C7H17O4PS2	+	261.0379	261.0377	0.8	1	0.9995
Phosalone	C12H15C NO4PS2	+	385.0207	385.0206	0.3	1	0.9945
Phosmet	C11H12NO4PS2	+	318.0018	318.0018	0.1	1	0.9938
Phosphamidon	C10H19C NO5P	+	317.1028	317.1026	0.4	1	0.9936
Phoxim	C12H15N2O3PS	+	299.0614	299.0613	0.4	1	0.9963
Picloram	C6H3Cl3N2O2	+	240.9333	240.9331	0.7	10	0.9594
Picoxystrobin	C18H16F3NO4	+	368.1104	368.1104	0.1	1	0.9981
Pinoxaden	C23H32N2O4	+	401.2435	401.2434	0.3	1	0.9968
Piperonyl butoxide	C19H30O5	+	356.2432	356.2433	0.3	1	0.9872
Piperophos	C14H28NO3PS2	+	354.1321	354.132	0.3	1	0.9932
Pirimicarb	C11H18N4O2	+	239.1503	239.1503	0.1	1	0.9992
Pirimiphos-ethyl	C13H24N3O3PS	+	334.1349	334.1348	0.2	1	0.9977
Pirimiphos-methyl	C11H20N3O3PS	+	306.1036	306.1034	0.7	1	0.9952
Pretilachlor	C17H26C NO2	+	329.199	329.1989	0.3	1	0.9928
Probenazole	C10H9NO3S	+	224.0376	224.0378	0.9	1	0.9989
Prochloraz	C15H16Cl3N3O2	+	376.0381	376.0379	0.4	1	0.9933
Profenophos	C11H15BrClO3PS	+	372.9424	372.9424	0.1	1	0.9939
Prohexadione	C10H12O5	-	211.0612	211.0613	0.4	1	0.9936
Promecarb	C12H17NO2	+	208.1332	208.1333	0.4	1	0.9972
Prometon	C10H19N5O	+	226.1662	226.1664	0.7	1	0.9991
Prometryn	C10H19N5S	+	242.1434	242.1434	0.2	1	0.9985
Propachlor	C11H14C NO	+	212.0837	212.0839	0.8	1	0.9962
Propamocarb	C9H20N2O2	+	189.1598	189.1597	0.5	1	0.9992
Propanil	C9H9Cl2NO	-	215.9988	215.9987	0.4	1	0.9855
Propargite	C19H26O4S	+	368.189	368.1891	0.1	1	0.9961
Propazine	C9H16ClN5	+	230.1167	230.1168	0.5	1	0.9976
Propetamphos	C10H20NO4PS	+	299.1189	299.1188	0.3	1	0.9929
Propham	C10H13NO2	+	180.1019	180.1019	0.1	1	0.9131
Propiconazole	C15H17Cl2N3O2	+	342.0771	342.077	0.1	1	0.9885
Propisochlor	C15H22C NO2	+	284.1412	284.1411	0.1	1	0.9981
Propoxur	C11H15NO3	+	210.1125	210.1126	0.7	1	0.9949
Prothioconazole	C14H15Cl2N3OS	-	342.024	342.0245	1.4	1	0.9864
Prothoate	C9H20NO3PS2	+	286.0695	286.0693	0.8	1	0.9982
Pymetrozine	C10H11N5O	+	218.1036	218.1037	0.5	1	0.9985

Compound	Formula	Polarity	Theoretical Mass (*m/z*)	Experimental Mass (*m/z*)	Mass Deviation (ppm)	LOQ (ppb)	R^2
Pyracarbolid	C13H15NO2	+	218.1176	218.1177	0.6	1	0.9986
Pyraclofos	C14H18ClN2O3PS	+	361.0537	361.0537	0.1	1	0.9969
Pyraclostrobin	C19H18ClN3O4	+	388.1059	388.1057	0.5	1	0.9951
Pyraflufen-ethyl	C15H13Cl2F3N2O4	+	430.0543	430.0527	3.7	1	0.9833
Pyrasulfotole	C14H13F3N2O4S	-	361.0475	361.0476	0.2	1	0.9926
Pyrazone (Chloridazon)	C10H8ClN3O	+	239.0694	239.0687	3.1	50	0.9448
Pyrazophos	C14H20N3O5PS	+	374.0934	374.0933	0.3	1	0.9958
Pyridaben	C19H25ClN2OS	+	365.1449	365.145	0.3	1	0.9881
Pyridalyl	C18H14Cl4F3NO3	+	489.9753	489.9755	0.4	1	0.9958
Pyridaphenthion	C14H17N2O4PS	+	341.0719	341.0721	0.3	1	0.9938
Pyridate	C19H23ClN2O2S	+	379.1242	379.1242	0.2	1	0.9902
Pyrifenox	C14H12Cl2N2O	+	295.04	295.0397	0.7	1	0.9979
Pyrimethanil	C12H13N3	+	200.1182	200.1183	0.2	1	0.9977
Pyriproxyfen	C20H19NO3	+	322.1438	322.1438	0	1	0.9977
Pyroquilon	C11H11NO	+	174.0913	174.0913	0.5	1	0.9992
Pyroxsulam	C14H13F3N6O5S	+	435.0693	435.0693	0.1	1	0.9962
Quinalphos	C12H15N2O3PS	+	299.0614	299.0613	0.4	1	0.9963
Quinclamine	C10H6ClNO2	+	208.016	208.0158	1	1	0.9879
Quinoxyfen	C15H8Cl2FNO	+	308.004	308.0039	0.4	1	0.9980
Resmethrin	C22H26O3	+	339.1955	339.1955	0.1	1	0.9948
Rotenone	C23H22O6	+	395.1489	395.1489	0.1	1	0.9948
Saflufenacil	C17H17ClF4N4O5S	+	518.0883	518.0883	0	1	0.9868
Schradan	C8H24N4O3P2	+	287.1396	287.1389	2.7	1	0.9937
Secbumeton	C10H19N5O	+	226.1662	226.1664	0.7	1	0.9991
Sethoxydim	C17H29NO3S	+	328.1941	328.1939	0.5	1	0.9977
Siduron	C14H20N2O	+	233.1648	233.165	0.5	1	0.9996
Simazine	C7H12ClN5	+	202.0854	202.0855	0.3	1	0.9963
Simeconazole	C14H20FN3OSi	+	294.1432	294.1431	0.5	1	0.9949
Simetryn	C8H15N5S	+	214.1121	214.1122	0.6	1	0.9986
Spinetoram	C42H69NO10	+	748.4994	748.4992	0.3	1	0.9878
Spinetoram 1	C43H69NO10	+	760.4994	760.4995	0.1	1	0.9934
Spinosad A	C41H65NO10	+	732.4681	732.468	0.2	1	0.9960
Spinosad D	C42H67NO10	+	746.4838	746.4836	0.3	1	0.9932
Spirodiclofen	C21H24Cl2O4	+	428.139	428.1389	0.2	1	0.9991
Spiromefisen	C23H30O4	+	388.2482	388.2482	0	1	0.9934
Spirotetramat	C21H27NO5	+	374.1962	374.1963	0.3	1	0.9990
Spiroxamine	C18H35NO2	+	298.2741	298.2739	0.4	1	0.9910
Sulcotrione	C14H13ClO5S	+	346.0511	346.0519	2.6	10	0.9706
Sulfentrazone	C11H10Cl2F2N4O3S	+	386.9892	386.9906	3.8	1	0.9906
Sulfotep-ethyl	C8H20O5P2S2	+	323.03	323.03	0.1	1	0.9950
Sulfuramid	C10H6F17NO2S	-	525.9775	525.9779	0.7	1	0.9828
Sulprofos	C12H19O2PS3	+	340.0623	340.0636	3.7	1	0.9950
Tebuconazole	C16H22ClN3O	+	308.1524	308.1522	0.7	1	0.9924
Tebufenozide	C22H28N2O2	+	353.2224	353.2223	0.3	1	0.9946
Tebufenpyrad	C18H24ClN3O	+	334.1681	334.1679	0.4	1	0.9968
Tebupirimphos	C13H23N2O3PS	+	319.124	319.124	0.1	1	0.9953
Tebuthiuron	C9H16N4OS	+	229.1118	229.1119	0.5	1	0.9947
Teflubenzuron	C14H6Cl2F4N2O2	-	378.967	378.9675	1.3	1	0.9785
Tefluthrin	C17H14ClF7O2	+	419.0643	419.0635	1.9	50	0.9203
Tembotrione	C17H16ClF3O6S	+	458.0647	458.0649	0.5	10	0.9866
Temephos	C16H20O6P2S3	+	484.0236	484.0236	0.1	1	0.9953
Tepraloxydim	C17H24ClNO4	-	340.1321	340.1322	0.2	1	0.9947
Terbacil	C9H13ClN2O2	-	215.0593	215.0596	1.3	1	0.9911
Terbufos	C9H21O2PS3	+	289.0514	289.052	2	1	0.9928
Terbufos oxon sulfoxide	C9H21O4PS2	+	289.0692	289.0691	0.4	1	0.9927
Terbufos sulfone	C9H21O4PS3	+	338.0678	338.0678	0.1	1	0.9963
Terbumeton	C10H19N5O	+	226.1662	226.1664	0.7	1	0.9991
Terbuthylazine	C9H16ClN5	+	230.1167	230.1168	0.5	1	0.9976
Terbutryn	C10H19N5S	+	242.1434	242.1434	0.2	1	0.9985

LC/MS data for Pesticide Standards *(Table 1 continued)*

Compound	Formula	Polarity	Theoretical Mass (*m/z*)	Experimental Mass (*m/z*)	Mass Deviation (ppm)	LOQ (ppb)	R^2
Tetrachlorvinphos	C10H9Cl4O4P	+	381.9331	381.9331	0	1	0.9973
Tetraconazole	C13H11Cl2F4N3O	+	372.0288	372.0289	0.3	1	0.9967
Tetramethrin	C19H25NO4	+	332.1856	332.1856	0.3	1	0.9977
Thiabendazole	C10H7N3S	+	202.0433	202.0433	0.4	1	0.9967
Thiacloprid	C10H9ClN4S	+	253.0309	253.0309	0.3	1	0.9975
Thiamethoxam	C8H10ClN5O3S	+	292.0266	292.0266	0.1	1	0.9908
Thiazopyr	C16H17F5N2O2S	+	397.1004	397.1003	0.1	1	0.9972
Thidiazuron	C9H8N4OS	+	221.0492	221.0492	0.4	1	0.9922
Thiofanox	C9H18N2O2S	+	236.1427	236.1428	0.5	1	0.9923
Thiometon	C6H15O2PS3	+	264.031	264.0301	3.3	10	0.9594
Thiophanate-methyl	C12H14N4O4S2	+	343.0529	343.0531	0.4	1	0.9932
Tolclofos-methyl	C9H11Cl2O3PS	+	300.9616	300.9626	3.3	25	0.8855
Tolfenpyrad	C21H22C N3O2	+	384.1473	384.1475	0.3	1	0.9878
Topramezone	C16H17N3O5S	+	364.0962	364.0944	5	1	0.9250
Tralkoxydim	C20H27NO3	+	330.2064	330.2063	0.2	1	0.9918
Tralomethrin	C22H19Br4NO3	+	678.8437	678.8447	1.4	10	0.9880
Triadimefon	C14H16C N3O2	+	294.1004	294.1003	0.4	1	0.9973
Triadimenol	C14H18C N3O2	+	296.116	296.1161	0.3	1	0.9905
Tri-allate	C10H16Cl3NOS	+	304.0091	304.009	0.3	10	0.9673
Triazophos	C12H16N3O3PS	+	314.0723	314.0721	0.7	1	0.9984
Trichlamide	C13H16Cl3NO3	+	340.0269	340.026	2.6	1	0.9986
Trichlorfon	C4H8Cl3O4P	+	256.9299	256.9298	0.1	1	0.9983
Triclopyr	C7H4Cl3NO3	-	253.9184	253.9186	0.7	1	0.9891
Tricyclazole	C9H7N3S	+	190.0433	190.0433	0.4	1	0.9996
Tridemorph	C19H39NO	+	298.3104	298.3103	0.4	1	0.9972
Trietazine	C9H16ClN5	+	230.1167	230.1168	0.5	1	0.9976
Trifloxystrobin	C20H19F3N2O4	+	409.137	409.1367	0.8	1	0.9981
Triflumizole	C15H15C F3N3O	+	346.0929	346.0928	0.1	1	0.9957
Triflumuron	C15H10C F3N2O3	-	357.0259	357.0251	2.2	1	0.9914
Trifluralin	C13H16F3N3O4	+	353.1431	353.143	0.2	10	0.9871
Triforine	C10H14Cl6N4O2	+	449.9586	449.9587	0.1	10	0.9851
Trinexapac-ethyl	C13H16O5	+	253.1071	253.1071	0.2	1	0.9871
Triticonazole	C17H20C N3O	+	318.1368	318.1367	0.3	1	0.9943
Uniconazole	C15H18C N3O	+	292.1211	292.1211	0.2	1	0.9884
Validamycin	C20H35NO13	+	498.2181	498.2172	1.9	1	0.8371
Vamidothion	C8H18NO4PS2	+	288.0488	288.0484	1.3	1	0.9984
Vamidothion sulfone	C8H18NO6PS2	+	320.0386	320.0386	0.1	1	0.9986
Vernolate	C10H21NOS	+	204.1417	204.1417	0.1	1	0.9929
Warfarin	C19H16O4	+	309.1121	309.112	0.5	1	0.9871
Zoxamide	C14H16Cl3NO2	+	336.0319	336.0318	0.4	1	0.9975

Table 1: LC/MS data for 510 pesticide standards



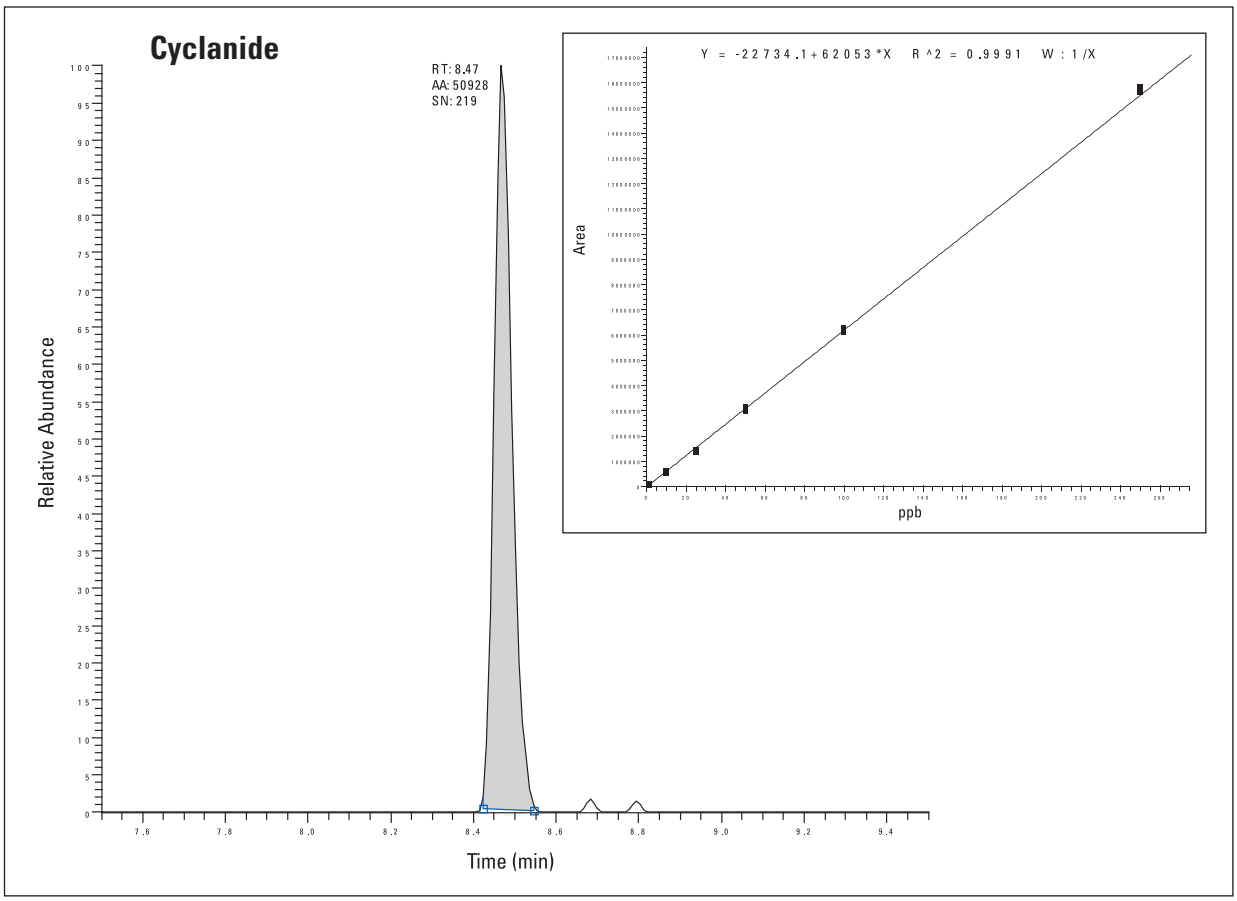
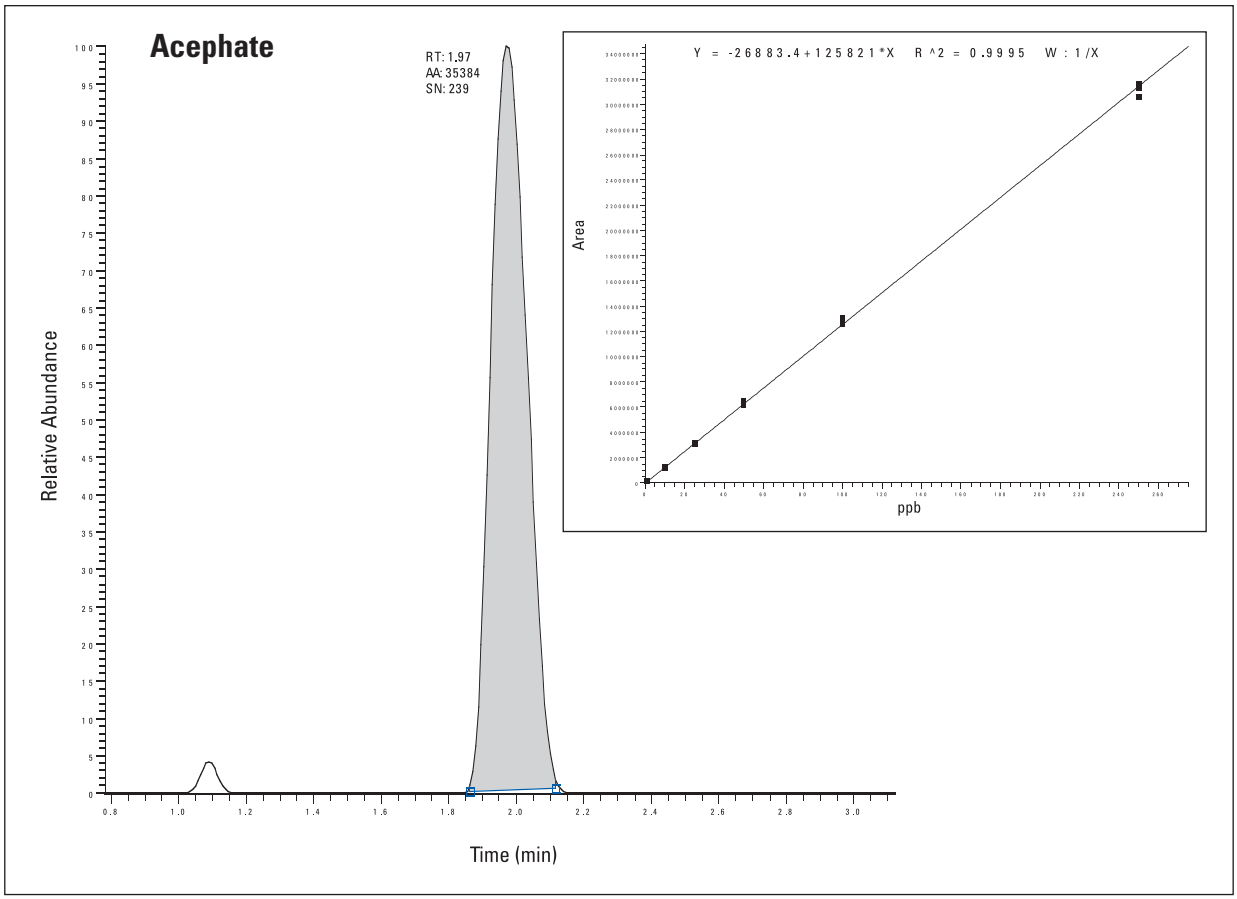


Figure 4: Extracted ion chromatograms (at 1 ppb level) and calibration curves (1-250 ppb) of eight pesticides



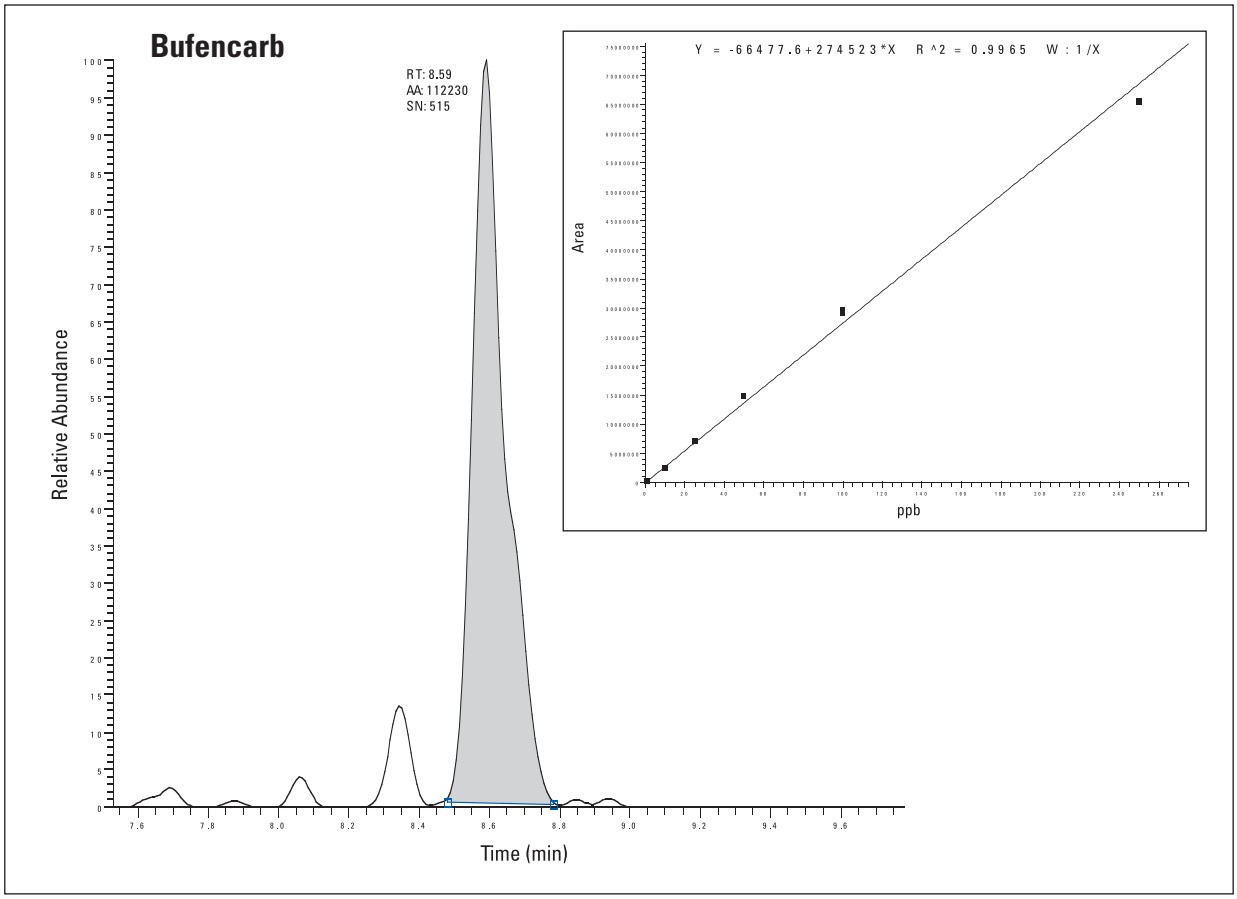
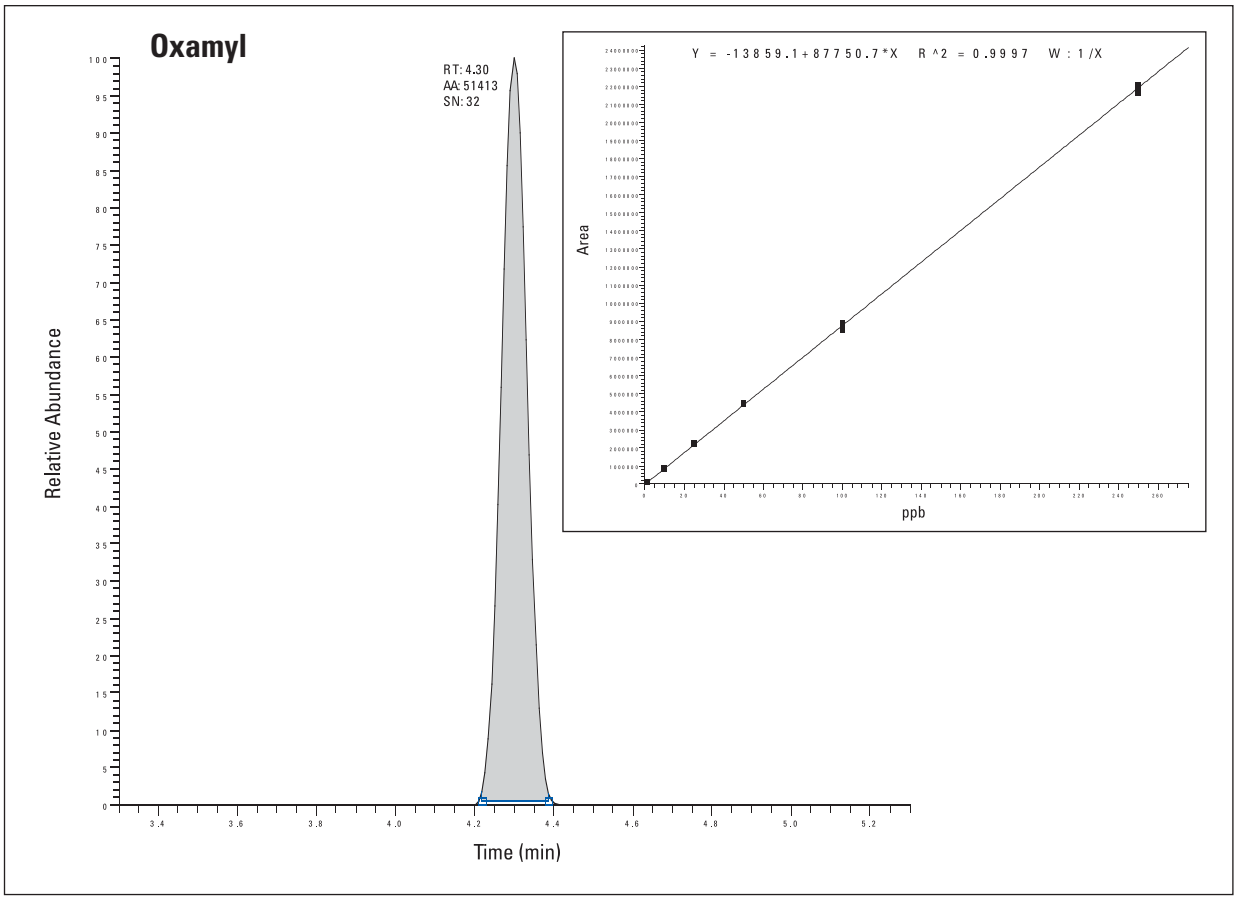


Figure 4 Continued: Extracted ion chromatograms (at 1 ppb level) and calibration curves (1-250 ppb) of eight pesticides



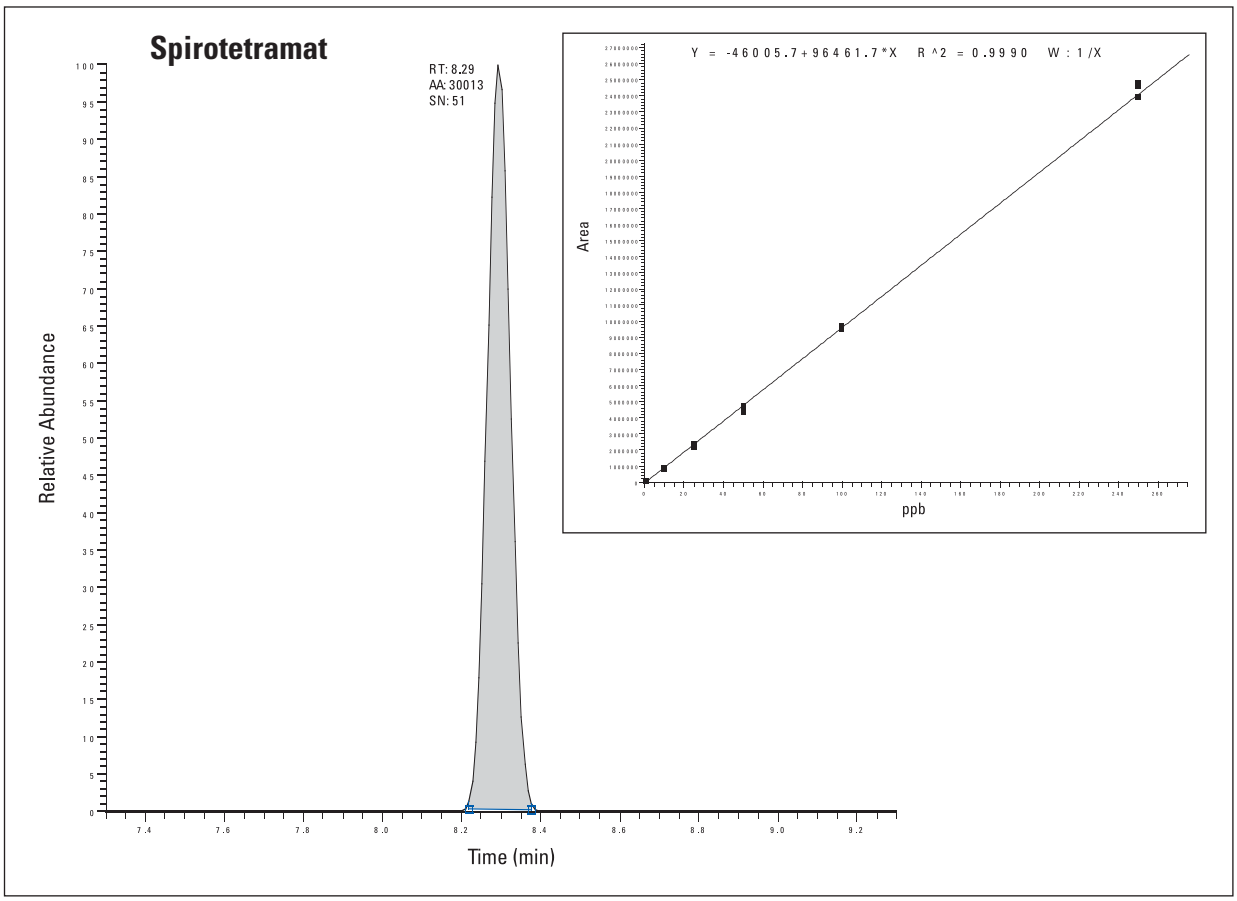
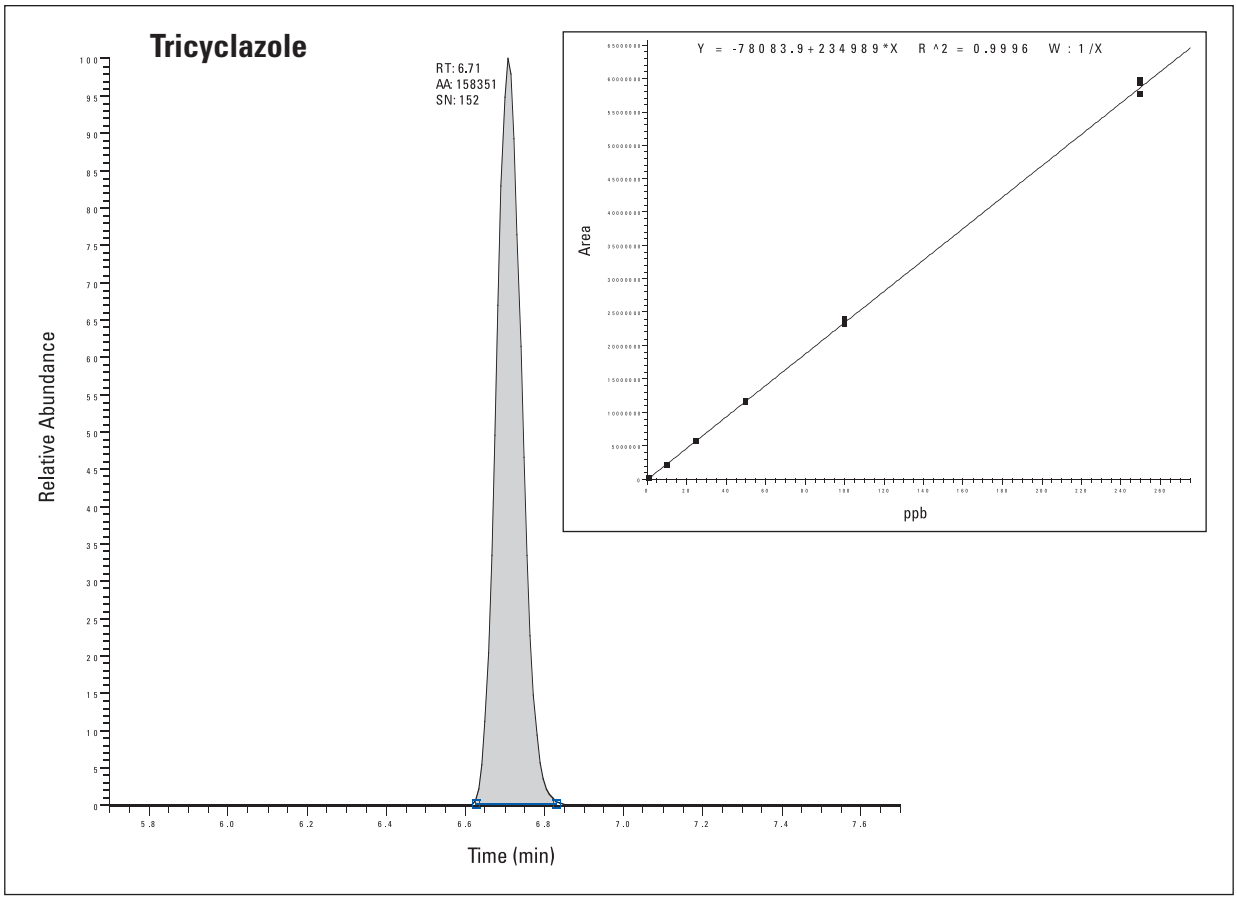


Figure 4 Continued: Extracted ion chromatograms (at 1 ppb level) and calibration curves (1-250 ppb) of eight pesticides



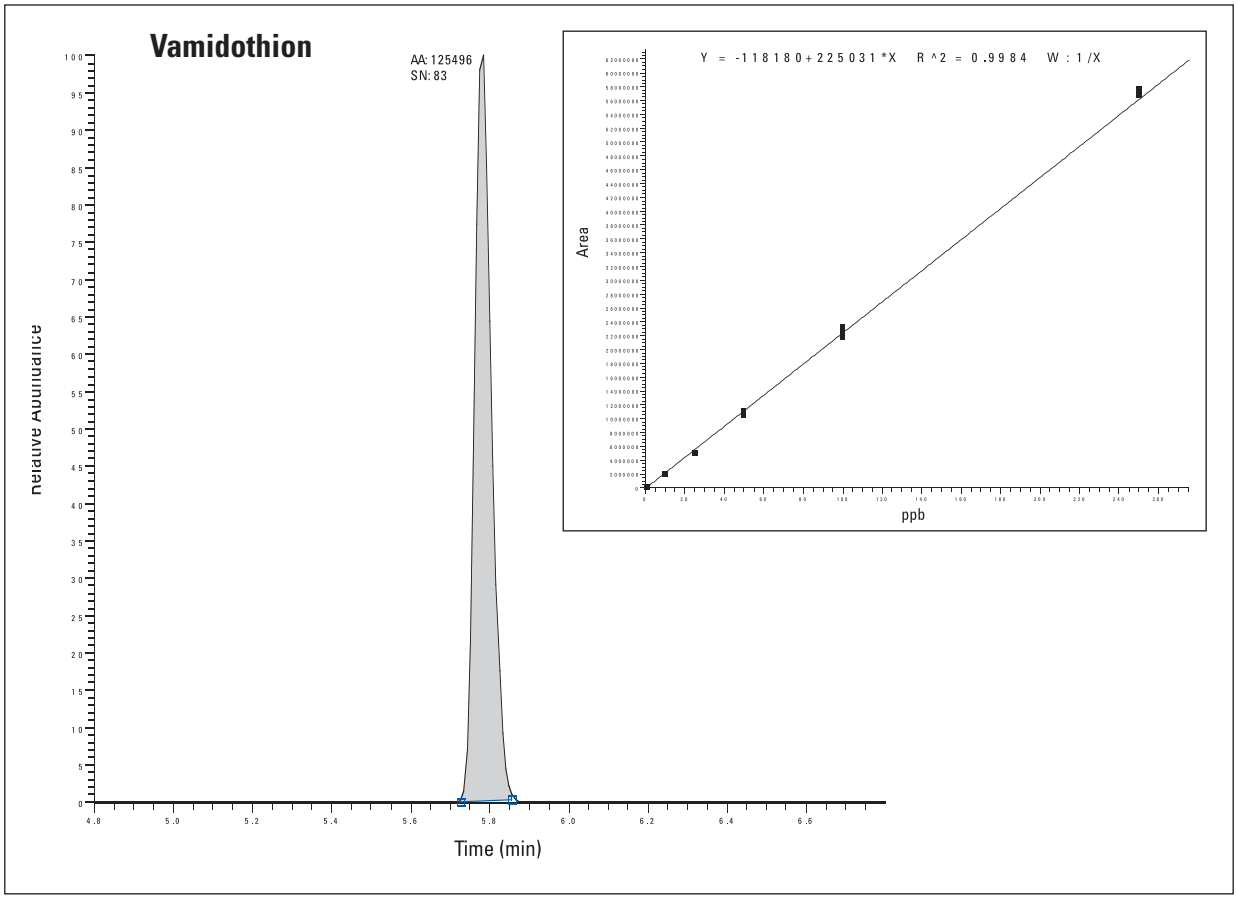


Figure 4 Continued: Extracted ion chromatograms (at 1 ppb level) and calibration curves (1-250 ppb) of eight pesticides

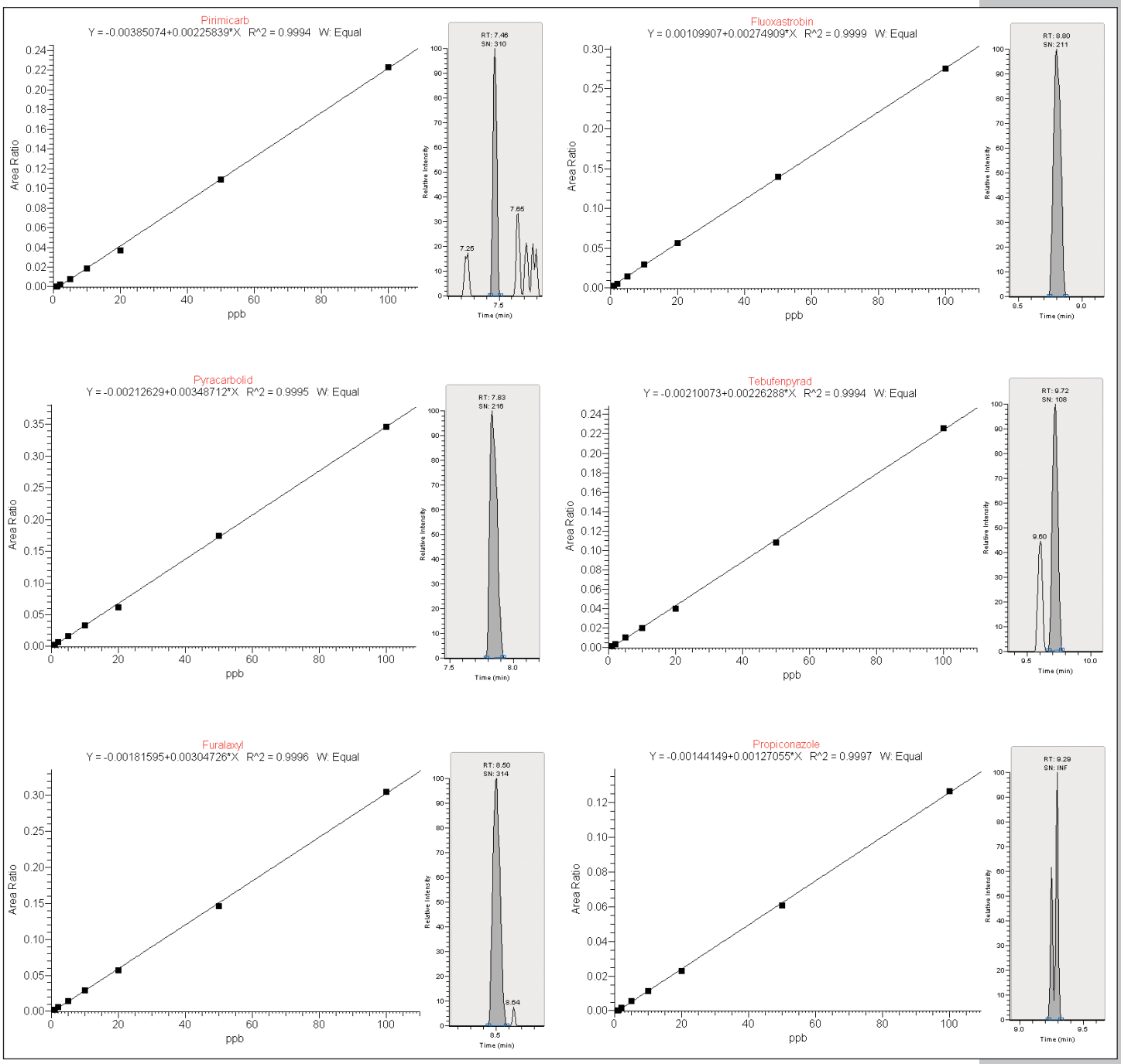


Figure 5: Extracted ion chromatograms (at 1 ppb) and calibration curves (1-250 ppb) of six pesticides extracted from spiked spinach sample

LC/MS data for representative pesticides extracted from spinach matrix *(Table 2)*

Compound	Formula	Theoretical Mass (*m/z*)	Experimental Mass (*m/z*)	Mass Deviation (ppm)	LOD (ppb)	LOQ (ppb)
Azoxystrobin	C22H17N3O5	404.1241	404.12466	1.4	0.2	0.7
Bendiocarb	C11H13NO4	224.09173	224.09169	0.2	0.2	0.7
Benthiavalicarb	C18H24FN3O3S	382.15952	382.1597	0.5	0.2	0.7
Benzoximate	C18H18ClNO5	364.09463	386.07663	0.2	0.2	0.5
Bifenazate	C17H20N2O3	301.15467	301.15457	0.3	0.3	0.9
Bupirimate	C13H24N4O3S	317.16419	317.16431	0.4	0.2	0.5
Buprofezin	C16H23N3OS	306.16346	306.16354	0.3	0.2	0.6
Butafenacil	C20H18ClF3N2	492.11437	492.11469	0.6	0.3	0.9
Carbaryl	C12H11NO2	219.1128	219.1127	0.5	0.3	0.9
Carbendazim	C9H9N3O2	192.07675	192.07684	0.5	0.2	0.7
Carbofuran	C12H15NO3	222.11247	222.11241	0.3	0.2	0.7
Carboxin	C12H13NO2S	236.07398	236.07358	1.7	0.2	0.5
Chlortoluron	C10H13ClN2O	213.07892	213.07925	1.6	0.2	0.6
Clethodim	C17H26ClNO3S	360.13947	360.13962	0.4	0.2	0.6
Clofentezine	C14H8Cl2N4	303.01988	303.01993	0.2	0.1	0.4
Cyazofamid	C13H13ClN4O2S	342.0786	342.077	4.7	0.3	0.8
Cycluron	C11H22N2O	199.18049	199.18054	0.3	0.2	0.7
Cyproconazole	C15H18ClN3O	292.12112	292.12115	0.1	0.2	0.7
Cyprodinil	C14H15N3	226.13387	226.13385	0.1	0.2	0.7
Diclobutrazol	C15H19Cl2N3O	328.09779	328.09781	0	0.2	0.5
Dicrotophos	C8H16NO5P	238.08389	238.08391	0.1	0.3	0.8
Difenoconazol	C19H17Cl2N3O3	406.07197	406.07251	1.3	0.2	0.6
Dimethoate	C5H12NO3PS2	230.0069	230.00685	0.2	0.3	0.8
Dimethomorph	C21H22ClNO4	388.13101	388.13113	0.3	0.3	0.9
Dimoxystrobin	C19H22N2O3	327.17032	327.17047	0.5	0.2	0.5
Dinotefuran	C7H14N4O3	203.11387	203.11389	0.1	0.2	0.7
Dioxacarb	C11H13NO4	203.11387	224.09169	0.2	0.2	0.7
Emamectin B1b	C49H75NO13	886.53112	886.53168	0.6	0.3	0.8
Epoxiconazole	C17H13ClFN3O	330.08039	330.08029	0.3	0.2	0.6
Etaconazole	C14H15Cl2N3O2	328.06141	328.06143	0.1	0.3	0.9
Ethiofencarb	C11H15NO2S	226.08963	226.08969	0.3	0.3	0.9
Etoxazole	C21H23F2NO2	360.17696	360.17715	0.5	0.1	0.4
Famoxadone	C22H18N2O4	392.16048	397.11591	0.1	0.2	0.7
Fenamidone	C17H17N3OS	312.11651	312.11652	0	0.2	0.6
Fenazaquin	C20H22N2O	307.18049	307.18039	0.3	0.3	0.8
Fenbuconazole	C19H17ClN4	337.12145	337.12128	0.5	0.2	0.6
Fenoxycarb	C17H19NO4	302.13868	324.12073	0.3	0.1	0.4
Fenpropimorph	C20H33NO	304.26349	304.26349	0	0.1	0.3
Fenpyroximate	C24H27N3O4	422.20743	422.20789	1.1	0.3	0.9
Fenuron	C9H12N2O	165.10224	165.10239	0.9	0.3	0.9
Flufenacet	C14H13F4N3O	364.07374	364.07401	0.7	0.2	0.6
Fluometuron	C10H11F3N2O	233.08962	233.08958	0.2	0.2	0.7
Fluoxastrobin	C21H16ClFN4O5	459.0866	459.08704	0.9	0.3	0.8
Flusiazole	C16H15F2N3Si	316.10761	316.10776	0.5	0.2	0.7
Flutolanil	C17H16F3NO2	324.12059	324.12073	0.4	0.3	0.9
Flutriafol	C16H13F2N3O	302.10995	302.10999	0.1	0.1	0.3
Forchlorfenuron	C12H10ClN3O	248.05852	248.05832	0.8	0.2	0.6
Formetanate	C11H15N3O2	239.15025	239.15018	0.3	0.2	0.5
Fuberidazole	C11H8N2O	185.07094	185.07108	0.7	0.3	0.9
Furalaxyl	C17H19NO4	302.13868	324.12073	0.3	0.1	0.4
Hexaconazole	C14H17Cl2N3O	314.08214	314.08206	0.3	0.2	0.7

Compound	Formula	Theoretical Mass (*m/z*)	Experimental Mass (*m/z*)	Mass Deviation (ppm)	LOD (ppb)	LOQ (ppb)
Hydramethylnon	C25H24F6N4	495.19779	495.19824	0.9	0.2	0.6
Imazalil	C14H14Cl2N2O	297.0556	297.05566	0.2	0.2	0.6
Iprovalicarb	C18H28N2O3	321.21727	321.21744	0.5	0.1	0.4
Isoproturon	C12H18N2O	207.14919	207.14932	0.6	0.2	0.5
Mefenacet	C16H14N2O2S	299.08487	299.08484	0.1	0.2	0.7
Mepanipyrim	C14H13N3	224.11822	224.11821	0.1	0.2	0.7
Mepronil	C17H19NO2	270.14886	270.14886	0	0.1	0.1
Metalaxyl	C15H21NO4	280.15433	280.15445	0.4	0.2	0.5
Methabenzhiazuron	C10H11N3OS	222.06956	222.06952	0.2	0.1	0.4
Methamidophos	C2H8NO2PS	142.00861	142.00865	0.3	0.2	0.5
Methiocarb	C11H15NO2S	226.08963	226.08969	0.3	0.3	0.9
Methomyl	C5H10N2O2S	163.05357	163.05357	0	0.2	0.6
Methoprotryne	C11H21N5OS	272.15396	272.15393	0.1	0.2	0.6
Methoxyfenozide	C22H28N2O3	369.21727	369.21738	0.3	0.1	0.2
Neburon	C12H16Cl2N2O	275.07125	275.07126	0	0.3	0.8
Oxadixyl	C14H18N2O4	279.13393	279.13397	0.1	0.1	0.4
Penconazole	C13H15Cl2N3	284.07158	284.07153	0.2	0.3	0.8
Pinoxaden	C23H32N2O4	401.24348	401.24393	1.1	0.1	0.1
Pirimicarb	C11H18N4O2	239.15025	239.15018	0.3	0.2	0.5
Promecarb	C12H17NO2	208.13321	208.13329	0.4	0.2	0.5
Prometon	C10H19N5O	226.16624	226.16623	0	0.2	0.5
Prometryn	C10H19N5S	242.14339	242.14348	0.4	0.2	0.5
Propamocarb	C9H20N2O2	189.15975	189.15988	0.7	0.1	0.4
Propargite	C19H26O4S	189.15975	368.18933	0.9	0.2	0.6
Propiconazole	C15H17Cl2N3O2	342.07706	342.077	0.2	0.3	0.9
Pyrimethanil	C12H13N3	200.11822	200.11826	0.2	0.2	0.6
Pyriproxyfen	C20H19NO3	322.14377	322.14392	0.5	0.2	0.6
Quinoxyfen	C15H8Cl2FNO	308.00397	308.00394	0.1	0.2	0.6
Rotenone	C23H22O6	395.14891	395.14923	0.8	0.2	0.6
Siduron	C14H20N2O	233.16484	233.16492	0.3	0.3	0.9
Simetryn	C8H15N5S	214.11209	214.11174	1.6	0.2	0.4
Spiroxamine	C18H35NO2	298.27406	298.27417	0.4	0.2	0.5
Tebuconazole	C16H22ClN3O	308.15242	308.15234	0.2	0.2	0.5
Tebufenozide	C22H28N2O2	353.22235	353.22247	0.3	0.1	0.2
Tebufenpyrad	C18H24ClN3O	334.16807	334.16821	0.4	0.2	0.7
Terbumeton	C10H19N5O	226.16624	226.16623	0	0.2	0.5
Terbutryn	C10H19N5S	242.14339	242.14348	0.4	0.2	0.5
Tetraconazole	C13H11Cl2F4N	372.02881	372.02902	0.6	0.3	0.8
Thiabendazole	C10H7N3S	202.04334	202.04344	0.5	0.2	0.6
Thiamethoxam	C8H10ClN5O3S	292.02656	292.02655	0	0.3	1
Thiobencarb	C12H16ClNOS	258.07139	280.05246	3.1	0.3	0.8
Triadimefon	C14H16ClN3O2	294.10038	294.10031	0.2	0.3	0.8
Tricyclazole	C9H7N3S	190.04334	190.04356	1.2	0.1	0.4
Trifloxystrobin	C20H19F3N2O4	409.13697	409.13745	1.2	0.2	0.6
Triflumizole	C15H15ClF3N3O	346.09285	346.09302	0.5	0.1	0.2
Triticonazole	C17H20ClN3O	318.13677	318.13687	0.3	0.3	0.8
Uniconazole	C15H18ClN3O	292.12112	292.12115	0.1	0.2	0.6
Vamidothion	C8H18NO4PS2	288.04876	288.04883	0.2	0.2	0.5
Zoxamide	C14H16Cl3NO2	336.03194	336.03189	0.1	0.3	0.9

Table 2: LC/MS data for representative pesticides extracted from spiked spinach matrix. All MS data reported below was obtained with Orbitrap MS operating in positive ion mode. LODs and LOQs were assessed using the EPA method detection limit (MDL) procedure.[9]

Conclusion

A rapid and robust U-HPLC Exactive Orbitrap MS method for multiresidue pesticide screening was developed and validated. Screening of 510 pesticides at low ppb levels was achieved within 12 minutes, and the high mass resolution and accuracy of the Exactive mass spectrometer enabled identification of all compounds. LOQs for the majority of pesticides in a standard mixture and in spiked matrix were lower than MRLs established by the EU and Japan. The Exactive LC/MS platform is ideally suited for the routine monitoring of targeted and non-targeted pesticides by regulatory laboratories.

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Application Note: 477

Screening for 250 Pesticides in Orange Oil and Ginseng Extract by LC-MS/MS Using TraceFinder Software

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Key Words

- TraceFinder software
- TSQ Vantage
- Triple Quadrupole
- Food safety
- Pesticides

Introduction

Orange oil is widely used for its fragrance and flavoring in consumer products such as cosmetics, medications, and processed foods. In addition, as consumers demand more environmentally friendly cleaning options, orange oil is used increasingly in household cleaning products. Orange oil is derived from the outermost part of the orange; therefore, pesticide contamination is a concern.

Ginseng, an herb used to stimulate the adrenal gland and increase energy, has been used in various systems of medicine for centuries. The root of the ginseng plant contains active chemical components called ginsenosides, which are believed to be responsible for the medicinal properties of the herb. Therefore, the root is commonly dried and made into tablets, extracts, and teas to be taken internally or made into creams for external use. Pesticide contamination in these products is also a concern because the ginseng plant may carry residuals of environmentally persistent pesticides.

Analyzing orange oil and ginseng for pesticides is challenging in part because of the extensive exporting and importing of produce. Pesticides that are approved in one country may be banned in another, and approved pesticides may have different restrictions on the permissible levels of exposure.

Thermo Scientific TraceFinder, a software program with built-in workflows, has been developed to assist routine analysis in environmental and food residue applications. It includes a methods database pre-loaded with the appropriate *m/z* and optimized parameters of contaminants commonly encountered in environmental and food samples, which can be customized by the user to include unique compounds. An LC-MS/MS library of commonly found contaminants, organized in National Institute of Standards and Technology (NIST) format, helps to confirm the compounds being analyzed. Data collection, analysis, and report generation can be performed using the same software program. To demonstrate the software capabilities, a mixture of 250 pesticides spiked into orange oil samples and ginseng extract samples were analyzed using both negative and positive ionization modes on a Thermo Scientific TSQ Vantage Extended Mass Range (EMR) mass spectrometer.

Goal

To develop a quick and efficient LC-MS/MS method for screening pesticides in orange oil and ginseng extract using TraceFinder™ software.

Experimental Conditions

Method

Orange oil and ginseng extract were spiked with a mixture of 250 pesticides (Table 1) to give solutions containing 1 ppb and 10 ppb of each pesticide. A 5 µL sample of the spiked orange oil or ginseng extract was injected directly onto the HPLC column. A simple gradient was used with a retention time of 18 minutes. Using the TraceFinder software, Timed-Selective Reaction Monitoring (T-SRM) was used to create the instrument method, collect and process the data. In a T-SRM experiment, using prior knowledge of the retention times of the compounds, the method is set to look for specific transitions only during the expected retention-time window. This increases the number of SRM transitions that can be monitored effectively per experiment. It also increases the dwell time and duty cycle for monitoring individual compounds per experiment. The result is more accurate and sensitive quantitation.

Sample Preparation

Samples were prepared by a modified QuEChERS procedure.[1] Mixtures of 250 pesticides were prepared in acetonitrile at concentrations of 20 ppb and 200 ppb. For the 10 ppb experiment, a solvent standard was made by mixing 50 µL of the 200 ppb pesticide mixture, 150 µL of acetonitrile, and 800 µL of buffer. The 10 ppb spiked sample was prepared by adding 50 µL of the 200 ppb pesticide mixture, 50 µL of acetonitrile, and 800 µL of water to orange oil or ginseng that has been extracted with 100 µL of acetonitrile. The sample was filtered with a 0.2 µm nylon membrane to remove any particulates.

Similarly, for the 1 ppb experiment, the solvent standard was prepared by mixing 50 µL of the 20 ppb pesticide mixture, 150 µL of acetonitrile, and 800 µL of buffer. The 1 ppb spiked sample was prepared by adding 50 µL of the 200 ppb pesticide mixture, 50 µL of acetonitrile, and 800 µL of water to orange oil or ginseng that has been extracted with 100 µL of acetonitrile. The sample was filtered to remove any particulates.

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Table 1. 250 pesticides and SRM transitions

Pesticide Name	Precursor Ion $[M+H]^+$	Product Ions
Methamidophos	142.0	95.0, 125.0
Naphthol, 1-	143.2	115.1, 143.2
Methomyl	163.1	88.1, 106.1
Fenuron	165.0	46.3, 72.1
o-phenylphenol	169.0	115.3, 141.3
Phropham	180.0	120.0, 138.0
Acephate	184.1	95.2, 143.0
Fuberidazole	185.1	130.1, 157.0
Propamocarb	189.0	102.1, 144.0
Tricyclazole	190.1	136.1, 163.1
Carbendazim	192.1	132.1, 160.1
Isoprocarb	194.1	95.0, 137.0
Cymoxanil	199.1	111.1, 128.1
Cycluron	199.1	72.2, 89.1
Pyrimethanil	200.1	82.0, 107.0
Diamidafos (Nellite)	201.1	82.4, 107.2
Thiabendazole	202.0	131.0, 175.0
Carbaryl	202.1	12.0, 145.0
Dinotefuran	203.2	114.0, 129.0
Aldicarb_Sulfoxide	207.0	89.0, 132.0
Isoproturon	207.1	72.0, 165.2
Promecarb	208.1	109.0, 151.0
Aldicarb+NH_4	208.1	89.2, 116.1
Butocarboxin	208.1	91.4, 109.2
Aminocarb	209.1	137.1, 152.1
Propoxur	210.1	111.1, 168.1
Acibenzolar-S-methyl	211.1	136.0, 140.0
Chlortoluron	213.1	140.0, 168.0
Omethoate	214.1	155.0, 183.0
Simetryne	214.1	96.0, 124.0
Monolinuron	215.1	99.0, 126.0
Metribuzin	215.1	131.0, 187.1
Pymetrozine	218.0	79.0, 105.0
Pyracarbolid	218.2	96.9, 124.9
Thidiazuron	221.1	94.2, 102.1
Formetanate	222.1	120.0, 165.0
Bufencarb	222.1	77.2, 95.2
Methabenzhiazuron	222.1	150.0, 165.0
Carbofuran	222.1	123.1, 165.1
Acetamiprid	223.1	90.2, 126.1
Butoxycarboxin	223.1	86.2, 106.1
Mexacarbate	223.2	151.0, 166.0
Monocrotophos	224.1	127.0, 193.1
Dioxacarb	224.1	123.1, 167.1
Mepanipyrim	224.1	77.0, 106.0
Bendiocarb	224.2	106.0, 109.1
Aldicarb sulfoxide + NH_4	224.2	89.0, 131.7
Mevinphos	225.1	127.1, 192.8
Cyprodinil	226.0	93.0, 108.0
Methiocarb	226.1	121.0, 169.0
Ethiofencarb	226.1	107.0, 106.0
Secbumeton	226.2	99.9, 169.9
Prometon	226.2	141.9, 184.0
Terbumeton	226.2	113.9, 169.9
Ametryn	228.2	96.0, 185.9
Tebuthiuron	229.2	116.1, 172.1
Dimethoate	230.1	125.1, 199.1
Flonicamid	230.1	174.1, 203.1
Fluometuron	233.1	46.3, 72.1
Diuron	233.1	46.3, 72.0
Siduron	233.1	94.0, 137.0
Carboxin	236.0	87.0, 143.0
Thiofanox + NH_4	236.1	57.2, 76.1
Oxamyl + NH_4	237.1	72.1 90.1
Carbetamide	237.1	118.1, 192.0
Carbofuran-3-hydroxy	238.1	181.1, 220.1
Dicrotophos	238.1	112.1, 193.1

Pesticide Name	Precursor Ion $[M+H]^+$	Product Ions
Bentazone_neg	239.1	132.0, 197.0
Pirimicarb	239.1	72.0, 182.0
Butoxycarboxin + NH_4	240.1	86.2, 106.1
Aldicarb sulfone + NH_4	240.1	86.2, 148.0
Prometryn	242.2	157.9, 199.9
Terbutryn	242.2	91.0, 185.9
Ethoprophos	243.1	97.1, 131.1
Cyanophos	244.0	125.1, 212.0
Fonophos	247.0	109.1, 137.1
Fludioxinil	247.1	126.0, 180.0
Forchlorfenuron	248.1	93.0, 129.0
Linuron	249.1	160.0, 182.0
Clothianidin	250.1	132.1, 169.1
Thiacloprid	253.1	90.2, 126.1
Imidacloprid	256.1	175.1, 209.1
Thiobencarb	258.1	100.2, 125.0
Demeton-S	259.0	61.2, 89.2
Metobromuron	259.1	148.0, 170.0
Phorate	261.0	75.1, 143.0
Parathion-methyl	264.0	109.1, 124.9, 232.1
Diethofencarb	268.2	180.1, 226.0
Thiometon + Na	268.9	61.1, 89.1
Mepronil	270.1	119.0, 228.0
Nitenpyram	271.2	225.0, 237.0
Methoprotryne	272.2	198.0, 240.0
Disulfoton	274.9	61.3, 89.3
Neburon	275.1	57.2, 88.0
Bromoxynil	276.1	79.0, 81.0
Fenitrothion	278.0	108.8, 125.1, 246.0
Fenthion	279.0	169.1, 247.0
Oxadixyl	279.0	132.0, 219.0
Metalaxyl	280.1	192.1, 220.1
Propetamphos	282.0	138.1, 156.0
Penconazole	284.1	70.1, 159.0
Ethofumesate	287.0	120.9, 258.9
Vamidothion	288.1	118.1, 146.0
Terbufos	289.0	57.5, 103.1
Iprobenfos	289.0	91.2, 205.0
Myclobutanil	289.1	70.2, 125.0
Chloroxuron	291.1	46.2, 72.2
Parathion	292.0	97.0, 236.0
Uniconazole	292.1	70.2, 125.0
Cyproconazole	292.1	93.2, 125.0
Thiamethoxam	292.2	132.0, 211.1
Amitraz	294.1	122.2, 163.1
Paclobutrazole	294.1	70.0, 125.0
Triadimefon	294.2	197.1, 225.1
Triadimenol	296.1	70.0, 99.0
Imazalil	297.2	159.0, 201.0
Spiroxamine	298.2	100.0, 144.0
Quinalphos	298.9	163.1, 243.0
Mefenacet	299.2	120.1, 148.0
Ditalimfos	300.1	144.2, 145.3
Phenmedipham	301.2	136.0, 168.0
Bifenazate	301.2	152.0, 170.0
Fenhexamid	302.1	55.0, 97.0
Furalaxyl	302.1	95.0, 242.1
Flutriafol	302.2	70.1, 123.0
Fenoxycarb	302.2	88.0, 116.0
Methidathion	302.9	85.2, 144.9
Clofentezine	303.1	102.0, 138.0
Fenamiphos	304.0	217.0, 234.0
Fenpropimorph	304.4	130.1, 147.1
Diazinon	305.0	153.1, 169.1
Pirimiphos-methyl	306.0	108.2, 164.1
Buprofezin	306.2	116.0, 201.0
Fenazaquin	307.2	57.2, 160.9

Table 1. 250 pesticides and SRM transitions (continued)

Pesticide Name	Precursor Ion [M+H]+	Product Ions
Quinoxyfen	307.9	161.9, 196.8
Tebuconazole	308.2	70.2, 125.0
Diflubenzuron	308.9	156.0, 289.0
Fensulfothion	309.2	163.0, 251.0
Edifenphos	311.0	109.1, 283.0
Fenamidone	312.2	236.2, 264.2
Triazophos	314.0	119.2, 162.1
Kresoxim-methyl	314.1	222.1, 267.1
Hexaconazole	314.1	70.2, 159.0
DEF	315.0	169.0, 259.1
Nuarimol	315.1	81.0, 251.9
Flusiazole	316.2	165.0, 247.1
Bupirimate	317.3	108.1, 166.1
Phosmet	317.9	133.1, 160.1
Azinphos-methyl	317.9	125.0, 261.0
Triticonazole	318.1	70.0, 125.0
Desmedipham + NH_4	318.2	136.0, 182.0
Tebupirimfos	319.1	166.1, 210.2
Metconazole	320.2	70.1, 124.9
Phenthoate	320.9	79.3, 247.0
Iprovalicarb	321.2	119.0, 203.0
Pyriproxyfen	322.2	96.0, 185.3
Sulprofos	322.9	218.9, 247.0
Sulfotep-ethyl	323.2	219.0, 247.1
EPN	324.0	157.0, 296.0
Flutolanil	324.2	242.0, 262.0
Cyazofamid	325.2	108.0, 261.0
Famphur	326.0	217.0, 281.0
Diniconazole	326.2	70.2, 148.2
Benalaxyl	326.2	148.0, 208.0
Dimoxystrobin	327.1	116.0, 205.0
Diclobutrazol	328.1	70.2, 159.0
Etaconazole	328.2	123.0, 159.0
Epoxiconazole	330.2	121.0, 123.0
Malathion	330.9	99.2, 285.0
Fenarimol	331.1	81.0, 268.0
Pirimiphos ethyl	334.1	182.1, 198.1
Ipconazole	334.1	70.2, 125.0
Tebufenpyrad	334.2	117.0, 145.2
Zoxamide	336.2	159.0, 187.0
Fenbuconazole	337.0	70.4, 125.1
Bitertanol	338.1	99.0, 269.0
Mesotrione	340.2	185.9, 228.0
Pyridaphenthion	341.1	189.0, 205.0
Prothioconazole	342.0	100.0, 306.0
Propiconazole	342.2	69.2, 159.0
Thiophanate-methyl	343.2	151.1, 311.2
Boscalid	343.2	271.0, 307.0
Azinphos-ethyl	346.0	132.1, 160.1
Isofenfos	346.0	217.0, 245.0
Triflumizole	346.1	73.0, 278.1
Tebufenozide	353.1	133.0, 297.0
Hexythiazax	353.2	168.1, 228.2
Piperonyl butoxide	356.2	119.0, 177.0
Triflumuron	359.1	139.0, 156.0
Clethodim	360.2	164.0, 268.0
Etoxazole	360.2	141.0, 177.1
Isoxaflutole	360.2	220.0, 251.0
Topramezone	364.2	124.9, 333.9
Flufenacet	364.2	152.0, 194.0
Benzoximate	364.4	105.2, 199.2
Pyridaben	365.2	147.0, 309.1
Methoxyfenozide	367.3	105.0, 149.0
Propargite	368.2	174.9, 231.0
Picoxystrobin	368.2	145.0, 205.1
Loxynil	369.9	127.0, 215.0, 242.9
Spiromefesin	371.3	255.3, 273.3
Tetraconazole	372.2	70.0, 159.0
Famoxadone	373.1	282.4, 329.6
Pyrazophos	374.0	194.0, 222.1
Fluquinconazole	376.2	307.0, 349.2
Prochloraz	376.2	266.0, 308.0
Bromuconazole 46	378.0	70.2, 159.0
Teflubenzuron	379.2	196.0, 339.0
Benthiavalicarb	382.1	116.0, 180.0
Furathiocarb	383.2	195.0, 252.0
Ethion	384.9	97.1, 143.0
Dimethomorph	388.1	165.0, 301.0
Pyraclostrobin	388.2	163.0, 194.0
Famoxadone + NH_4	392.1	238.0, 331.2
Rotenone	395.3	192.1, 213.2
Ethiprole	397.1	255.0, 351.0
Flucarbazone	397.1	115.0, 129.9
Alanycarb	400.3	91.0, 238.0
Pinoxaden	401.2	57.1, 317.0
Sulfentrazone	404.0	307.0, 387.0
Azoxystrobin	404.1	329.1, 372.1
Difenoconazole	406.2	111.0, 251.0
Trifloxystrobin	409.3	186.0, 206.1
Spirodiclofen	411.0	213.1, 313.1
Benfuracarb	411.1	195.1, 252.0
Mandipropamid	412.1	327.9, 355.9
Carfentrazone-ethyl	412.2	366.2, 384.0
Fenpyroximate	422.2	214.0, 366.0
Fipronil	437.2	330.2, 368.0
Hexaflumuron	458.9	175.0, 439.0
Fluoxastrobin	459.2	188.0, 427.1
Fluazinam	463.2	398.0, 416.0
Temephos	466.9	405.1, 419.1
Dioxathion	474.0	153.0, 271.1
Flufenoxuron	487.2	156.0, 304.0
Novaluron	491.2	305.0, 471.0
Butafenacil + NH_4	492.3	180.0, 331.0
Novaluron	493.3	141.0, 158.0
HydramethylNon	495.3	150.9, 323.0
Lufenuron_neg	509.2	175.0, 326.0
Lufenuron	511.3	141.0, 158.0
Milbemycin A3	511.4	475.2, 493.2
Milbemycin A4 - H_2O	525.4	489.2, 507.2
Noviflumuron	527.0	193.0, 344.0
Indoxacarb	528.3	203.0, 293.0
Chlorfluazuron	539.7, 541.9	383.0, 385.0
Milbemycin A4 + NH_4	560.4	507.2, 525.2
Moxidectin	640.2	498.5, 528.5
Spinosyn A	732.5	98.0, 142.0
Spinosyn D	746.5	98.0, 142.0
Emamectin B1b	872.4	158.2, 302.3
Avermectin B1b + NH_4	876.5	145.0, 291.0
Emamectin	886.7	158.0, 302.0
Avermectin B1a + NH_4	890.4	305.3, 307.0, 567.4
Ivermectin B1a + NH_4	892.5	307.0, 569.0
Avermectin B1a + Na	895.4	183.1, 751.5
Doramectin	916.4	331.4, 593.5
Eprinomectin B1a	936.5	352.1, 490.2

HPLC

Chromatographic analysis was performed using the Thermo Scientific Accela HPLC pump and Accela™ autosampler. The chromatographic conditions were as follows:

Column:	Thermo Scientific Hypersil GOLD PFP (100 mm × 2.1 mm, 1.9 µm)		
Injection volume:	5 µL		
Column temperature:	45 °C		
Mobile phase A:	5 mM ammonium formate in water		
Mobile phase B:	5 mM ammonium formate in methanol		
Flow rate:	0.3 mL/min		
Gradient:	Time (min)	A%	B%
	0	95	5
	2	75	25
	30	0	100
	35	0	100

MS

MS analysis was carried out on a TSQ Vantage EMR™ triple stage quadrupole mass spectrometer with a HESI-II heated electrospray ionization source.

The MS conditions were as follows:

Ion source polarity:	Positive and negative ion mode
Spray voltage:	3500 V
Vaporizer temperature:	400 °C
Ion sweep gas:	2.0 units
Ion transfer tube temperature:	200 °C
Sheath gas pressure (N_2):	55 units
Auxiliary gas pressure (N_2):	15 units
Resolution:	0.7 amu (FWHM) on Q1 and Q3
Scan Width:	0.002 Da
Chrom Filter:	10.0 ms
Collision Gas Pressure:	1.5 mTorr
Scan Type:	Timed SRM (T-SRM)
Cycle Time:	0.4 s

Two SRM transitions per pesticide were monitored for confirmation (Table 1).

Software

Data collection and processing was handled by TraceFinder environmental and food safety software. TraceFinder includes several methods applicable to the environmental and food safety markets, as well as a comprehensive Compound Datastore (CDS). The CDS includes SRM transitions and collision energies for several hundred pesticides, herbicides, personal care products, and pharmaceutical compounds that are of interest to the environmental and food safety industries. A user can select one of the included methods in TraceFinder or quickly develop new or modified methods by using the pre-existing SRM transition information in the CDS, thus eliminating time-consuming compound optimizations.

Results and Discussion

Method Development

The method development section of the software allows the user to choose the compounds that will be analyzed. In this experiment, the appropriate SRMs for the 250 pesticides were chosen from the CDS (Figure 1) and inserted into the instrument method for detection (Figure 2). No compound optimization is necessary for compounds already in the CDS.

Additionally, the calibration levels, QC levels, and peak detection settings are defined in the method development section. Results can be flagged based on user-defined criteria. For example, a flag can be set for a compound whose calculated concentration is beyond the upper limit of linearity, above a defined reporting limit, or below a limit of detection. This allows for faster reviewing of the data after collection; positive samples can be quickly identified.

Acquisition

The Acquisition section provides a step-by-step process to acquire data. The overall progress is followed in an overview section on the left side of the screen (Figure 3). A green check box indicates that the step has been completed and that there are no errors. The steps include template selection (pre-defined sample lists, which are helpful in routine analysis), method selection, sample list definition, report selection, and instrument status.

A final status page summarizes the method and all of the samples to be run. In addition, it gives an overall summary of the status of the instrument (Figure 4). Three colored dots are shown: green indicates an “ok” status; yellow indicates that the attached device is in standby; and red indicates that the attached device is not ready. From the final status page, the batch can be acquired or saved to be run at a later date. A previously saved calibration curve can be used, so that a calibration need not be run every day.

Data Review

The targeted screening analysis of 250 pesticides in a ginseng extract sample was reviewed in the Data Review section of TraceFinder software. In this section, calibration lines, ion ratios, peak integration, and MS spectra (if applicable) can all be viewed (Figure 5). In addition, the Data Review section can flag samples that meet certain user-set criteria. For example, if a tolerance is specified for the ion ratio, a green flag means that the criteria has been met, while a red or yellow flag indicates that it has not. As another example, flags can be used to alert for the presence of carry-over in a blank sample. A red flag indicates that there is a significant issue with the blank sample. In this experiment, the two-point calibration was sufficient to show the calculated amount of the different pesticides found in ginseng extract.

The Data Review section allows user adjustments, such as peak reintegration. The effects of the changes on the results are instantly updated in the results grid.

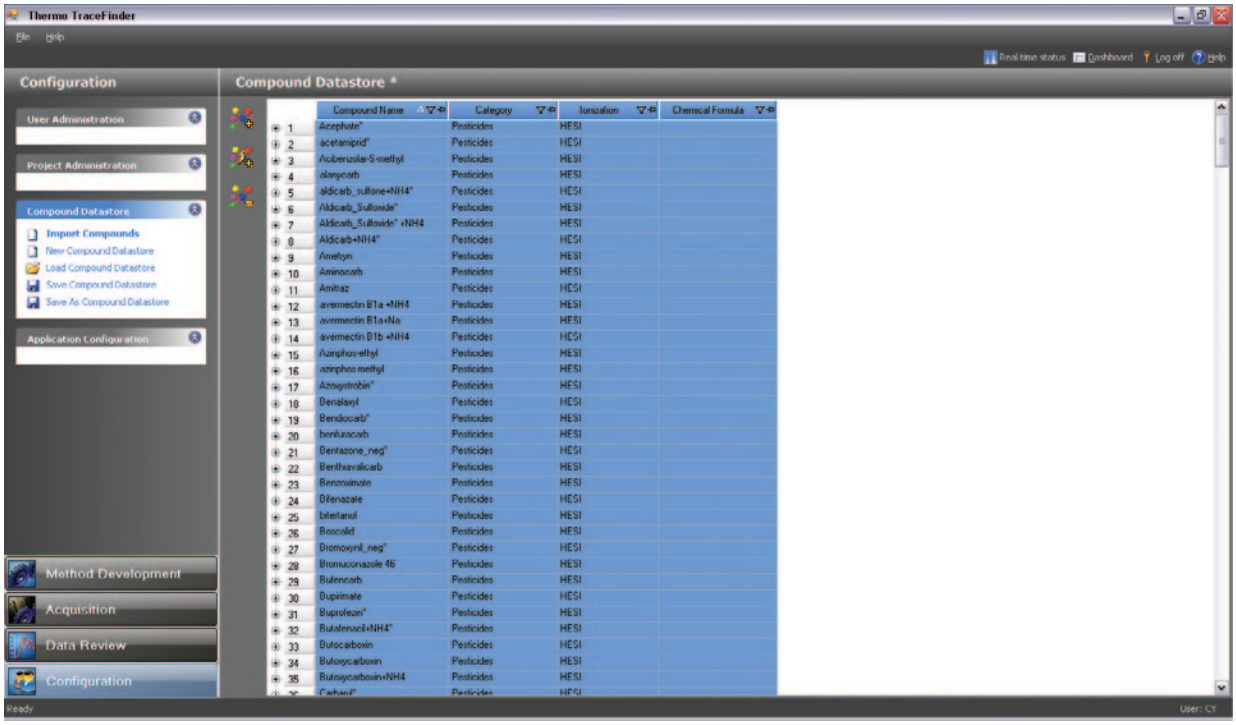

Figure 1. TraceFinder Compound Datastore (CDS)

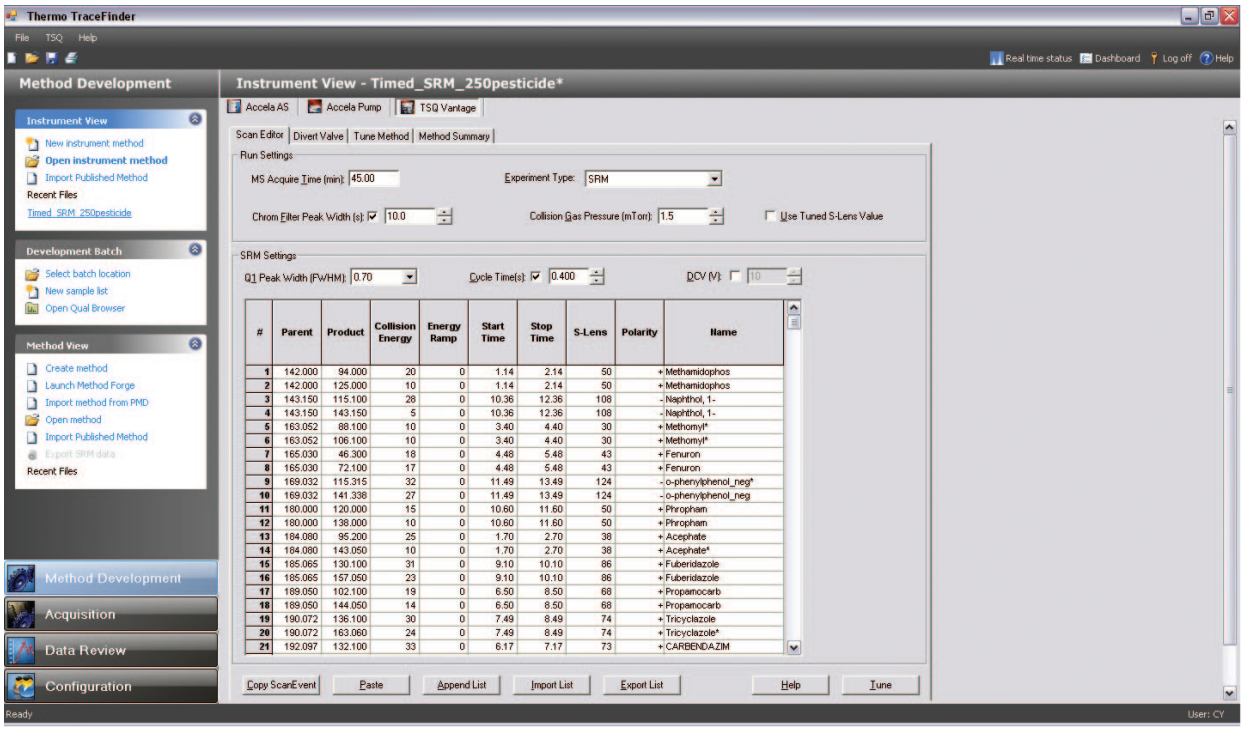

Figure 2. The Compound Datastore is easily inserted into the Instrument Method page.

Reporting

A large number of report templates are available in TraceFinder software. The user has the option of creating PDF reports, printing reports directly to the printer, or saving reports in an XML format, which is useful with laboratory information management systems (LIMS). The user can decide which reports are most applicable to each particular method. In this manner, a supervisor or lab director can set up methods and reports, lock the method, and make it non-editable by technicians. In this way, the integrity of a method is preserved, which is especially useful in controlled environments.

Two examples of the reports generated by TraceFinder software are shown in Figures 6 and 7. This view shows

the on-screen preview function. Figure 6 shows the Calibration Density Report, which displays calibration curves for each compound on one page. Figure 7 shows the Quantitation Report for 1 ppb level in ginseng extract. In this report, the sample summary is provided at the top of the page, and the quantified results follow beneath the chromatogram. TraceFinder can generate results for the entire batch with one click, or the user can view reports individually and print only those of interest.

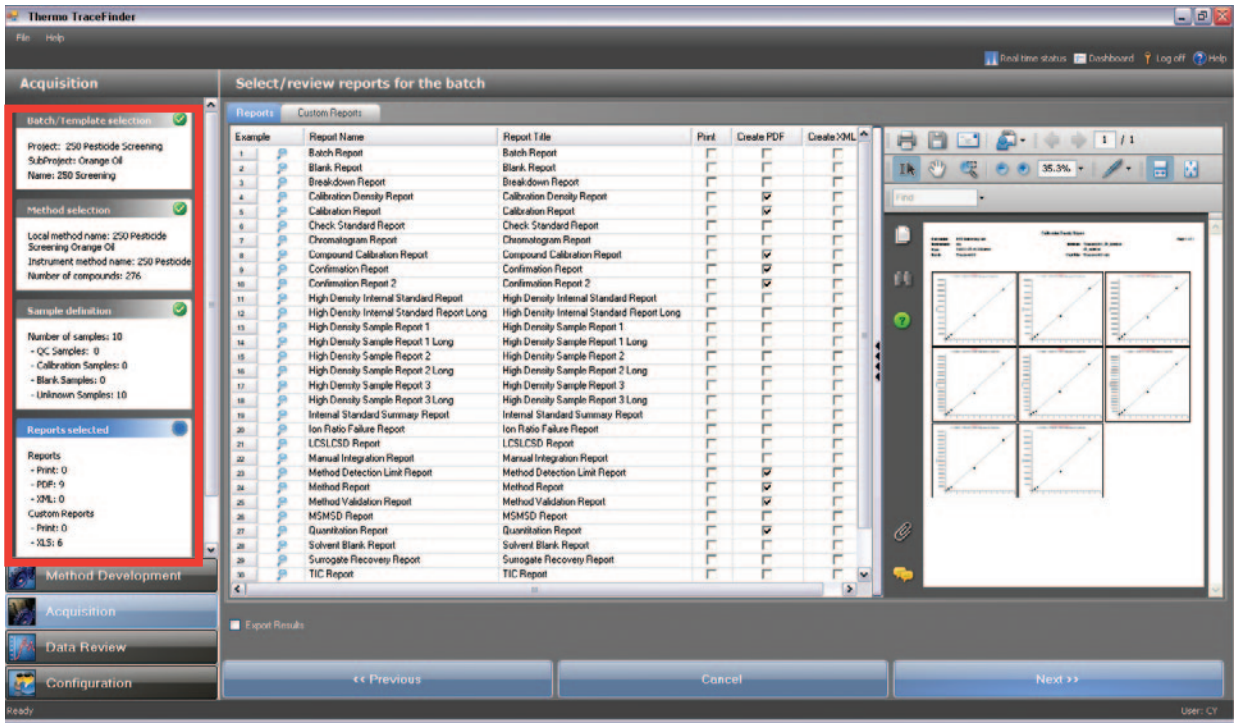

Figure 3. Acquisition section, showing the reporting templates and report preview. The red box at the left outlines the overall progress.

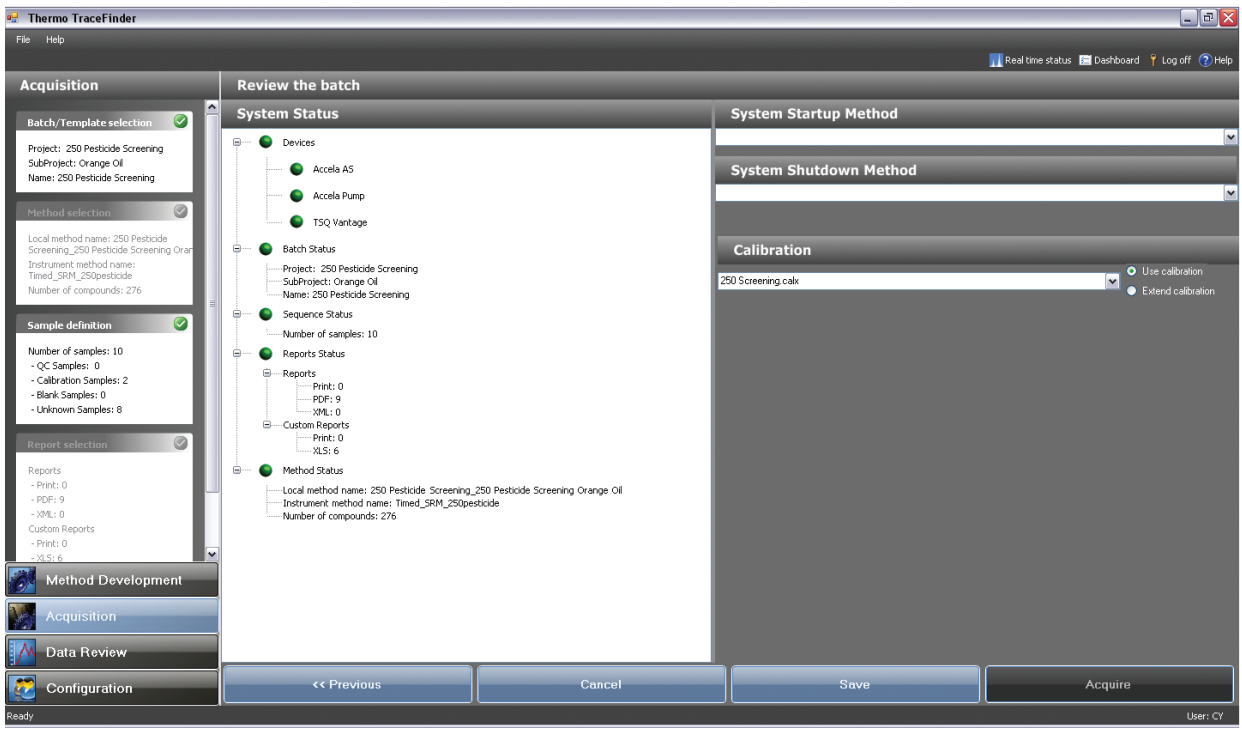

Figure 4. TraceFinder Acquisition status page. This is the final view before submitting a batch for analysis, providing the user instant instrument and method feedback.

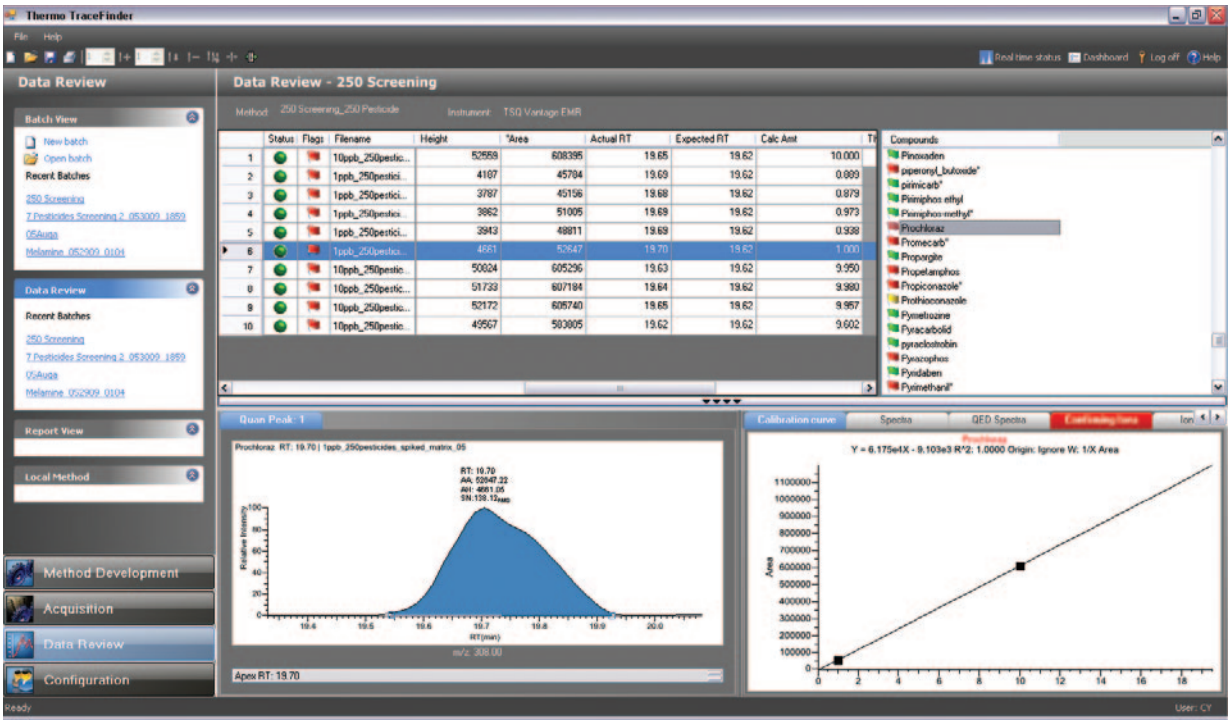

Figure 5. TraceFinder Data Review section. The red and yellow flags indicate that there are certain issues with the compound. For example, the ion ratio may be off or the value may be below the specified limit of detection.

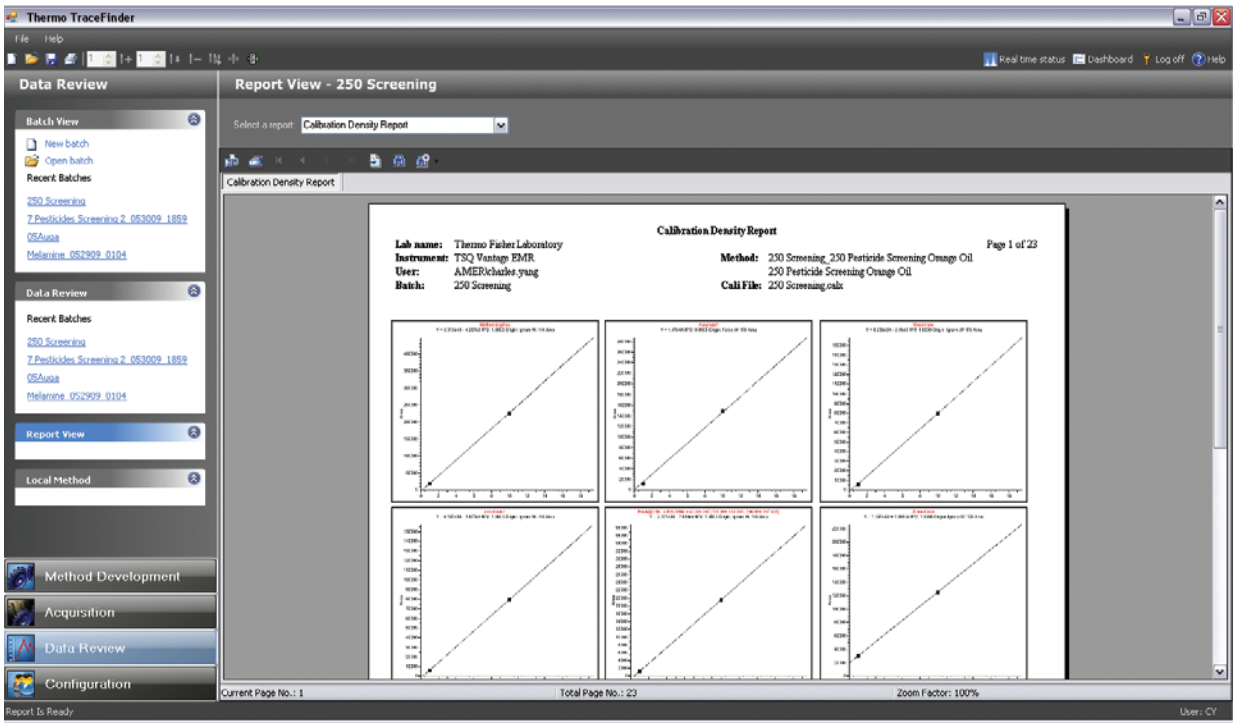

Figure 6. Report View section of TraceFinder, showing calibration curves.

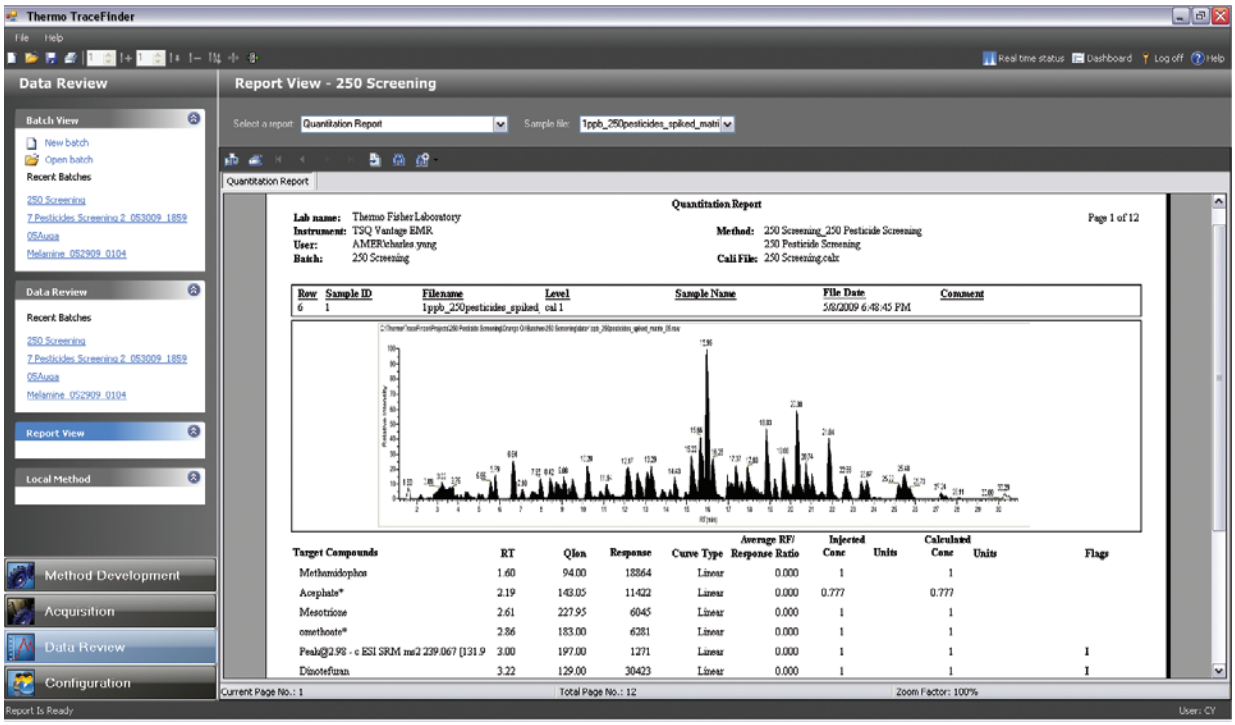

Figure 7. Report View section of TraceFinder showing quantitation results of ginseng.

Conclusion

A new software package, TraceFinder, with an extensive menu of preconfigured methods and report formats, was used to simplify method development for the screening of 250 pesticides in orange oil and ginseng extract. The results from this experiment show positive confirmation of approximately 220 pesticides in orange oil and 250 pesticides in ginseng extract based on the tolerances set in the method for quantitation and confirmation. The method development capabilities and Compound Datastore of TraceFinder software allowed for the quick creation of a method for the analysis of these compounds. In addition, the ability to flag problematic samples in the data review section helped to reduce the overall analysis time by filtering out samples that did not meet predefined criteria.

References

1. Wong, J.W., Hennessy, M.K., Hayward, D.G., Krynitsky, A.J., Cassias, I., Schenck, F.J. (2007) J. Agric. Food Chem. 55, 1117-1128.

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Application Note: 514

Screening Method for 30 Pesticides in Green Tea Extract Using Automated Online Sample Preparation with LC-MS/MS

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Key Words

- Transcend TLX-1 System
- TurboFlow Technology
- TSQ Access MAX
- Food Safety

Introduction

Analysis of pesticide residues has been one of the most important tasks of food safety laboratories. Mass spectrometers (MS), with liquid chromatography coupled to triple stage quadrupole mass spectrometers (LC-MSMS), have been the main tools used in pesticide residue analysis. There is a consensus that sample preparation is becoming the bottleneck to the entire workflow. Traditional sample preparation methods, usually involving liquid-liquid extraction (LLE) or solid phase extraction (SPE), can be time-consuming and labor-intensive. In addition, low recovery, matrix interference and poor reproducibility are among other major concerns. In recent years, a rapid processing method, QuEChERs, has gained popularity. The QuEChERs method makes it easier and less expensive for analytical chemists to examine pesticide residues in various food matrices[1]. However, some reports show matrix interference tends to be severe after QuEChERs, and the mass spectrometer is more vulnerable to contamination by highly complex food matrices[2].

In this study, we describe an easy, comprehensive, online screening LC method using a Thermo Scientific Transcend TLX-1 system powered by Thermo Scientific TurboFlow technology to analyze multiple pesticide residues in green tea extract. Figure 1 illustrates a typical Transcend™ TLX-1 system with the Thermo Scientific TSQ Access MAX triple stage quadrupole mass spectrometer.

Goal

Develop a rapid and sensitive automated online sample preparation LC-MS/MS method to screen for multiple pesticides in green tea extract.

Experimental

The matrix standard curve

One gram of Chinese green tea was extracted using 10 mL HPLC grade acetonitrile followed by 15 minutes of ultra-sonication. The extract was then filtered through a 0.45 µm membrane filter. The resultant solution was used to prepare the matrix calibrators and QC samples. The matrix calibrant concentrations are 6.25 µg/L, 12.5 µg/L, 25 µg/L, 50 µg/L and 100 µg/L, respectively. The matrix QC sample concentration is 10 µg/L.

TurboFlow™ Method Parameters

System:	Transcend TLX-1 system controlled by Thermo Scientific Aria OS 1.6.3 software
Column:	TurboFlow Cyclone 0.5 x 50 mm
Injection Volume:	10 µL
Loading Solvent:	0.1% formic acid in water
Loading Flow Rate:	1.5 mL/min
Eluting Solvent:	0.1% formic acid in methanol

Figure 1. Typical layout of a Transcend TLX-1 system with a TSQ Access MAX™ triple stage quadrupole mass spectrometer.

Thermo SCIENTIFIC

HPLC Method Parameters

Analytical Column:	Thermo Scientific Hypersil GOLD 2.1 x 100 mm, 3 µm
Solvent A:	0.1% formic acid in water
Solvent B:	0.1% formic acid in methanol

Mass Spectrometer Parameters

MS:	TSQ Quantum Access MAX
MS Ionization Source:	Heated Electrospray Ionization (H-ESI)
Ion Polarity:	Positive ion mode
Spray Voltage:	2 KV
Sheath Gas Pressure (N_2):	30 arbitrary units
Auxiliary Gas Pressure (N_2):	15 arbitrary units
Vaporizer Temperature:	300 °C
Capillary Temperature:	300 °C
Collision Gas Pressure:	1.5 mTorr

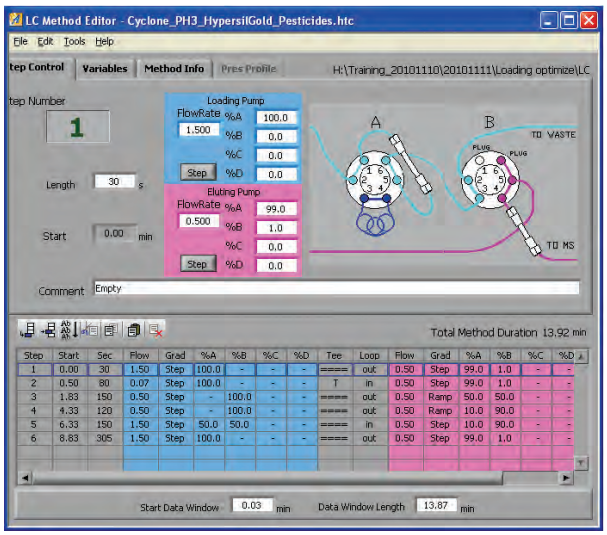

Figure 2. TurboFlow method schematic as viewed in the Aria OS software.

Results and Discussion

The Extraction and Separation of 30 Pesticide Residues

In 2006, Japan released the most stringent pesticide-related regulation in history entitled "Positive List System for Agricultural Chemical Residues in Foods"[3]. Since Japan is China's major tea importer, the limits discussed in the current study follow this regulation. As described in the Experimental section, the tea matrix standard samples are 6.25 µg/L, 12.5 µg/L, 25 µg/L, 50 µg/L and 100 µg/L, respectively. The matrix QC samples are 10 µg/L. Figure 3 shows the representative chromatograms at 6.25 µg/L, which has been determined as the lower limit of quantitation (LLOQ). The data demonstrate that 30 pesticides were well separated with good peak shape. The peaks' signal to noise ratios are far greater than the required 10:1 at the LLOQ. Table 1 shows the linear curve for these 30 analytes. All R^2 values are between 0.993-0.999. The relative standard deviation (RSD) for 6 consecutive injections of 6.25 µg/L calibrator was in the range of 2.85% -7.48%.

Background Reduction Effects using TurboFlow Technology

By using the Transcend TLX system with TurboFlow technology, the background noise and interference peaks are reduced significantly. Figure 4 compares chromatograms of Clomazone at 6.25 µg/L in tea extract using standard HPLC (top) and the TurboFlow method (bottom). The left panel (A-1 and B-1) shows the primary transition of *m/z* 240 > 125. The right panel (A-2 and B-2) shows the secondary transition of *m/z* 240 > 89. It clearly shows the effectiveness of background reduction using TurboFlow technology while the signal to noise ratio increased by 3 and 4 times for *m/z* 125 and 89 transitions, respectively. The area responses of both peaks also increase by more than 50% due to the minimization of ion suppression incurred by matrix. We also noticed the mass spectrometry response become more stable across the entire tested concentration range, thus improving the method reliability.

A Simple Method Optimization Process

During TurboFlow method development, the sample loading condition, elution solvents and many other parameters may need to be optimized. Aria™ OS 1.6.3 operation software for Transcend systems offers a method variable function. By utilizing this unique tool, different parameters can be easily tried using the same method in a single batch. For example, in this study, one of the critical steps was to find the optimal solvent content in the transfer loop to elute the target analytes completely from the TurboFlow column without introducing unnecessarily high organic solvent into the analytical column. We compared 5 different concentration ratios of 0.1% formic acid in acetonitrile to 0.1% formic acid in water (10:90, 30:70, 50:50, 70:30 and 90:10). The results indicated that with the increase of organic content, the target compounds were more completely washed off from TurboFlow column. However, once the organic concentration reached 50%, the elution strength was approaching a balance. Therefore, we chose 50:50 as the optimal elution ratio of organic to aqueous solvent in the transfer loop. Another example of method optimization appears in Figure 5, showing the effects of the loading flow rate on Dimethametryn's elution peak shape. All these tests were done in just one sample batch without writing multiple methods, which simplified the method development process and improved method reliability.

The Comparison of TurboFlow Technology with Two of the Most Popular Pesticides Sample Preparation Methods

As shown in Figure 6, we compared a TurboFlow method and two currently popular methods for pesticide residue sample preparation, SPE and QuEChERs. A typical SPE method involves equilibrating the cartridge, loading, washing and eluting analytes. It usually takes about 1 week to process 100 samples. Although QuEChERs was designed to simplify sample preparation, it still requires two-step centrifugation and concentration. A few days are typically required to prepare 100 samples with QuEChERs. TurboFlow technology minimizes preparation of 100 samples to less than 3 hours, dramatically improving the efficiency and throughput of this routine lab test.

Table 1: Standard curve linearity and QC results for the 30 pesticides in tea extract.

Compound	RT (min)	Parent ion *(m/z)*	Product ion *(m/z)*	Collision Energy (V)	Linear Curve	R^2	CV% (n = 6) QC = 10 µg/L
Prometon	4.82	226.0	184.0 142.1	20 27	Y=167343+396533X	0.999	2.99%
Ametryn	5.07	228.0	186.0 96.0	26 34	Y=83264.1+194461X	0.999	2.85%
Dimethametryn	5.68	256.1	186.1 158.1	21 27	Y=166875+605055X	0.999	3.29%
Mefenoxam	5.79	280.0	220.0 192.0	17 20	Y=460109+272420X	0.998	4.23%
Monolinuron	5.85	215.0	126.0 99.0	17 36	Y=-10985.6+18335.3X	0.998	6.51%
Isoprocarb	5.94	194.0	95.0 137.0	16 11	Y=-18662+12428.2X	0.999	6.43%
Dimethachlor	6.01	256.0	224.0 148.0	15 28	Y=-23531.9+96341.3X	0.997	5.53%
Clomazone	6.05	240.0	125.0 89.0	20 37	Y=-43447.5+42181.6X	0.998	6.37%
Furalaxyl	6.21	302.0	242.0 270.0	15 10	Y=358101+267257X	0.998	4.85%
Azoxystrobin	6.33	404.0	372.0 329.0	15 33	Y=538988+377945X	0.997	4.00%
Triadimefon	6.39	294.0	197.0 225.0	19 19	Y=-20167.4+16685.8X	0.997	7.31%
Ethoprophos	6.41	243.0	131.0 97.0	21 33	Y=-13814+14313.8X	0.997	7.48%
Iprobenfos	6.52	289.0	205.0 91.0	12 23	Y=53008.6+137376X	0.999	6.28%
Isoprothiolane	6.57	291.0	189.0 231.0	22 12	Y=123106+87284X	0.998	6.00%
Flutolanil	6.60	324.0	242.0 262.0	26 18	Y=6077+47866X	0.998	6.56%
Propiconazole	6.65	342.0	159.0 69.0	30 31	Y=-17113.2+36428.7X	0.997	6.74%
Benalaxyl	6.78	326.0	148.0 208.0	25 20	Y=172291+126493X	0.997	5.92%
Pirimiphos-methyl	6.81	306.0	164.0 108.0	22 33	Y=227752+204491X	0.994	4.73%
Picoxystrobin	6.82	368.0	145.1 205.0	22 7	Y=320093+78661.3X	0.993	4.03%
Diazinon	6.90	305.0	169.0 153.0	24 26	Y=182248+386247X	0.998	4.96%
Thiazopyr	6.95	397.0	335.0 275.0	30 40	Y=-5052.12+18434.8X	0.997	6.47%
Piperophos	7.09	354.0	171.0 143.0	25 33	Y=142671+143459X	0.996	4.68%
Trifloxystrobin	7.13	409.0	186.0 206.0	21 16	Y=-18755.2+43150.6X	0.998	6.22%
Tebufenpyrad	7.16	334.0	145.0 117.0	28 36	Y=-3267.09+9390.51X	0.998	7.01%
Piperonyl butoxide	7.25	356.0	177.0 119.0	13 33	Y=-300922+175066X	0.996	4.16%
Pyriproxyfen	7.34	322.0	96.0 185.2	16 27	Y=-19160.9+56881.6X	0.999	4.73%
Tralkoxydim	7.39	330.0	284.0 138.0	15 20	Y=-8119.47+46536.4X	0.997	5.01%
Fenazaquin	7.55	307.0	161.0 57.0	18 23	Y=-56587.3+97365.3X	0.998	2.76%
Butralin	7.58	296.0	240.0 222.0	15 20	Y=-2485.92+25777.9X	0.998	4.72%
DEF	7.85	315.0	169.0 113.0	15 25	Y=-8658.91+7992.12X	0.998	3.89%

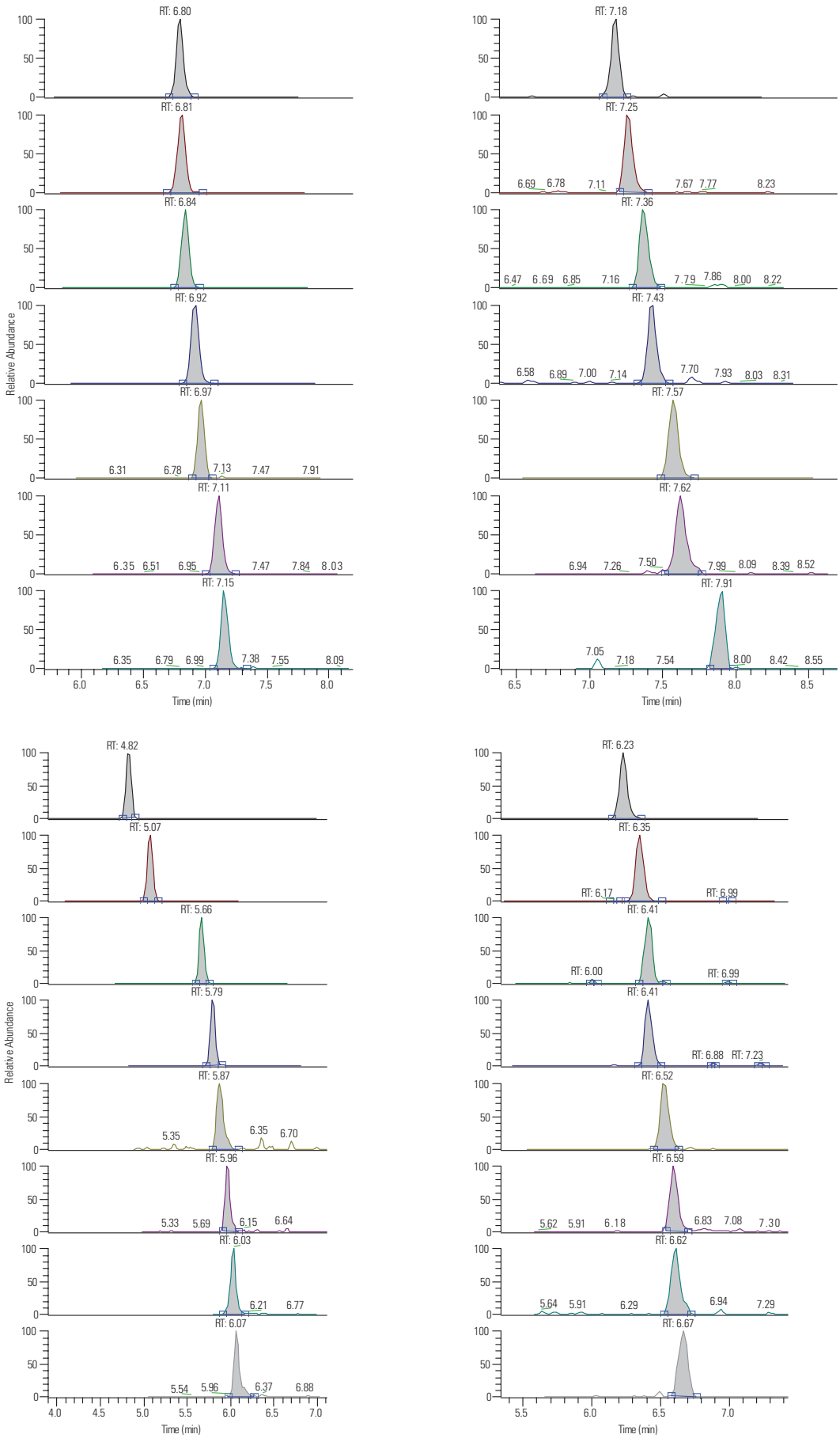

Figure 3. Selected ion chromatograms at LLOQ of 6.25 µg/L for all 30 analytes (same as the order in Table 1).

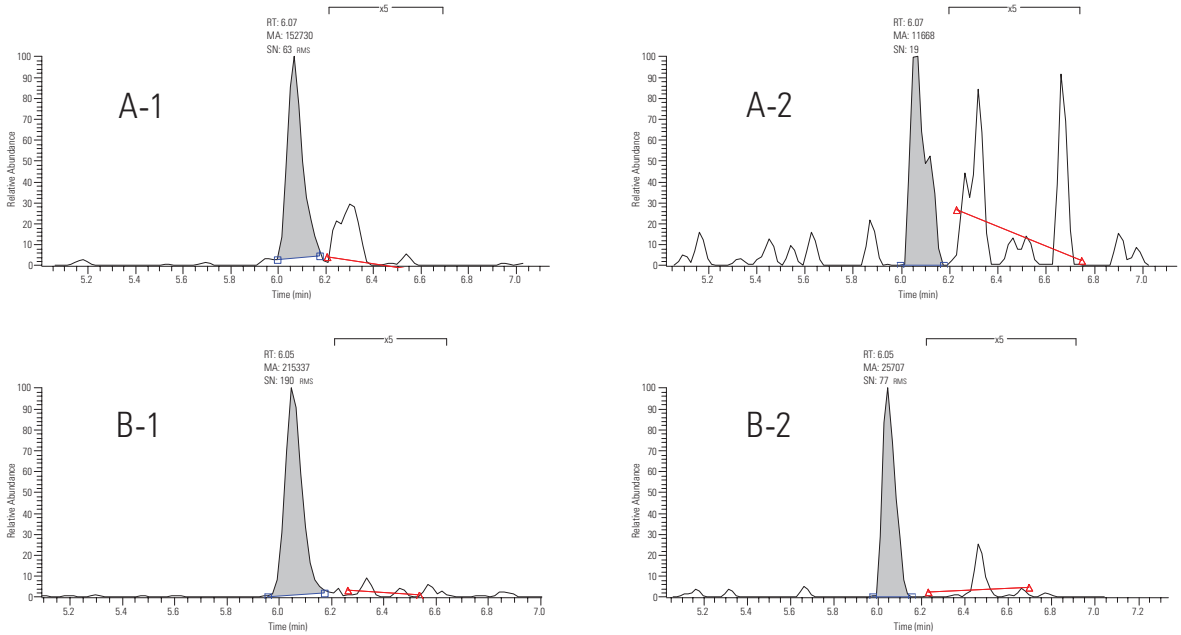

Figure 4: Comparison of chromatograms of Clomazone at 6.25 µg/L in tea extract using standard HPLC (top) and the TurboFlow method (bottom).

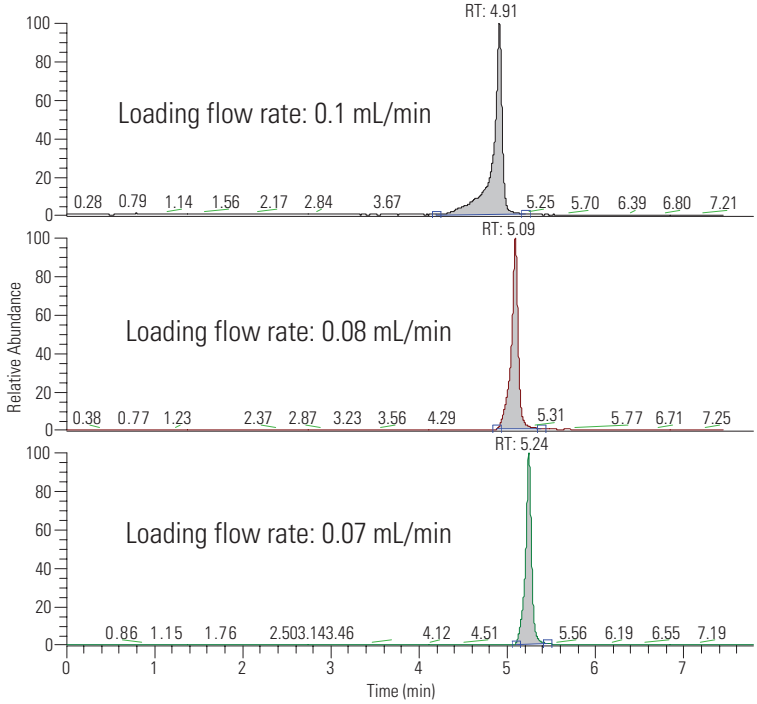

Figure 5. Effect of the loading flow rate on Dimethametryn's elution peak shape.

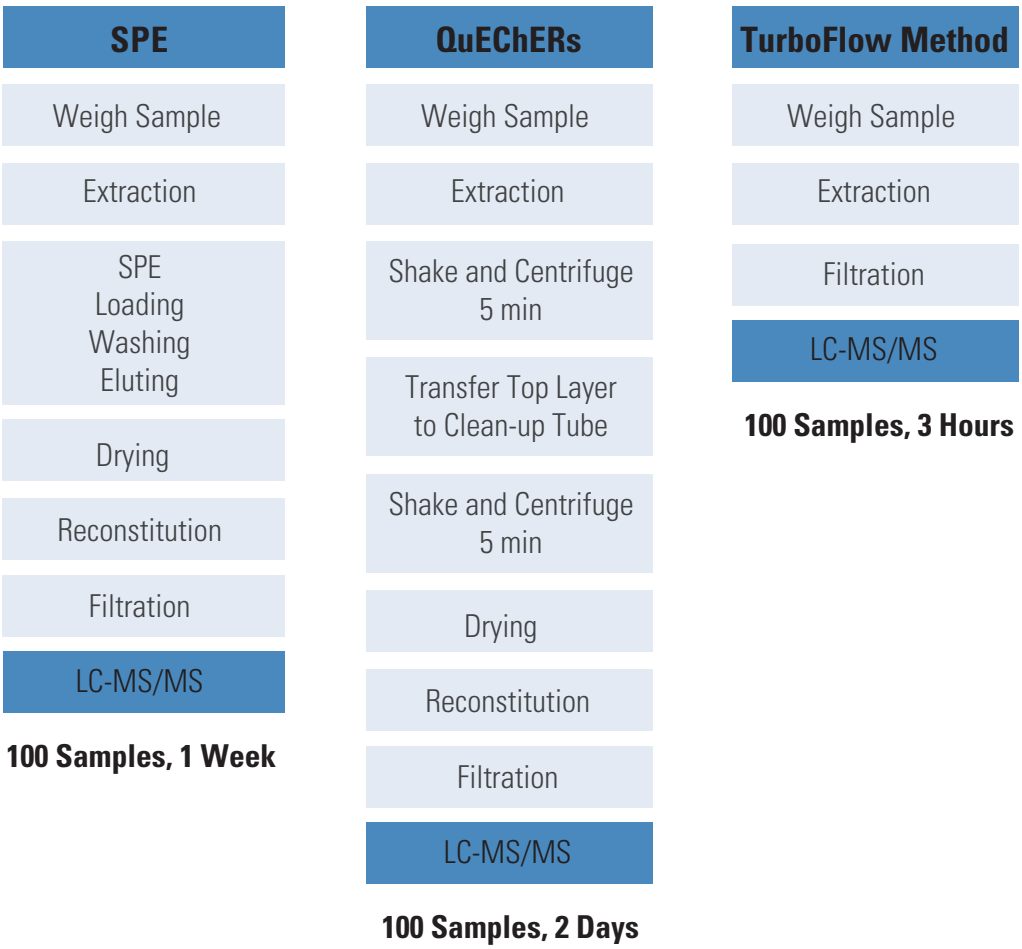

Figure 6. Comparison of the TurboFlow method to SPE and QuEChERs.

Conclusion

A quick, automated online sample preparation LC-MS/MS method has been developed that is sensitive enough to screen the tested pesticides in tea extracts. The method detection and quantitation limits are significantly lower than the strictest limits set by the Japanese government. TurboFlow technology eliminates the need for time-consuming sample preparation procedures such as SPE and QuEChERs. By using Aria OS software, the method development and optimization process is greatly simplified.

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4. GB/T 19648-2005 (466 Pesticides analysis method in Fruit and Vegetable GC-MS and LC-MS-MS)

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Application Note: 525

Streamlined Analysis of 400+ Pesticides in a Single Run Using the TSQ Quantum Access MAX Mass Spectrometer and TraceFinder Software

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Key Words

- TSQ Quantum Access MAX
- TraceFinder software
- T-SRM
- Pesticide analysis
- Food safety

Introduction

Growing concerns over food safety and the expanding world agricultural trade have lead to the enforcement of stricter pesticide regulations. In 2006, Japan introduced the Positive List System that established maximum residue levels (MRLs) for hundreds of agricultural chemicals in food, including approximately 400 pesticides, and set a uniform limit of 10 µg/kg (ppb) for chemicals for which MRLs have not been determined.[1] In 2008, the European Parliament implemented Regulation (EC) No. 396/2005, which harmonized all pesticide MRLs for European Union (EU) member states and set default limits of 10 µg/kg for all pesticide/commodity combinations for which no MRLs have been set.[2] A pesticide safety review of about 1,000 active substances on the market was mandated by EU Directive 91/414/EEC and, upon its completion in 2009, led to the approval of only about 250 substances and effectively set the permissible levels of over 700 de-listed pesticides to the default limit.[3] The EU and Japanese regulations are among the most stringent in the world and have fueled the need for faster and more sensitive analytical methods for cost-efficient, high-throughput screening and quantitation of multi-class pesticide residues.

Liquid chromatography-triple quadrupole tandem mass spectrometry (LC/MS/MS) enables highly selective, targeted, and sensitive quantitation and confirmation of hundreds of target pesticides in a single run. A multi-residue method was developed for screening and quantitation of 437 pesticides in one 45-minute run using Thermo Scientific TraceFinder software and a Thermo Scientific TSQ Series LC-MS/MS system. At least one, and often two or three, ion ratios were used to confirm each analyte. In addition, the use of the Quantitation-Enhanced Data-Dependent scan mode (QED-MS/MS) provided MS/MS mass spectra that was used for structural confirmation.

Goal

To analyze large numbers of pesticides in a single run on a triple quadrupole mass spectrometer using TraceFinder™ software with built-in workflows for streamlining method development and routine analysis.

Experimental Conditions

Sample Preparation

Pesticide standards were obtained from the U.S. Food and Drug Administration (FDA). The stock solution was prepared at a concentration of 3 mg/L. Calibration solutions, with concentrations of 0.1-250 µg/L (ppb), were prepared by serial dilution of the stock solution in 50:50 (v/v) acetonitrile/water.

Apple, orange, and asparagus matrices were prepared for analysis by using a modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method, which is a sample preparation procedure used to extract pesticides from food.[4] The QuEChERS extracts were obtained from California Department of Food and Agriculture. For the QuEChERS extraction, 15 g of homogenized sample and 15 mL of acetonitrile were used. Then, 200 µL of final QuEChERS extract, 300 µL of acetonitrile, and 500 µL of water were transferred into an autosampler vial, spiked with 20 µL of the pesticides standard, and mixed well.

HPLC

Chromatographic analysis was performed using the Thermo Scientific Accela 1250 U-HPLC system. The autosampler was an HTC-PAL Autosampler (CTC Analytics, Zwingen, Switzerland). The chromatographic conditions were as follows:

Column:	Thermo Scientific Hypersil GOLD aQ column (100 x 2.1 mm, 1.9 µm particle size)		
Mobile Phase A:	Water with 0.1% formic acid and 4 mM ammonium formate		
Mobile Phase B:	Methanol with 0.1% formic acid and 4 mM ammonium formate		
Flow Rate:	300 µL/min		
Column Temperature:	40 °C		
Sample Injection Volume:	10 µL		
Gradient:	Gradient Time (min)	%A	%B
	0.00	98	2
	0.25	70	30
	35.00	0	100
	40.00	0	100
	40.01	98	2
	45.00	98	2

MS

All samples were analyzed on the Thermo Scientific TSQ Quantum Access MAX triple stage quadrupole mass spectrometer with a heated electrospray ionization (HESI) source. To maximize the performance of the mass spectrometer, time-specific SRM windows were employed at the retention times of the target compounds. In addition, Quantitation-Enhanced Data-Dependent scanning, which delivers SRM-triggered MS/MS data, was used for structural confirmation. Alternating positive and negative polarity switching was utilized in the method. The MS conditions were as follows:

Sheath Gas Flow Rate:	55 units
Aux Gas Flow Rate:	15 units
Spray Voltage:	3500 V
Capillary Temp:	280 °C
Heater Temp:	295 °C
Cycle Time:	0.2 s

Software

Method development, data acquisition, and data processing were performed with TraceFinder software. TraceFinder software streamlines method development, acquisition, and data review. It provides a comprehensive system incorporating processing methods, library searching capabilities, data review, reporting, and built-in methods for commonly found contaminants. The Compound Data Store (CDS) in TraceFinder software includes selective reaction monitoring (SRM) transitions and collision energies for several hundred pesticides, herbicides, personal care products, and pharmaceutical compounds (Figure 1).

In this experiment, the appropriate SRM transitions of the pesticides were chosen from the CDS and inserted into the method for detection. No compound optimization was necessary for compounds that were included in the Compound Data Store.

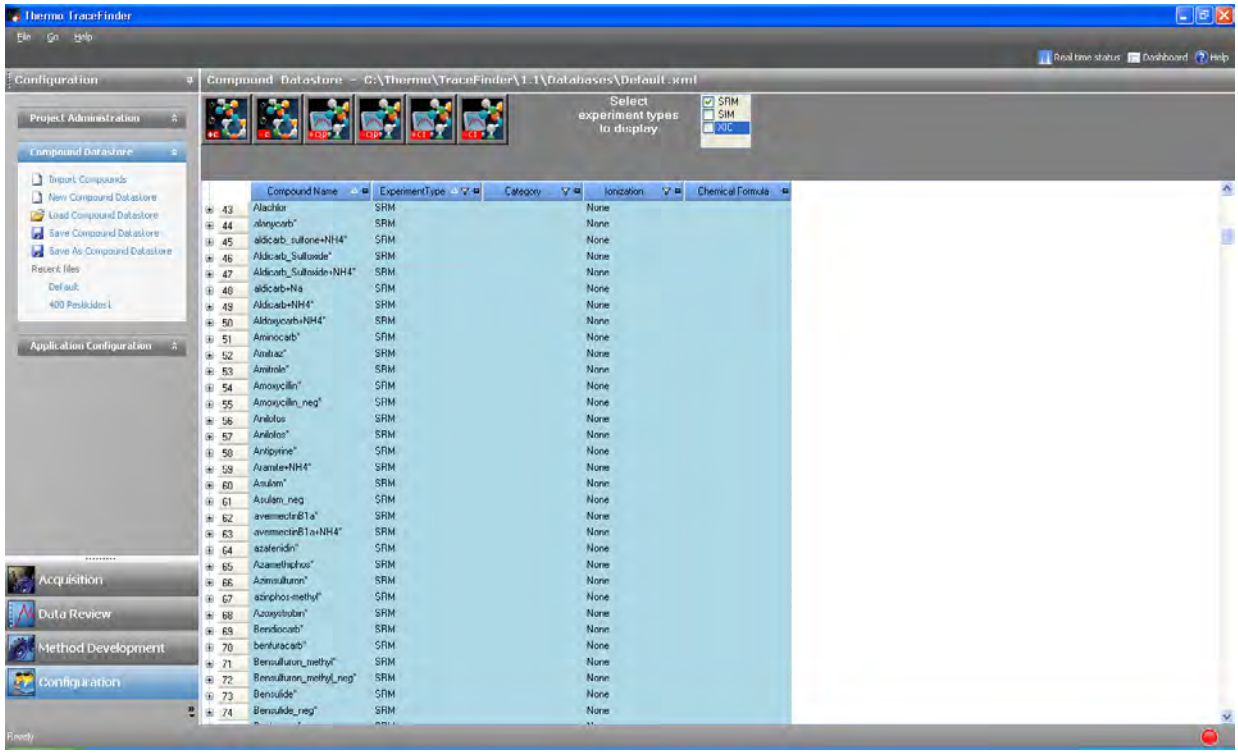


Figure 1. TraceFinder software Compound Data Store (CDS)

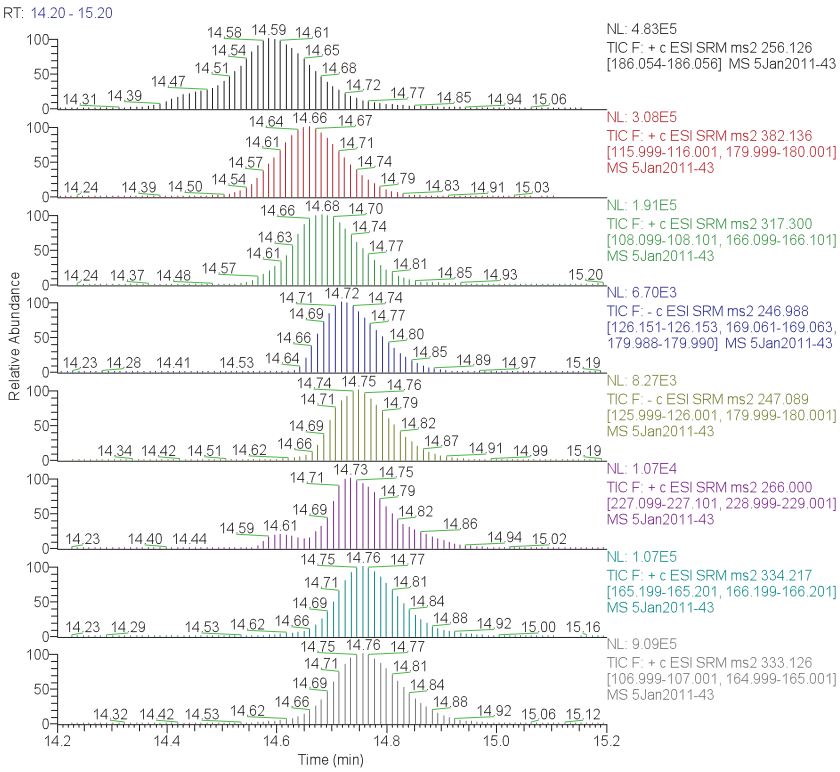


Figure 2. Eight extracted ion chromatograms showing number of scans with positive and negative switching

Results and Discussion

Multi-residue screening studies can generate very large SRM transition lists in a single experiment. T-SRM can be a useful tool to enhance qualitative and quantitative analyses. In a T-SRM experiment, using prior knowledge of the pesticide retention times, the method is set to look for specific transitions only during the expected retention time window. This increases the number of SRM transitions that can be monitored effectively per experiment. T-SRM also increases the scan time and duty cycle for monitoring individual compounds per experiment, providing more accurate and sensitive quantitation. In this screening, after retention times were determined by standard SRM run, a T-SRM method containing a total of 933 T-SRMs was constructed to analyze the compounds in one single mix. For most compounds, the time window was 60 s. Figure 2 shows that by using T-SRM, enough scans were obtained for closely and overlapping peaks with positive and negative polarity switching. T-SRM enabled the efficient detection of a large list of SRM transitions without compromising the scan time for each SRM.

A mixture of 437 pesticides representing a broad spectrum of chemical classes was separated and detected within 45 minutes (Table 1). For the concentration range studied (0.1-250 µg/L), limits of detection (LOD) were estimated from standard solutions. The LOD ranged from 0.1 to 50 µg/L, depending on the analytes.

Table 1. LC-MS/MS data for 437 pesticide standards

Compound	Precursor Ion	Quantitation Ion	CE	Confirming Ion 1	CE	Confirming Ion 2	CE	RT (min)	Polarity
Acephate	184.08	143.05	10	95.20	25			2.11	+
Acetamiprid	223.10	126.10	22	90.20	36			3.87	+
Acibenzolar-S-methyl	211.09	136.00	32	140.00	24			13.17	+
Acifluorfen	360.00	316.00	10					15.26	-
Acrinathrin+NH_4	559.00	208.00	16	181.00	33	317.00	12	27.78	+
Akton	374.80	304.90	20	97.10	40			22.05	+
Alachlor	270.10	162.00	19					15.86	+
Aldicarb sulfone+NH_4	240.12	86.20	22	148.05	12			2.38	+
Aldicarb sulfoxide	207.00	132.00	10	89.00	16			2.3	+
Aldicarb sulfoxide +NH_4	224.20	89.00	19	131.70	15			2.3	+
Aldicarb+NH_4	208.10	116.10	10	89.20	17			4.97	+
Allethrin	303.16	135.05	13	123.11	18	91.16	33	23.14	+

Table 1. LC-MS/MS data for 437 pesticide standards (continued)

Compound	Precursor Ion	Quantitation Ion	CE	Confirming Ion 1	CE	Confirming Ion 2	CE	RT (min)	Polarity
Allidochlor	174.09	98.23	12	41.44	23	39.44	45	5.24	+
Ametryn	228.20	185.90	19	96.00	26			8.94	+
Amicarbazone	242.18	143.10	12	85.20	32			6.21	+
Aminocarb	209.12	137.10	25	152.10	15			2.2	+
Amitraz	294.08	122.19	33					2.77	+
Ancymidol	257.11	135.05	26	81.21	26	77.20	45	7.44	+
Anilofos	368.00	199.00	16	171.00	23	125.00	34	18.96	+
Aramite+NH_4	352.00	191.00	12	255.00	10			23.2	+
Aspon	378.90	210.90	21	115.10	33			25.28	+
Asulam	231.00	156.00	12	92.00	25			2.29	+
Atrazine	216.00	174.00	16					9.32	+
Avermectin B1a +NH_4	890.45	305.28	22	307.00	29	567.41	11	27.65	+
Avermectin B1a+Na	895.39	751.50	45	183.08	50			27.65	+
Avermectin B1b +NH_4	876.45	291.00	30	553.40	15	145.00	35	26.77	+
Azaconazole	300.00	158.93	27	230.92	17	122.99	51	11.07	+
Azafenidrin	338.11	264.03	30	302.10	17	298.98	20	10.94	+
Azamethiphos	324.98	182.91	17	112.04	36	138.96	23	6.41	+
Azinphos-ethyl	345.96	132.10	16	160.10	7			16.14	+
Azinphos-methyl	317.93	260.98	8	125.03	19			11.9	+
Azoxystrobin	404.12	372.14	14	329.11	32			13.86	+
Benalaxyl	326.18	148.00	22	208.00	15			18.7	+
Bendiocarb	224.16	167.06	10	109.10	20			6.94	+
Benodanil	324.01	241.98	25	261.96	18	132.03	19	14.84	+
Benoxacor	260.03	148.69	17	133.98	13			11.31	+
Bensulide	398.00	314.00	12	158.00	25	218.00	18	18.34	+
Bentazone	239.07	132.00	28	197.00	22			6.51	-
Benthiavalicarb	382.14	180.00	33	116.00	23			14.65	+
Benzoximate	364.35	199.20	11	105.20	33			20.06	+
Bifenazate	301.23	170.00	20	152.00	40			16.02	+
Bifenox	342.00	310.00	15					18.99	+
Bifenthrin+NH_4	440.00	181.00	14	166.00	42			29.35	+
Bispyribac-sodium	453.14	296.96	19					13.92	+
Bitertanol	338.08	269.00	10	99.00	16			20.15	+
Boscalid	343.24	307.00	19	271.00	34			14.21	+
Brodifacoum	522.88	335.00	23	178.20	35			28.91	+
Bromadiolone	525.07	249.96	37	263.27	40	218.93	50	23.67	-
Bromoxynil	276.07	81.00	36	79.00	36			8.86	+
Bromuconazole1	377.92	158.92	28	160.88	28	123.02	35	15.16	+
Bromuconazole2	377.92	158.92	28	160.88	28	123.02	35	17.83	+
Bufencarb	222.11	95.20	34	77.20	43			17.19	+
Bupirimate	317.30	166.10	25	108.10	27			14.68	+
Buprofezin	306.21	201.00	12	116.00	18			20.78	+
Butachlor	312.20	238.00	11					22.92	+
Butafenacil+NH_4	492.31	331.00	26	180.00	46			16.26	+
Butocarboxin	208.10	109.20	15	91.40	39			13.82	+
Butoxycarboxin	223.11	106.10	10	86.20	20			2.35	+
Butoxycarboxin+NH_4	240.11	86.20	18	106.10	25			2.36	+
Butralin	296.14	240.03	14	222.03	22	208.00	28	24.95	+
Butylate	218.20	156.00	11					21.14	+
Cadusafos	270.97	158.90	16	97.00	36			20.21	+
Carbaryl	202.08	145.00	12	127.00	30			8.13	+
Carbendazim	192.10	160.06	20	132.10	33			2.75	+

Table 1. LC-MS/MS data for 437 pesticide standards (continued)

Compound	Precursor Ion	Quantitation Ion	CE	Confirming Ion 1	CE	Confirming Ion 2	CE	RT (min)	Polarity
Carbetamide	237.12	192.05	10	118.10	15			5.83	+
Carbofuran	222.14	165.06	14	123.10	25			6.91	+
Carbofuran-3-hydroxy	238.08	220.08	9	181.08	11			3.64	+
Carboxin	235.95	142.97	17	86.98	24			7.6	+
Carfentrazone-ethyl	412.19	384.00	15	366.20	19			17.94	+
Carpropamid	334.00	139.00	22	196.00	14	103.00	38	18.84	+
Chlorantraniliprole	482.13	450.89	21	283.81	19			11.79	+
Chlorbromuron	292.91	203.88	20	181.95	19	124.94	33	13.73	+
Chlordimeform	197.02	117.20	29	89.00	50			3.15	+
Chlorfenvinphos	358.81	155.20	14	99.10	33			19.16	+
Chlorfluazuron	541.90	385.00	25					26.79	+
Chlorfluazuron	539.70	383.00	20					26.79	+
Chloroxuron	291.11	72.20	20	46.20	19			15.97	+
Chlorpropham	214.00	172.00	12	154.00	19			9.96	+
Chlorpyrifos	350.00	198.00	18	97.00	35			23.81	+
Chlortoluron	213.08	140.00	22	168.00	20			9.18	+
Clethodim	360.19	164.00	20	268.00	14			21.38	+
Clofentezine	303.07	138.00	18	102.00	36			20.42	+
Clothianidin	250.12	169.06	14	132.10	18			3.38	+
Coumaphos	363.02	226.90	25					19.38	+
Coumaphos oxon	347.02	290.92	18	210.92	28	318.93	14	12.72	+
Crotoxyphos	332.07	126.99	23	99.04	27			14.36	+
Dumyluron	303.00	185.00	14	125.00	34	119.00	22	15.44	+
Cyanazine	241.10	214.00	17					6.18	+
Cyazofamid	325.22	108.00	15	261.00	10			17.23	+
Dycloate	216.00	154.00	12	134.00	14	83.00	18	19.81	+
Cyclohexamide	299.18	264.16	14	246.12	19	159.16	30	5.5	+
Cycluron	199.11	89.10	16	72.20	24			10.42	+
Cyflufenamid	413.00	295.00	16	241.00	25	203.00	42	20.34	+
Cyfluthrin	434.10	191.00	17					26.68	+
Cyhalothrin+NH_4	467.00	225.00	18	450.00	10			26.79	+
Cymoxanil	199.06	128.10	10	111.10	20			4.07	+
Cyphenothrin	393.08	315.89	23	376.00	10			20.84	+
Cyproconazole	292.13	125.00	32					15.58	+
Cyromazine	167.09	85.17	19	68.23	28	81.21	26	1.97	+
Daimuron	269.00	151.00	14	91.00	45	119.00	25	14.55	+
DEF	315.02	169.00	17	259.09	13			26.36	+
Deltamethrin	506.10	281.00	11					26.9	+
Demeton S-methyl	231.01	89.16	10	61.26	32			7.06	+
Demeton-O	259.00	89.10	11	61.21	29			11.72	+
Demeton-S	259.00	89.25	12	61.20	47			11.72	+
Desmedipham+NH_4	318.16	182.00	15	136.00	28			11.72	+
Desmetyrn	214.11	172.07	18	82.21	30	57.34	33	6.63	+
Di-allate	269.99	86.15	17	109.04	30	143.03	20	20.67	+
Diamidafos (Nellite)	201.10	107.20	28					3.96	+
Diazinon	305.03	169.10	25	153.13	23			18.51	+
Diazinon Oxon	289.00	233.00	20					16.12	+
Dichlorfenthion	314.98	258.82	16					26.36	+
Dichlormid	208.04	81.26	13	98.18	13	41.47	20	6.85	+
Dichlorvos	221.00	109.00	18	145.00	14	127.00	10	6.72	+
Dichlorvos+NH_4	238.00	109.00	24	221.00	18	127.00	24	6.72	+
Diclobutrazol	328.14	159.00	35	70.20	25			16.24	+

Table 1. LC-MS/MS data for 437 pesticide standards (continued)

Compound	Precursor Ion	Quantitation Ion	CE	Confirming Ion 1	CE	Confirming Ion 2	CE	RT (min)	Polarity
Dicrotophos	238.10	193.10	10	112.10	14			3.04	+
Diethofencarb	268.21	226.00	13	180.10	18			12.43	+
Difenacoum	445.13	179.00	30	256.97	21	177.83	59	26.63	+
Difenoconazole	406.17	251.00	25	111.00	55			21.12	+
Difenoxuron	287.09	123.04	22	72.22	26	95.15	30	11.19	+
Dimepiperate	264.12	146.08	11	119.13	17	91.15	35	20.36	+
Dimethametryn	256.13	186.05	22					14.59	+
Dimethenamid	276.00	243.97	14	168.02	23	111.15	33	12.76	+
Dimethoate	230.11	199.10	12	125.10	23			3.68	+
Dimethomorph	388.14	301.00	22	165.00	34			15.25	+
Dimethylvinphos1	331.00	127.04	13	99.06	26			14.47	+
Dimethylvinphos2	331.00	127.04	13	99.06	26			15.55	+
Dimetilan	241.10	72.20	21					4	+
Dimoxystrobin	327.13	205.00	12	116.00	25			17.73	+
Diniconazole	326.17	148.20	27	70.20	35			18.7	+
Dinotefuran	203.02	129.00	10	114.00	15			2.31	+
Dioxacarb	224.08	167.06	10	123.10	18			6.94	+
Dioxathion	473.99	271.09	10	153.04	28			22.8	+
Diphenamid	240.12	134.13	21	167.09	24	165.09	48	11.18	+
Diphenylamine	170.09	114.09	17	100.13	22	69.21	26	7.91	+
Dipropetryn	256.15	214.06	19	144.06	29	172.03	21	14.46	+
Disulfoton	274.94	89.27	5	61.28	34			19.59	+
Ditalimfos	300.10	145.30	22	144.20	21			14.47	+
Dithiopyr	402.10	354.00	20	272.30	32			21.84	+
Diuron	233.11	72.00	20	46.30	35			8.81	+
DNOC	199.14	117.10	28	89.00	53			3.15	+
Dodemorph	282.23	116.16	20	98.22	25	69.29	31	11.66	+
Doramectin	916.40	331.40	35	593.50	25			28.79	+
Edifenphos	310.98	283.00	12	109.11	35			18.62	+
Emamectin	886.70	158.00	33	302.00	20			24.99	+
Emamectin B1b	872.40	158.20	33	302.30	20			24.02	+
Epoxiconazole	330.20	121.00	21	123.00	20			16.84	+
Eprinomectin B1a	936.53	490.22	52	352.13	57			27.15	+
EPTC	190.07	128.20	13	86.20	14			16.67	+
Esprocarb	266.20	91.00	24	71.10	17			22.34	+
Etaconazole	328.19	159.00	32	123.00	58			16.62	+
Ethaboxam	321.00	183.10	24	200.10	28			8.89	+
Ethalfluralin	334.22	166.20	21	165.20	20			14.76	+
Ethidimuron	265.09	208.20	16	114.20	20			3.32	+
Ethiofencarb	226.09	107.00	16					13.16	+
Ethiolate	162.10	132.16	23	147.16	15	117.14	30	22.92	+
Ethion	384.92	142.97	29	97.09	49			23.56	+
Ethion monoxon	368.85	199.20	13	142.90	27			17.7	+
Ethiprole	397.12	351.00	20	255.00	34			14.03	+
Ethirimol	210.20	140.10	23	98.10	28			4.82	+
Ethofumesate	286.96	258.90	11	120.90	20			12.86	+
Ethoprophos	243.07	97.10	30	131.10	40			15.93	+
Ethoxyquin	218.00	174.00	34	160.00	34			8.81	+
Etobenzanid	340.13	179.10	20	121.00	33			19.13	+
Etofenprox	394.15	177.07	14	107.11	38	135.03	28	28.5	+
Etoxazole	360.21	177.10	22					19.06	+
Etrimfos	293.10	265.00	17					17.81	+

Table 1. LC-MS/MS data for 437 pesticide standards (continued)

Compound	Precursor Ion	Quantitation Ion	CE	Confirming Ion 1	CE	Confirming Ion 2	CE	RT (min)	Polarity
Famoxadone+NH_4	392.11	331.22	8	238.03	18			20.08	+
Famphur	325.96	217.03	21	280.98	13			10.36	+
Famphur oxon	327.14	201.00	26	265.00	19	186.01	35	4.91	+
Fenamidone	312.20	236.20	16	264.20	12			13.57	+
Fenamiphos	304.03	217.01	24	234.03	8			17.47	+
Fenamiphos sulfone	336.09	279.87	17	199.98	28			16.95	+
Fenarimol	331.12	268.00	23	81.00	34			16.32	+
Fenazaquin	307.20	57.20	23	160.90	18			20.77	+
Fenbuconazole	337.04	125.14	35	70.41	27			17.8	+
Fenhexamid	302.09	97.00	26	55.00	36			15.84	+
Fenitrothion	277.95	245.95	17	125.10	21			12.76	+
Fenoxycarb	302.17	116.00	13	88.00	20			18.07	+
Fenpiclonil	254.07	172.01	17					7	+
Fenpropathrin	350.20	97.00	34	125.00	16			23.82	+
Fenpropathrin+NH_4	367.20	125.00	18	97.00	34			25.65	+
Fenpropimorph	304.40	147.10	31	130.10	26			13.16	+
Fenpyroximate	422.21	366.00	15	214.00	34			25.9	+
Fensulfothion	309.18	251.00	21	163.00	18			14.17	+
Fenthion	278.95	247.01	13	169.06	20			12.76	+
Fenthion sulfone	328.09	311.04	9	109.12	37			9.11	+
Fenthion sulfoxide	294.90	108.90	32	114.90	27			8.39	+
Fenuron	165.03	72.10	17	46.30	18			3.53	+
Flonicamid	230.12	174.10	18					13.18	+
Florasulam	360.00	129.00	26	192.00	18			4.98	+
Florasulam+NH_4	377.00	129.00	30					4.98	+
Fluazinam	463.19	416.00	20	398.00	17			23.95	-
Flubendiamide	681.00	253.94	29	273.93	19	271.89	19	19.03	+
Flucarbazone	397.13	129.90	21	115.00	48			5.01	+
Fludioxinil	266.00	229.00	17	227.10	10			14.74	+
Fludioxonil	246.99	179.99	34	169.06	32	126.15	34	14.74	-
Flufenacet	364.23	194.00	12	152.00	20			16.23	+
Flufenoxuron	487.16	304.00	20	156.00	16			25.95	-
Flumetsulam	326.00	109.00	53					3.46	+
Flumioxazin	355.06	170.81	24	212.82	17	142.87	29	20.84	+
Fluometuron	233.08	72.10	18	46.30	17			8.81	+
Fluopicolide	383.01	172.94	23	144.95	47	365.01	17	14.44	+
Fluorochloridone	329.11	302.04	12	188.98	20			17.61	+
Fluoxastrobin	459.20	427.10	18	188.00	37			16.67	+
Fluquinconazole	376.17	349.20	21	307.00	20			15.8	+
Flusiazole	316.18	247.10	19	165.00	34			18.02	+
Flutolanil	324.21	242.00	26	262.00	18			14.84	+
Flutriafol	302.16	70.10	19	123.00	33			10.18	+
Fluvalinate	503.00	181.00	34	208.00	12			28.24	+
Fonophos	246.98	109.10	23	137.10	12			18.44	+
Forchlorfenuron	248.14	129.00	18	93.00	26			10.77	+
Formetanate	222.10	165.00	30					10.01	+
Fosthiazate	284.00	228.00	12	104.00	23			8.77	+
Fuberidazole	185.05	157.05	23	156.03	29	130.18	23	3.41	+
Furalaxyl	302.11	242.10	17	95.00	35			13.23	+
Furathiocarb	383.19	195.00	20	252.00	14			22.38	+
Griseofulvin	353.10	215.00	19	285.06	18	165.03	19	10.97	+
Halofenozide	329.10	121.14	22	77.33	37	155.15	29	13.57	+

Table 1. LC-MS/MS data for 437 pesticide standards (continued)

Compound	Precursor Ion	Quantitation Ion	CE	Confirming Ion 1	CE	Confirming Ion 2	CE	RT (min)	Polarity
Haloxyfop-methyl	376.03	315.96	17	287.98	25	91.13	31	20.84	+
Hexaconazole	314.14	70.20	20	159.00	20			19.39	+
Hexaflumuron	458.92	439.00	12	175.00	39			22.79	-
Hexazinone	253.09	171.05	17	85.19	29	71.27	30	7	+
Hexythiazax	353.24	228.20	18	168.10	25			24.03	+
Hydramethylnon	495.27	323.00	35	150.90	55			23.22	+
Imazalil	297.18	159.00	24	201.00	18			10.18	+
Imazamox	306.09	261.10	23	193.10	27			4.05	+
Imazapyr	262.06	216.98	19	201.97	27			9.64	+
Imazaquin	312.00	267.00	22	199.00	30	252.00	27	7.29	+
Imibenconazole	411.00	125.00	36	171.00	21			23.76	+
Imidacloprid	256.12	209.10	18	175.10	20			3.29	+
Inabenifide	339.26	80.20	38	78.90	55			13.09	+
Indanofan	341.00	187.00	14	175.00	17			16.23	+
Indoxacarb	528.30	203.00	40	293.00	15			21.9	+
Ipconazole	334.13	70.20	22	125.00	42			21.54	+
Iprobenfos	289.02	204.96	11	91.23	24			17.82	+
Iprovalicarb	321.16	119.00	20	203.00	10			15.55	+
Isocarbamid	186.08	145.05	22					21.54	+
Isocarbophos	307.12	230.93	17	171.12	22			10.71	+
Isofenfos	346.04	216.94	23	244.99	12			19.79	+
Isofenfos O-analog	330.15	121.10	43					16.84	+
Isoprocarb	194.09	95.00	16	137.00	11			9.5	+
Isopropalin	310.15	225.94	20	222.07	20	210.01	19	26.19	+
Isoprothiolane	291.00	189.00	22	231.00	12			14.5	+
Isoproturon	207.10	72.00	19	165.15	14			10.09	+
Isoxaben	333.13	165.00	20	107.00	61			14.76	+
Isoxaflutole	360.25	220.00	42					19.04	+
Isoxathion	314.00	286.00	10	105.00	18	258.00	12	20.09	+
Isozophos	314.03	162.01	16	97.03	34	120.02	28	15.81	+
Ivermectin B1a +NH_4	892.50	307.00	28	569.00	17			29.92	+
Kresoxim-methyl	314.07	267.14	8	222.13	15			17.77	+
Lactofen+NH_4	479.00	344.00	15	223.00	36			23.62	+
Linuron	249.10	182.00	18	160.00	17			12.87	+
Loxynil	369.86	242.95	28		28			11.26	-
Lufenuron	509.21	326.00	18	175.00	37			24.97	-
Malathion	330.97	126.99	13	99.02	25	124.98	32	14.48	+
Mandipropamid	412.10	327.90	15	355.90	11			15.16	+
Matoxuron	229.02	72.22	25	156.03	24			5.25	+
Mefenacet	299.17	148.00	14	120.10	31			15.4	+
Mefluidide	328.09	311.04	14	135.12	30	121.10	41	7.58	+
Mepanipyrim	224.14	106.00	27	77.00	40			15.48	+
Mephospholan	270.03	139.98	25	196.02	14	167.96	17	6.7	+
Mepronil	270.15	228.00	16	119.00	21			14.37	+
Mesotrione	340.16	227.95	16					4.72	+
Metaflumizone	505.15	302.04	22	285.10	52	117.15	34	24.67	-
Metalaxyl	280.11	220.10	16	192.10	16			10.36	+
Metazachlor	278.02	134.07	24	105.11	41			9.96	+
Metconazole	320.20	70.10	22	124.90	41			19.62	+
Methabenzhiazuron	222.13	165.00	17					6.91	+
Methacrifos	258.05	209.01	12	125.04	25	79.21	32	11.44	+
Methamidophos	142.00	94.00	20	125.00	10			1.95	+

Table 1. LC-MS/MS data for 437 pesticide standards (continued)

Compound	Precursor Ion	Quantitation Ion	CE	Confirming Ion 1	CE	Confirming Ion 2	CE	RT (min)	Polarity
Methidathion	302.90	85.20	23	144.92	5			10.92	+
Methiocarb	226.09	169.00	10					8.28	+
Methomyl	163.05	106.10	10	88.10	10			2.63	+
Methoprotryne	272.20	240.00	10	198.00	15			9.55	+
Metobromuron	259.10	170.00	20	148.00	25			9.34	+
Metolachlor	284.14	252.10	17	148.20	24			16.14	+
Metominostrobin	285.08	193.96	17	166.02	28	139.95	41	11.15	+
Metosulam	418.00	174.88	27	139.96	45	189.68	24	8.21	+
Metrafenone	409.03	209.10	16	227.10	20			20.13	+
Metribuzin	215.09	187.07	17	130.97	17			6.23	+
Mevinphos1	225.09	127.10	15	192.80	8			3.63	+
Mevinphos2	225.09	127.10	15	192.80	8			4.57	+
Mexacarbate	223.15	151.00	26	166.00	16			3.07	+
Milbemycin A3	511.40	493.20	10	475.20	10			26.77	+
Milbemycin A4+NH_4	560.40	525.20	10	507.20	12			27.95	+
Milbemycin A4-H_2O	525.40	507.20	10	489.20	10			27.96	+
Molinate	188.06	126.20	16	83.10	20			13.75	+
Monocrotophos	224.08	127.05	28	193.10	19			2.83	+
Monolinuron	215.08	126.00	17	99.00	36			8.31	+
Moxidectin	640.20	528.50	15	498.50	20			29.19	+
Myclobutanil	289.13	125.00	31	70.20	19			15.58	+
Naled	396.12	324.13	20	308.15	22			16.22	+
Naphthol	145.11	115.10	18	102.12	22			18.1	+
Napropamide	272.14	171.07	20	129.15	16	114.17	22	16.4	+
Naptalam sodium	331.14	105.16	18	139.04	19			13.57	+
Neburon	275.10	57.20	35	88.00	30			17.82	+
Nitenpyram	271.22	225.00	12	237.00	20			2.53	+
Nitralin	346.12	303.98	15	241.87	17	196.00	36	17.44	+
Nitrothal-isopropyl	313.03	148.95	15	91.14	41			15.23	+
Norflurazon	304.07	284.00	25	88.00	39			11.01	+
Novaluron	493.26	158.00	18	141.00	42			23.17	+
Novaluron	491.23	471.00	15	305.00	19			23.18	-
Noviflumuron	527.00	344.00	15	193.00	35			25.7	+
Nuarimol	315.11	251.90	26	81.00	36			13.33	+
Octhilinone	214.14	102.12	16	57.36	17			16.78	+
Ofurace	299.09	254.05	17	236.04	21	160.09	28	7.25	+
Omethoate	214.07	183.00	13	155.00	18			2.23	+
Orbencarb	258.06	125.05	28	100.15	13	89.13	43	19.38	+
Oryzalin	345.00	281.00	19	147.00	30	78.00	38	16.82	-
Oxadiazon	362.06	302.93	18	219.69	25	184.89	35	23.09	+
Oxadixyl	279.00	219.00	15	132.00	25			5.92	+
Oxamyl+NH_4	237.10	72.08	15	90.09	10			2.44	+
Paclobutrazole	294.10	70.00	20	125.00	35			14.26	+
Parathion	292.00	236.00	15	97.00	30			17.68	+
Parathion-methyl	263.94	232.07	18	109.13	20	124.90	25	12.11	+
Penconazole	284.12	159.00	35	70.10	17			18.43	+
Pencycuron	329.00	125.00	30	218.00	16			20.49	+
Pendimethalin	282.09	212.00	11	194.11	18	119.07	25	24.1	+
Penoxsulam	484.06	195.20	29	194.70	36			9.33	+
Permethrin+NH_4	408.00	183.00	22	355.00	10			28.45	+
Phenmediphame	301.17	136.00	22	168.00	10			12.23	+
Phenothrin	368.20	183.00	24	237.04	12	165.03	42	28.24	+

Table 1. LC-MS/MS data for 437 pesticide standards (continued)

Compound	Precursor Ion	Quantitation Ion	CE	Confirming Ion 1	CE	Confirming Ion 2	CE	RT (min)	Polarity
Phenthoate	320.93	247.02	11	79.26	46			18.01	+
Phorate	260.97	75.08	14	142.94	19			19.08	+
Phorate oxon sulfone	276.98	142.92	22	97.00	36	152.97	16	9.2	+
Phorate sulfone	276.05	94.15	35	173.97	21			9.85	+
Phosalone	368.00	182.00	17					20	+
Phosmet	317.91	160.05	15	133.15	39			12.18	+
Phosphamiden	317.08	300.01	10	127.04	25	226.93	19	6.07	+
Phoxim	299.00	129.00	10	77.00	20			19.81	+
Phropham	180.00	138.00	10	120.00	15	92.00	26	9.11	+
Picloram	241.00	195.00	24					2.67	+
Picoxystrobin	368.20	145.00	23	205.10	11			18.1	+
Pinoxaden	401.19	317.00	23	57.10	34			20.09	+
Piperonyl butoxide	356.19	177.00	13	119.00	33			22.74	+
Piperophos	354.09	170.85	22	212.83	16	142.90	32	20.84	+
Pirimicarb	239.09	182.00	16	72.00	21			4.59	+
Pirimiphos ethyl	334.07	198.11	24	182.14	26			21.8	+
Pirimiphos-methyl	306.01	164.12	24	108.18	34			18.17	+
Pretilachlor	312.20	252.00	17					22.53	+
Prochloraz	376.21	308.00	14	266.00	18			18.99	+
Profenophos	372.90	302.80	19	143.86	36	127.97	40	22.05	+
Prohexadione	211.07	167.19	17	123.24	17	111.18	23	4.83	+
Promecarb	208.09	151.00	10	109.00	17			13.82	+
Prometon	226.21	184.00	21	141.90	24			7.65	+
Prometryn	242.21	157.90	24	199.90	20			11.65	+
Propachlor	212.06	169.99	15	94.13	25	77.18	41	9.95	+
Propamocarb	189.05	102.10	19	144.05	14			2.32	+
Propanil	215.99	160.02	21					12.9	+
Propargite	368.18	231.00	11	174.90	18			24.9	+
Propazine	230.00	124.00	17					15.09	+
Propetamphos	282.04	138.08	18	156.00	10			15.22	+
Propiconazole	342.20	159.00	29	69.20	21			18.91	+
Propoxur	210.07	111.10	16	168.06	10			6.62	+
Prothioconazole	341.98	306.00	16	100.00	30			19.09	+
Prothoate	286.04	97.02	35					10.73	+
Pymetrozine	218.00	105.00	25	79.00	30			2.18	+
Pyracarbolid	218.20	124.90	21	96.90	31			7.03	+
Pyraclofos	361.10	257.00	23					20	+
Pyraclostrobin	388.22	194.00	14	163.00	26			20.01	+
Pyraflufen-ethyl	413.10	339.00	19					19.46	+
Pyrasulfotole	361.06	159.08	46	64.35	61	79.25	18	24.99	+
Pyrazophos	374.04	222.10	22	194.04	36			19.73	+
Pyridaben	365.20	309.10	13	147.00	23			26.81	+
Pyridalyl	489.95	109.00	29	163.90	38			30.53	+
Pyridaphenthion	340.94	189.09	23	205.04	22			15.62	+
Pyridate	379.20	207.00	19					28.28	+
Pyrifenox	294.97	93.12	26	92.07	52	67.19	50	12.88	+
Pyrimethanil	200.07	107.00	26	82.00	30			9.74	+
Pyriproxyfen	322.22	96.00	16	185.30	27			23.49	+
Pyroquilon	174.10	132.13	23	117.15	31	130.13	38	6.77	+
Pyrosulam	434.95	195.20	28	194.10	39			7.42	+
Quinalphos	299.05	163.01	23	147.06	24	38.00		17.63	+
Quinoxyfen	307.88	196.80	33	161.90	47			23.92	+

Table 1. LC-MS/MS data for 437 pesticide standards (continued)

Compound	Precursor Ion	Quantitation Ion	CE	Confirming Ion 1	CE	Confirming Ion 2	CE	RT (min)	Polarity
Resmethrin	356.16	171.01	15	143.01	26	128.03	43	27.36	+
Rotenone	395.30	213.20	23	192.10	26			17.69	+
Salflufenacil	518.19	348.94	30	459.00	16			12.36	+
Schradan	287.12	242.02	14	135.08	26	92.15	40	4.25	+
Secbumeton	226.21	169.90	19	99.90	33			7.91	+
Sethoxydim	328.00	178.00	20					7.58	+
Siduron	233.12	137.00	20	94.00	38			12.55	+
Simazine	202.10	132.00	20	104.00	27			6.7	+
Simetryne	214.10	124.00	20	96.00	26			6.56	+
Spinetoram1	748.32	141.92	30	98.03	37			22.65	+
Spinetoram2	760.2	141.88	31					24.11	+
Spinosyn A	732.50	142.00	35	98.00	47			21.19	+
Spinosyn D	746.50	142.00	34	98.00	47			22.6	+
Spirodiclofen	411.00	313.10	15	213.10	25			25.6	+
Spiromefesin	371.30	273.30	15	255.30	25			24.73	+
Spirotetramat	374.20	330.20	17	302.20	19			16.21	+
Spiroxamine	298.22	144.00	21	100.00	35			14.74	+
Sulfentrazone	404.00	387.00	10	307.00	15			7.9	+
Sulfotep-ethyl	323.19	219.00	16	247.10	15			24.39	+
Sulfuramid	525.99	219.02	26	168.94	27	269.07	23	25.97	-
Sulprofos	322.93	218.95	17	246.95	12			24.39	+
Tebuconazole	308.22	70.20	21	125.00	34			18.57	+
Tebufenozide	353.12	133.00	19	297.00	10			17.95	+
Tebufenpyrad	334.21	145.20	28	117.00	36			22.77	+
Tebupirimfos	319.10	210.20	22					14.7	+
Tebuthiuron	229.16	172.06	18	116.10	28			7.29	+
Teflubenzuron	379.16	339.00	13	196.00	22			23.82	-
Tefluthrin	419.03	174.85	27	140.72	47			8.21	+
Temephos	466.95	419.13	20	405.08	14			24.23	+
Tepraloxydim	340.00	220.00	34	248.00	18			8.38	-
Terbufos	288.97	103.10	12	57.50	21			22.23	+
Terbufos sulfone	338.08	171.00	16	115.01	31	97.06	42	12.39	+
Terbumeton	226.22	169.90	20	113.90	25			7.66	+
Terbutryn	242.22	185.90	20	91.00	28			12.03	+
Tetrachlorvinphos-a	365.00	204.00	40	127.00	16			17.79	+
Tetrachlorvinphos-a+NH_4	382.00	127.00	20					17.79	+
Tetrachlorvinphos-b	366.87	127.03	16	205.96	37	240.74	23	17.79	+
Tetrachlorvinphos-b+NH_4	383.88	126.95	19	205.81	49	240.88	24	17.79	+
Tetraconazole	372.19	159.00	39	70.00	24			17.13	+
Tetramethrin	332.10	127.04	28	174.03	19	226.92	18	14.29	+
Thiabendazole	202.04	175.05	28	131.05	35			3.2	+
Thiacloprid	253.13	126.10	22	90.20	37			4.68	+
Thiamethoxam	292.15	211.10	14	132.05	24			2.76	+
Thiazopyr	397.05	377.04	22	335.00	26			18.67	+
Thidiazuron	221.13	102.10	16	94.20	14			7.13	+
Thiobencarb	258.07	125.00	18	100.20	15			19.38	+
Thiofanox+NH_4	236.09	57.20	16	76.10	12			8.52	+
Thiometon+Na	268.88	89.10	25	61.10	36			14.52	+
Thiophanate-methyl	343.21	151.06	24	311.20	12			6.78	+
Tolclofos-methyl	301.00	175.00	22					6.16	+
Tolfenpyrad	384.08	196.95	29	181.69	30			23.59	+
Tralkoxydim	330.00	284.00	13	138.00	22			16.13	+

Table 1. LC-MS/MS data for 437 pesticide standards (continued)

Compound	Precursor Ion	Quantitation Ion	CE	Confirming Ion 1	CE	Confirming Ion 2	CE	RT (min)	Polarity
Tralomethrin+NH_4	682.80	440.60	18	665.80	10	412.60	22	27.59	+
Triadimefon	294.17	197.10	16	225.10	16			14.86	+
Triadimenol	296.10	70.00	15					14.26	+
Triazophos	313.99	162.10	21	119.17	36			15.82	+
Trichlamide	340.00	121.00	22					19.14	+
Trichlorfon	256.90	127.00	19	109.10	19			4.57	+
Tricyclazole	190.07	163.06	24	136.10	30			5.33	+
Tridemorph	298.00	130.00	28	98.00	32			19.42	+
Trifloxystrobin	409.30	186.00	21	206.10	16			21.54	+
Triflumizole	346.16	278.10	12	73.00	18			21.4	+
Triflumuron	359.10	156.20	17	139.00	31			20.24	+
Triforine-a	434.90	390.00	12					12.45	+
Triforine-b	432.90	388.00	12					12.46	+
Triforine-c	436.90	392.00	12					12.45	+
Trinexapac-ethyl	253.11	207.02	11	69.27	20	165.02	17	10.28	+
Triconazole	318.12	70.00	25	125.00	30			16.16	+
Uniconazole	292.13	70.20	25	125.00	32			17.32	+
Vamidothion	288.07	146.05	14	118.10	27			3.6	+
Vernolate	204.15	128.21	11	86.22	13	43.47	19	19.47	+
Warfarin	307.03	160.94	20					26.95	+
Zoxamide	336.22	187.00	23	159.00	38			18.7	+

Excellent linearity in detector response was observed over the calibration range. The correlation coefficients of 319 analytes were greater than 0.99, and those of 52 analytes were greater than 0.98. The total ion chromatogram is shown in Figure 3.

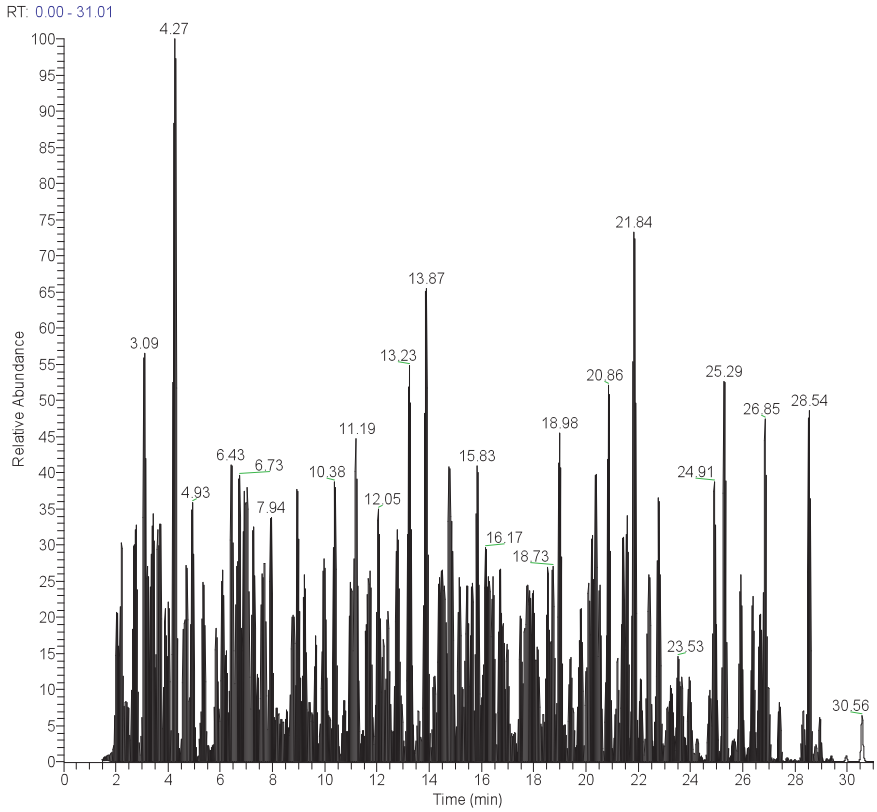
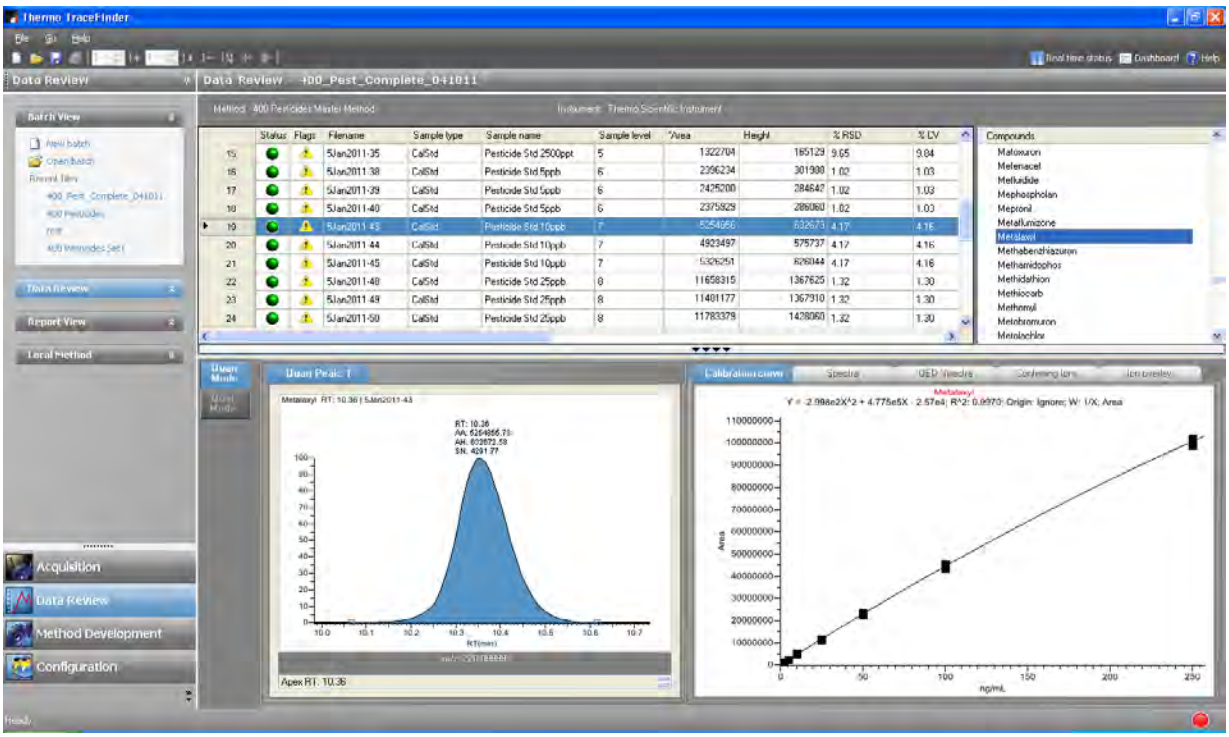


Figure 3. Chromatogram of 437 pesticides (10 µg/L standard solution)

The analysis of the pesticides was reviewed in the Data Review section of TraceFinder software (Figure 4). In this section, calibration curves, ion ratios, peak integration, and MS spectra can be monitored, and samples that meet user-set criteria can be flagged. In addition, user adjustments, such as peak re-integration, are permitted. The effects of the changes on the results are instantly updated in the results grid and standard reports. The extracted ion chromatogram and solvent standard calibration curve for two example pesticides, metalaxyl and pyridaben, are shown in Figure 4. Three replicates of each calibration standard were injected at each level.

(A) Metalaxyl



(B) Pyridaben

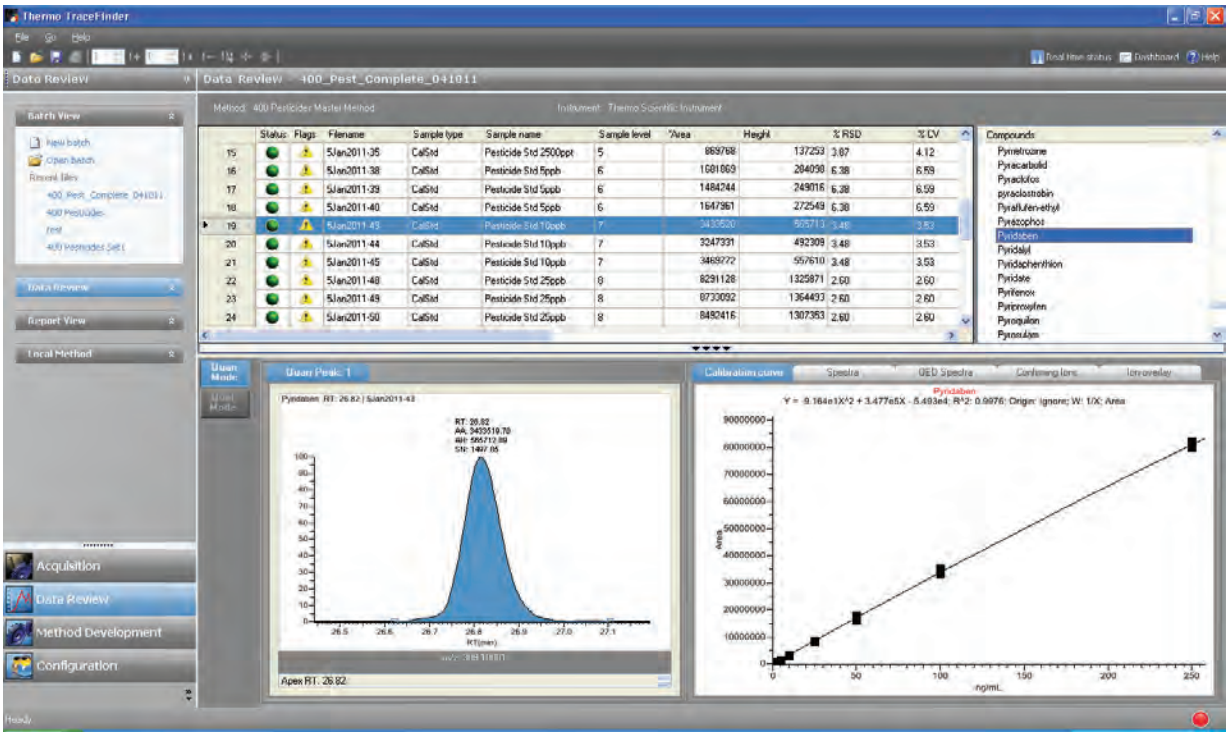


Figure 4. TraceFinder software view of extracted ion chromatogram and solvent standard calibration curve [metalaxyl (A) and pyridaben (B), 10 µg/L, 3 replicates, quadratic curve fit]

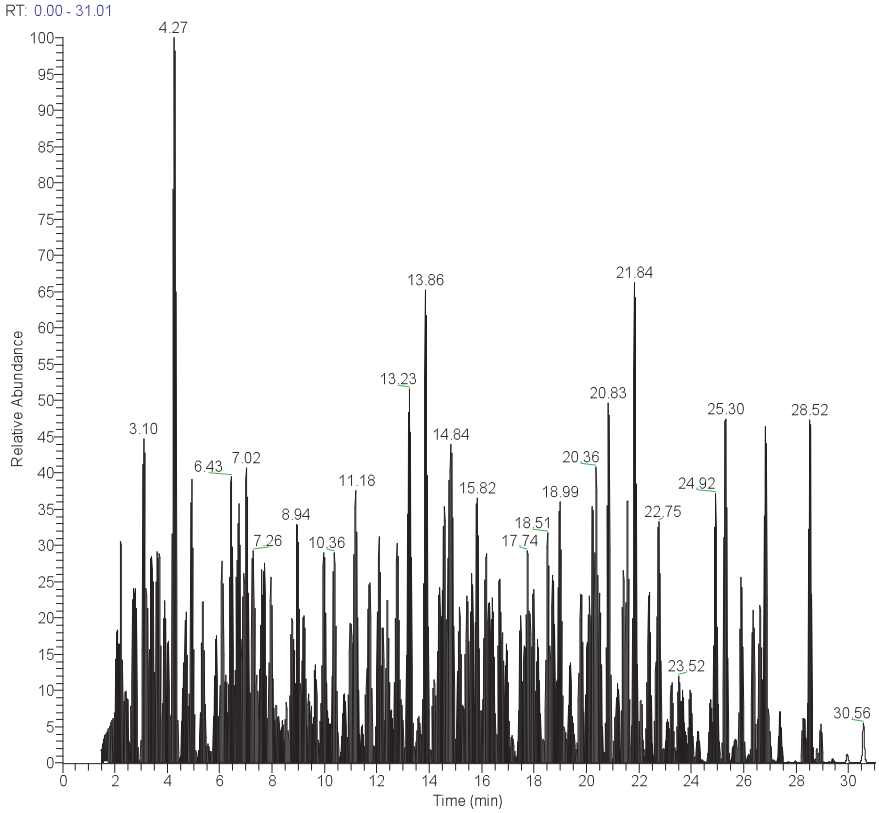
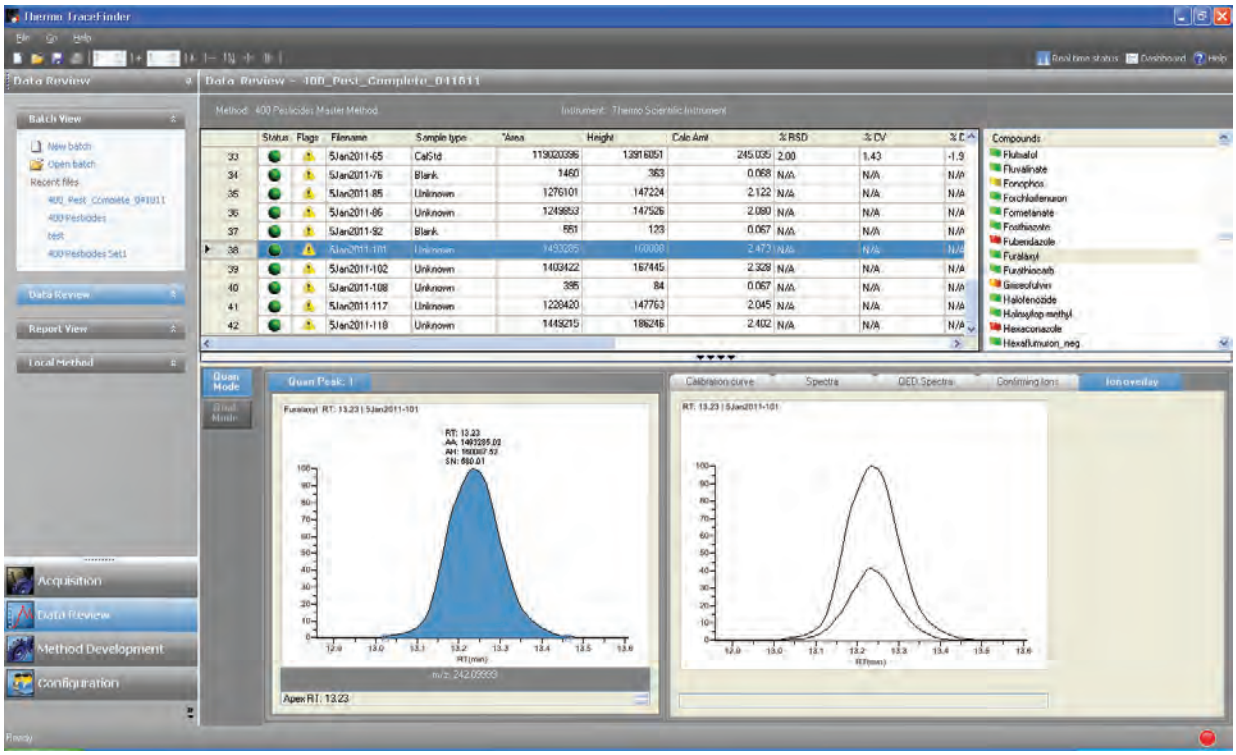


Figure 5. Chromatogram of 437 pesticides in orange extract at 2 µg/kg



Green flag – Compound found, above limit of reporting (LOR), all criteria passed.
Orange flag – Compound close to limit of detection (LOD) or LOR. User may want to double check results.
Yellow flag – Compound not found.
Red flag – Error, such as ion ratio, linearity, carryover, etc.

Figure 6. TraceFinder software view of quantitation peak and ion overlay of confirmation by secondary SRM (furalaxyl in orange extract 2 µg/kg)

To evaluate the applicability of this technique to complex food samples, the pesticide mixture was spiked into apple, orange, and asparagus matrices and analyzed. Figure 5 shows the chromatogram of 437 pesticides at 2 µg/kg in the orange matrix. The majority of the pesticides were detected at 2 µg/kg. The confirmation of target analytes was achieved by the second or third SRM. In Figure 6, the quantification ion and two qualification ions for furalaxyl are displayed in the Data Review section of TraceFinder software. The acceptance criteria percentage can be set for the ion ratio confirmation. If the ion ratio fails, the Confirmation Ion box is flagged in red by the software.

QED-MS/MS experiments were also applied to pesticide analysis in orange, asparagus, and apple extract to confirm the existence of compounds while they were being quantified. A full-scan MS/MS mass spectrum was obtained by data dependent scanning for confirmatory analysis during the SRM experiment. After a particular SRM transition reached the specified intensity threshold, the instrument automatically triggered the QED-MS/MS scan using the Reverse Energy Ramp (RER) scan function. The collision energy was linearly ramped from a high to a low value while Q3 was scanned from low *m/z* to high *m/z*. A highly sensitive, fragment-rich spectrum that was used to positively confirm the existence of a compound was collected. An example of a QED-MS/MS full scan spectrum is shown in Figure 7 for the compound fenamiphos. This QED-MS/MS scan function fragmented the precursor ion *m/z* 304 for fenamiphos over a reversed energy ramp of 10 to 50 eV.

TraceFinder software includes a large number of report templates. Reports can be created in PDF format, printed directly to the printer, or saved in XML format, which is useful for LIMS systems. Figure 8 shows the onscreen preview function of a report generated by TraceFinder software. The chromatogram shown is an apple sample spiked with 437 pesticides at 2 µg/kg. The top of the page contains a sample summary, and the quantitated results follow beneath the chromatogram. TraceFinder software can generate results for the entire batch with the click of a button, or the user can choose to view reports individually and print only those of interest.

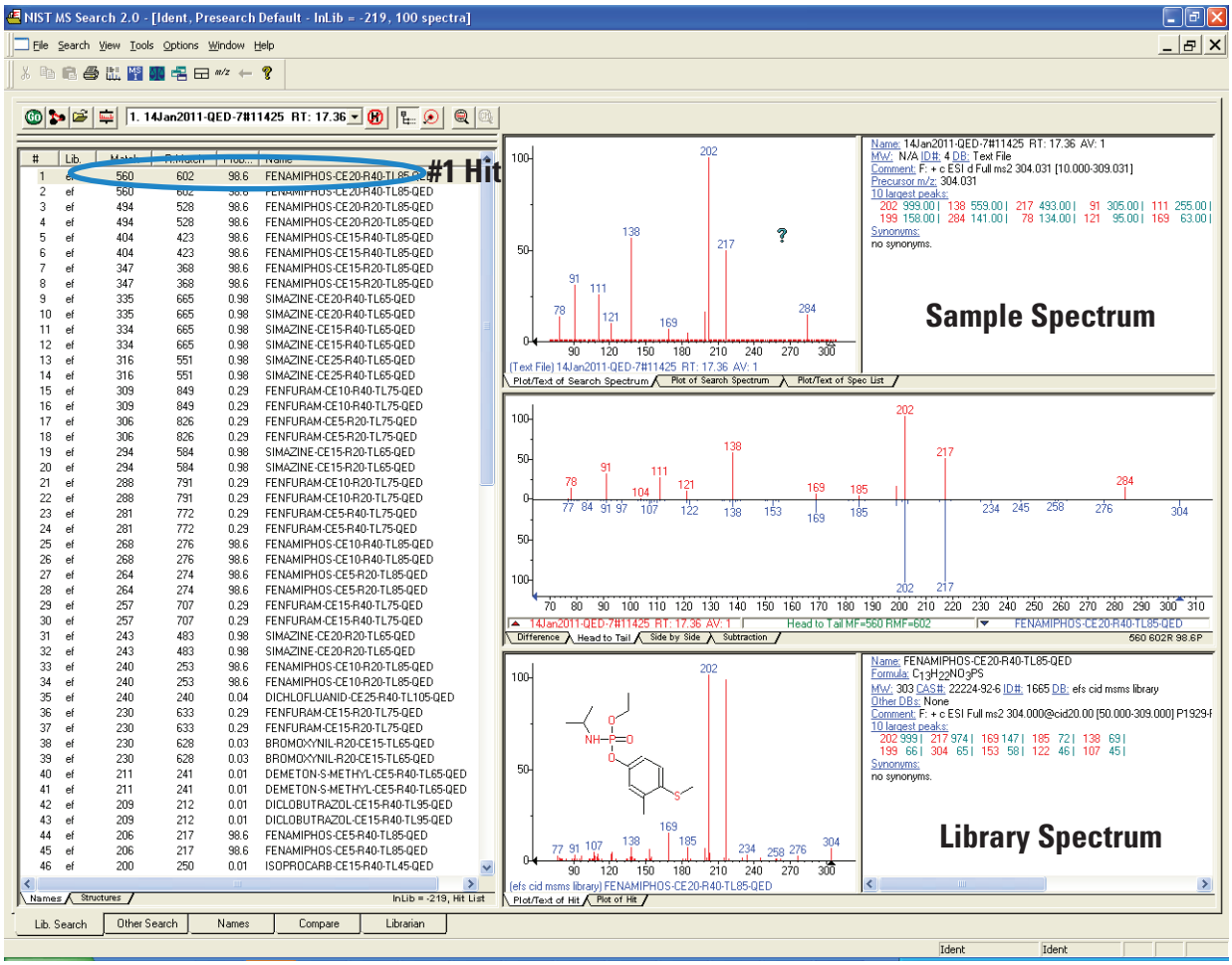


Figure 7. QED spectrum of fenamiphos at 2 µg/kg in asparagus. Searching against the standard library available on the TSQ Quantum Access MAX™ instrument platform yields a positive confirmation.

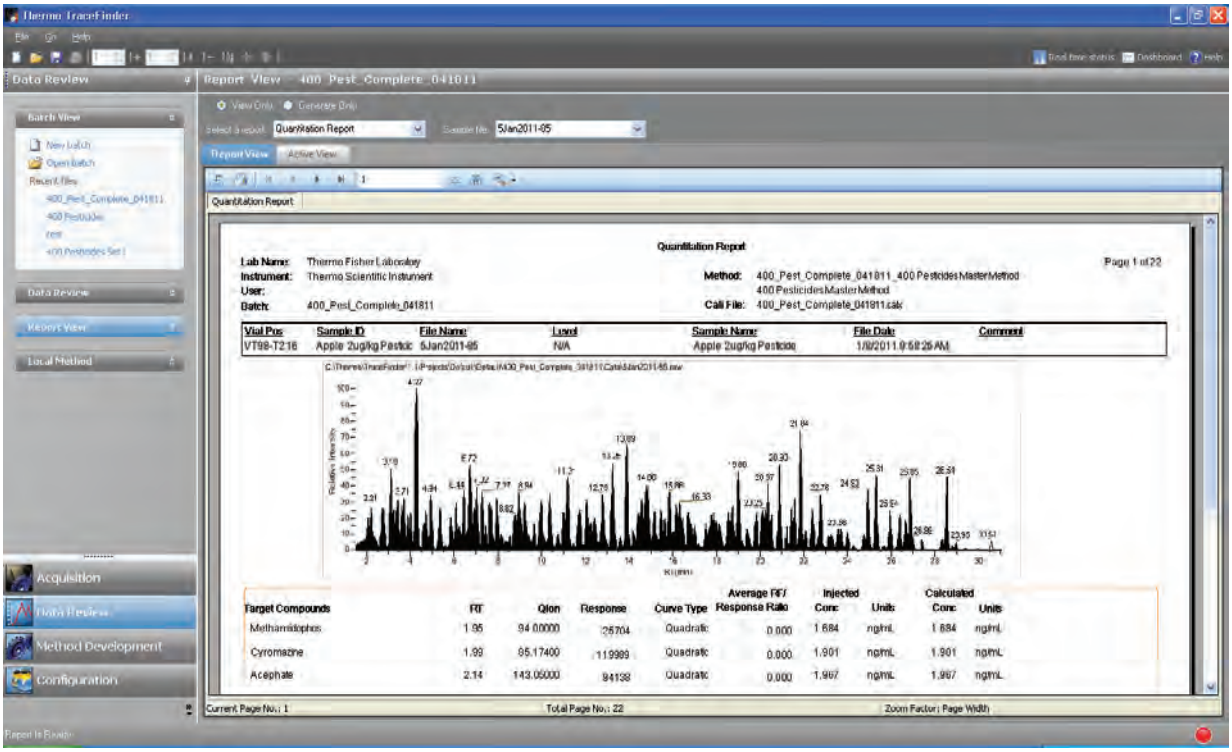


Figure 8. Report View section of TraceFinder software showing quantitative results

Conclusion

A multi-residue method was developed for the screening and determination of 437 pesticides in 45 minutes in a single run on a triple quadrupole mass spectrometer. Data analysis was streamlined by using TraceFinder software, which is ideally suited for quantitation of large amounts of data. For this large-scale multi-pesticide residue study, a timed SRM experiment provided accurate and sensitive analysis, without compromising the dwell time (and duty cycle) for detecting each compound per experiment. Quantitation-Enhanced Data-Dependent scanning provided confirmatory data following quantitative analysis. The majority of the pesticides were detected in the spiked matrices at concentrations lower than the MRLs established by EU and Japan.

Acknowledgment

The authors wish to thank Dr. Jon Wong at the FDA for providing the pesticide standards used in this study. The authors are also grateful to Roland Carlson at CDFA for providing the QuEChERS extracts for this study.

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Application Note: 373

Testing LC-MS System Robustness with Automated Sample Cleanup Using Red Wine as a Matrix

Jonathan Beck, Thermo Fisher Scientific, San Jose, CA, USA

Key Words

- TSQ Quantum Ultra™
- EQuan™ System
- Hypersil GOLD™ Columns
- Pesticides

Introduction

Achieving low limits of detection (LODs) of pesticides, antibiotics and veterinary residues in food residues and drinking water is of paramount importance in order to monitor the regulatory levels as stated by the US, Japanese and EU directives. These substances pose a significant health threat and therefore, need to be accurately detected at the lowest levels, typically at part per trillion (ppt). Traditionally, LC-MS/MS has been used by the environmental and food industries for the identification and quantitation of these residues. However, this methodology typically requires extensive offline sample preparation, which can be time consuming and expensive.

The Thermo Scientific EQuan environmental quantitation system consists of a Thermo Scientific TSQ Quantum™ series mass spectrometer, two Thermo Scientific Surveyor™ HPLC pumps with a preconcentration column, an analytical column, and a CTC autosampler. The unique capabilities of EQuan for online preconcentration and cleanup of samples result in improved sensitivity and precision, as well as unmatched throughput.

In previous experiments, using the EQuan system for online sample preconcentration and detection of pesticides in ground water yielded lower limits of detection compared to standard injection techniques. See Table 1.

Typically, when red wine is analyzed using LC-MS/MS, some form of sample preparation and/or extraction is necessary prior to injection. In this application note, the EQuan system was tested for robustness using a matrix of neat red wine spiked with a mixture of pesticides using large volume (1000 µL) injections.

Goal

To test the robustness of an LC-MS system for an automated online preconcentration system using a dirty matrix.

Experimental Conditions

Sample Preparation

Red Burgundy wine was spiked with a mixture of nine herbicides and six fungicides at a level of 500 pg/mL (500 ppt). The following herbicides were analyzed: atrazine, cyanazine, simazine, propazine, trietazine, metazachlor, propachlor, pendimethalin, and propyzamide. The following fungicides were analyzed: flutriafol, triadimefon, epoxiconazole, flusilazole, tebuconazole, and propiconazole. No other sample treatment was performed prior to injection.

HPLC

HPLC analysis was performed using an HTC PAL™ Autosampler with two LC quaternary pumps and two LC columns, the first for preconcentration of the sample and the second for the analytical analysis. A sample of 1000 µL of the spiked neat wine was injected directly onto the Thermo Scientific Hypersil GOLD 20 × 2.1 mm, 12 µm loading column in a high aqueous mobile phase (see Figure 1a). After 1 minute, a six-port valve on the mass spectrometer was switched by LCQUAN™ 2.5 instrument control software. This enabled the load column to be back flushed onto the analytical column (Thermo Scientific Hypersil GOLD 50 × 2.1 mm, 3 µm), where the

	1 mL Injection Area	100 µL Injection Area	Gain Factor	1 mL Injection Area	100 µL Injection Area	Gain Factor	1 mL Injection Area	100 µL Injection Area	Gain Factor	1 mL Injection Area	100 µL Injection Area	Gain Factor
	Propham			Isoproturon			Diuron			Linuron		
1 ppt				5.53E+04	NA		1.97E+04	1.73E+03	11			
5 ppt	2.17E+04			3.35E+05	3.17E+04	11	4.15RE+04	5.65E+03	7	6.96E+03		
10 ppt	2.71E+04			6.68E+05	4.90E+04	14	8.25E+04	1.18E+04	7	1.99E+04		
50 ppt	5.09E+04			3.33E+06	2.82E+05	12	4.47E+05	3.72E+04	12	5.91E+04	7.98E+03	7
100 ppt	6.51E+04			6.54E+06	5.24E+05	12	8.83E+05	7.60E+04	12	1.34E+05	2.50E+04	5
500 ppt	2.47E+05	3.00E+04	8	3.11E+07	2.60E+06	12	4.65E+06	3.80E+05	12	7.36E+05	1.28E+05	6
1000 ppt	5.29E+05	5.69E+04	9	5.81E+07	5.23E+06	11	9.39E+06	7.63E+05	12	1.43E+06	2.47E+05	6
5000 ppt	2.59E+06	2.82E+05	9	2.58E+08	2.44E+07	11	4.95E+07	3.68E+06	13	9.49E+06	1.25E+06	8

Table 1: Calculations demonstrating the gain in peak areas due to larger injection volumes in ground water samples

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compounds were separated prior to introduction into the mass spectrometer (see Figure 1b). After all of the compounds were eluted from the analytical column, the 6-port valve was switched back to the starting position, and the loading and analytical columns were cleaned with a high organic phase before being re-equilibrated to their starting conditions. The total run time for each analysis was 22 minutes. The mobile phases for the analysis were water and methanol, both with 0.1% formic acid.

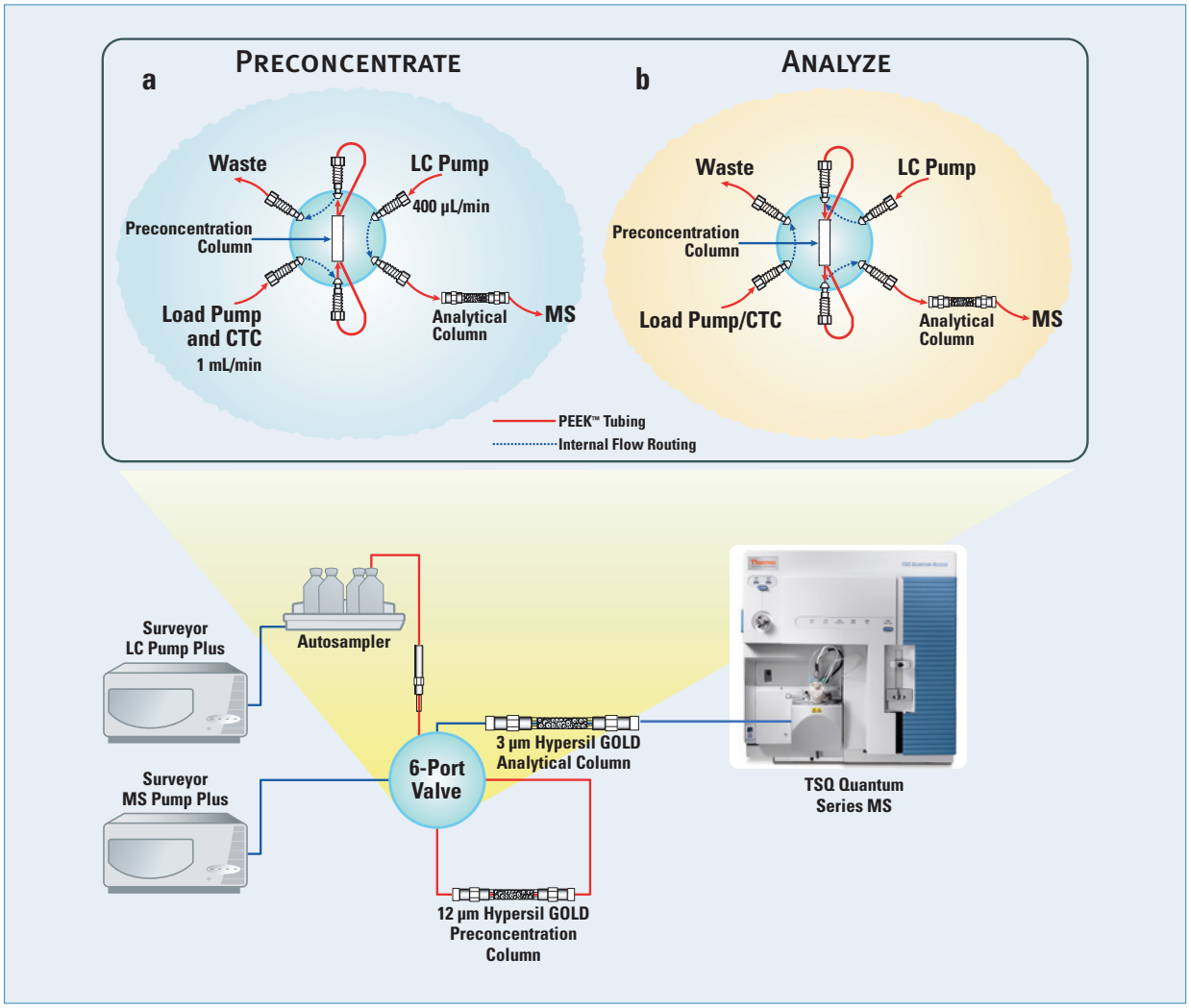

Figure 1: The schematic of the EQuan system used for this assay

Figure 2: Ion sweep cap after several hundred injections, showing contamination from red wine

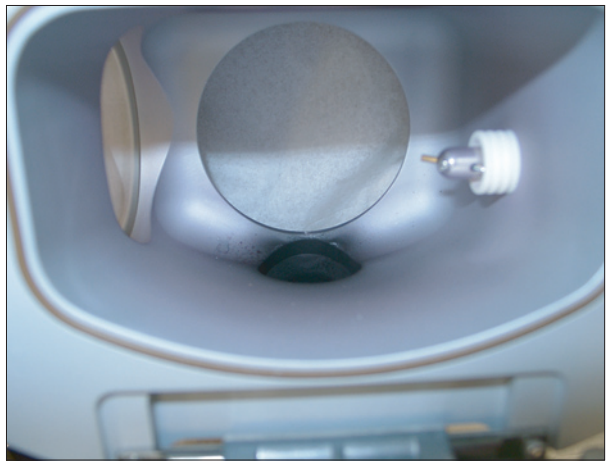

Figure 3: Electrospray ionization source with the electrospray probe removed, showing the main spray pattern directed towards the drain

MS

MS analysis was carried out on a Thermo Scientific TSQ Quantum Ultra triple quadrupole mass spectrometer with an electrospray ionization source. The MS conditions were as follows:

Electrospray ionization: Positive
Spray voltage: 3.0 kV
Ion transfer tube temperature: 350 °C
Sheath gas pressure: 45 arbitrary units
Auxiliary gas pressure: 5 arbitrary units
Ion sweep gas pressure: 3 arbitrary units
Collision gas (Ar): 1.0 mTorr
Q1/Q3 peak resolution: 0.7 Da
Scan width: 0.002 Da

The source of the mass spectrometer was adjusted so that the ESI probe was off axis to prevent contamination of the ion transfer tube. The position of the probe was set so that the main spray pattern of the electrospray hit the Ion Sweep™ cone below the center line and off to the left by about 0.5 cm. The probe depth was set to position "C" on the electrospray probe. An ion sweep gas of three arbitrary units was set to prevent any large droplets from entering the ion transfer tube of the mass spectrometer.

Results and Discussion

The back pressure of the loading column and the analytical column were monitored over the course of the wine injections to determine if the columns were becoming clogged with any particulates from the wine. Over 600 injections, the back pressure on the 12 µm loading column remained at approximately 20 bar under the starting conditions of the analytical run, while the back pressure on the 3 µm analytical column remained at approximately 72 bar.

The resulting spray pattern of the electrospray can be seen in Figure 2. A thick deposit of red wine residue is clearly visible from just below the center of the sweep cone to the outside radius. The red wine spray can also be seen on the inside of the electrospray housing in Figure 3. In the picture, the drain is dark purple in color, illustrating that the main excess spray of the red wine was directed to the bottom of the ion source and away from the main orifice of the mass spectrometer. Additionally, the ESI probe can be adjusted to be closer to the ion transfer tube, which increases robustness by allowing less side scatter from the electrospray beam, thus focusing the main spray pattern lower on the ion sweep cap.

The reproducibility of the method is shown in Figure 4. The graph plots the peak area for metazachlor for 164 injections of red wine. The first four injections were excluded from the %RSD calculation. Because the loading column was new at the beginning of the runs, several injections were required to condition the column before a stable peak area was achieved. A representative chromatogram is shown in Figure 5.

As shown in Figure 6, after several hundred injections of the spiked red wine matrix, no degradation in column performance or source robustness was observed. In total, over 600 injections were made on the system with no loss in column performance.

Conclusion

This application note demonstrates the robustness of the TSQ Quantum Ultra triple quadrupole mass spectrometer and an online extraction and preconcentration method. The described sample cleanup technique improves signal-to-noise ratios by a factor of 10 to 100 (based on injection volume) for low concentration samples in red wine matrices. Preliminary results using onion and tobacco matrices have yielded similar results in terms of column performance and mass spectrometer robustness. Further studies will be conducted in other matrices, as well as with other pesticides, herbicides, and insecticides.

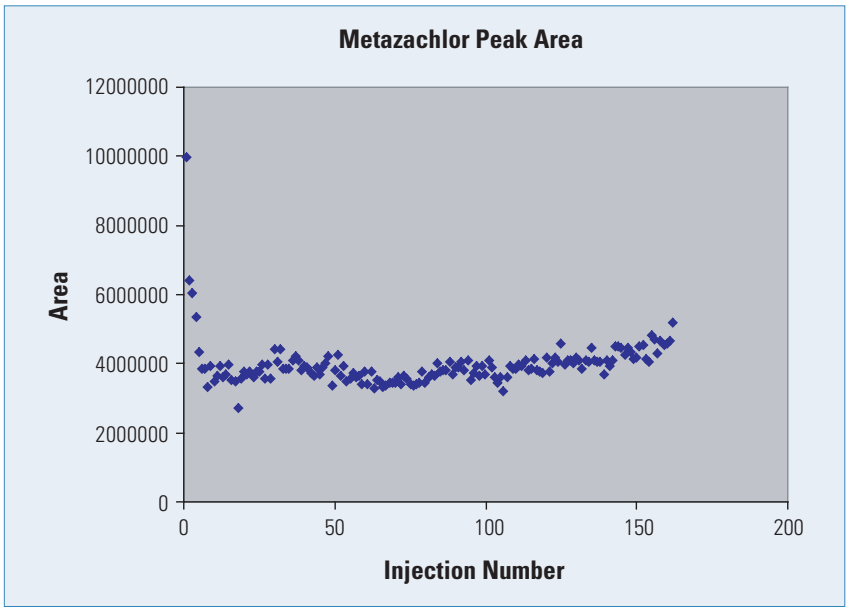

Figure 4: Scatter plot of the peak area for 164 injections (1000 µL) of metazachlor spiked in red wine. The %RSD is 9% when the first four points are excluded.

References

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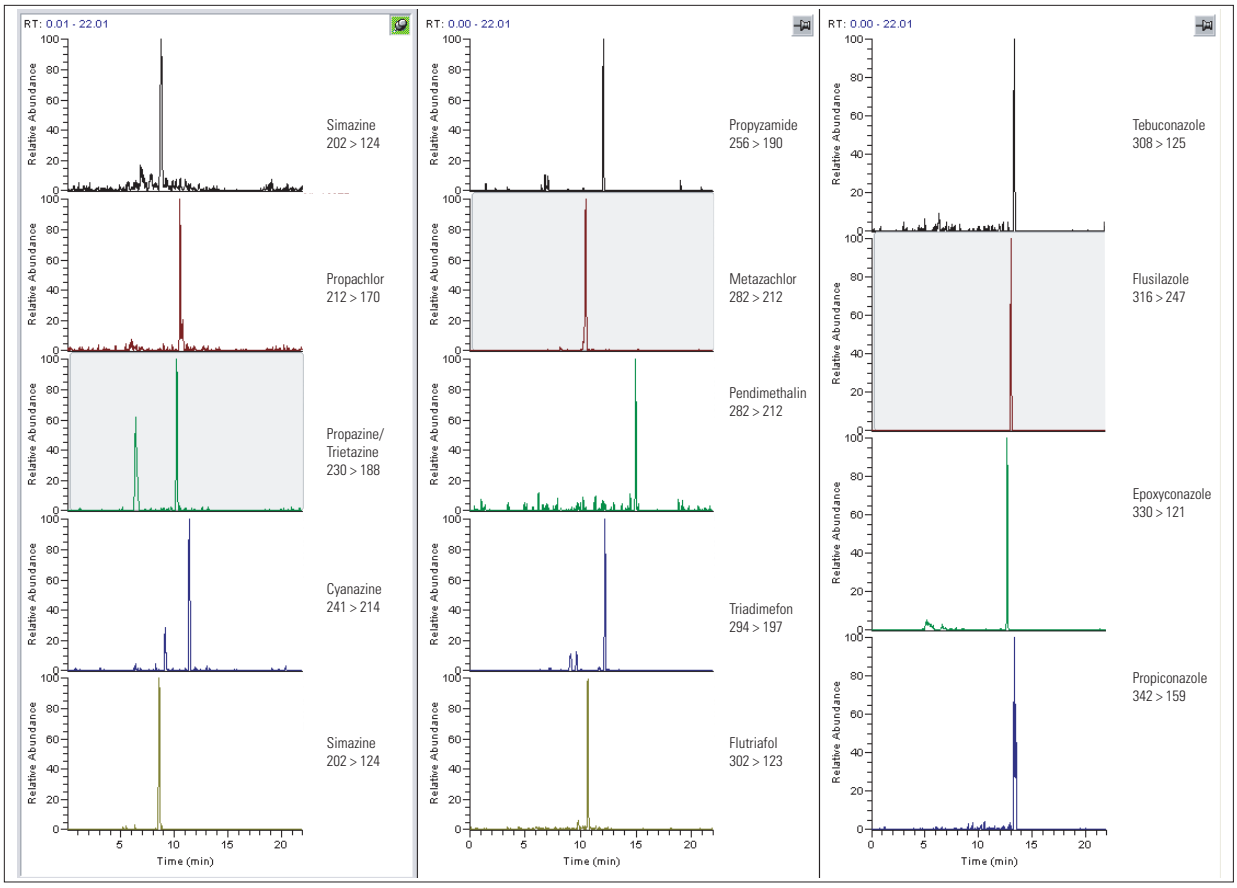

Figure 5: Example chromatograms for a 1000 µL injection of spiked red wine

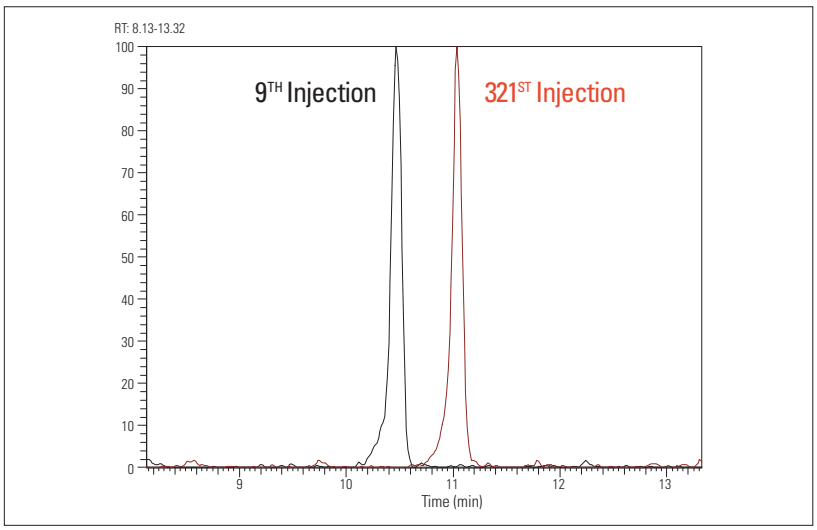

Figure 6: Different injections of metazachlor (retention times have been offset for greater visibility)

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The Thermo Scientific Exactive Benchtop LC/MS Orbitrap Mass Spectrometer

Andreas Wieghaus, Alexander Makarov, Ulf Froehlich, Markus Kellmann, Eduard Denisov, Oliver Lange, Thermo Fisher Scientific, Bremen, Germany

Key Words

- Exactive™
- Accurate Mass
- High Resolution
- Polarity Switching
- Scan Speed

Overview

Review of a new benchtop mass spectrometer based on a stand-alone Thermo Scientific Orbitrap™ mass analyzer. Key features of the instrument layout, analytical parameters and typical applications are described.

Introduction

Over the past three years, the combination of Orbitrap technology with a linear ion trap has become an established platform for high resolution, accurate mass LC/MS^n analysis. The high resolving power, mass accuracy and dynamic range of the Orbitrap analyzer allow rigorous characterization of complex mixtures even in the absence of precursor ion mass selection. We now describe the development of a non-hybrid mass spectrometer comprising of an atmospheric-pressure ion source (API) and a standalone Orbitrap mass analyzer.

Methods

All experiments were performed on a prototype of the new Thermo Scientific Exactive™ mass spectrometer using an electrospray ionization (ESI) source.

Instrument Layout Overview

Figure 1 shows the schematic layout of the instrument. Samples can be introduced introduced into the API source by a variety of methods including direct infusion or an U-HPLC system (Thermo Scientific Accela™).
The source is similar to the commercial source of the Thermo Scientific TSQ Quantum Ultra™.

Ions are transferred from the source through four stages of differential pumping using RF-only multipoles into a curved RF-only trapping quadrupole (the C-trap). In the C-trap, ions are accumulated and their energy dampened using a bath gas (nitrogen). Ions are then injected through three further stages of differential pumping using a curved lens system into the Orbitrap analyzer where mass spectra are acquired via image current detection. The vacuum inside the Orbitrap mass analyzer is maintained below 1E-09 mBar.

Automatic Gain Control (AGC)

Automatic control of the number of ions in the Orbitrap is performed by measuring the total ion charge using a pre-scan and by calculating the ion injection time for the analytical scan from this. For very high scan rates, the previous analytical scan is used as a prescan to optimize the scan cycle time without compromising automatic gain control. Ion gating is performed using a fast split lens setup that ensures the precise determination of the ion injection time.

Higher Energy Collision Induced Dissociation (HCD)

In a HCD experiment, ions are passed through the C-trap into a multipole collision cell where they are fragmented. After that, the HCD cell voltages are ramped, and ions are transferred back into the C-trap from where they are injected into the Orbitrap for detection.

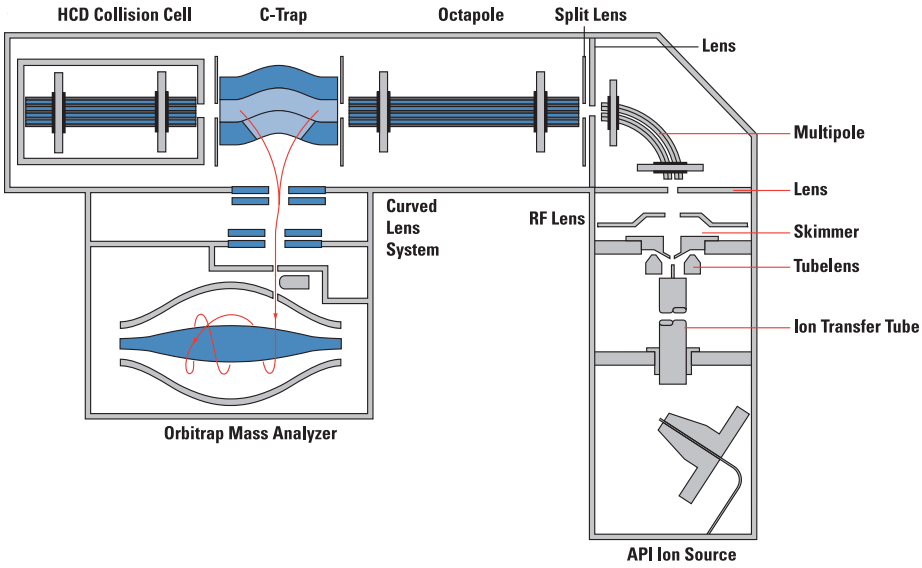

Figure 1: Schematic layout of the instrument.

Results

Automatic Gain Control (AGC)

A requirement of any ion trap device is the ability to control the ion population within the trap. When the ion population is not accurately maintained, it can result in large variations in the quality of data. The correct AGC functionality of the Exactive instrument is exemplified in Figure 2 by two mass spectra acquired in the middle and at the end of an eluting LC peak of Buspirone.

In both, cases the mass resolution, mass accuracy and signal-to-noise ratio are excellent. The AGC feature, in combination with the precise determination of the ion injection time, allows the instrument to be used for accurate quantitative analyses.

Scan Speed

The use of a single mass analyzer with very high transmission characteristics, in combination with the use of fast digital and analog electronics, allow high resolution mass spectra to be detected, processed and recorded at high scan rates of up to 10 Hz. This is compatible with the narrow peak widths observed in fast chromatography analyses (Figure 2).

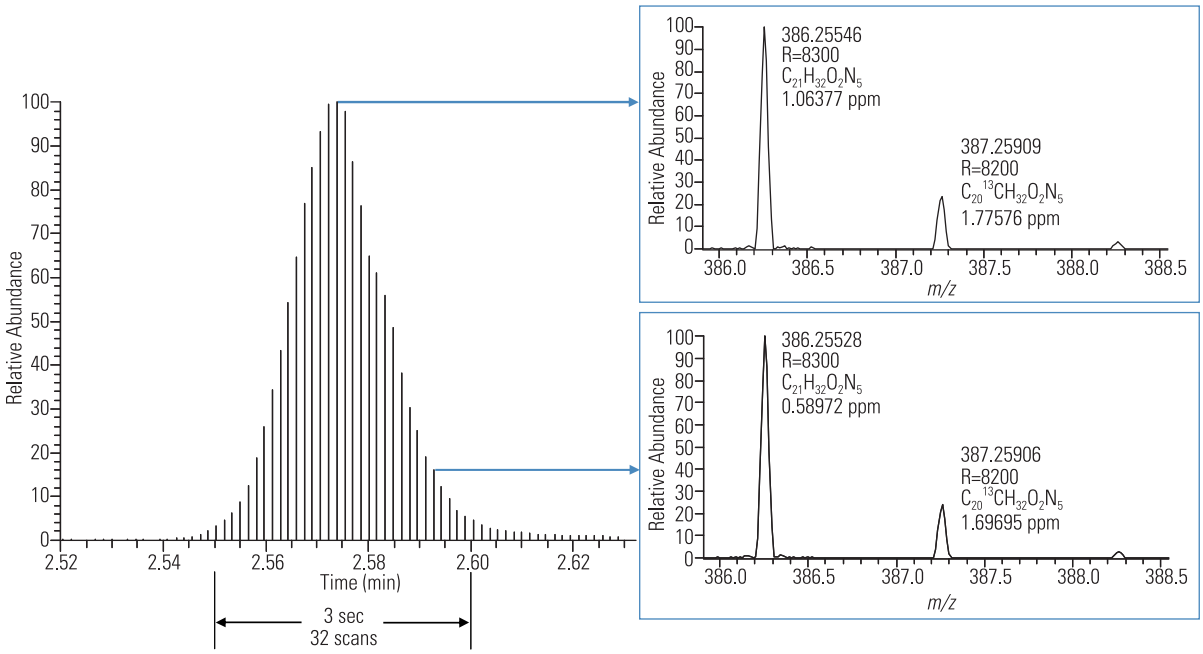

Figure 2: LC peak and mass spectra of Buspirone acquired at a scan rate of 10 scans per second.

Mass Resolution

At a scan rate of 10 Hz, the resolving power of the instrument is > 10,000 at *m/z* 200. Increasing the transient detection time by a factor of 10 (corresponding to a scan rate of 1 Hz), the mass resolution can be increased beyond 100,000.

To demonstrate the resolving power of the instrument, a pesticide mixture was measured showing well resolved isobaric peaks of Dimethon (*m/z* 231.0273) and Asulam (*m/z* 231.0434) within a full scan spectrum (Figure 3).

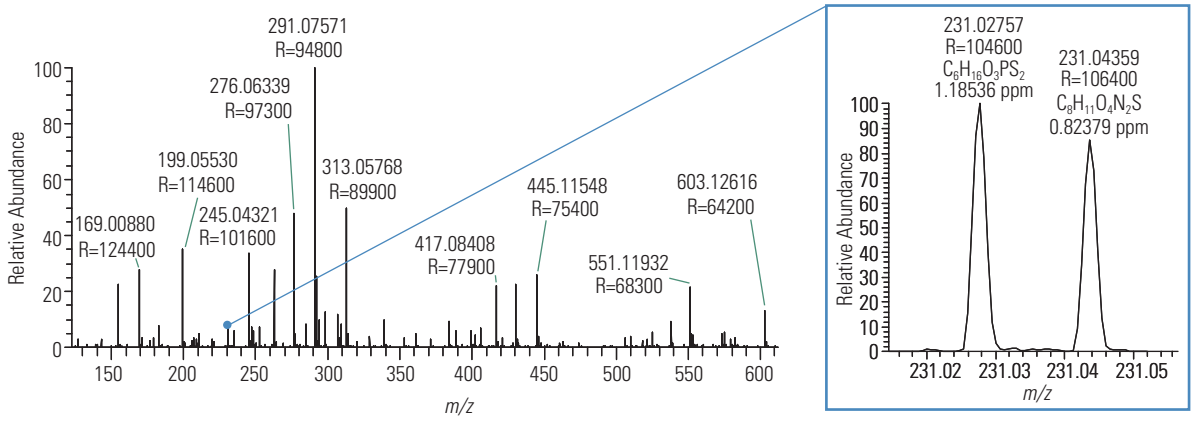

Figure 3: Full scan spectrum of a pesticide mixture demonstrating a resolving power of up to 100,000.

Mass Accuracy and Stability

Using fully automated AGC and mass calibration procedures, mass spectra with high mass accuracy are recorded. The mass accuracy, precision and stability is equally as good as that obtained in ion trap based hybrid instruments, i.e. Thermo Scientific LTQ Orbitrap™ or LTQ FT Ultra™.

Figure 4 shows the mass accuracy and its stability over time for different molecular ions of an ESI calibration mixture. The full scan spectra were acquired at a resolution setting of 100,000 in an infusion experiment applying an external calibration, i.e. no lock masses were used.

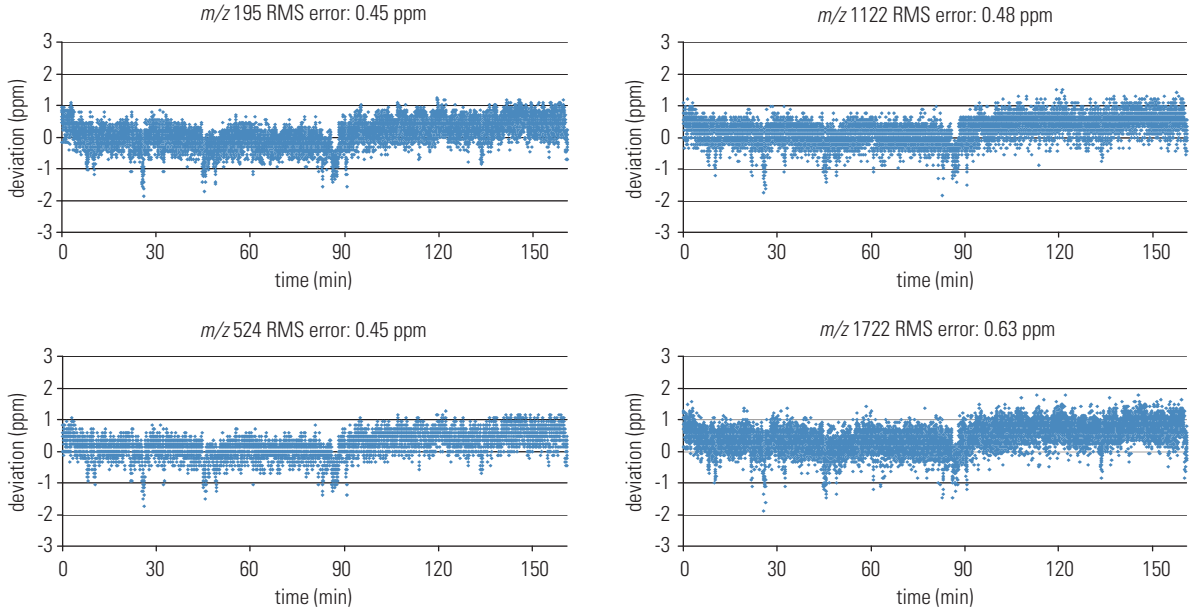

Figure 4: Mass accuracy and stability of ions at different *m/z* values acquired in an infusion experiment without using lock masses.

Fast Polarity Switching

Due to the use of a novel power supply design, it is possible to perform fast polarity switching without sacrificing mass accuracy in any scans. Figure 5 demonstrates this feature by means of two experiments. In the first experiment, the polarity was changed from scan to scan to check mass accuracy at fast alternating polarity switching corresponding to a full cycle of 1 positive and 1 negative scan within 1 second. In the second experiment, the polarity was switched every 5 minutes to check for potential drift effects. In both cases, full scan spectra were acquired at a resolution setting of 30,000 in an infusion experiment using an ESI calibration solution applying an external calibration, i.e. no lock masses were used.

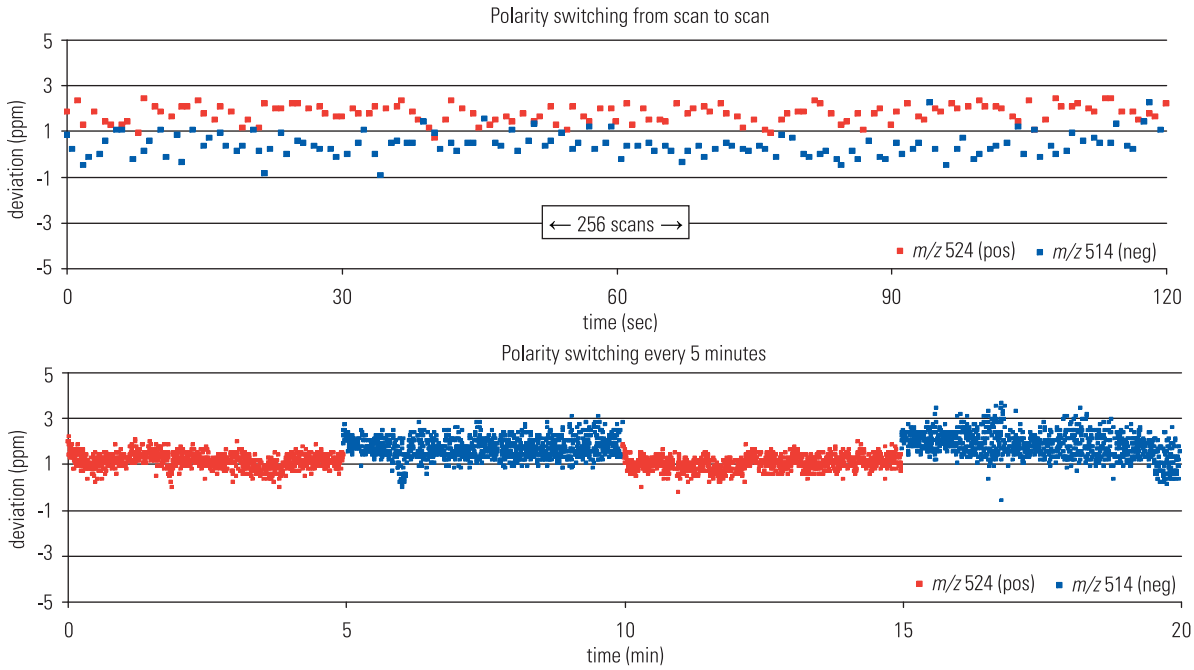

Figure 5: Mass deviations of *m/z* 524 (positive ions) and *m/z* 514 (negative ions) observed in polarity switching experiments.

Dynamic Range

The dynamic range of the instrument varies by sample and with the instrument settings, but it is typically about 3 to 4 orders of magnitude. Figure 6 shows that it is possible to acquire full scan spectra with an in-scan dynamic range of more than 13,000. The spectrum was acquired in an infusion experiment using a mix of Buspirone (*m/z* 386) and Caffeine (*m/z* 195).

The ratio of the Buspirone signal to the Caffeine signal is greater than 13,000. Both peaks show mass accuracies of less than 1 ppm. Thus, this spectrum demonstrates not only the high in-scan dynamic range in terms of signal, but also the high dynamic range in terms of mass accuracy of this instrument – analogous to the performance of a hybrid LTQ Orbitrap mass spectrometer.

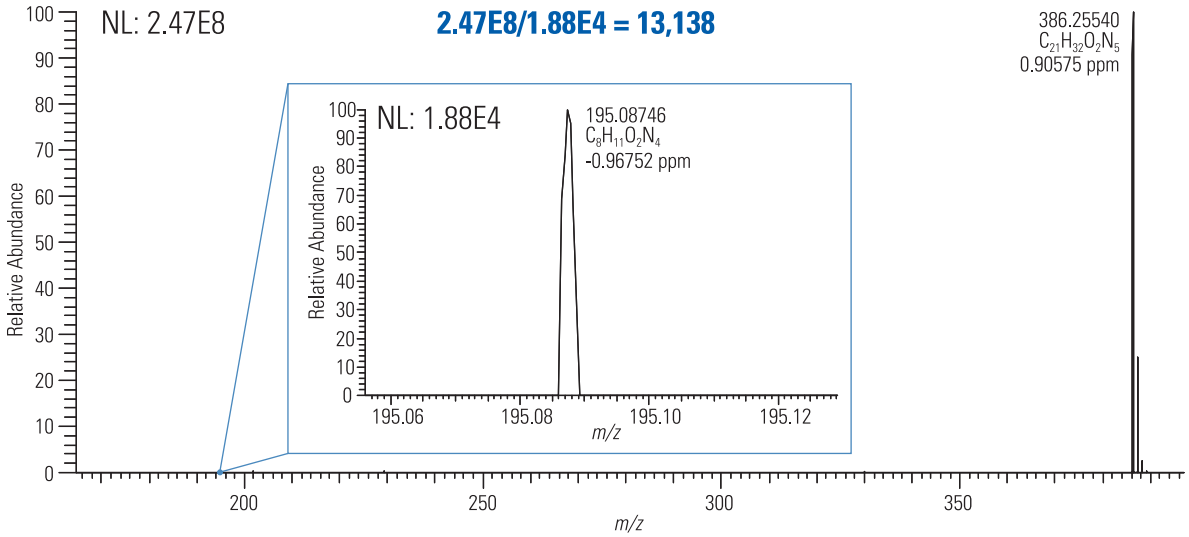

Figure 6: Spectrum of a mixture of Buspirone (*m/z* 386) and Caffeine (*m/z* 195) showing an in-scan dynamic range of > 13,000 and sub-ppm mass accuracies.

All Ion Fragmentation (HCD)

The instrument design allows high efficiency "All Ion Fragmentation" experiments by means of Higher Energy Collision Induced Dissociation (HCD).

As an example, Figure 7 shows full scan spectra of Verapamil with and without HCD fragmentation and demonstrates the high fragmentation efficiency and the excellent mass accuracy of the HCD fragments.

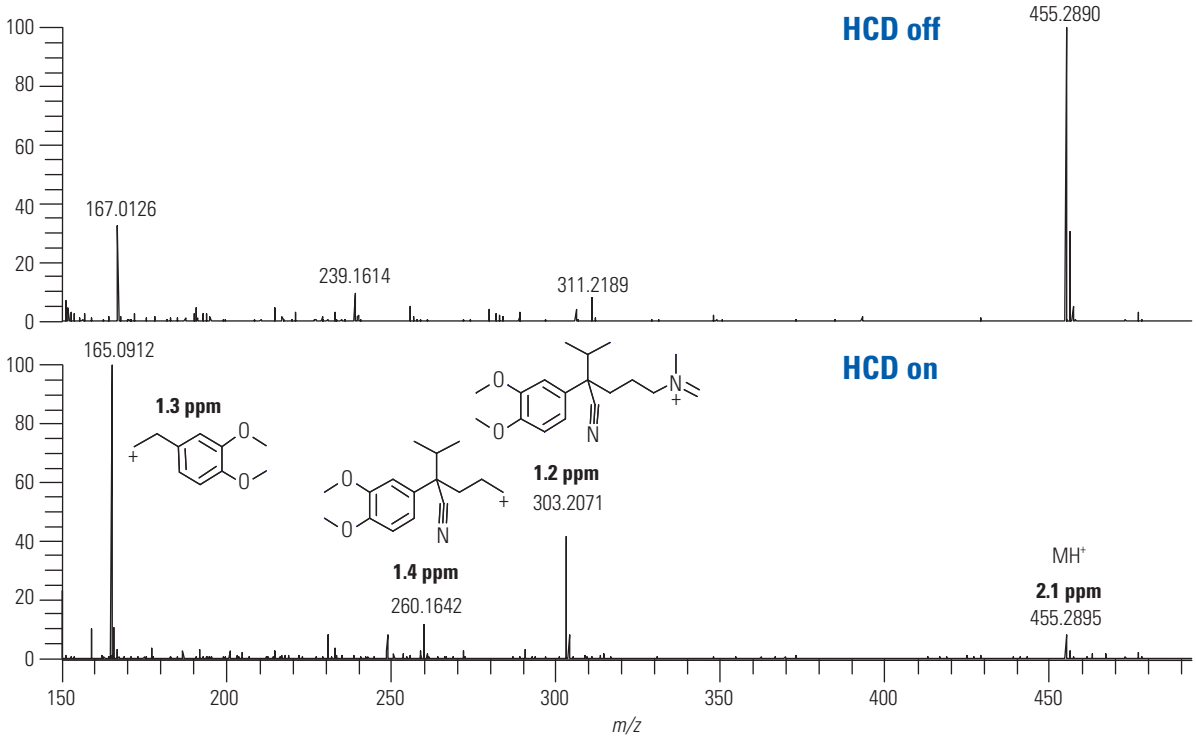

Figure 7: Full scan spectra of Verapamil with and without HCD fragmentation.

Applications

As a result of the described performance characteristics of this new benchtop Orbitrap mass spectrometer, several key applications are ideally suited to the use of the Exactive mass spectrometer. Some of these are:

1. Exact mass measurements of organic compounds
2. Early drug discovery metabolism and pharmacokinetics (DMPK)
3. General unknown screening
4. Multiple residue analysis (Pesticides, Mycotoxins, veterinary drugs)
5. Metabolomics

For all of these applications, high resolution, accurate mass measurements, together with high dynamic range, is required for unequivocal results in full MS mode. Where it is needed, additional information can be provided by use of high resolution/high mass accuracy MS/MS experiments in an "All Ion Fragmentation" mode. Figure 8 shows an extracted ion chromatogram of 116 pesticides and mycotoxins at a level of 50 ppb in a very complex matrix of horse feed extract at a mass resolution of 50,000. This exemplifies the high selectivity and sensitivity of the instrument working in full scan mode, which is a prerequisite for a successful screening approach, since resolving matrix interferences from the target analytes is essential.

Conclusions

A new benchtop mass spectrometer has been developed based on an API ion source combined with a stand-alone Orbitrap mass analyzer. The key performance features are as follows:

- Mass resolutions of up to 100,000
- Scan speeds of up to 10 Hz
- High in-scan dynamic range (4 orders of magnitude)
- Mass accuracies of better than 2 ppm in full scan and "All Ion Fragmentation" mode
- Fast polarity switching (full cycle of 1 positive and 1 negative scan within 1 second)
- High efficiency "All Ion Fragmentation" Higher Energy Collision Induced Dissociation (HCD)

The instrument is very easy to operate and its performance characteristics are ideally suited for discovery work, screening applications, quantitative analyses and elemental composition determinations.

Acknowledgements

We would like to thank the other Exactive project team members Frank Czemper, Florian Grosse-Coosmann, Thomas Heise, Oliver Hengelbrock, Sebastian Kanngiesser, Alexander Kholomeev, Sascha Moehring, Uwe Rickens, Ronald Seedorf and Stevan Horning.

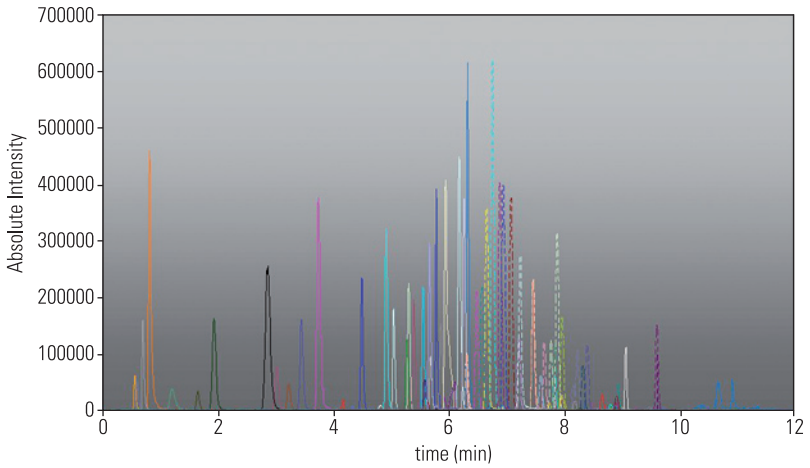

Figure 8: Extracted ion chromatogram of 116 pesticides and mycotoxins at a level of 50 ppb in a complex matrix of horse feed extract.

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Application Note: 52027

Fast GC-MS/MS for High Throughput Pesticides Analysis

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Key Words

- Fast GC
- GC-MS/MS
- Matrix Selectivity
- Multi-component analysis
- Pesticides
- Productivity
- Quantitation
- Timed-SRM

Introduction

A wide variety of phytosanitary products are commonly applied in agricultural crops in order to increase production yield and obtain high quality products. Consequently, the control of pesticide residue levels by the performance of monitoring programs is currently an increasing concern for producers, traders, and consumers.

The QuEChERS method is the most diffused analytical procedure for preparing samples of fruits and vegetables while performing a multi-residues pesticides analysis in combination with GC-MS and LC-MS systems.[1-3] The analytical benefit of the QuEChERS method is the quick procedure with only a short clean-up step to cover a wide variety of polar and less polar pesticide compounds in a multi-component approach. Due to the reduced clean-up however, the produced sample extracts carry a high concentration of vegetal matrix, that raise a particular challenge to the GC and MS systems.

The application of structure selective MS/MS detection for the quantitation of pesticides residues using multiple reaction monitoring methods (MRM) in fruits and vegetables has been proven to overcome matrix effects.[4-5] The next challenge for commercial routine laboratories is to increase sample throughput to keep pace with the steady increase of the demand for food safety analysis.

In this work the Fast GC and tandem mass spectrometry combination is presented as the analytical system to solve the requirement for matrix robustness with high sample throughput. The Fast GC-MS/MS analytical approach requires a robust, selective and sensitive instrumental system in order to quantify thousands samples/year with short run times. The goal is to obtain a reliable pesticide compound detection and quantification at ppb levels while avoiding breakdown phenomena for the more reactive compounds. The analytical setup and results for the screening and quantitation of 233 pesticides in one Fast GC run is described. For each pesticides compound 2 SRM transitions have been used to comply with EU regulations for compound confirmation gaining 5 EU identification points.[6]

Experimental Conditions

Sample Preparation

10 g of sample was processed in according with the QuEChERS procedures.[1-3] In the clean-up step the graphitized carbon black treatment was not utilized in order to avoid the loss of planar compounds as there are the coplanar PCBs and pyrethroids. 1 mL of final volume of the extracts was reconstituted using acetone/hexane 1:1

after evaporation of the acetonitrile extraction solvent. 10 µL of a solution of Fenchlorphos (5 ppm in hexane) as volumetric standard has been added before injection (1 µL).

A Thermo Scientific TSQ Quantum GC GC-MS/MS system with a Thermo Scientific TRACE GC Ultra gas chromatograph and TriPlus AS liquid autosampler was used for analysis of the samples extracts, equipped and programmed for a Fast GC analysis method, using the following analytical parameters.

TRACE GC Ultra™ Conditions

Carrier Gas:	He, constant flow 1 mL/min
Injector:	PTV splitless mode with Siltek baffled liner 2 mm ID (p/n 453T2120)
PTV Temp. Program:	70 °C, 0.02 min, 12 °C/s to 280 °C, 1.2 min, 14.5 °C/s to 320 °C, 6 min, clean flow 80 mL/min.
Split:	splitless injection, splitflow 50 mL/min at 1.3 min
Column Type:	Restek Rxi-5Sil MS, 20 m, 0.18 mm, 0.18 µm (Restek p/n 43602)
Transfer Line Temp.:	280 °C
GC Oven Program:	80 °C, 1.5 min 30 °C/min to 210 °C 20 °C/min to 320 °C, 2 min

TSQ Quantum GC™ Acquisition Parameter Setting

Source Temperature:	260 °C
Emission Current:	25 µA
Ionisation Mode:	EI, 70 eV
Mass Resolution:	Q1, Q3 at 0.7 Da (FWHM)
Collision Gas:	Ar, 1.5 mTorr
Cycle Time:	0.30 s
Acquisition Mode:	Timed-SRM

Mass Table

467 Timed-SRM transition/Fast GC run, see Table 1

Fast GC-MS/MS Data for Pesticide Standards *(Table 1)*

Precursor	Product	CE	Rention Time	Pesticide
212.02	182.02	10	8.09	Aclonifen
264.03	194.02	15	8.09	Aclonifen
181.02	152.04	25	8.85	Acrinathrin
208.05	181.04	8	8.85	Acrinathrin
161.07	146.06	12	6.54	Alachlor
188.08	160.07	10	6.54	Alachlor
292.91	222.92	20	6.94	Aldrin
292.91	257.91	20	6.94	Aldrin
293.19	147.10	15	9.24	Amitraz
293.19	162.10	10	9.24	Amitraz
215.09	200.09	10	5.95	Atrazine
215.09	173.08	10	5.95	Atrazine
132.01	77.01	20	9.43	Azinphos-ethyl
160.02	104.01	10	9.43	Azinphos-ethyl
132.02	77.02	20	9.15	Azinphos-Methyl
160.03	104.02	10	9.15	Azinphos-Methyl
344.10	329.10	20	10.96	Azoxystrobin
388.11	345.10	15	10.96	Azoxystrobin
234.12	174.09	10	8.24	Benalaxyl
266.14	148.08	10	8.24	Benalaxyl
166.06	151.06	15	3.94	Bendiocarb
223.08	166.06	15	3.94	Bendiocarb
292.10	160.05	21	5.63	Benfluralin
292.10	264.09	10	5.63	Benfluralin
164.08	149.07	10	9.35	Benfuracarb
190.09	144.07	10	9.35	Benfuracarb
180.91	144.93	15	5.82	BHC, A+B+C+D
218.89	182.91	15	5.82	BHC, A+B+C+D
154.08	152.08	15	4.51	Bifenil
154.08	153.08	15	4.51	Bifenil
181.05	141.04	22	8.77	Bifenthrin
181.05	153.05	6	8.77	Bifenthrin
170.09	115.06	25	9.62	Bitertanol
170.09	141.07	20	9.62	Bitertanol
342.01	140.01	20	10.12	Boscalid (Nicobifen)
344.01	140.01	20	10.12	Boscalid (Nicobifen)
328.86	313.87	20	7.04	Bromophos-methyl
330.86	315.87	20	7.04	Bromophos-methyl
358.89	302.91	20	7.38	Bromophos-ethyl
358.89	330.90	10	7.38	Bromophos-ethyl
340.96	184.98	15	8.81	Bromopropylate
342.96	184.98	20	8.81	Bromopropylate
273.14	193.10	10	7.73	Bupirimate
316.16	208.10	10	7.73	Bupirimate
104.94	104.00	9	7.75	Buprofezin
249.13	193.10	10	7.75	Buprofezin
174.12	146.10	10	4.67	Butylate (Sutan)
217.15	156.11	5	4.67	Butylate (Sutan)
149.96	78.98	15	8.58	Captafol
310.92	78.98	10	8.58	Captafol
123.05	79.03	15	4.95	Captafol-captan Met. (THPI)
151.06	122.05	10	4.95	Captafol-captan Met. (THPI)
148.97	69.98	8	7.30	Captan
148.97	104.98	8	7.30	Captan
164.01	149.00	10	5.91	Carbofuran
221.01	164.00	5	5.91	Carbofuran
341.97	156.99	10	8.27	Carbophenothion
341.97	295.98	5	8.27	Carbophenothion
330.03	310.03	20	8.20	Carfentrazone-ethyl
340.03	312.03	20	8.20	Carfentrazone-ethyl
372.81	265.87	15	7.47	Chlordane
374.81	267.87	15	7.47	Chlordane

Precursor	Product	CE	Rention Time	Pesticide
246.98	226.98	20	7.80	Chlorfenapyr
248.98	228.98	20	7.80	Chlorfenapyr
174.98	110.98	10	7.62	Chlorfenson
301.96	174.98	10	7.62	Chlorfenson
266.98	158.99	15	7.09	Chlorfenvinphos-E+Z
322.97	266.98	15	7.09	Chlorfenvinphos-E+Z
220.04	166.03	23	8.38	Chloridazon
220.04	158.03	25	8.38	Chloridazon
153.98	120.98	5	4.72	Chlormephos
233.97	120.98	14	4.72	Chlormephos
263.88	167.92	25	6.18	Chlorothalonil
265.88	169.92	25	6.18	Chlorothalonil
213.06	127.03	15	5.61	Chlorpropham
213.06	171.04	10	5.61	Chlorpropham
313.93	257.95	15	6.85	Chlorpyrifos-ethyl
315.93	259.95	12	6.85	Chlorpyrifos-ethyl
285.91	92.97	20	6.49	Chlorpyrifos-methyl
285.91	270.91	25	6.49	Chlorpyrifos-methyl
300.91	222.93	25	6.90	Chlorthal-dimethyl
331.90	300.91	15	6.90	Chlorthal-dimethyl
259.01	188.01	15	7.15	Chlozolinate
188.01	147.01	20	7.15	Chlozolinate
349.05	266.04	15	8.33	Clodinafop-propargyl
349.05	238.04	15	8.33	Clodinafop-propargyl
304.01	138.01	10	9.54	Clofentezine
304.01	132.01	10	9.54	Clofentezine
321.00	304.00	22	7.51	Clorfluazuron
323.00	306.00	20	7.51	Clorfluazuron
251.02	139.02	22	7.97	Clorpropilato
253.01	139.00	15	7.97	Clorpropilato
226.01	198.00	12	9.72	Coumaphos
226.01	163.01	20	9.72	Coumaphos
225.08	198.07	10	6.86	Cyanazine
225.08	189.07	10	6.86	Cyanazine
206.03	151.02	20	9.90	Cyfluthrin
226.03	206.03	17	9.90	Cyfluthrin
181.04	152.03	23	9.22	Cyhalothrin, lambda
197.04	141.03	15	9.22	Cyhalothrin, lambda
163.03	127.02	12	10.07	Cypermethrin+alfametrina
181.03	152.03	17	10.07	Cypermethrin+alfametrina
222.09	125.05	20	7.89	Cyproconazole
224.09	127.05	20	7.89	Cyproconazole
224.13	208.12	20	7.14	Cyprodinil
225.13	210.12	18	7.14	Cyprodinil
234.98	164.98	20	7.75	DDD, o,p
236.98	164.98	20	8.05	DDD, o,p
234.97	198.97	18	8.05	DDD, p,p
234.97	164.98	20	7.75	DDD, p,p
245.96	175.97	25	7.42	DDE o,p
317.94	245.95	20	7.42	DDE o,p
245.95	175.97	25	7.68	DDE p,p
247.95	175.97	20	7.68	DDE p,p
234.95	164.96	15	7.74	DDT o,p
236.94	164.96	20	7.74	DDT o,p
234.94	198.95	15	8.04	DDT p,p
234.94	164.96	20	8.04	DDT p,p
252.93	171.95	10	10.89	Deltamethrin+Tralometrina
252.93	173.95	10	10.89	Deltamethrin+Tralometrina
199.06	93.03	15	6.09	Diazinon
304.10	179.06	15	6.09	Diazinon
222.98	204.98	10	6.43	Dichlofenthion
278.97	222.98	15	6.43	Dichlofenthion

Precursor	Product	CE	Rention Time	Pesticide
223.97	122.99	15	6.79	Dichlofluanid
225.97	122.99	15	6.79	Dichlofluanid
205.97	175.97	10	5.92	Dichloran
207.96	177.97	10	5.92	Dichloran
184.95	92.98	17	3.82	Dichlorphos
219.95	184.95	10	3.82	Dichlorphos
270.07	159.04	15	7.80	Diclobutrazol
272.08	161.04	15	7.80	Diclobutrazol
138.97	110.97	20	6.98	Dicofol (1st, 2nd degr.)
250.94	138.97	15	6.98	Dicofol (1st, 2nd degr.)
276.92	206.93	20	7.83	Dieldrin
276.92	240.92	10	7.83	Dieldrin
267.15	225.12	8	6.83	Diethofencarb
267.15	168.09	10	6.83	Diethofencarb
323.05	265.04	15	10.75	Difenoconazole 1+2
325.05	267.04	20	10.75	Difenoconazole 1+2
266.05	246.05	10	8.48	Diflufenican
394.07	266.05	10	8.48	Diflufenican
125.00	79.00	15	5.92	Dimethoate
229.01	87.01	5	5.92	Dimethoate
301.10	165.05	10	11.04	Dimethomorph 1+2
387.12	301.10	12	11.04	Dimethomorph 1+2
268.06	232.05	15	8.02	Diniconazole
270.06	234.05	15	8.02	Diniconazole
305.08	244.07	15	6.17	Dinitramine
307.08	216.06	15	6.17	Dinitramine
167.09	165.09	20	7.04	Diphenamid
239.13	167.09	10	7.04	Diphenamid
167.10	166.09	25	5.51	Diphenylamine
169.10	168.09	20	5.51	Diphenylamine
142.01	109.01	10	6.20	Disulfoton
186.02	153.02	5	6.20	Disulfoton
271.03	243.03	5	7.51	Ditalimfos
299.04	243.03	10	7.51	Ditalimfos
273.88	238.89	15	7.54	Endosulfan A+B
271.88	236.89	15	7.54	Endosulfan A+B
280.91	244.92	5	8.07	Endrin
344.88	280.90	8	8.07	Endrin
192.04	138.03	10	8.26	Epoxiconazole
192.04	111.02	10	8.26	Epoxiconazole
128.08	86.05	5	4.35	EPTC
189.12	128.08	5	4.35	EPTC
245.04	173.03	15	8.00	Etaconazole 1+2
245.04	191.03	10	8.00	Etaconazole 1+2
230.99	129.01	20	8.03	Ethion
230.99	174.99	15	8.03	Ethion
202.14	145.10	20	5.91	Ethoxyquin
202.14	174.12	15	5.91	Ethoxyquin
163.09	135.07	10	10.21	Etofenprox
163.09	107.06	16	10.21	Etofenprox
158.04	130.03	10	5.53	Etoprofos
200.05	158.04	10	5.53	Etoprofos
210.93	182.94	15	4.78	Etridiazole (Terrazole)
210.93	139.95	15	4.78	Etridiazole (Terrazole)
292.06	153.03	10	6.21	Etrimfos
292.09	181.04	10	6.21	Etrimfos
238.08	209.07	20	8.88	Fenamidone
238.08	237.08	20	8.88	Fenamidone
288.10	260.09	10	7.54	Fenamiphos
303.11	260.09	15	7.54	Fenamiphos
139.01	111.01	15	9.38	Fenarimol
251.03	139.01	15	9.38	Fenarimol

Precursor	Product	CE	Rention Time	Pesticide
145.08	117.07	15	8.97	Fenazaquin
160.09	117.07	20	8.97	Fenazaquin
129.04	102.03	15	9.91	Fenbuconazole
198.07	129.04	10	9.91	Fenbuconazole
284.82	269.97	12	6.61	Fenchlorphos (VS)
286.72	272.08	12	6.61	Fenchlorphos (VS)
177.04	113.02	15	8.39	Fenhexamid
301.06	97.02	15	8.39	Fenhexamid
277.02	109.01	8	6.72	Fenitrothion
277.02	260.02	10	6.72	Fenitrothion
265.13	89.04	10	8.86	Fenpropathrin
265.13	210.10	15	8.86	Fenpropathrin
145.13	117.11	10	6.72	Fenpropidin
274.25	98.09	10	6.72	Fenpropidin
128.11	70.06	15	6.89	Fenpropimorph
128.11	110.09	15	6.89	Fenpropimorph
267.98	77.00	20	7.03	Fenson
267.98	141.00	10	7.03	Fenson
293.03	125.01	10	7.97	Fensulfothion
293.03	97.01	16	7.97	Fensulfothion
278.02	109.01	18	6.88	Fenthion
278.02	169.01	20	6.88	Fenthion
167.05	125.04	10	10.52	Fenvalerate 1+2
419.13	225.07	10	10.52	Fenvalerate 1+2
419.94	350.95	15	7.03	Fipronil
421.94	352.95	15	7.03	Fipronil
383.13	254.09	20	7.85	Fluazifop-P-butyl
383.13	282.10	15	7.85	Fluazifop-P-butyl
388.90	352.20	12	7.72	Fluazinam
388.90	354.20	12	7.72	Fluazinam
167.00	77.00	15	7.52	Flubenzimine
186.00	77.00	25	7.52	Flubenzimine
199.07	107.04	22	10.14	Flucitrinate 1+2
199.07	157.06	10	10.14	Flucitrinate 1+2
248.04	154.02	20	7.60	Fludioxonil
248.04	182.03	15	7.60	Fludioxonil
211.04	183.03	10	6.89	Flufenacet
211.04	123.02	10	6.89	Flufenacet
346.95	171.93	26	8.36	Fluopicolide
346.95	176.02	26	8.36	Fluopicolide
313.01	174.01	15	6.96	Fluorocloridone I+II
313.01	187.01	15	6.96	Fluorocloridone I+II
340.01	286.01	25	9.73	Fluquinconazole
340.01	298.01	22	9.73	Fluquinconazole
233.07	152.05	20	7.72	Flusilazole
233.07	165.05	20	7.72	Flusilazole
123.04	75.03	15	7.55	Flutriafol
219.07	123.04	15	7.55	Flutriafol
250.06	200.05	20	10.57	Fluvalinate tau
252.06	200.05	20	10.57	Fluvalinate tau
261.60	129.80	15	7.35	Folpet
261.60	234.40	5	7.35	Folpet
146.98	103.24	15	4.86	Folpet met.
146.98	104.39	15	4.86	Folpet met.
137.02	109.01	10	6.11	Fonofos
246.03	137.02	10	6.11	Fonofos
224.01	125.01	15	6.34	Formothion
224.01	196.01	10	6.34	Formothion
242.11	95.04	15	7.26	Furalaxyl
301.13	225.10	10	7.26	Furalaxyl
375.05	316.04	10	7.33	Haloxyfop-methyl
375.05	288.04	20	7.33	Haloxyfop-methyl

Fast GC-MS/MS Data for Pesticide Standards *(Table 1 continued)*

Precursor	Product	CE	Rention Time	Pesticide
273.87	238.88	15	6.63	Heptachlor
271.87	236.89	15	6.63	Heptachlor
182.91	154.93	15	7.26	Heptachlor epoxide B
134.93	98.95	15	7.26	Heptachlor epoxide B
352.83	262.87	15	7.23	Heptachlor epoxide A
352.83	281.86	16	7.23	Heptachlor epoxide A
124.01	89.01	10	5.26	Heptenophos
250.02	89.01	25	7.62	Heptenophos
283.81	213.86	20	7.62	Hexachlorobenzene (HCB)
283.81	248.84	20	7.47	Hexachlorobenzene (HCB)
214.05	172.04	20	7.47	Hexaconazole
214.05	187.04	15	5.26	Hexaconazole
184.05	149.04	10	7.62	Hexythiazox
227.07	149.04	10	7.62	Hexythiazox
173.00	145.00	20	10.79	Imazalil
175.00	147.00	16	10.79	Imazalil
203.03	106.01	20	8.72	Indoxacarb
203.03	134.02	20	8.72	Indoxacarb
314.03	245.03	15	8.00	Iprodione
316.03	247.03	15	8.00	Iprodione
243.88	187.00	16	6.08	Iprodione degr.
243.88	188.00	16	6.08	Iprodione degr.
213.07	121.04	17	7.05	Isofenphos
213.07	185.06	10	7.05	Isofenphos
280.15	180.10	15	7.72	Isopropalin
280.15	238.13	10	7.72	Isopropalin
131.06	116.05	20	8.34	Kresoxim-methyl
206.09	131.06	15	8.34	Kresoxim-methyl
153.09	82.05	15	5.00	Lenacil
153.09	136.08	15	5.00	Lenacil
175.99	120.99	20	4.60	Lufenuron 1
175.99	147.99	20	4.60	Lufenuron 1
352.99	173.99	25	6.77	Lufenuron 2
352.99	202.99	25	6.77	Lufenuron 2
173.02	99.01	10	8.60	Malathion
173.02	127.01	10	8.60	Malathion
253.04	190.03	20	7.49	Mefenpyr-diethyl
253.04	189.03	20	7.49	Mefenpyr-diethyl
222.11	207.10	15	6.58	Mepanipyrim
223.11	208.10	15	6.58	Mepanipyrim
234.11	174.11	10	7.81	Metalaxyl
249.13	190.10	10	7.81	Metalaxyl
202.09	174.07	5	7.11	Metamitron
202.09	186.08	10	7.11	Metamitron
133.05	117.04	20	5.71	Metazachlor
209.07	132.05	12	5.71	Metazachlor
164.05	136.04	20	7.38	Methabenzthiazuron
164.05	135.04	20	7.38	Methabenzthiazuron
144.98	57.99	15	8.48	Methidathion
144.98	84.99	10	8.48	Methidathion
227.01	169.01	20	6.85	Methoxychlor I
227.01	212.01	15	6.85	Methoxychlor I
162.08	133.06	15	6.48	Metolachlor
238.11	162.08	15	6.48	Metolachlor
198.08	82.03	20	4.64	Metribuzin
198.08	110.05	20	4.64	Metribuzin
127.04	109.02	10	9.37	Mevinphos
192.04	127.03	12	9.37	Mevinphos
269.81	234.84	15	5.18	Mirex
271.81	236.84	15	5.18	Mirex
126.07	55.03	10	5.67	Molinate (Ordram)
187.10	126.07	10	5.67	Molinate (Ordram)

Precursor	Product	CE	Rention Time	Pesticide
127.03	95.03	20	7.72	Monocrotophos
192.05	127.03	10	7.72	Monocrotophos
179.07	125.05	15	3.82	Myclobutanil
179.07	90.00	30	7.58	Myclobutanil
109.00	79.00	12	7.58	Naled
128.07	72.04	10	7.91	Napropamide
271.16	128.07	5	7.91	Napropamide
201.99	138.99	21	8.48	Nitrofen
282.98	252.98	15	8.48	Nitrofen
235.05	139.03	15	5.09	Nuarimol
314.06	139.03	15	5.09	Nuarimol
170.07	115.05	20	7.68	Ortho-phenylphenol
170.07	141.06	20	7.68	Ortho-phenylphenol
258.05	175.04	10	8.02	Oxadiazon
304.06	260.05	10	8.02	Oxadiazon
163.07	117.05	40	7.71	Oxadixyl
163.07	132.06	10	7.71	Oxadixyl
300.03	223.02	10	7.46	Oxyfluorfen
361.03	300.03	12	7.46	Oxyfluorfen
236.10	125.06	15	7.01	Paclobutrazol
236.10	167.07	15	7.01	Paclobutrazol
149.03	119.02	10	6.19	Paraoxon-ethyl
220.05	174.04	10	6.19	Paraoxon-ethyl
230.02	136.01	10	6.92	Paraoxon-methyl
230.02	200.02	10	6.92	Paraoxon-methyl
291.03	109.01	15	6.53	Parathion-ethyl
291.03	137.02	10	6.53	Parathion-ethyl
262.99	109.00	15	7.18	Parathion-methyl
262.99	246.00	15	7.18	Parathion-methyl
248.06	157.04	25	5.75	Penconazole
248.06	192.04	15	5.75	Penconazole
125.05	89.04	12	7.12	Pencycuron
180.07	125.05	12	7.12	Pencycuron
252.13	162.08	12	9.64	Pendimethalin
252.13	191.09	12	9.64	Pendimethalin
183.04	153.03	15	7.24	Permethrin 1+2
183.04	165.03	15	7.24	Permethrin 1+2
274.03	246.02	10	5.75	Phenthoate
274.03	121.01	7	5.75	Phenthoate
231.01	203.01	10	9.11	Phorate
260.01	75.01	5	9.11	Phorate
181.99	111.00	15	8.79	Phosalone
366.99	181.99	10	8.79	Phosalone
160.01	77.01	20	6.40	Phosmet
160.01	133.01	15	6.40	Phosmet
227.05	127.03	15	6.29	Phosphamidon I+II
264.06	127.03	15	6.29	Phosphamidon I+II
166.10	137.08	10	7.00	Pirimicarb
238.14	166.10	15	7.00	Pirimicarb
304.12	168.06	15	6.68	Pirimiphos-ethyl
333.13	318.12	15	6.68	Pirimiphos-ethyl
290.09	233.07	10	9.76	Pirimiphos-methyl
305.10	290.09	15	9.76	Pirimiphos-methyl
180.01	138.01	15	7.29	Prochloraz
308.03	70.01	10	7.29	Prochloraz
283.02	255.02	10	7.66	Procymidone
285.02	257.02	10	7.66	Procymidone
336.94	266.95	20	6.01	Profenofos
338.94	268.95	20	6.01	Profenofos
318.10	198.05	15	5.91	Profluralin
330.10	302.10	5	5.91	Profluralin
225.16	183.13	10	6.60	Prometon

Precursor	Product	CE	Rention Time	Pesticide
225.16	210.15	10	6.60	Prometon
226.13	184.10	12	5.43	Prometryn
241.15	184.10	15	5.43	Prometryn
176.06	120.04	10	6.44	Propachlor
196.07	120.04	10	6.44	Propachlor
217.01	161.00	10	8.49	Propanil
219.01	163.00	10	8.49	Propanil
135.06	107.05	15	6.03	Propargite
173.08	105.05	12	6.03	Propargite
236.07	166.05	15	4.79	Propetamphos
236.07	194.06	5	4.79	Propetamphos
137.07	93.05	8	8.30	Propham
179.09	93.05	15	8.30	Propham
259.02	173.02	20	5.41	Propiconazole 1+2
261.02	175.02	20	5.41	Propiconazole 1+2
110.00	64.00	10	6.10	Propoxur
152.00	110.00	10	6.10	Propoxur
145.01	109.01	15	7.63	Propyzamide
173.01	109.01	18	7.63	Propyzamide
266.97	238.97	10	10.56	Prothiofos
308.97	238.97	5	10.56	Prothiofos
132.03	77.02	15	9.33	Pyraclostrobin
325.08	132.03	20	9.33	Pyraclostrobin
221.05	193.04	10	9.74	Pyrazophos
232.05	204.05	10	9.74	Pyrazophos
147.06	117.04	20	8.69	Pyridaben
309.12	147.06	15	8.69	Pyridaben
340.06	109.02	10	7.20	Pyridaphenthion
340.06	199.04	10	7.20	Pyridaphenthion
262.03	192.02	20	6.16	Pyrifenox 1+2
262.03	200.02	20	6.16	Pyrifenox 1+2
198.11	118.07	35	7.25	Pyrimethanil
198.11	183.10	15	7.25	Pyrimethanil
146.03	91.02	15	8.34	Quinalphos
146.03	118.02	15	8.34	Quinalphos
237.05	208.00	20	6.03	Quinoxyfen
272.01	237.00	20	6.03	Quinoxyfen
292.84	234.87	15	6.68	Quintozene (PCNB)
294.84	236.87	20	6.68	Quintozene (PCNB)
129.93	94.95	22	8.53	S421
131.93	96.95	22	8.53	S421
178.01	81.00	24	5.65	Sethoxydim
178.01	107.95	21	5.65	Sethoxydim
202.01	146.01	15	7.31	Sulfotep
322.02	202.01	15	7.31	Sulfotep
255.78	159.87	12	8.50	Sulphur
255.78	95.83	24	8.50	Sulphur
250.12	125.06	20	8.97	Tebuconazole
252.12	127.06	20	8.97	Tebuconazole
145.00	117.00	10	8.91	tebufenozide
160.00	145.00	15	8.91	tebufenozide
276.13	171.08	15	5.35	Tebufenpyrad
318.15	145.07	15	5.35	Tebufenpyrad
260.88	202.90	15	6.19	Tecnazene
258.88	200.90	15	6.19	Tecnazene
177.02	127.02	20	6.07	Tefluthrin
197.03	141.02	15	6.07	Tefluthrin
231.04	175.03	15	6.02	Terbufos
231.04	203.03	10	6.02	Terbufos
214.10	104.05	10	6.70	Terbuthylazine
214.10	132.06	10	6.70	Terbuthylazine
241.14	185.10	10	7.42	Terbutryn
241.14	170.10	15	7.42	Terbutryn
328.91	108.97	22	6.89	Tetrachlorvinphos
330.91	108.97	22	6.89	Tetrachlorvinphos
336.02	218.01	20	9.06	Tetraconazole
336.02	204.01	20	9.06	Tetraconazole
226.93	198.94	18	8.72	Tetradifon
355.88	228.93	10	8.72	Tetradifon
164.09	107.06	17	6.55	Tetramethrin
164.09	135.07	10	6.55	Tetramethrin
264.96	92.99	20	7.20	Tolclofos-methyl
264.96	249.96	15	7.20	Tolclofos-methyl
238.09	137.05	15	6.93	Tolylfluanid
240.09	137.05	15	6.93	Tolylfluanid
208.07	111.04	25	7.28	Triadimefon
208.07	181.06	10	7.28	Triadimefon
128.05	100.04	10	8.15	Triadimenol
168.06	70.03	10	8.15	Triadimenol
161.03	105.02	13	3.82	Triazophos
257.05	162.03	10	3.82	Triazophos
161.94	160.93	8	7.69	Triciclazole
188.98	160.93	20	7.69	Triciclazole
116.04	89.03	15	8.24	Trifloxystrobin
131.04	130.04	10	8.24	Trifloxystrobin
306.10	160.05	15	5.61	Trifluralin
306.10	264.09	15	5.61	Trifluralin
145.02	87.01	10	7.43	Vamidothion
145.02	112.02	10	7.43	Vamidothion
284.97	212.00	15	6.51	Vinclozolin
286.97	214.00	15	6.51	Vinclozolin
187.01	159.01	15	7.67	Zoxamide
258.02	187.01	15	7.67	Zoxamide
187.02	159.01	15	7.30	Zoxamide-metab.
242.01	214.01	15	7.30	Zoxamide-metab.

Table 1: 467 Timed-SRM transitions used in one run for pesticide compound detection and quantitation

Sample Measurements

More than 3,500 samples were analysed in 6 months. A weekly calibration curve for each of the pesticide components in the assay and volumetric standard quantification has been performed.

Figure 1 shows the highly overlapped elution of the compounds in a single run. The SRM distribution in Figure 2 shows the typical low homogeneity of the retention time distribution of a Fast GC run. The unique acquisition mode “Timed-SRM” of the TSQ Quantum series instruments meets the optimum acquisition conditions for each compound by only monitoring the pesticides compound in a small window around the compound retention time. With a short retention time window of 18 seconds for every compound, even in the highest density elution zone, there are up to 80 SRM scans with a scan time lower than 4 ms. For the other areas of the chromatogram, scan times of up to 30 ms are resulting.

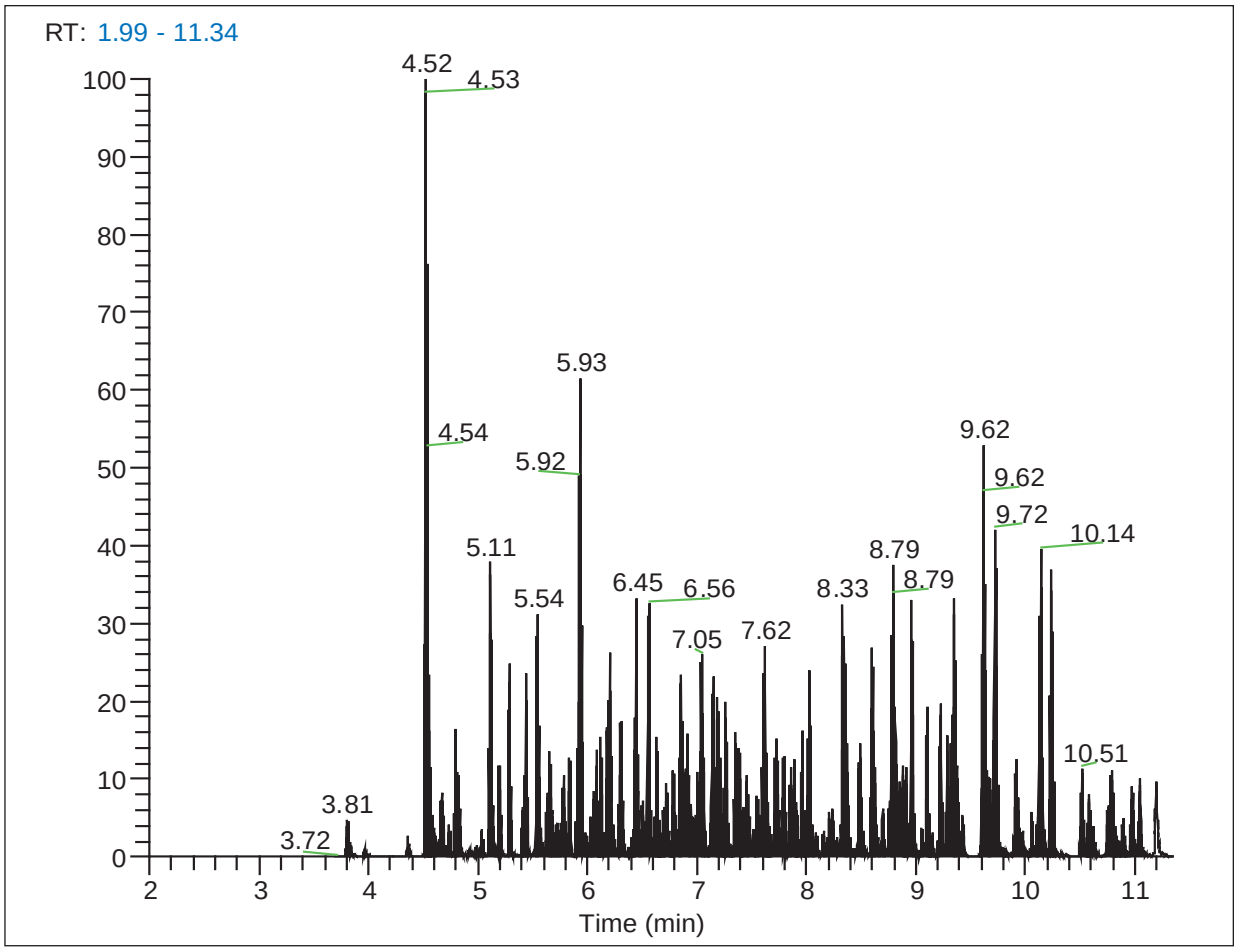

Figure 1: Highly overlapped elution of the compounds in a single run

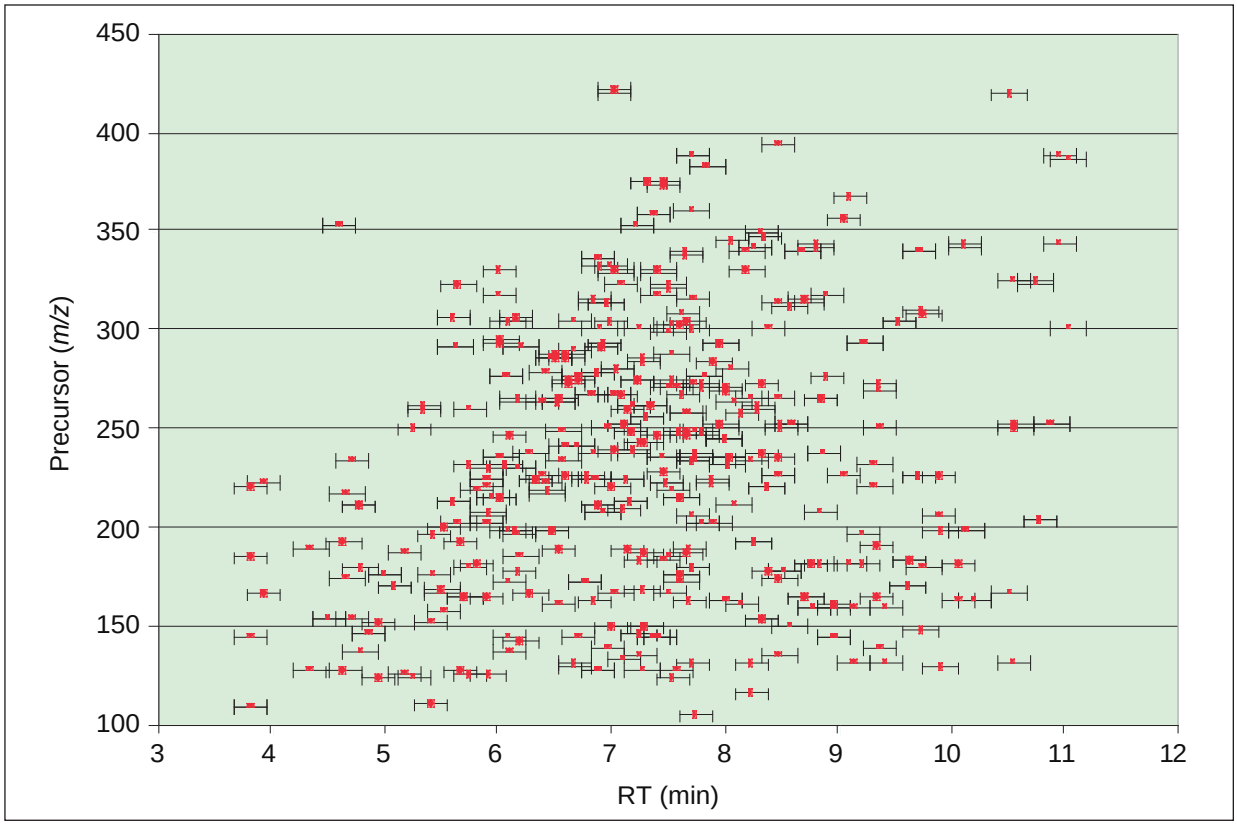

Figure 2: Timed-SRM distribution during the Fast GC chromatography

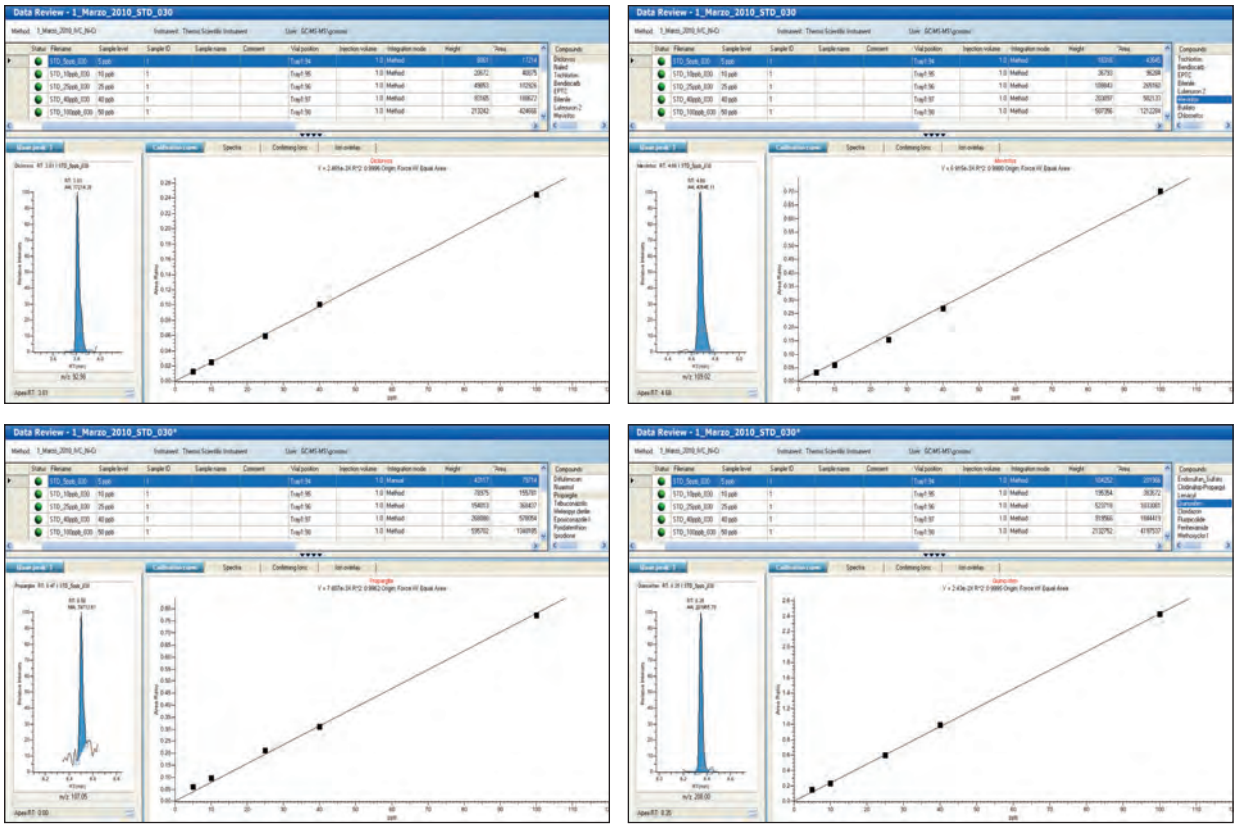

Figure 3: Calibration curve and integrated peak area of the lowest level (5 ppb) in apple matrix (Dichlorvos, Mevinphos, Propargite, Quinoxifen)

Results

Figure 3 shows the operative calibration curves and integrated peak area of the lowest calibration level (5 ppb) in apple matrix. The correlation factor of the linear calibration was always higher than 0.9950. A great sensitivity could be shown at the 5 ppb level with a S/N better than 15 for the compounds investigated.

The analysis was run at the increased mass resolution set to 0.7 Da peak width (FWHM) in Q1 and Q3. The hyperbolic rods of the mass separating quadrupoles produce a high precision quadrupolar electrical field that allow a high ion transmission coupled with increased selectivity against the unspecific matrix of the extracts. The curved square rods of the collision cell provide increased efficiency of the fragmentation especially with high ion transmission for high sensitivity. The often observed high background of neutral compounds is efficiently removed by the 90° bended collision cell and the off axis multiplier for low noise detection with high S/N values at low pesticides concentrations in these matrix samples.

For the large number of pesticide compounds in a Fast GC separation Figure 4 shows the sampling rate with the chromatographic profile of Flusilazole at the lowest calibration point of 5 ppb in apple matrix. The high statistics of sampling is the instrumental characteristics that allow the high repeatability and precision of peak integration.

With the Timed-SRM acquisition setting the two mass separating quadrupoles Q1 and Q3 increase the efficiency of sampling with only short acquisition windows around the expected compound retention time of every eluted compound. This acquisition mode is ideally suited for Fast GC separations.

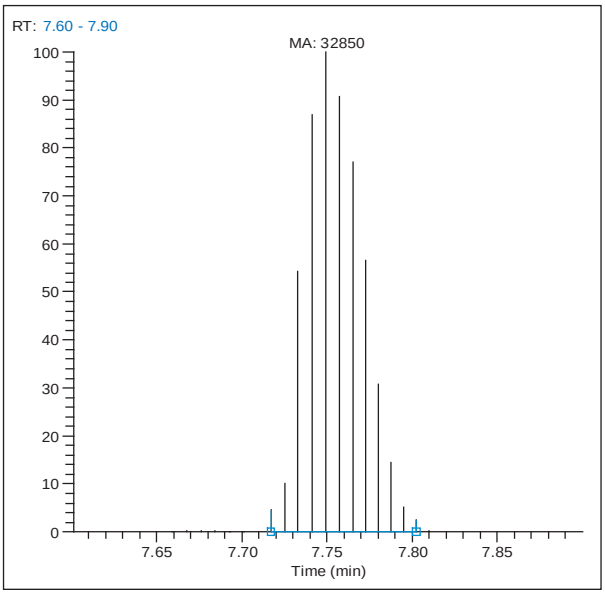

Figure 4: Flusilazole at 5 ppb in apple matrix

Figure 5 shows the superimposed chromatographic profiles of 5 repeated injections of Flucythrinate at 5 ppb in apricot matrix. The coefficients of variation (CV%) of the area integration was in the range and below of 10%. These results demonstrate the compatibility of the Fast GC solution with the TSQ Quantum GC for a true fast and reliable quantification.

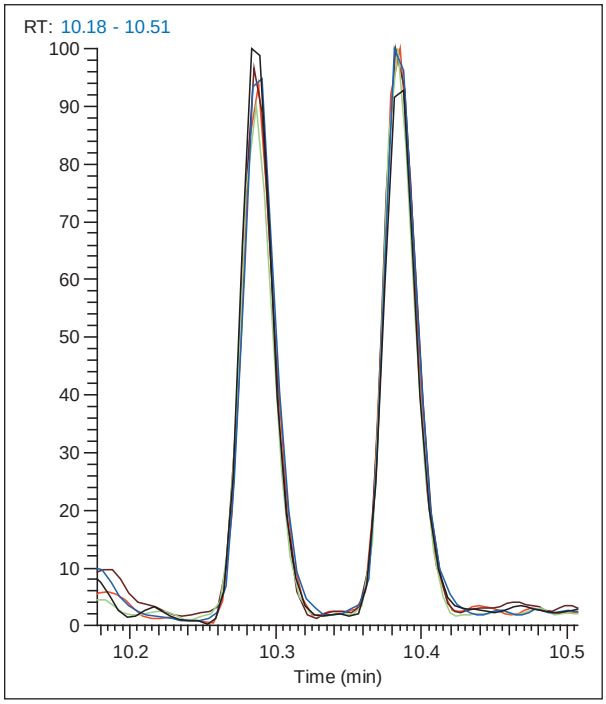

Figure 5: 5 injections of Flucythrinate at 5 ppb in apricot matrix

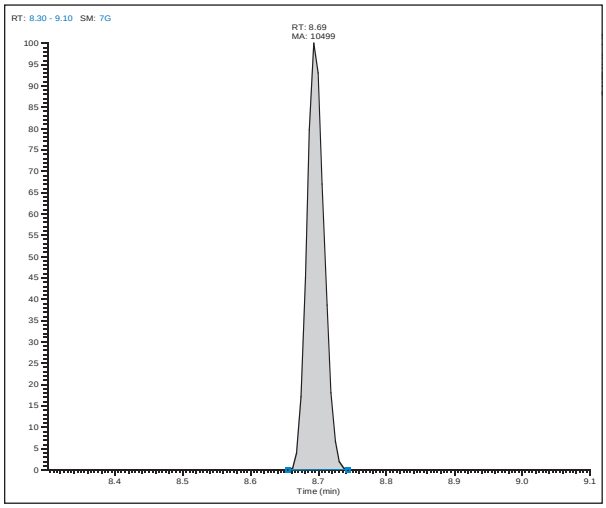

Figure 6: Iprodione at 15 ppb in onion sample

As one of the compounds with most critical chromatographic behavior, Figure 6 shows the elution profile of Iprodione at the 15 ppb level in an onion sample. A very symmetrical peak shape is associated with a very good sensitivity demonstrating the inertness and integrity of the chromatographic system from injector to transfer line and ion source. The high speed of the analysis additionally decreases the residence.

Conclusion

With the described method, a very good linearity, sensitivity and robustness have been obtained at the sensitivity levels required for being fully compatible with the reliable quantification of pesticides in vegetal matrix, with very limited breakdown phenomena and without any tailing chromatographic peaks.

The Fast GC-MS approach using the TSQ Quantum GC-MS/MS system is not only a faster method to obtain high throughput of analysis, but also the productive solution to improve the general quality of the analytical results. The Thermo Fisher TSQ Quantum GC-MS/MS system has proven to provide fast data acquisition for reliable integration of short Fast GC peaks with high selectivity and sensitivity.

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Application Note: 30163

High Resolution and Precise Mass Accuracy: A Perfect Combination for Food and Feed Analysis in Complex Matrices

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Key Words

- Exactive
- Food Analysis
- Pesticide Screening
- High Resolution MS

Overview

Purpose:
To demonstrate the analytical advantages of using high resolution (> 40,000) for the accurate screening of pesticides in complex matrices using a new benchtop Thermo Scientific Orbitrap detector.

Methods:
Use of a Ultra High Pressure Liquid Chromatography (U-HPLC) coupled with Orbitrap™ detector (Thermo Scientific Exactive) operating in high resolution mode.

Results:
The combination of high resolution (15,000 – 50,000) accurate mass is required for the detections of pesticides and mycotoxins.

Introduction

Screening of pesticides, mycotoxins and veterinary drugs is of great importance in regulated environments, such as food and animal feed analysis. Traditionally, these type of experiments have been carried out using triple quadrupole instruments. This approach has certain limitations:

- no post acquisition re-interrogation of data
- limited number of compounds per analysis
- cannot screen unidentified unknowns

Because of these limitations, there is currently a trend towards full scan MS experiments in residue analysis. Current screening approaches are performed using high performance ToF instruments, with mass accuracies of < 5ppm and resolutions of about 15,000, coupled to Ultra High Performance Liquid Chromatography (U-HPLC).

In complex sample matrices (e.g. food, feed, hair, honey) this limited resolution leads to inaccurate mass measurements caused by unresolved background matrix interferences. In this work, we show a full scan screening approach using a novel single stage Orbitrap mass spectrometer coupled to U-HPLC, capable of providing high mass accuracy at resolutions of up to 100,000.

Additionally, we will discuss two aspects of the analysis which also greatly benefit from very high resolution:

- resolving co-eluting, isobaric target compounds
- elemental composition determination

Methods

A new non-hybrid single stage Orbitrap mass spectrometer (Exactive™, Thermo Fisher Scientific, Bremen, Germany) coupled to a U-HPLC chromatograph (Thermo Scientific Accela™, Thermo Fisher Scientific, San Jose, USA) was used to evaluate a highly complex mixture of 116 pesticides, mycotoxins and plant toxins in different concentrations. A 12 min gradient was applied to a 50 x 2 mm RP C18 column (Thermo Scientific Hypersil GOLD™ 1.9 um particles, Thermo Fisher Scientific, Madison, USA) with water/acetonitrile eluents. The method developed was evaluated with respect to sensitivity, selectivity and linearity in standard solutions and extracts from animal feed. Mass measurements were performed at different resolution settings (R = 15,000 and R = 50,000) to enable comparisons to data acquired by ToF instruments and to demonstrate the advantage of ultra high resolution. Orbitrap detection was carried out using automatic control of the number of ions entering the detector (AGC, target value = 10^6).

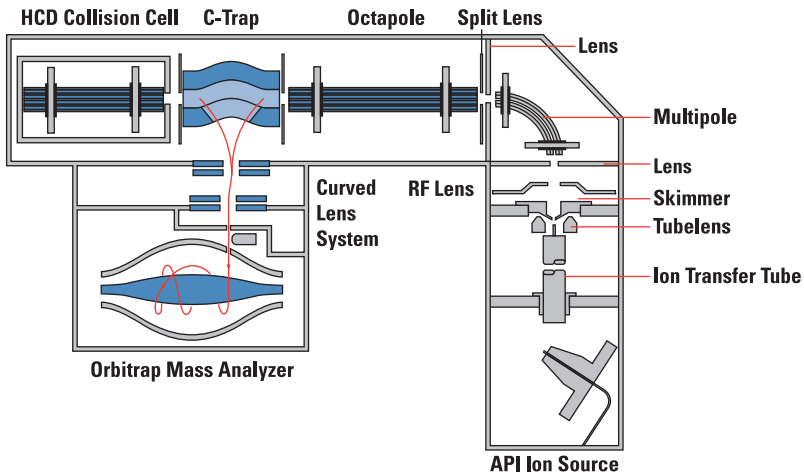

Figure 1: Schematic of the new Orbitrap benchtop mass spectrometer, including HCD collision cell for "All Ion Fragmentation".

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Results

Resolution of Isobaric Pesticides

In cases where isobaric compounds co-elute, erroneous mass accuracy and elemental composition assignment will occur if the resolving power of the mass spectrometer is insufficient to separate these compounds. Figure 2 shows two pesticides Thiamethoxam ($C_8H_{10}ClN_5O_3S$) and Parathion ($C_{10}H_{14}NO_5PS$), which have protonated molecular ions (MH^+) at 292.02656 and 292.04031, respectively. A resolution higher than 40,000 is needed to resolve the protontated molecular ion of these two compounds completely. This is a pre-requisite for analysis of low concentration compounds in the presence of higher abundant ones. The example in Figure 2 shows an approximate 1:3 mixture of both pesticides measured and simulated.

Influence on Elemental Composition Determination

A limited resolution of 15,000 results in two major limitations. First, the detection of unresolved doublets may result in significant mass errors, which are outside the characteristic accuracy specification of the Exactive instrument. As a consequence, at lower resolution settings, the mass windows for elemental composition determination have to be increased, resulting in much larger number of elemental composition proposals for the unknown or targeted compounds. This can be seen for the example (Figure 3) of Pirimicarb at *m/z* 239.1503. Due to the presense of an isobaric interference, the peak at 239 shows a mass error of 6.5 ppm. At a resolution of 15,000, the underlying interference causes an apparent shift to higher mass, whereas at higher resolution (here 80,000), the doublet is clearly resolved, and the mass accuracy is well within instrument specifications.

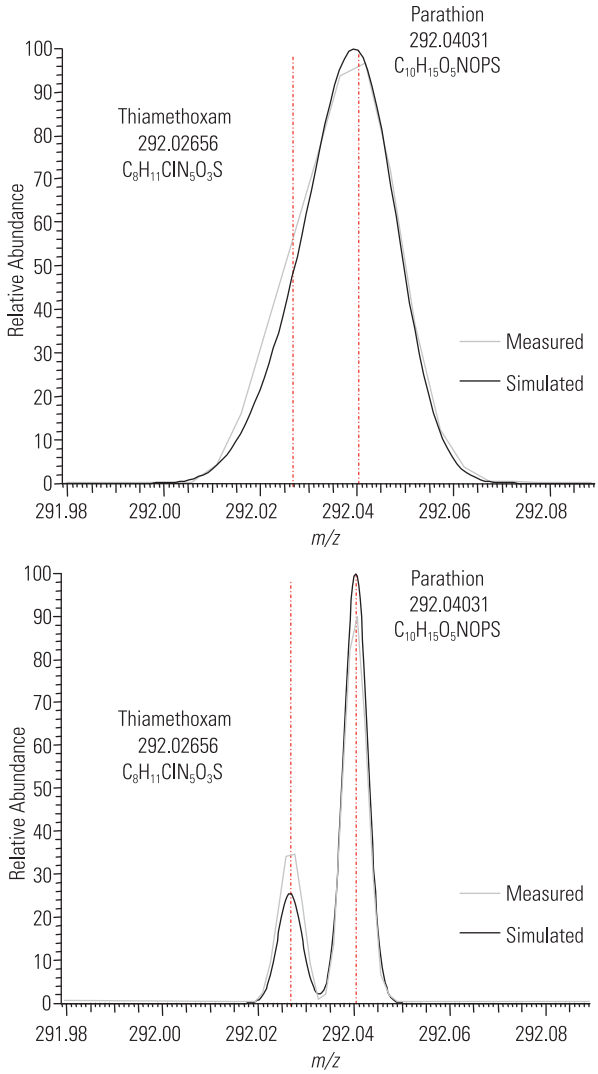

Figure 2: Mass chromatogram of two isobaric pesticides measured at a resolution of 15,000 (left) and 50,000 (right). Superimposed is the simulated mass trace at each resolution setting.

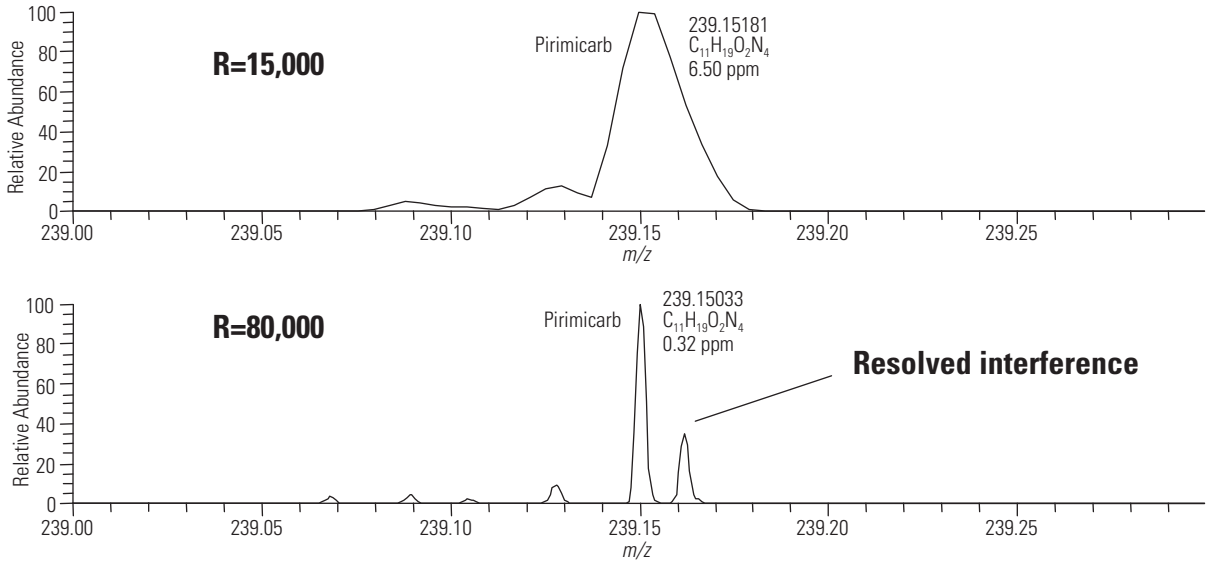

Figure 3: Improved mass accuracy simply by increasing the resolution in order to resolve the doublet. The peak at 15,000 resolution splits up into 2 by increasing the resolution to 80,000.

In order to limit the number of candidate elemental compositions to a single confident assignment, a sophisticated software algorithm is used. It takes into account the peak height and mass accuracy of the monoisotopic peak and its isotopes. However, in order to function correctly, all of the accurate mass values for the isotopic peaks must be within specified limits. The absence of interference peaks can only be assured by use of high resolution, (as can be seen in Figure 4). Here the fungicide, Azoxystrobin, is shown at resolutions of 15,000 and 80,000. The medium resolution spectrum shows very good mass accuracy for the monoisotopic peak, but gives unusually high mass errors for the A+1 and A+2 ions. This is due to an interference at *m/z* 405.1452, which cannot be resolved at medium resolution. Whereas, the high resolution spectrum shows exellent mass accuracy for all three measured isotopes. Determining elemental compositions using data acquired at ~15,000 resolution will result in misleading or incorrect data. Only sufficient high resolution allows the determination of the accurate mass of the complete molecular ion cluster, and therefore allows automated assignment of an elemental formula with a high degree of confidence.

Analyzing highly complex samples such as extracts from food or animal feed, and the screening of regulated substances including pesticides, mycotoxins and veterinary drugs is a major analytical challenge for mass spectrometry. On one hand, the methodology must have a high intra scan dynamic range in order to detect low concentrated compounds in presence of high abundant matrix ions, on the other hand high selectivity and high sensitivity is needed to avoid false positive, or even worse, false negative results. In our procedure, we analyzed an extract from horse feed as an example of extremely complex matrix, spiked with a mixture of 116 pesticides and mycotoxins. A dilution series ranging from 2 to 250 ppb (for each compound) was measured in duplicates at two different resolution settings. In addition, a 100 ppb sample of the same mixture was analyzed at a resolution of 50,000 in order to determine the maximum number of detectable substances for this method.

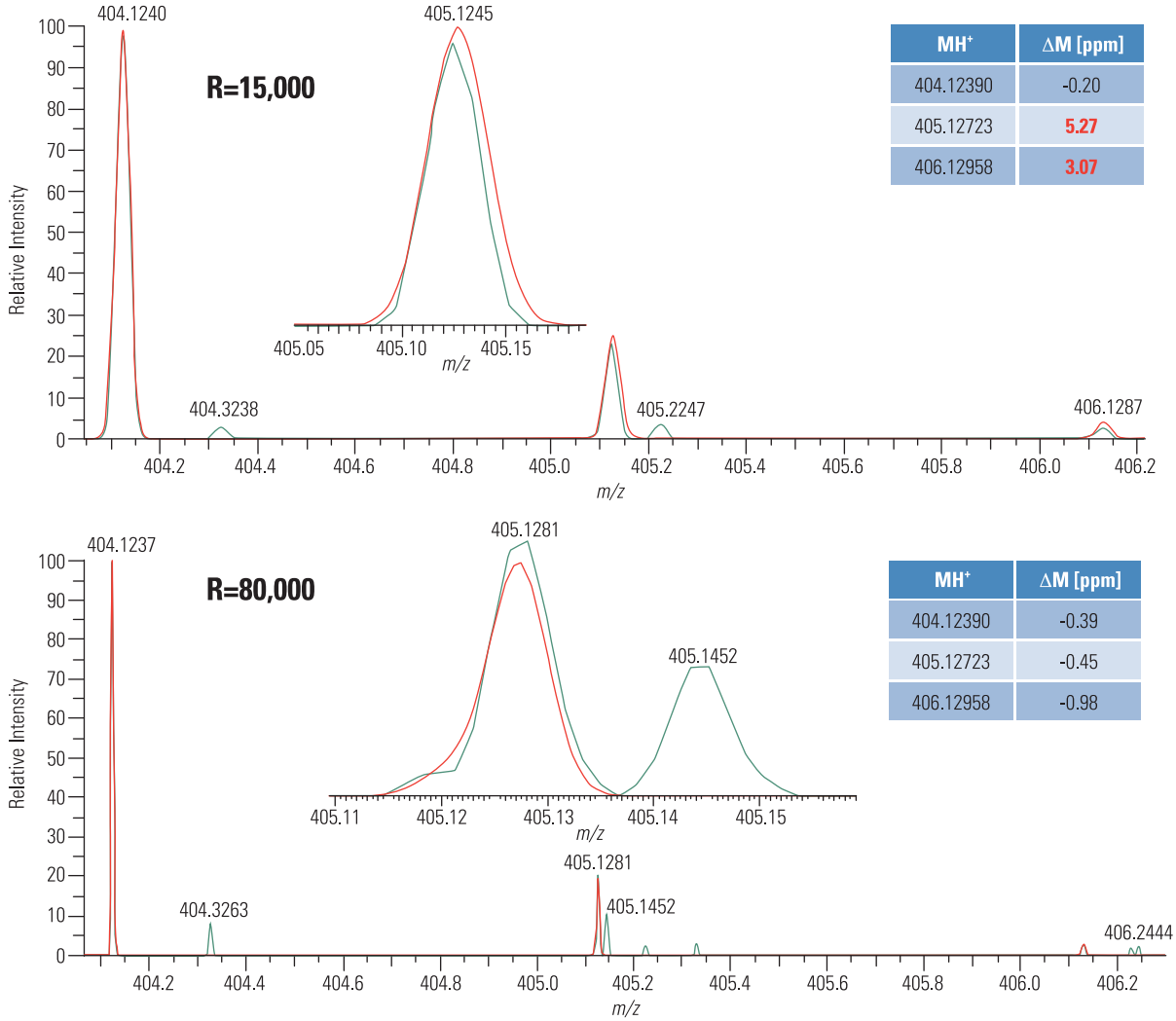

Figure 4: The importance of resolution for the molecular ion AND its isotopes. At high resolution the complete molecular ion cluster is correctly detected.

LC-MS analysis of the extracted spiked samples showed the presence of 95 out of 116 compounds at 100 ppb in matrix. Figure 5 shows the overlaid ion chromatograms for all 116 compounds (3 ppm window) at 50 ppb (in matrix). The number of recovered pesticides in different concentrations is shown in Figure 5. The data illustrates that a greater number of detected compounds (higher sensitivity) with an extraction window of 3 ppm at higher resolution setting. This is exemplified in Figure 6, where extracted ion chromatograms of Sulcotrion at 50 ppb for R = 15,000 and R = 50,000 are shown. The higher resolved spectrum displays two peaks, of which the smaller one is Sulcotrion.

The lower resolved peak masks the pesticide signal completely. The only indication for the presence of Sulcotrion is a slightly broader peak or shoulder and a mass shift of the interfering ion towards higher masses. This would lead to a false negative result, if the analysis was only performed at a resolution of 15,000, and is the major reason why the number of identified components in the case of R = 15,000 decreases disproportionately to the measurements at higher resolving power (diagram, Figure 5).

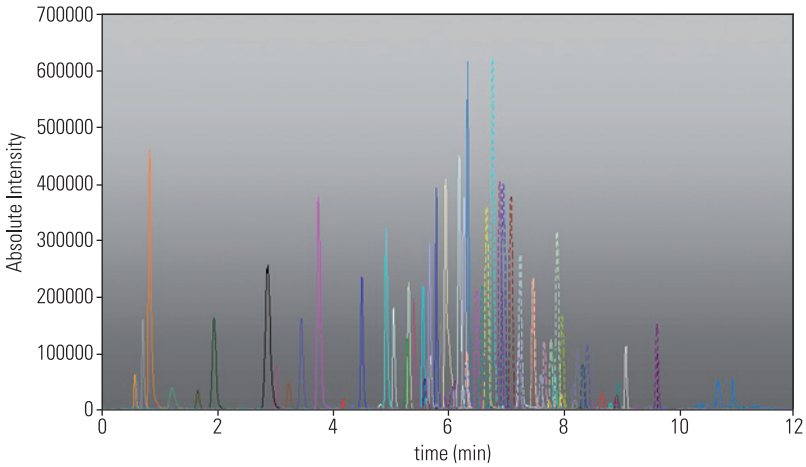

Figure 5: Overlaid extracted ion chromatograms from a mixture of 116 pesticides and mycotoxins at a 100 ppb level. Extraction was done with 3 ppm mass window. The inset chart shows the number of detected compounds at different concentrations (in matrix) at two different resolution settings.

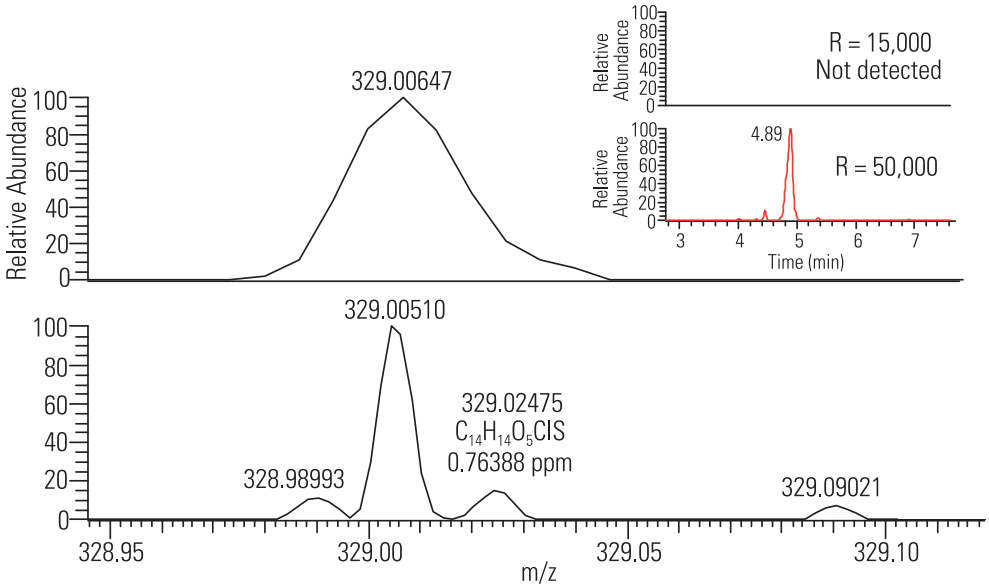

Figure 6: Expanded view of the pesticide mixture at different resolution settings (top: 15,000 and bottom: 50,000). Pesticide Sulcotrion (*m/z* 328.02475) is masked under background ions at a resolution of 15,000 but is easily detected at 50,000 resolution (see also mass chromatogram inset).

For this reason, a dilution series was measured at higher resolution settings. One example (Metabenthiazuron) is shown in Figure 7. It demonstrates excellent linearity and sensitivity down to 2 ppb level (2 ng/mL).

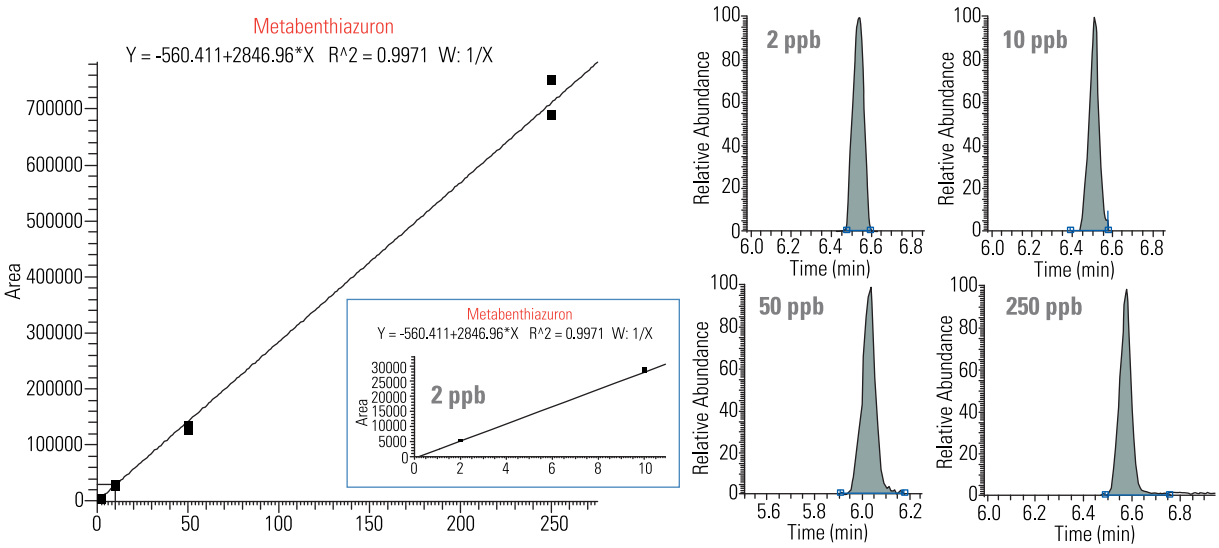

Figure 7: Quantitation curve for Metabenthiazuron ranging from 2 to 250 ppb. The quantified peak for each concentration level demonstrates the high quality data even at the lowest level.

Conclusions

- New benchtop Orbitrap mass spectrometer demonstrates superior mass resolving power compared to that obtained using TOF instruments.
- High resolving power (up to 100,000) provides precise mass accuracy for complex sample analysis.
- High resolving power provides excellent sensitivity, linearity and selectivity in multi-residue screening of complex matrices.
- Fast scan speeds (10 Hz) are fully compatible with the use of U-HPLC fast chromatography methods.

For the analysis of very complex samples, it is advantageous to select the appropriate scan speed and resolution in order to avoid unresolved isobaric compounds (matrix ions from analyte ions) and still allow unambiguous detection of low abundant species.

Acknowledgements

We would like to thank Paul Zomer and Hans Mol from the RIKILT Institute for Food Safety in Wageningen, The Netherlands, for providing the samples.

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Analysis of Early Eluting Pesticides in a C18-Type Column Using a Divert Valve and LC-MS/MS

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Application Note 572

Key Words
TSQ Quantum Access MAX, Divert Valve, Split Peaks, Reversed-Phase Liquid Chromatography, Pesticides

Goal
To demonstrate the ability to override the solvent effects from a sample extract using gradient solvents with liquid chromatography. Additionally, to increase injection volume without overloading the column.

Introduction
Many pesticide analyses are based on the QuEChERS extraction method, which uses acetonitrile (ACN) in the final extraction step. However, injecting a solvent stronger than the HPLC mobile phase can cause peak shape problems, such as peak splitting or broadening, especially for the early eluting analytes (low capacity factor, k). The common practice is to exchange the solvent of the final extraction step for one similar to the mobile phase, for example methanol / water, but this procedure is laborious and can lead to analyte losses.

There are several possible causes of peak splitting or broadening. This study presents the peak shape differences between acetonitrile and methanol / water [1:1 v/v] solutions due to the interaction of gradient and sample solvent, as indicated in Figure 1. The lowest detection limit is achieved when an analyte is in as compact a band as possible within the flow stream of mobile phase and with larger injection volumes. However, this is limited by maximum loop volume and column capacity.

Mobile phase composition and the use of a divert valve have been evaluated for the analysis of seven selected pesticides in acetonitrile solutions (Table 1). The sample solutions were chosen to represent both low and high analyte levels for compounds that elute either early or middle-early from a C18 column. Performance was evaluated in terms of linearity (injection volume range 1–8 µL), robustness (RSD), and sensitivity as measured by signal-to-noise ratio (S/N) and peak area reproducibility.

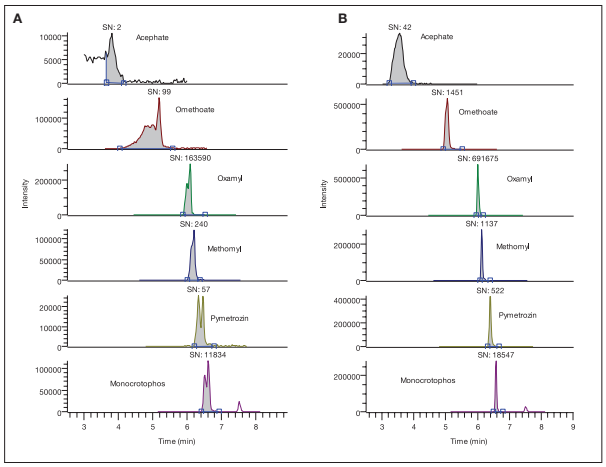

Figure 1. Chromatograms of 5 µL injections of acephate, omethoate, oxamyl, methomyl, pymetrozin, and monocrotophos in 50 µg/L acetonitrile (A) and methanol / water [1:1 v/v] solution (B), with no divert valve used

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Table 1. List of studied pesticides and their physicochemical properties

Name	Pesticide Class	Chemical Formula	Water Solubility [mg/L] / pKow	Vapor Pressure [Pa]	Molecular Weight [g/mol]
Acephate	Organophosphorous	$C_4H_{10}NO_3PS$	790,000 / -0.85	2.26 x 10^{-4} (24 °C)	183.165862
Aldicarb sulfone	Oxime carbamate	$C_7H_{14}N_2O_4S$	10,000 (25 °C) / -0.57 (calculated)	0.012 (25 °C)	222.26206
Metamitron	Triazinone	$C_{10}H_{10}N_4O$	1770 (25 °C; pH 5) / 0.85 (21 °C, not pH dependent)	7.44 x 10^{-7} (25 °C)	202.2126
Methomyl	Oxime carbamate	$C_5H_{10}N_2O_2S$	55,000 (25 °C, pH 7) / 0.09 (25 °C, pH 4-10)	7.2 x 10^{-4} (25 °C)	162.210100
Monocrotophos	Organophosphorous	$C_7H_{14}NO_5P$	water miscible	2.9 x 10^{-4} (20 °C)	223.163522
Omethoate	Organophosphorous	$C_5H_{12}NO_4PS$	water-miscible / -0.74 (20 °C)	3.3 x 10^{-3} (20 °C)	213.191842
Oxamyl	Oxime carbamate	$C_7H_{13}N_3O_3S$	148,100 (20 °C, pH 5) / -0.44 (25 °C, pH 5)	5.12 x 10^{-5} (25 °C)	219.26142

Experimental Conditions

Sample Preparation

Individual stock solutions of pesticides were prepared at concentrations that were sufficient to evaluate the linearity of peak area versus injection volume at the same concentration e.g. 10 µg/L, but different injection volumes (e.g. 1, 2, 3, 4, 5, 6, 7 µL, etc.). Additional solutions with different concentrations (5, 10, 25, 50, 70, 100, 200 µg/L) were prepared to study the linearity of peak area versus compound concentration. Finally, solutions with different solvents (acetonitrile or methanol / water [1:1 v/v]) were prepared to study the solvent effect on the methanol / water gradient mobile phase during the injection.

HPLC

HPLC analysis was performed using a Thermo Scientific Accela UHPLC system. The chromatographic conditions were as follows:

HPLC Column	Thermo Scientific Hypersil GOLD, 100 mm x 2.1 mm, 1.9 µm particle size
Trap Column	Hypersil™ GOLD, 10 mm x 2.1 mm, 5 µm particle size
Column Temperature	40 °C
Mobile Phase A	Water with ammonium formate (5 mM) and formic acid (2 mM)
Mobile Phase B	Methanol with ammonium formate (5 mM) and formic acid (2 mM)

The trap column was used to trap the analytes, while the divert valve was switched to the waste position. A tee union between the trap column and the analytical column was connected to the divert valve. The two positions of the divert valve are shown in Figure 2.

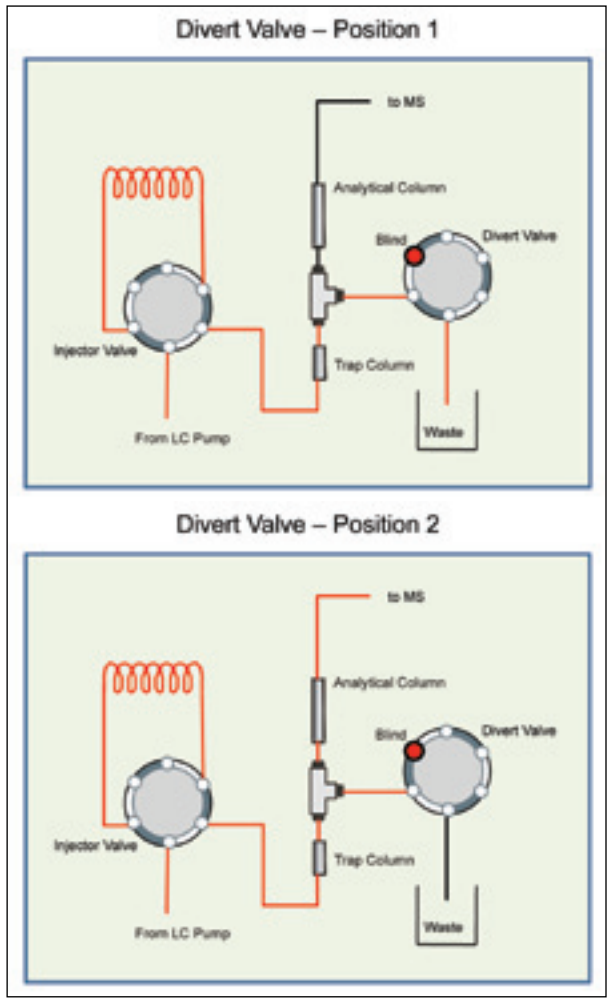

Figure 2. Divert valve positions

The gradient used is detailed in Table 2. The duration of the gradient was 21 minutes and the column equilibration time was 10 minutes. The flow rate increased at 21.10 min and decreased at 25.10 min to increase the speed of column equilibration for the next run (larger column volumes in less time). The maximum backpressure was 9,500 psi.

Table 2. HPLC Gradient. Mobile phase A is water with ammonium formate (5 mM) and formic acid (2 mM), and mobile phase B is methanol with ammonium formate (5 mM) and formic acid (2 mM).

No.	Time	A%	B%	µL/min
0	0.00	90.0	10.0	450.0
1	2.40	90.0	10.0	450.0
2	7.00	40.0	60.0	450.0
3	14.00	10.0	90.0	450.0
4	21.00	10.0	90.0	450.0
5	21.10	90.0	10.0	560.0
6	25.00	90.0	10.0	560.0
7	25.10	90.0	10.0	450.0
8	31.00	90.0	10.0	450.0

Mass Spectrometry

MS analysis was carried out on a Thermo Scientific TSQ Quantum Access MAX triple stage quadrupole mass spectrometer with an electrospray ionization (ESI) probe. The MS conditions were as follows:

Ion polarity	Positive
Q1 Resolution	0.7 Da
Spray Voltage	4000 V
Sheath/Auxiliary Gas	Nitrogen
Sheath Gas Pressure	40 (arbitrary units)
Auxiliary Gas Pressure	25 (arbitrary units)
Ion Transfer Tube Temperature	325 °C
Scan Type	Selected-Reaction Monitoring (SRM)
Collision Gas	Argon
Collision Gas Pressure	1.5 mTorr
Divert Valve	Rheodyne® model 7750E-185

The divert valve was connected to the front of the TSQ Quantum Access MAX™ and was fully controlled from the data system software.

Results and Discussion

The comparison of peak shapes between the acetonitrile and methanol / water sample solutions demonstrated that only early eluting analytes were altered by the mobile phase composition (Figure 3). Without the divert valve, the peak shape of omethoate, which elutes earlier than methomyl, was unacceptable in acetonitrile solution; whereas the peak shape of methomyl was better but not optimum (Figure 3a). The peak shape of metamitron, which elutes later than methomyl, was good in both acetonitrile and methanol / water sample solutions (Figures 3a, 3b). With the divert valve switched to the waste position for 1.30 minutes in the beginning of the run, the peak shapes of both omethoate and methomyl resembled those in the methanol / water sample solutions (Figure 3c).

The amount of time the valve was in the waste position affected the combination of peak shape and S/N ratio. As shown in Figure 4, the optimum combination of peak shape and RMS S/N ratio was achieved with a divert valve time of 1.30 minutes. Longer duration times were avoided, since the column equilibration was disturbed.

Figure 5 shows the range of injection volumes used. To assess the dependence between each compound peak area and the corresponding injection volume, eight injection volumes (1–8 µL) at a level of 10 µg/L were run three times each. The linear correlation coefficients (R^2 values) of the curve plots for all analytes studied were >0.99, and relative standard deviations were <20% (range 1%–14%). A S/N ratio greater than 10 for acephate and omethoate could not be achieved for injection volumes of 1 µL and 2 µL.

Figure 6 shows the curve of each compound's peak area versus concentration for a 5 µL injection volume. Seven different concentration levels (5, 10, 25, 50, 70, 100, 200 µg/L) with 5 µL injection volumes were run three times. The linear correlation coefficients (R^2 values) of the curve plots for all analytes studied were >0.99 and relative standard deviations were <20% (range 2%–16%). Using 5 µL injections of 5 µg/L acetonitrile solutions, RMS S/N ranged between 75 and 263,000.

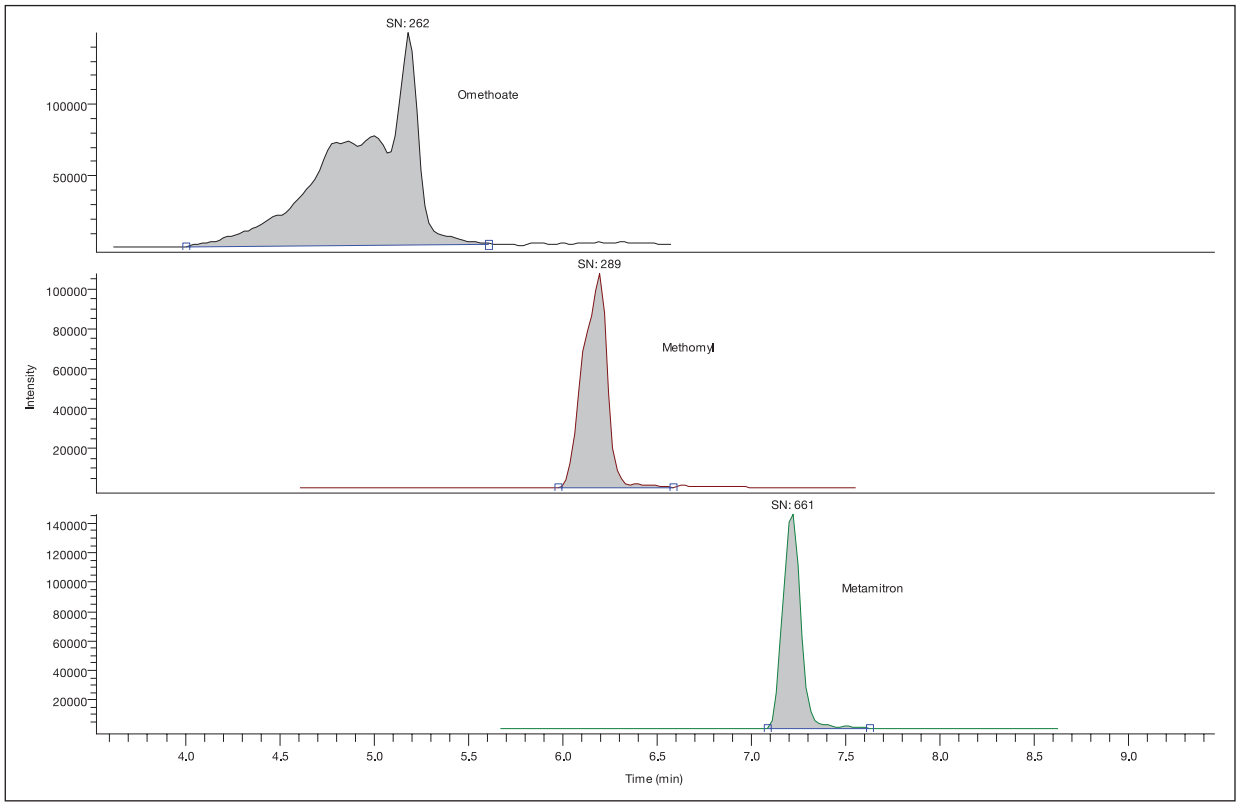

Figure 3a. Extracted chromatograms of 50 µg/L omethoate, methomyl, and metamitron in acetonitrile solution with no divert valve

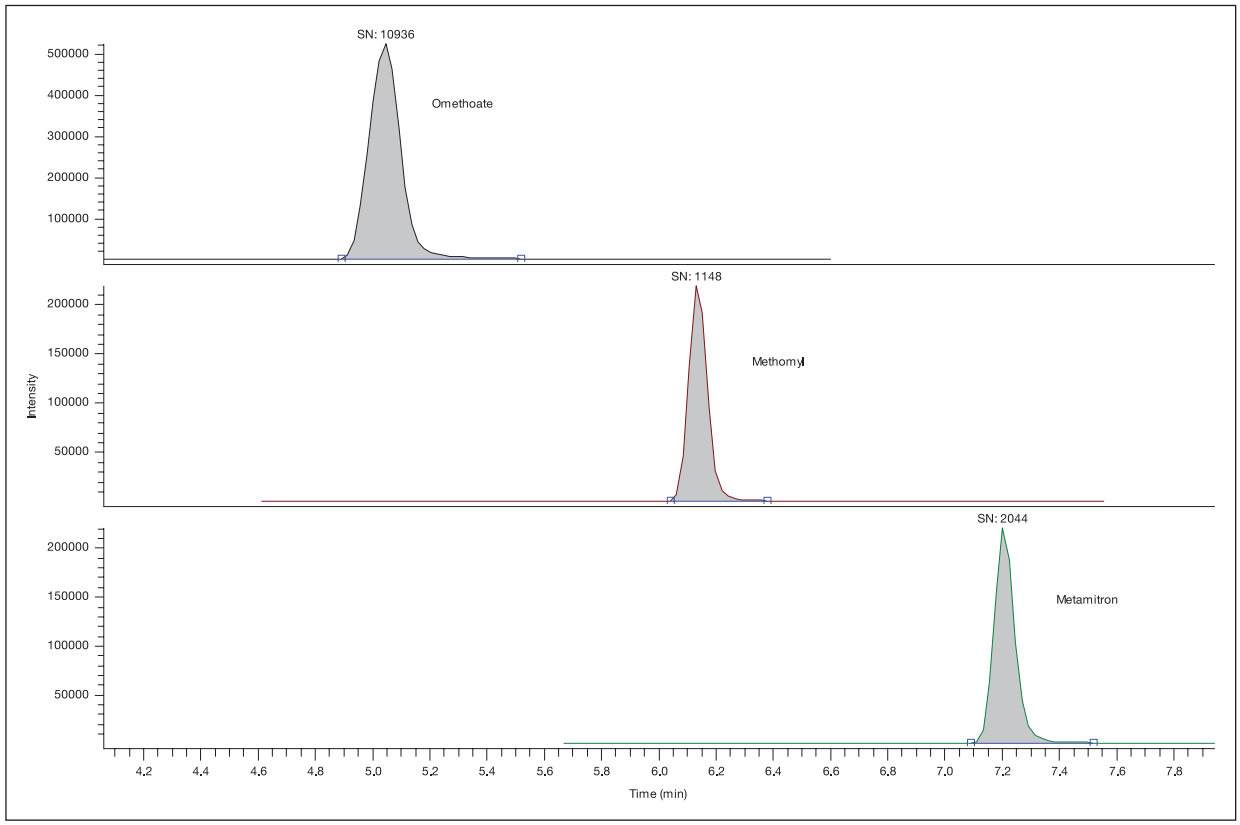

Figure 3b. Extracted chromatograms of 50 µg/L omethoate, methomyl, and metamitron in methanol / water [1:1 v/v] solution with no divert valve

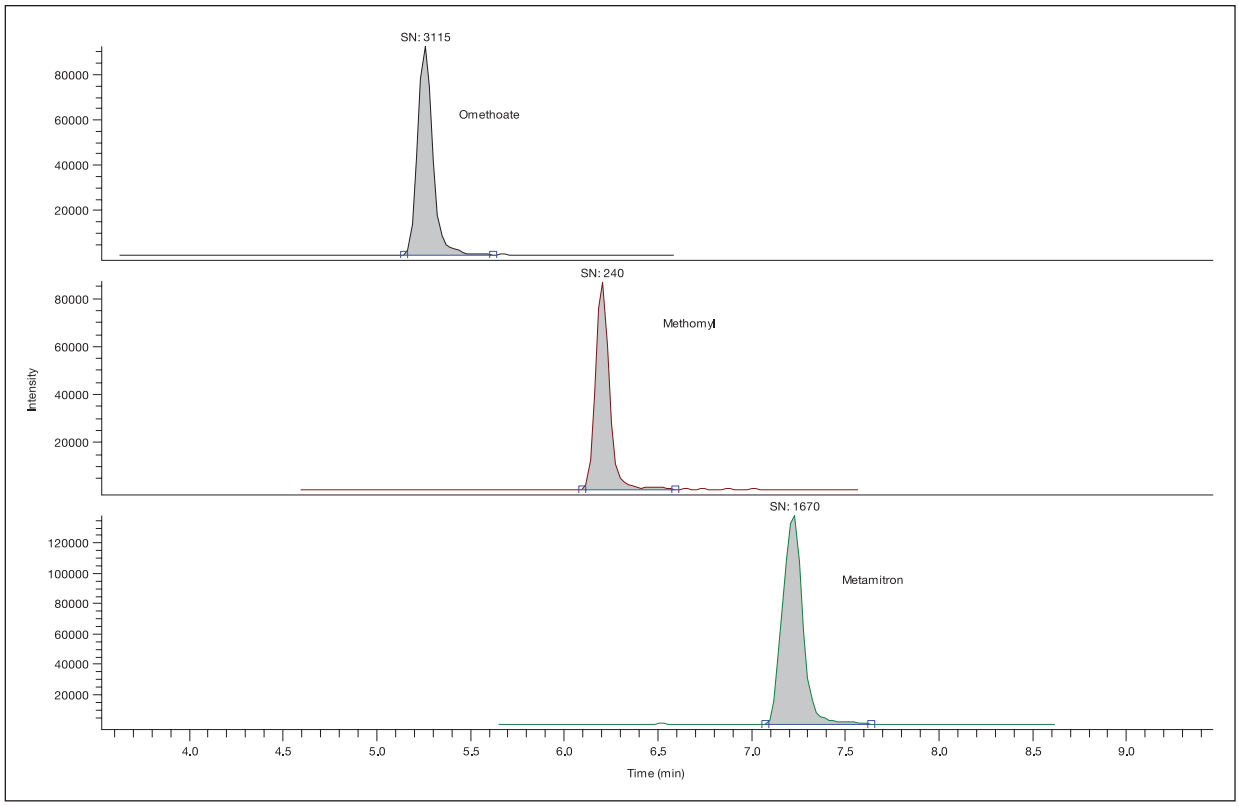

Figure 3c. Extracted chromatograms of 50 µg/L omethoate, methomyl, and metamitron in acetonitrile solution with divert valve open for 1.30 minutes

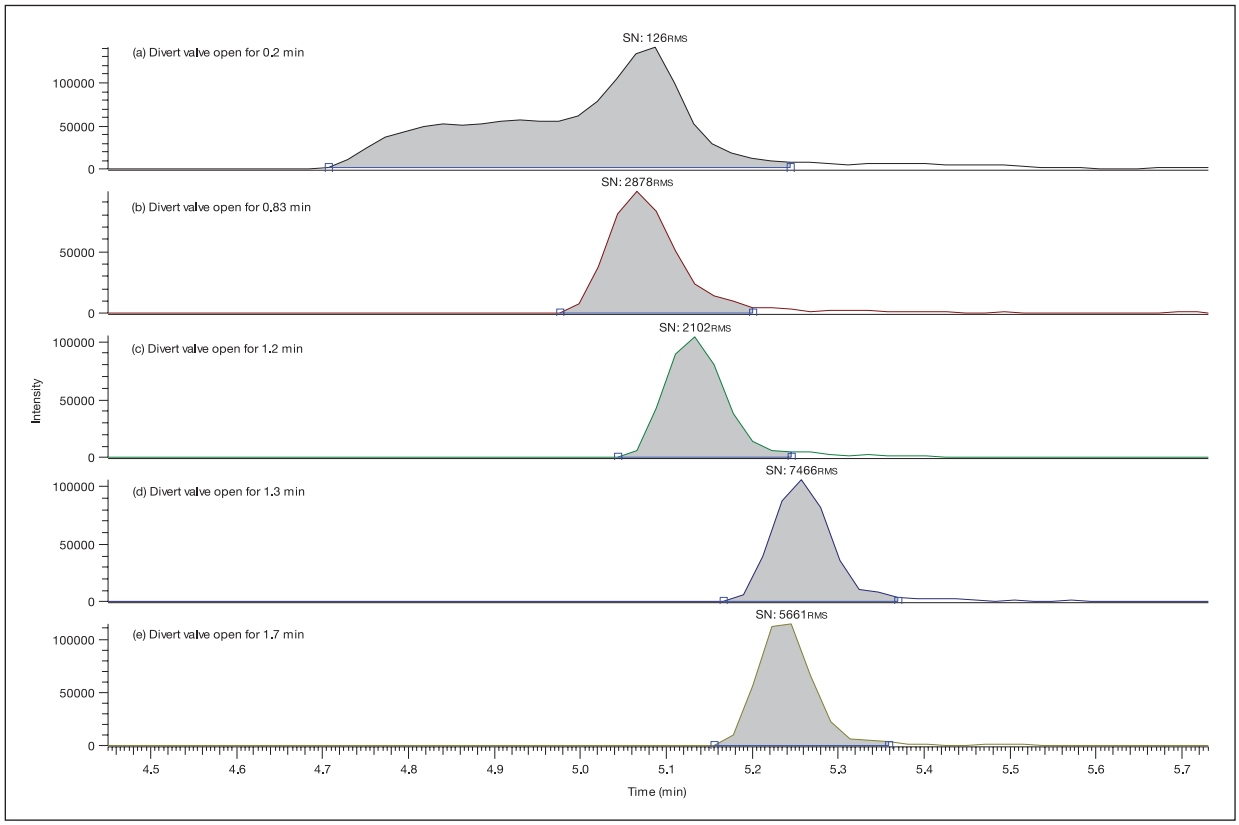

Figure 4. Extracted chromatograms of 5 µL injections of omethoate in 50 µg/L acetonitrile solution with various divert valve duration times used

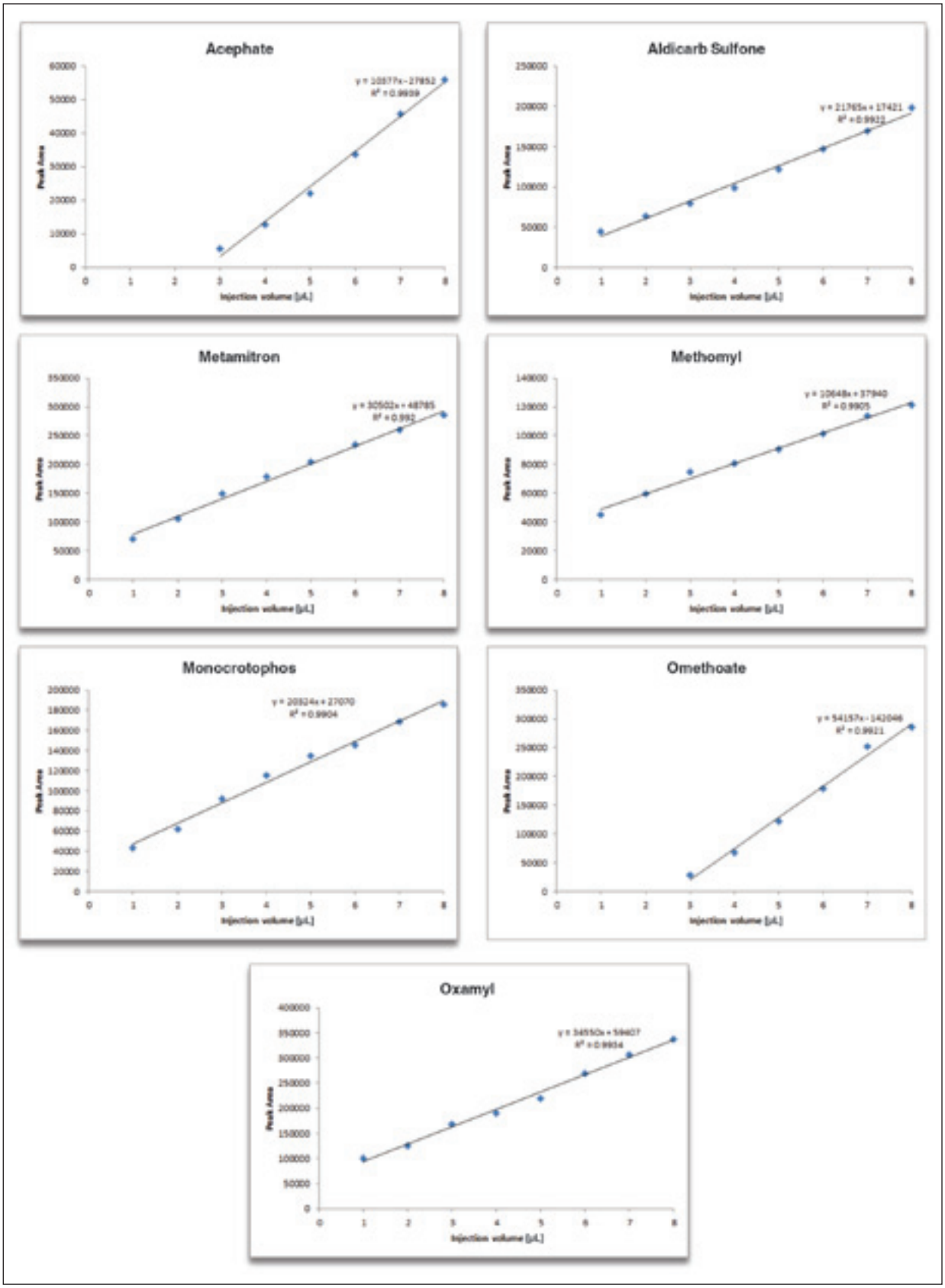

Figure 5. Curves for analyte peak area versus injection volumes 1-8 µL in 10 µg/L acetonitrile solution

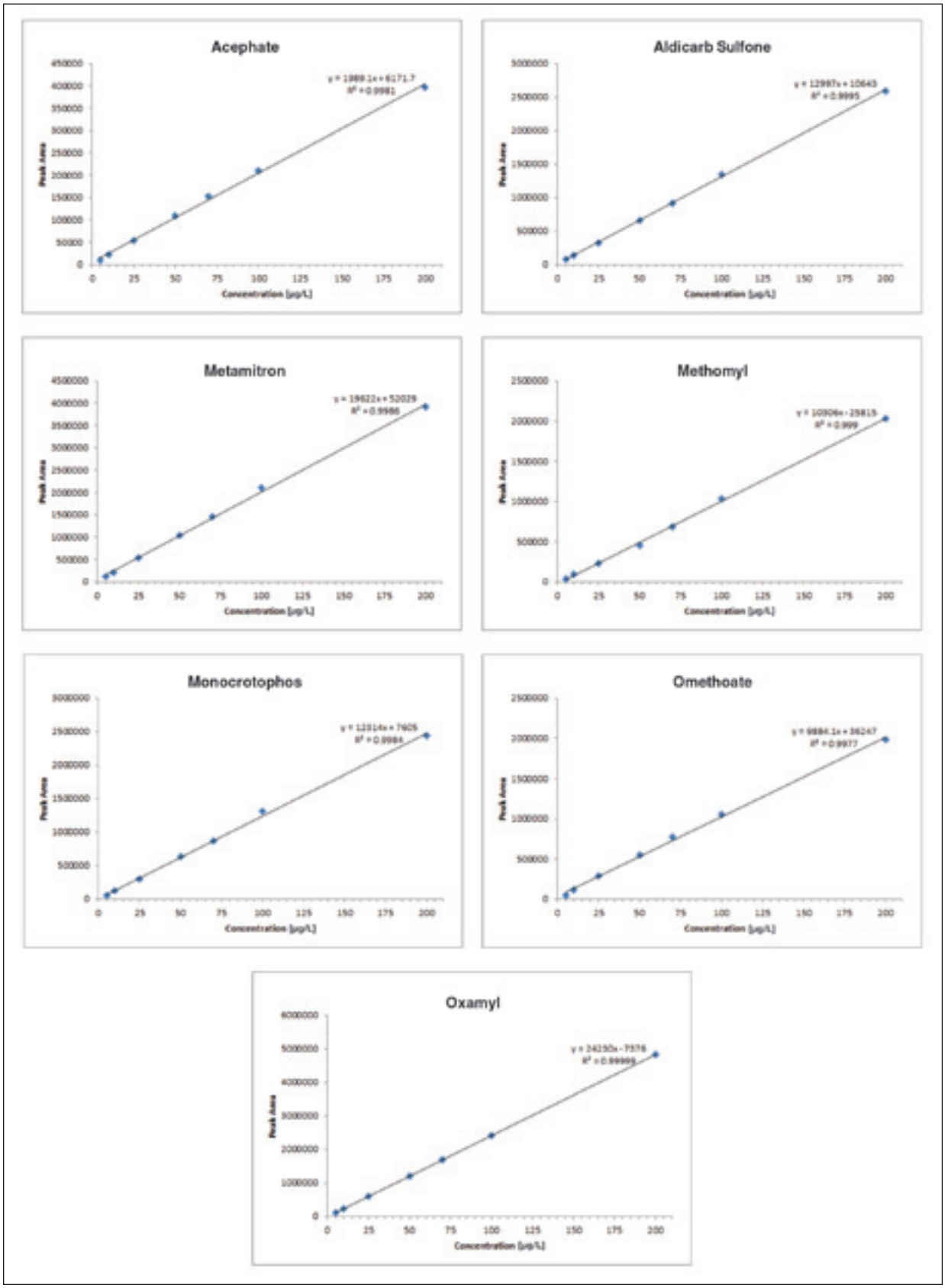

Figure 6. Curves for analyte peak area versus concentration 5-200 µg/L acetonitrile solution with 5 µL injection volume

Conclusion

The use of a divert valve proved suitable for the analysis of early eluting pesticides in acetonitrile solutions. Good peak shapes and S/N ratios were achieved and chromatographic problems, such as peak splitting or broadening, were overcome. In addition, the injection volume was increased up to 8 µL, reaching low detection limits with good linearity and repeatability, even for a sample concentration of 5 µg/L. It may be possible to increase the injection volume to 10 µL, and in some cases up to 15 µL, but with a larger loop volume. After the initial experiments, we concluded that a 5 µL injection volume is sufficient to achieve RMS S/N ratio greater than 10.

This technique resolves chromatographic issues involving interactions of gradient and sample solvent in a simple way and offers an increased laboratory sample capacity by avoiding solvent exchange in the final extract.

Reference

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Application Note 573

Qualitative and Quantitative Analysis of Pesticides in Horse Feed Matrix Using Orbitrap MS

Olaf Scheibner and Maciej Bromirski, Thermo Fisher Scientific, Bremen, Germany

Key Words

Exactive Plus, UHPLC, high resolution, accurate mass, high throughput, Orbitrap, Data-Dependent All-Ion Fragmentation, ExactFinder

Goal

To test the ability of a high-resolution, accurate-mass benchtop Orbitrap™ mass spectrometer to achieve high sensitivity and selectivity when analyzing modern, very-short-gradient UPHLC separations of complex samples.

Introduction

Productivity of a liquid chromatograph-mass spectrometer (LC-MS) system is measured in samples per day. To achieve higher productivity, modern ultra-high-performance LC-MS (UHPLC-MS) methods use very short gradients. Chromatographic peak widths are often below 5 seconds at the base. A high-resolution, accurate-mass (HR/AM) mass spectrometer operating in full-scan mode must be able to provide a sufficient number of scans (≥10) across the chromatographic peak without compromising sensitivity and selectivity. As reported earlier, a resolving power in excess of 50,000 (FWHM at *m/z* 200) combined with a mass extraction window of 5 ppm is necessary to ensure selectivity comparable to established MS/MS techniques.[1]

The Thermo Scientific™ Exactive™ Plus Orbitrap mass spectrometer (Figure 1) is the second generation of the Exactive product family. It features two major changes over the first generation instrument. First, in the ion optics the tube-lens / skimmer assembly has been replaced by an S-Lens (Figure 2) that provides significantly higher ion transmission, increasing the instrument's sensitivity. Second, the Orbitrap mass analyzer and related electronics have been improved,[2] resulting in higher scan speed and resolution, as well as improved polarity switching. As a result, the range of resolving power is from 17,500 to 140,000 at *m/z* 200, with a maximum scan rate of 12 Hz.

In this research, the Exactive Plus instrument was used to analyze extracts of horse feed spiked with common pesticides.

Figure 1. Exactive Plus mass spectrometer with Accela 1250 UHPLC

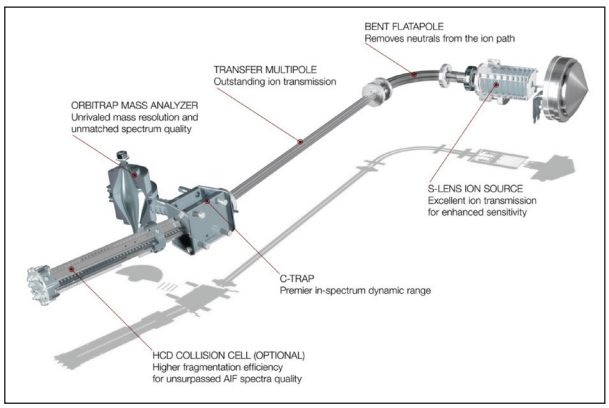

Figure 2. Exactive Plus ion optics and mass analyzer components

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Experimental

Sample Preparation

QuEChERS extracts of horse feed were spiked with 85 common pesticides (Table 1) at levels of 10 and 100 ppb, and diluted 1:1 with acetonitrile. Six calibration standards with the 85 pesticides in acetonitrile were mixed 1:1 with horse feed matrix that, through previous analysis, was proven to be free of pesticides. The final calibration levels were 5, 10, 25, 50, 100, and 150 ppb (5–150 µg/kg).

Table 1. Pesticides spiked into QuEChERS extracts

Pesticide	Chemical Formula
Acephate	$C_4H_{10}NO_3PS$
Acetamiprid	$C_{10}H_{11}ClN_4$
Aldicarb	$C_7H_{14}N_2O_2S$
Aldicarb-sulfone	$C_7H_{14}N_2O_4S$
Azinphos-ethyl	$C_{12}H_{16}N_3O_3PS_2$
Azinphos-methyl	$C_{10}H_{12}N_3O_3PS_2$
Azoxystrobin	$C_{22}H_{17}N_3O_5$
Bromacil	$C_9H_{13}BrN_2O_2$
Bromuconazole	$C_{13}H_{12}BrCl_2N_3O$
Carbaryl	$C_{12}H_{11}NO_2$
Carbendazim	$C_9H_9N_3O_2$
Carbofuran	$C_{12}H_{15}NO_3$
Carbofuran-3-hydroxy	$C_{12}H_{15}NO_4$
Chlorfluazuron	$C_{20}H_9Cl_3F_5N_3O_3$
Clofentezine	$C_{14}H_8Cl_2N_4$
Cymiazole	$C_{12}H_{14}N_2S$
Cymoxanil	$C_7H_{10}N_4O_3$
Cyproconazole	$C_{15}H_{18}ClN_3O$
Cyromazine	$C_6H_{10}N_6$
Demeton-S-methyl-sulfone	$C_6H_{15}O_5PS_2$
Dichlorvos	$C_4H_7Cl_2O_4P$
Diethofencarb	$C_{14}H_{21}NO_4$
Difenoconazole	$C_{19}H_{17}Cl_2N_3O_3$
Diflubenzuron	$C_{14}H_9ClF_2N_2O_2$
Dimethoate	$C_5H_{12}NO_3PS_2$
Disulfoton	$C_8H_{19}O_2PS_3$
Disulfoton-sulfone	$C_8H_{19}O_4PS_3$
Diuron	$C_9H_10Cl_2N_2O$
Ethiofencarb	$C_{11}H_{15}NO_2S$
Fenamiphos	$C_{13}H_{22}NO_3PS$
Fenazaquin	$C_{20}H_{22}N_2O$
Fenhexamid	$C_{14}H_{17}Cl_2NO_2$
Fenobucarb	$C_{12}H_{17}NO_2$
Fenoxycarb	$C_17H_{19}NO_4$
Fenthion	$C_{10}H_{15}O_3PS_2$
Flucycloxuron	$C_{25}H_{20}ClF_2N_3O_3$
Flufenoxuron	$C_{21}H_{11}ClF_6N_2O_3$
Formetanate	$C_{11}H_{15}N_3O_2$
Furathiocarb	$C_{18}H_{26}N_2O_5S$
Hexaflumuron	$C_{16}H_8Cl_2F_6N_2O_3$
Hexythiazox	$C_{17}H_{21}ClN_2O_2S$
Imazalil	$C_{14}H_{14}Cl_2N_2O$
Imidacloprid	$C_9H_{10}ClN_5O_2$

Pesticide	Chemical Formula
Indoxacarb	$C_{22}H_{17}ClF_3N_3O_7$
Iprovalicarb	$C_{18}H_{28}N_2O_3$
Isofenphos-methyl	$C_{14}H_{22}NO_4PS$
Isofenphos-oxon	$C_{15}H_{24}NO_5P$
Isoprothiolane	$C_{12}H_{18}O_4S_2$
Isoproturon	$C_{12}H_{18}N_2O$
Linuron	$C_9H_{10}Cl_2N_2O_2$
Mepanipyrim	$C_{14}H_{13}N_3$
Metconazole	$C_{17}H_{22}ClN_3O$
Methiocarb	$C_{11}H_{15}NO_2S$
Methiocarb-sulfone	$C_{11}H_{15}NO_4S$
Methoxyfenozide	$C_{22}H_{28}N_2O_3$
Metobromuron	$C_9H_{11}BrN_2O_2$
Monocrotophos	$C_7H_{14}NO_5P$
Napropamide	$C_{17}H_{21}NO_2$
Nitenpyram	$C_{11}H_{15}ClN_4O_2$
Omethoate	$C_5H_{12}NO_4PS$
Oxamyl	$C_7H_{13}N_3O_3S$
Pencycuron	$C_{19}H_{21}ClN_2O$
Phenmedipham	$C_{16}H_{16}N_2O_4$
Pirimicarb	$C_{11}H_{18}N_4O_2$
Prochloraz	$C_{15}H_{16}Cl_3N_3O_2$
Propamocarb	$C_9H_{20}N_2O_2$
Propoxur	$C_{11}H_{15}NO_3$
Prosulfocarb	$C_{14}H_{21}NOS$
Prosulfuron	$C_{15}H_{16}F_3N_5O_4S$
Pymetrozine	$C_{10}H_{11}N_5O$
Pyraclostrobin	$C_{19}H_{18}ClN_3O_4$
Pyridaphenthion	$C_{14}H_{17}N_2O_4PS$
Spinosyn-A	$C_{41}H_{65}NO_{10}$
Spinosyn-D	$C_{42}H_{67}NO_{10}$
Spiroxamine	$C_{18}H_{35}NO_2$
Tebufenozide	$C_{22}H_{28}N_2O_2$
Tebufenpyrad	$C_{18}H_{24}ClN_3O$
Teflubenzuron	$C_{14}H_6Cl_2F_4N_2O_2$
Tetraconazole	$C_{13}H_{11}Cl_2F_4N_3O$
Thiabendazole	$C_{10}H_7N_3S$
Thiacloprid	$C_{10}H_9ClN_4S$
Thiodicarb	$C_{10}H_{18}N_4O_4S_3$
Trichlorfon	$C_4H_8Cl_3O_4P$
Trifloxystrobin	$C_{20}H_{19}F_3N_2O_4$
Triflumuron	$C_{15}H_{10}ClF_3N_2O_3$

Liquid Chromatography

A Thermo Scientific Accela™ UHPLC system consisting of an Accela open autosampler in combination with an Accela 1250 UHPLC pump was used. A 2 minute chromatographic gradient of water and methanol, both spiked with 0.1% formic acid, was applied resulting in a total chromatographic cycle time of 5 minutes (Figure 3). Ten microliters of each sample were injected onto a Thermo Scientific Hypersil™ GOLD PFP column (50 x 2.1 mm, 1.9 µm particle size) with a flow rate of 800 µL/min. This resulted in peak widths of 3–6 seconds for the analytes of interest.

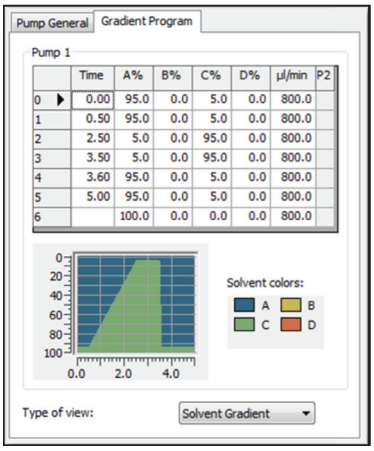

Figure 3. Chromatographic gradient

Mass Spectrometry

Given that resolution in excess of 50,000 was needed for this application, the Exactive Plus system was set to a resolving power of 70,000 at *m/z* 200, resulting in a scan rate of 3.7 Hz. As shown in Figure 4, this provided 13 scans across a 3.2 second peak.

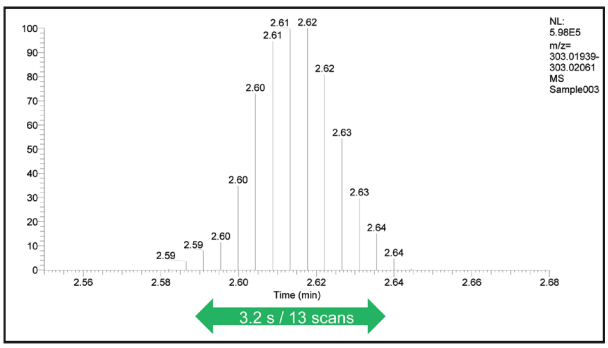

Figure 4. Scans achieved across a narrow chromatographic peak

For improved component identification, it would have been useful to have fragmentation scans on the analytes of interest. However, continual switching between full-scan and all-ion fragmentation scan modes (FS/AIF) would have required resolution to be reduced to maintain the number of scans. As an optimal solution, data-dependent AIF scans (dd-AIF) were introduced into the full scans (FS/dd-AIF) by means of a mass inclusion list containing the masses of the spiked components. One AIF scan was triggered for each target compound as soon as the abundance of the target compound crossed a given intensity threshold in a full scan. This significantly reduced the number of fragmentation scans and kept the overall data rate close to what could have been achieved in full-scan-only mode. Method details are shown in Figure 5.

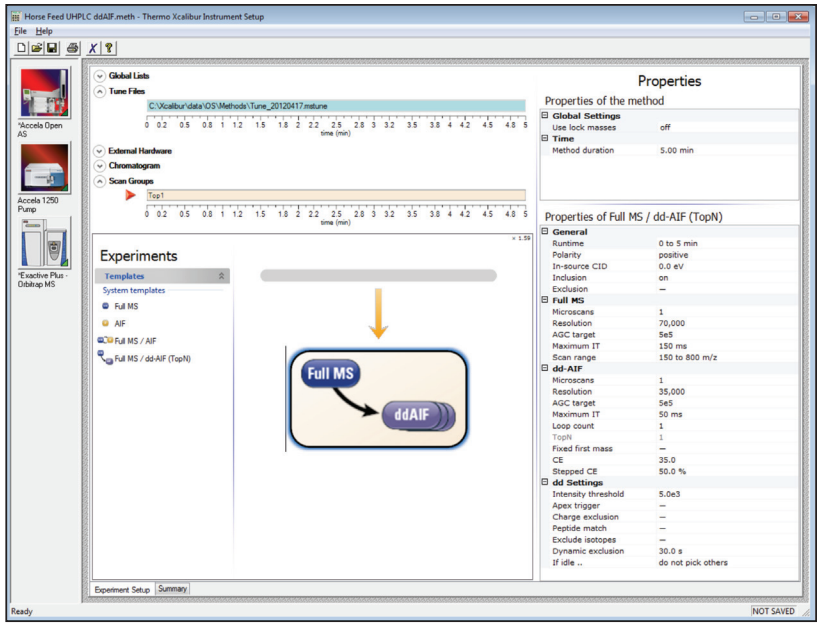

Figure 5. Exactive Plus instrument method setup

Data Analysis

The same data set was used for quantitative and qualitative data processing. Thermo Scientific ExactFinder™ software version 2.0 was used to process the data. Qualitative processing included targeted screening in combination with general unknown screening. The 85 common pesticides were selected using built-in databases from ExactFinder software. These selection could be exported directly into the mass inclusion list used by the Exactive Plus instrument method to trigger the dd-AIF scans. No further optimization of the LC-MS system was needed.

Results and Discussion

Quantitative Analysis

The six calibration standards, with spike levels ranging from 5 to 150 µg/kg, were analyzed to establish calibration curves for each of the target pesticides. The majority of pesticides eluted at between 1.3 and 3.0 minutes, so a number of target components and matrix components coeluted (Figure 6). However, the extracted ion chromatograms of most target components were free from additional peaks, demonstrating that the 5 ppm extraction window combined with the resolving power of the mass spectrometer provided very high selectivity. Linear calibration curves were achieved for nearly all target pesticides (example shown in Figure 7), confirming that the compounds could be clearly distinguished from the matrix.

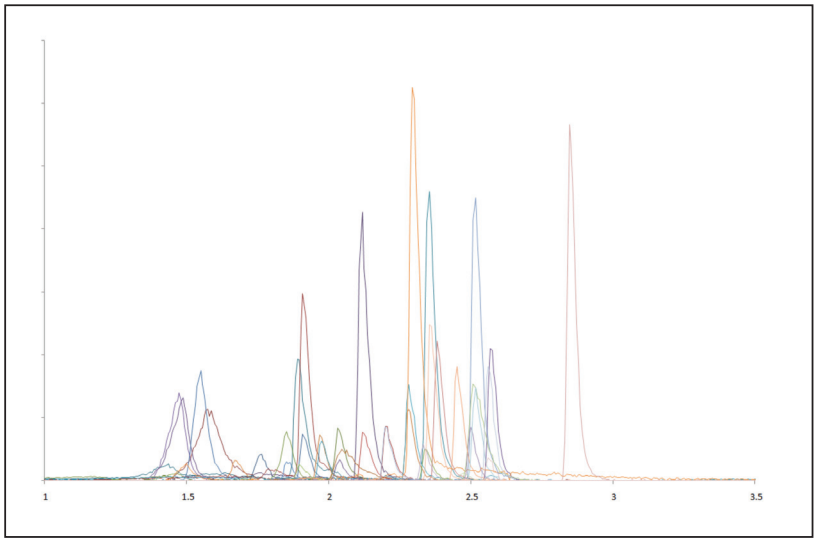

Figure 6. Extracted chromatograms demonstrate coelution of target and matrix compounds (only 20 traces compound shown)

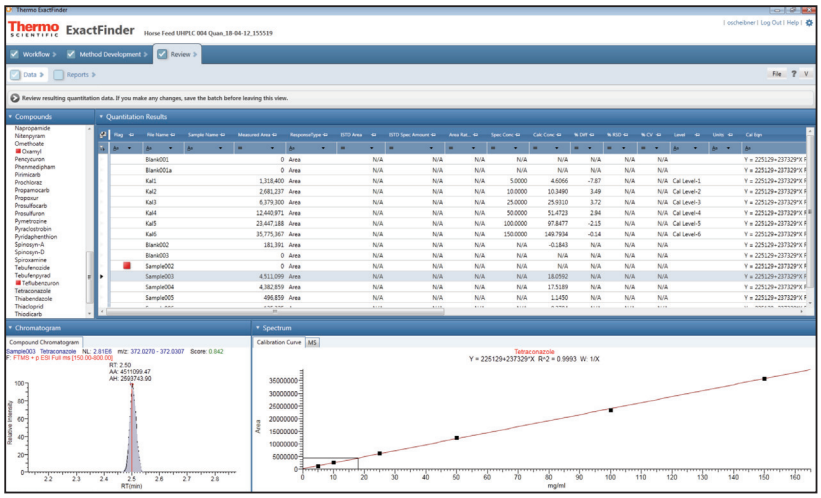

Figure 7. Example of quantitative results from one target compound (tetraconazole)

Qualitative Analysis

Qualitative analysis was carried out as a combination of targeted analysis and general unknown screening. In a first step, targeted analysis was carried out. In a second step, all peaks not identified in the targeted search were automatically forwarded for general unknown screening.

The same list of analytes used for quantitative analysis (Table 1) was applied for the targeted search. Retention time, isotopic pattern match, fragment search, and library search were used as confirmation criteria for targeted search. The fragment information for the analytes of interest and the fragmentation spectra for the library search were taken from databases included with the ExactFinder software. Even at the lower end of the concentration range, most components quantified could be easily confirmed on all four stages of confirmation (see Figure 8). With its built-in reporting capabilities, the ExactFinder software version 2.0 provided a quick, easy overview of the screening results.

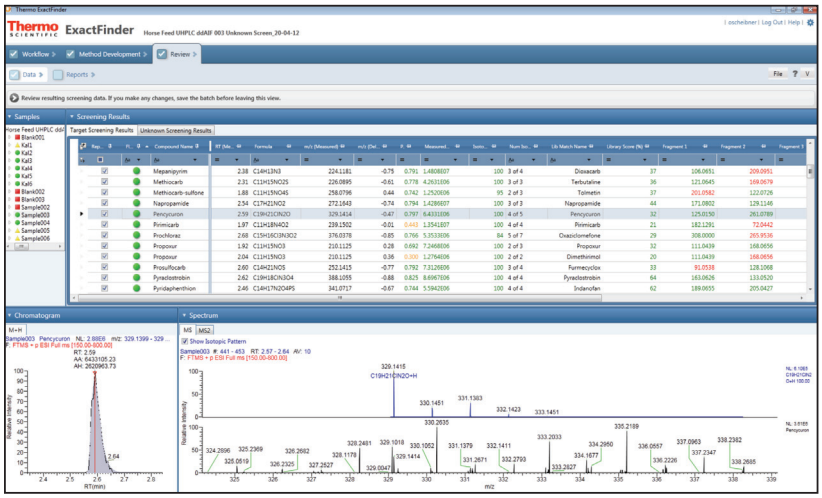

Figure 8. Qualitative results as displayed by the ExactFinder software

It quickly became clear that sufficient resolution was the key to successful full-scan quantitation and screening of complex samples like the ones analyzed in this work. As shown in Figure 9, most analyte signals were surrounded by numerous matrix signals. Only sufficient resolving power ensured proper separation of analyte and matrix signals. This applies to the monoisotopic signals used for analysis as well as for the isotopic signals used for confirmation. The peaks of interest showed a resolution of close to 60,000. It was apparent that significantly lower resolving power at these masses would have led to interference and merged signals, causing significant mass shifts. The mass shifts would have led to false negatives or would have required to widening of the extraction window. Widening the extraction window would have lowered the selectivity of the analysis and resulted in false positives.

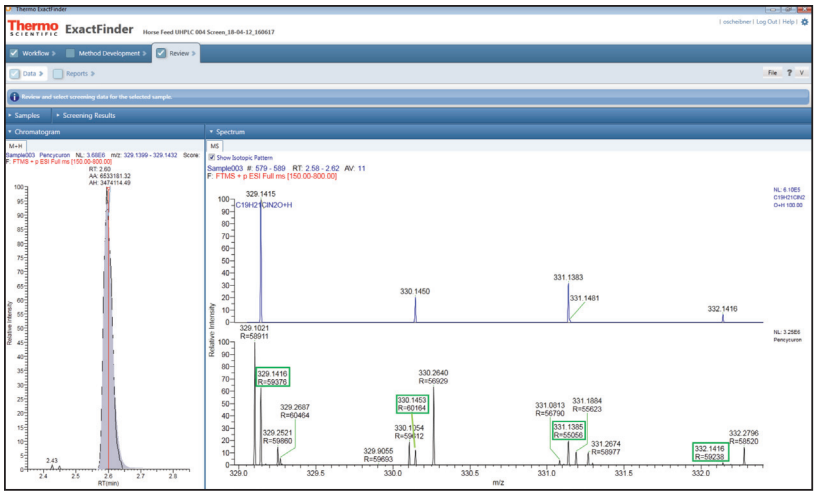

Figure 9. Isotopic pattern match of pencycuron. Green boxes mark the isotope signals surrounded by matrix signals

The general unknown screening carried out on the remaining peaks offers several options for automatic identification of the found peaks: database search, elemental composition determination based on isotopic pattern matching, spectral library search, and internet search. For the samples, roughly 15,000 components were detected; all of them went through the identification process. Database and spectral library searches were carried out using built-in resources. Internet search was carried out using a selection of databases listed in the ChemSpider® online search portal. Numerous additional contaminants could be identified, especially pesticides and a selection of aflatoxins (results not shown).

Conclusion

HR/AM analysis is a versatile method for residue analysis. It offers full quantitation capabilities in combination with unrestricted target and unknown screening options. Ultra-high resolution delivered by the Orbitrap mass analyzer in the Exactive Plus mass spectrometer provides reliability and selectivity comparable to established MS/MS techniques. The Exactive Plus mass spectrometer is compatible with UHPLC without compromising resolution or mass accuracy.

References

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Application Note 579

Use of UHPLC and High-Resolution MS for Quantitative Analysis of Pesticides in Onion Matrix

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Key Words

Exactive Plus, TraceFinder, high resolution, accurate mass, food safety, UltiMate UHPLC

Goal

To demonstrate the ability of a high-resolution, accurate-mass UHPLC-MS system, combined with appropriate application-specific workflow software, to provide fast, confident, and precise screening and quantitative analysis of pesticides in onion matrix.

Introduction

Monitoring for pesticide and other chemical residues in produce is essential to maintaining a safe food supply. Monitoring data can also be used to better understand the relationship of pesticide residues to agriculture practices, enhance integrated pest management, and support the export of U.S. commodities. Monitoring is typically done by public agencies, but budget restrictions have increased pressure on these agencies to improve productivity while lowering costs.

Traditionally, triple quadrupole mass spectrometers have been used for the identification and quantitation of pesticide and chemical residues. However, MS/MS analysis with triple quadrupole mass spectrometers requires time-consuming selection of mass transitions and optimization of collision energies. The introduction of affordable benchtop, Orbitrap™-based, high-resolution, accurate-mass (HR/AM) mass spectrometers has provided an alternative method for unequivocal identification of trace contaminants without time-consuming MS/MS optimization.

A liquid chromatography/mass spectrometry methodology employing ultrahigh performance liquid chromatography (UHPLC) and HR/AM mass spectrometry makes it possible to identify, quantify, and confirm more trace-level contaminants in complex mixtures in a single analytical run. The results of this unique solution are improved sensitivity and precision, as well as unmatched throughput.

Experimental

Sample Preparation

Onion was prepared for analysis by using a modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method, which is a sample preparation procedure used to extract pesticides from food. For the QuEChERS extraction, 15 g of homogenized sample and 15 mL of acetonitrile were used. Then, 200 µL of final QuEChERS extract, 300 µL of acetonitrile, and 500 µL of water were transferred into an autosampler vial, spiked with 20 µL of the pesticides standard, and mixed thoroughly. A mixture of 120 pesticides with different starting concentrations was prepared in neat matrix (70:30 methanol/water) to make the standard calibration curve and spiked into onion matrix to determine if there was any ion suppression.

Liquid Chromatography

Chromatographic analysis was performed using a Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC UHPLC system with high-pressure mixing binary pump and 35 µL gradient mixing kit. High-purity Fisher Chemical LC/MS solvents were used.

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The chromatographic conditions were as follows:

Column:	Thermo Scientific Hypersil GOLD aQ™ column (50 x 2.1 mm, 1.9 µm)
Oven:	TCC-3300RS
Autosampler:	WPS-3000RS thermostated autosampler
Pump:	HPG3200RS binary with 35 µL gradient mixing kit, SRD-3400 solvent rack, and degasser
Mobile Phase A:	Water with 0.1% formic acid and 4 mM ammonium formate
Mobile Phase B:	Methanol with 0.1% formic acid and 4 mM ammonium formate
Flow Rate:	300 µL/min
Column Temperature:	40 °C
Sample Injection Volume:	5 µL

Gradient:	Gradient Time (min)	%A	%B
	-2.50	98	2
	0.00	98	2
	0.25	70	30
	10.00	0	100
	12.49	0	100
	12.50	98	2

Mass Spectrometry

All samples were analyzed on a Thermo Scientific Exactive™ Plus benchtop Orbitrap mass spectrometer.

The MS conditions were as follows:

Ion Source:	Heated electrospray (HESI-II)
Ion Mode:	Positive/Negative
Capillary Temperature:	280 °C
Vaporizer Temperature:	295 °C
Spray Voltage:	2200 V
Sheath Gas:	32 arbitrary units
Aux Gas:	7 arbitrary units
Scan Type:	Full MS scan
Mass Range:	*m/z* 120–1000
Mass Resolution:	70,000

Unlike triple quadrupole mass spectrometers, the high-resolution, accurate-mass Exactive Plus instrument required no optimization of mass transitions or collision energies for each analyte. Therefore, the effort for method development was significantly reduced. Table 1 lists the pesticides targeted in this analysis.

Data Analysis

Data processing was carried out with Thermo Scientific TraceFinder™ software for quantitation and targeted-screening workflows. Specificity of analysis was achieved by applying a mass extraction window of 5 ppm to the theoretical mass of the analytes.

Table 1. Targeted pesticides and their associated retention times (RT), actual and theoretical *m/z*, and calculated mass errors

Compound	RT	Formula	Theoretical *m/z*	Detected *m/z*	Delta (ppm)
Acetamiprid	2.27	$C_{10}H_{11}ClN_4$	223.0745	223.0746	0.51
Aldicarb	2.80	$C_7H_{14}N_2O_2S$	208.1114	208.1117	1.23
Aldicarb sulfone	1.49	$C_7H_{14}N_2O_4S$	240.1013	240.1013	0.14
Aldicarb sulfoxide	1.55	$C_7H_{14}N_2O_3S$	224.1063	224.1065	0.48
Atrazine	4.38	$C_8H_{14}ClN_5$	216.1010	216.1013	1.03
Azinphos methyl	5.01	$C_{10}H_{12}N_3O_3PS_2$	318.0130	318.0137	1.97
Azinphos methyl OA	2.90	$C_{10}H_{12}N_3O_4PS$	302.0359	302.0359	-0.10
Azoxystrobin	5.40	$C_{22}H_{17}N_3O_5$	404.1241	404.1245	1.03
Bendiocarb	3.52	$C_{11}H_{13}NO_4$	224.0917	224.0918	0.28
Benoxacor	4.97	$C_{11}H_{11}Cl_2NO_2$	260.0240	260.0241	0.57
Bifenazate	6.04	$C_{17}H_{20}N_2O_3$	301.1547	301.1550	0.99
Boscalid	5.61	$C_{18}H_{12}Cl_2N_2O$	343.0399	343.0403	1.07
Buprofezin	7.70	$C_{16}H_{23}N_3OS$	306.1635	306.1637	0.67
Carbaryl	3.88	$C_{12}H_{11}NO_2$	202.0863	202.0864	0.62
Carbofuran	3.52	$C_{12}H_{15}NO_3$	222.1125	222.1126	0.42
Carbofuran, 3-hydroxy	2.19	$C_{12}H_{15}NO_4$	255.1339	255.1339	-0.09
Carboxin	3.77	$C_{12}H_{13}NO_2S$	236.0740	236.0741	0.38
Carfentrazone ethyl	6.62	$C_{15}H_{14}Cl_2F_3N_3O_3$	429.0703	429.0706	0.70
Chlorpyrifos OA	6.37	$C_9H_{11}Cl_3NO_4P$	350.9830	350.9831	0.31
Clofentezine	7.27	$C_{14}H_8Cl_2N_4$	303.0199	303.0200	0.37
Clothianidin	2.03	$C_6H_8ClN_5O_2S$	250.0160	250.0162	0.82
Cymoxanil	2.53	$C_7H_{10}N_4O_3$	199.0826	199.0828	1.07
Difenoconazole	7.40	$C_{19}H_{17}Cl_2N_3O_3$	406.0720	406.0723	0.79
Diflubenzuron	6.50	$C_{14}H_9ClF_2N_2O_2$	311.0393	311.0395	0.39
Dimethomorph	5.80	$C_{21}H_{22}ClNO_4$	388.1310	388.1313	0.63
Dinotefuran	1.49	$C_7H_{14}N_4O_3$	203.1139	203.1140	0.50
Diuron	4.68	$C_9H_{10}Cl_2N_2O$	233.0243	233.0245	0.71
Famoxadone	7.04	$C_{22}H_{18}N_2O_4$	392.1605	392.1608	0.88
Fenamidone	5.46	$C_{17}H_{17}N_3OS$	312.1165	312.1167	0.51
Fenamiphos sulfone	3.77	$C_{13}H_{22}NO_4PS$	320.1080	320.1081	0.22
Fenamiphos sulfoxide	3.93	$C_{13}H_{22}NO_5PS$	336.1029	336.1029	0.09
Fenbuconazole	6.46	$C_{19}H_{17}ClN_4$	337.1215	337.1216	0.57
Fludioxonil	5.71	$C_{12}H_6F_2N_2O_2$	266.0736	266.0737	0.41
Fluridone	5.19	$C_{19}H_{14}F_3NO$	330.1100	330.1100	-0.12
Flutolanil	5.76	$C_{17}H_{16}F_3NO_2$	324.1206	324.1207	0.42
Formetanate	1.43	$C_{11}H_{15}N_3O_2$	222.1237	222.1238	0.48
Halosulfuron methyl	5.96	$C_{13}H_{15}ClN_6O_7S$	435.0484	435.0490	1.28
Hexaconazole	6.98	$C_{14}H_{17}Cl_2N_3O$	314.0821	314.0823	0.42
Hexythiazox	8.18	$C_{17}H_{21}ClN_2O_2S$	353.1085	353.1087	0.65
Imazalil	4.49	$C_{14}H_{14}Cl_2N_2O$	297.0556	297.0559	0.95
Imidacloprid	1.99	$C_9H_{10}ClN_5O_2$	256.0596	256.0596	-0.03
Indoxacarb	7.54	$C_{22}H_{17}ClF_3N_3O_7$	528.0780	528.0785	0.95
Isoprocarb	4.39	$C_{11}H_{15}NO_2$	194.1174	194.1178	1.33
Linuron	5.34	$C_9H_{10}Cl_2N_2O_2$	249.0192	249.0194	0.92
Metalaxyl	4.59	$C_{15}H_{21}NO_4$	280.1543	280.1546	0.85
Methidathion OA	2.70	$C_6H_{11}N_2O_5PS_2$	286.9920	286.9919	-0.43
Methiocarb	5.38	$C_{11}H_{15}NO_2S$	226.0896	226.0898	0.76
Methomyl	1.45	$C_5H_{10}N_2O_2S$	163.0536	163.0537	0.74

Table 1 (continued). Targeted pesticides and their associated retention times (RT), actual and theoretical *m/z*, and calculated mass errors

Compound	RT	Formula	Theoretical *m/z*	Detected *m/z*	Delta (ppm)
Methoxyfenozide	5.86	$C_{22}H_{28}N_2O_3$	369.2173	369.2176	0.79
Metribuzin	3.35	$C_8H_{14}N_4OS$	215.0961	215.0963	0.67
Monocrotophos	1.74	$C_7H_{14}NO_5P$	224.0682	224.0684	0.83
Myclobutanil	5.92	$C_{15}H_{17}ClN_4$	289.1215	289.1218	1.09
Norflurazon	4.78	$C_{12}H_9ClF_3N_3O$	304.0459	304.0461	0.80
Norflurazon desmethyl	4.27	$C_{11}H_7ClF_3N_3O$	290.0303	290.0305	0.92
Oxamyl	1.57	$C_7H_{13}N_3O_3S$	237.1016	237.1017	0.34
Oxamyl oxide	1.66	$C_5H_{10}N_2O_2S$	163.0536	163.0537	0.74
Oxydemeton methyl sulfone	4.41	$C_6H_{15}O_4PS_2$	247.0222	247.0224	0.82
Phorate sulfone	4.41	$C_7H_{17}O_4PS_3$	293.0099	293.0101	0.57
Phorate sulfoxide	4.25	$C_7H_{17}O_3PS_3$	277.0150	277.0153	0.97
Pirimicarb	2.80	$C_{11}H_{18}N_4O_2$	239.1503	239.1503	0.07
Promecarb	5.57	$C_{12}H_{17}NO_2$	208.1332	208.1335	1.26
Propamocarb	1.51	$C_9H_{20}N_2O_2$	189.1598	189.1599	0.75
Propargite	8.38	$C_{19}H_{26}O_4S$	368.1890	368.1893	0.88
Propiconazole	6.89	$C_{15}H_{17}Cl_2N_3O_2$	342.0771	342.0775	1.15
Propoxur	3.46	$C_{11}H_{15}NO_3$	210.1125	210.1125	0.23
Pyraclostrobin	7.08	$C_{19}H_{18}ClN_3O_4$	388.1059	388.1062	0.80
Pyridaben	8.90	$C_{19}H_{25}ClN_2OS$	365.1449	365.1452	0.78
Pyrimethanil	4.72	$C_{12}H_{13}N_3$	200.1182	200.1184	0.62
Pyriproxyfen	8.05	$C_{20}H_{19}NO_3$	322.1438	322.1439	0.37
Quinoxyfen	8.20	$C_{15}H_8Cl_2FNO$	308.0040	308.0042	0.67
Sethoxydim	7.72	$C_{17}H_{29}NO_3S$	328.1941	328.1942	0.28
Simazine	3.48	$C_7H_{12}ClN_5$	202.0854	202.0855	0.40
Spinosad A	7.27	$C_{41}H_{65}NO_{10}$	732.4681	732.4687	0.77
Spinosad D	7.66	$C_{42}H_{67}NO_{10}$	746.4838	746.4838	-0.01
Spiromesifen	8.36	$C_{23}H_{30}O_4$	388.2482	388.2485	0.62
Sulfentrazone	3.81	$C_{11}H_{10}Cl_2F_2N_4O_3S$	404.0157	404.0159	0.57
Tebuconazole	6.75	$C_{16}H_{22}ClN_3O$	308.1524	308.1526	0.65
Tebufenozide	6.58	$C_{22}H_{28}N_2O_2$	353.2224	353.2226	0.59
Tebuthiuron	3.62	$C_9H_{16}N_4OS$	229.1118	229.1119	0.39
Thiabendazole	1.95	$C_{10}H_7N_3S$	202.0433	202.0435	0.71
Thiabendazole, 5-hydroxy	1.65	$C_{10}H_7N_3OS$	218.0383	218.0384	0.61
Thiacloprid	2.60	$C_{10}H_9ClN_4S$	253.0309	253.0310	0.27
Thiobencarb	7.16	$C_{12}H_{16}ClNOS$	258.0714	258.0715	0.56
Triadimefon	5.82	$C_{14}H_{16}ClN_3O_2$	294.1004	294.1005	0.49
Triadimenol	5.96	$C_{14}H_{18}ClN_3O_2$	296.1160	296.1163	0.92
Trifloxystrobin	7.51	$C_{20}H_{19}F_3N_2O_4$	409.1370	409.1373	0.73
Triflumizole	7.56	$C_{15}H_{15}ClF_3N_3O$	346.0929	346.0930	0.39

Results and Discussion

The extracted ion chromatograms shown in Figure 1 illustrate the quality of the UHPLC separation at 1 ppb in onion matrix. All analytes gave very good linear response in the calibration range of 1.35–1280 ppb depending on the starting concentration in the mixture. The quantification data showed good reproducibility and recovery rates.

Table 2 shows the retention time, R^2, and LOQ for the pesticides analyzed in onion matrix. The mass accuracy of the LOQ (less than 2 ppm), as well as the retention times and curve fits, increase the confidence level for the analyst.

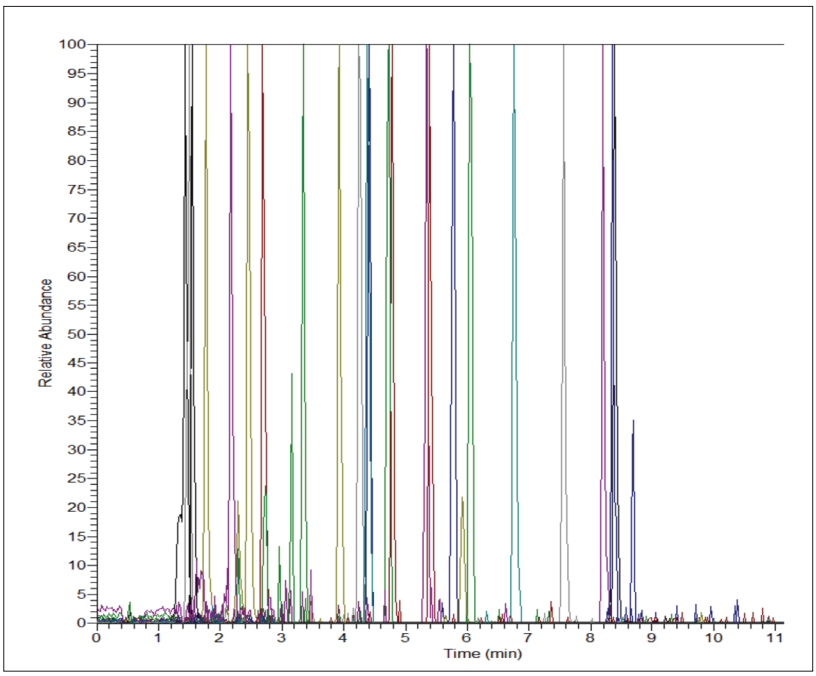

Figure 1. Extracted ion chromatograms showing peak shape and elution time at 1 ppb level in onion matrix

Table 2. Tabulated results of LOQs for each targeted compound, with retention times and curve fit R^2

Compound	RT	R^2	LOQ (ppb)
Acetamiprid	2.27	0.9990	0.125
Aldicarb	2.80	0.9956	2.000
Aldicarb sulfone	1.49	0.9986	0.500
Aldicarb sulfoxide	1.55	0.9979	0.500
Atrazine	4.38	0.9994	0.500
Azinphos methyl	5.01	0.9959	2.500
Azinphos methyl OA	2.90	0.9992	0.500
Azoxystrobin	5.40	0.9992	0.125
Bendiocarb	3.52	0.9991	0.250
Benoxacor	4.97	0.9986	0.500
Bifenazate	6.04	0.9706	0.500
Boscalid	5.61	0.9991	0.500
Buprofezin	7.70	0.9989	0.025
Carbaryl	3.88	0.9989	0.250
Carbofuran	3.52	0.9988	0.250
Carbofuran, 3-hydroxy	2.19	0.9985	0.500
Carboxin	3.77	0.9990	0.250
Carfentrazone ethyl	6.62	0.9991	0.250
Chlorpyrifos OA	6.37	0.9994	0.050
Clofentezine	7.27	0.9937	1.000
Clothianidin	2.03	0.9978	0.250
Cymoxanil	2.53	0.9029	0.500
Difenoconazole	7.40	0.9994	0.250
Diflubenzuron	6.50	0.9996	1.000
Dimethomorph	5.80	0.9993	0.250
Dinotefuran	1.49	0.9974	0.050
Diuron	4.68	0.9990	1.000
Famoxadone	7.04	0.9992	0.250
Fenamidone	5.46	0.9990	0.025
Fenamiphos sulfone	3.77	0.9992	0.250
Fenamiphos sulfoxide	3.93	0.9992	0.025
Fenbuconazole	6.46	0.9993	0.500
Fludioxonil	5.71	0.9991	0.500
Fluridone	5.19	0.9987	0.250
Flutolanil	5.76	0.9990	0.125
Formetanate	1.43	0.9983	0.050
Halosulfuron methyl	5.96	0.9866	0.500
Hexaconazole	6.98	0.9986	1.000
Hexythiazox	8.18	0.9990	0.250
Imazalil	4.49	0.9995	0.250
Imidacloprid	1.99	0.9965	0.050
Indoxacarb	7.54	0.9989	0.500
Isoprocarb	4.39	0.9971	0.500
Linuron	5.34	0.9989	0.500
Metalaxyl	4.59	0.9992	0.125

Table 2 (continued). Tabulated results of LOQs for each targeted compound, with retention times and curve fit R^2

Compound	RT	R^2	LOQ (ppb)
Methidathion OA	2.70	0.9991	2.000
Methiocarb	5.38	0.9990	0.500
Methomyl	1.45	0.9968	0.100
Methoxyfenozide	5.86	0.9996	0.250
Metribuzin	3.35	0.9992	0.250
Monocrotophos	1.74	0.9989	0.025
Myclobutanil	5.92	0.9988	0.500
Norflurazon	4.78	0.9992	0.500
Norflurazon desmethyl	4.27	0.9988	0.050
Oxamyl	1.57	0.9992	0.500
Oxamyl oxide	1.66	0.9966	1.000
Oxydemeton methyl sulfone	4.41	0.9979	0.250
Phorate sulfone	4.41	0.9984	0.025
Phorate sulfoxide	4.25	0.9990	0.050
Pirimicarb	2.80	0.9988	0.100
Promecarb	5.57	0.9990	0.250
Propamocarb	1.51	0.9981	0.500
Propargite	8.38	0.9993	0.025
Propiconazole	6.89	0.9992	0.500
Propoxur	3.46	0.9993	0.500
Pyraclostrobin	7.08	0.9990	0.125
Pyridaben	8.90	0.9991	0.125
Pyrimethanil	4.72	0.9995	0.250
Pyriproxyfen	8.05	0.9990	0.125
Quinoxyfen	8.20	0.9993	0.125
Sethoxydim	7.72	0.9964	0.250
Simazine	3.48	0.9999	0.250
Spinosad A	7.27	0.9995	0.420
Spinosad D	7.66	0.9994	0.080
Spiromesifen	8.36	0.9987	0.250
Sulfentrazone	3.81	0.9986	0.500
Tebuconazole	6.75	0.9994	0.050
Tebufenozide	6.58	0.9989	0.500
Tebuthiuron	3.62	0.9990	0.125
Thiabendazole	1.95	0.9986	0.250
Thiabendazole, 5-hydroxy	1.65	0.9993	0.250
Thiacloprid	2.60	0.9993	0.125
Thiodicarb	4.23	0.9953	20.000
Triadimefon	5.82	0.9990	0.500
Triadimenol	5.96	0.9981	1.500
Trifloxystrobin	7.51	0.9989	0.125
Triflumizole	7.56	0.9994	0.251

TraceFinder software comes with many features including user-customizable flagging. A green flag next to the name of the compound (Figure 2) indicates the compound was found in the unknown sample, whereas a yellow flag indicates the compound was not found. A red flag indicates the compound has an issue with the calibration curve and that it exceeded the flagging threshold (Figures 3 and 4). A yellow triangle caution sign indicates there is an above-threshold quantitation error with a single or multiple compounds in the sample that needs to be checked.

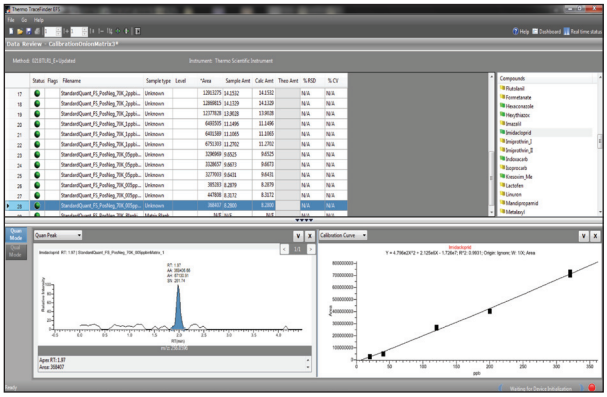

Figure 2. TraceFinder software displays imidacloprid calibration curve plot of matrix, R^2, list of compounds, and chromatogram. A green flag in the compound list indicates the compound was found in the unknown sample, whereas a yellow flag indicates the compound was not found.

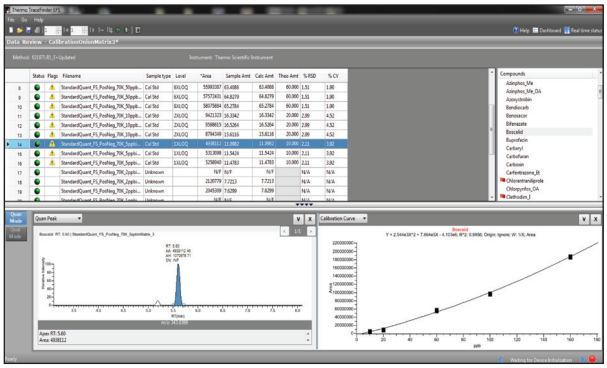

Figure 3. TraceFinder software displays boscalid calibration curve plot of matrix, R^2, list of compounds, and chromatogram. The red flag indicates the compound has an issue with the calibration curve and that it did not meet the flagging requirement. The yellow triangle caution sign indicates there is an issue with a single or multiple compounds in the sample that needs to be checked.

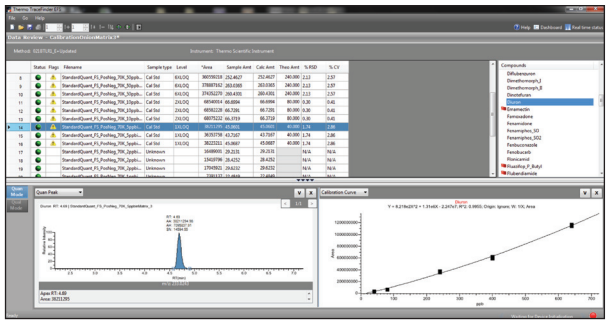

Figure 4. TraceFinder software displays diuron calibration curve plot of matrix, R^2, list of compounds, and chromatogram. The highlighting of diuron in the upper right section indicates that the compound was found within the calibration curve. Therefore, there are no flags present next to the name.

Conclusion

The Exactive Plus benchtop mass spectrometer paired with TraceFinder software provided easy access to full quantitative and targeted screening data in one package. The results showed good linearity with excellent sensitivity at very low LOQs, which will assist in detecting pesticides. The Exactive Plus instrument's exceptionally high mass resolution helped resolve matrix compounds that would otherwise interfere with detection of low-level analytes. The measured mass errors showed high confidence in the data acquired with regard to mass accuracy.

Acknowledgements

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Increasing Speed of UHPLC-MS Analysis Using Single-stage Orbitrap Mass Spectrometer

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Overview

Purpose: Improve the performance of Orbitrap HR/AM systems for high throughput sample analysis

Methods: Full scan / all ion fragmentation MS analysis of complex samples in combination with UHPLC sample separation

Results: Significant increase of data quality and processing time could be accomplished with second generation MS hardware and processing software

Introduction

Productivity of a liquid chromatography-mass spectrometry (LC-MS) system is measured in samples per day. To accomplish high productivity, modern ultrahigh pressure LC-MS (UHPLC-MS) methods increasingly deal with very short gradients, leading to chromatographic signals with peak widths below 5 seconds at the base. It is still a challenge for high-resolution, accurate mass (HR/AM) systems to provide a sufficient number of scans (≥10) across the chromatographic peak in full scan mode without compromising sensitivity and selectivity. As reported earlier, a resolution in excess of 50,000 (FWHM@ m/z 200) is necessary to ensure a selectivity comparable to established MS/MS techniques[1], combined with a mass extraction window of 5 ppm. With this work we show data acquisition and processing by using the capabilities of the Thermo Scientific Exactive Plus mass spectrometer in combination with Thermo Scientific ExactFinder 2.0 processing software.

Methods

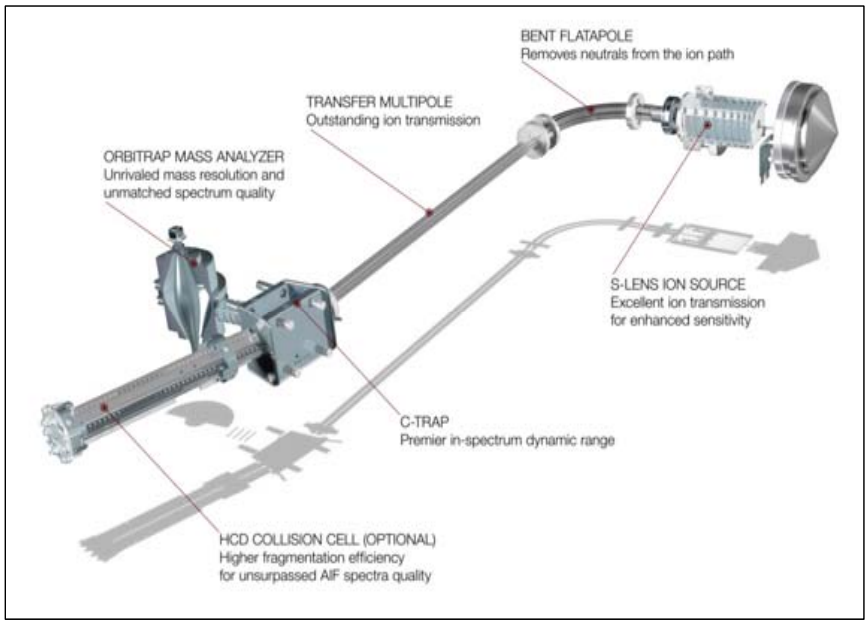
Sample Preparation: Quick, easy, cheap, effective, rugged, and safe (QuEChERS) extracts of horse feed were taken and spiked with common pesticides.

Liquid Chromatography (or more generically Separations): A Thermo Scientific Accela UHPLC system was used, consisiting of an Accela™ open autosampler in combination with an Accela 1250 UHPLC pump. For analyte separation, a Thermo Scientific Hypersil GOLD PFP column (50 x 2.1mm, 1.9µm particle size) was used and a flow rate of 800 µL/min was applied to generate a 2 min gradient (5 min full chromatographic cycle) of water and methanol, both spiked with 0.1% formic acid.

Mass Spectrometry: An Exactive™ Plus mass spectrometer was operated at 70,000 resolving power (FWHM) with full scan / data dependent AIF (FS / ddAIF) setting to generate all ion fragmentation scans based on an inclusion list containing the masses of the spiked components. The schematic of the Exactive Plus mass spectrometer is illustrated in Figure 1.

Data Analysis: The same data set was used for quantitative and qualitative data processing with ExactFinder™ 2.0 software. Qualitative processing included targeted screening in combination with general unknown screening.

FIGURE 1. Exactive Plus schematics



Results

Method Improvements: In the new Exactive Plus MS, two major changes have been implemented for performance improvement. First, the tube-lens / skimmer assembly has been replaced by the S-Lens, which serves for significantly higher ion transmission, increasing the instrument's sensitivity. Second, the assembly and the electronics of the Thermo Scientific Orbitrap mass analyzer have been improved[2], resulting in higher scan speed and resolution, together with improved pos / neg switching performance. As a result, the range of resolution is from 17,500 to 140,000 @ m/z 200, with a maximum scan rate of 12 Hz. Given that a resolution in excess of 50,000 is needed, the system was set to a resolution of 70,000 @ m/z 200, resulting in a scan rate of 3.7 Hz. As shown in Figure 2, for a 3.2 seconds wide peak, 13 scans across the peak were achieved. For improved component identification, it would be desirable to have fragmentation scans on the analytes of interest, but permanent switching between full scan and fragmentation scan mode (Full Scan / AIF) would decrease the data rate significantly, leading to the need to reduce the resolution setting. As a compromise, data dependant AIF scans were introduced into the full scans (FS / ddAIF) by means of a mass inclusion list, on which only one AIF scan for each target compound is triggered as soon as it crosses a given intensity threshold in a full scan. By means of this, the number of fragmentation scans is significantly reduced, keeping the overall data rate close to full scan only mode. Method details are shown in Figure 3. The Exactive Plus MS is shown in Figure 4.

FIGURE 2. Zoom on peak of Clofentezine.

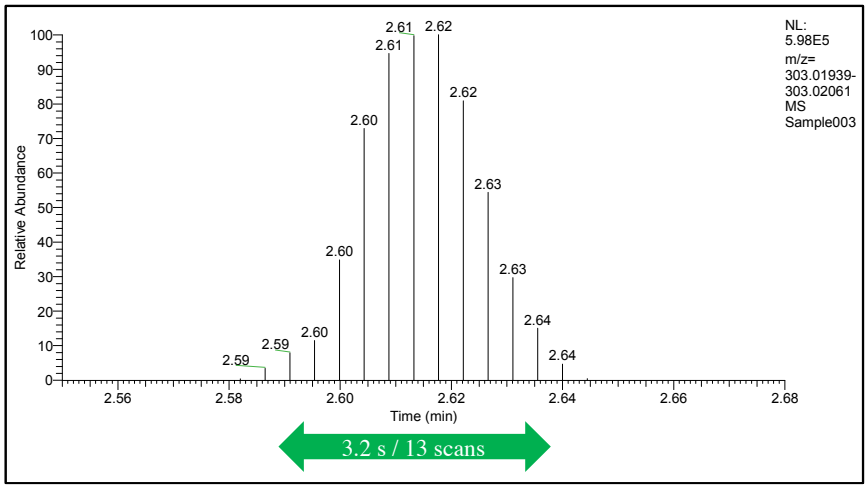
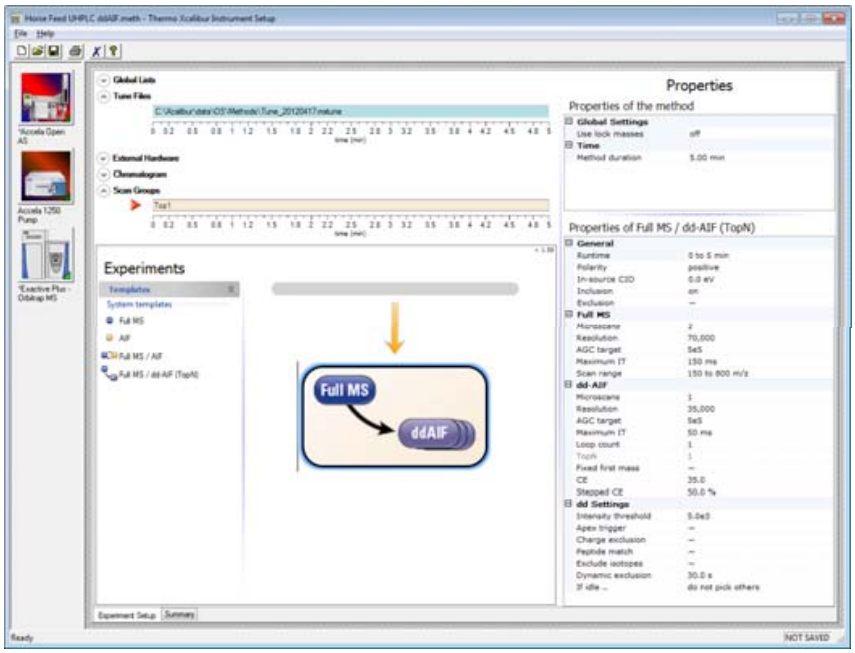


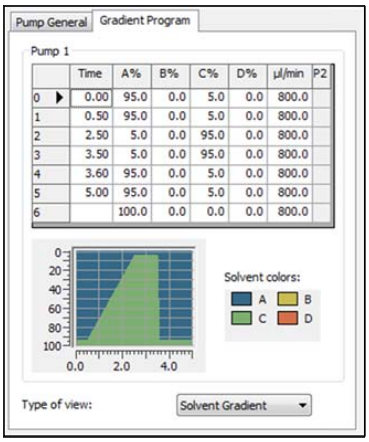
FIGURE 3. Exactive Plus Instrument Method.



Sample Analysis: For chromatographic separation, a 2-minute chromatographic gradient was applied to QuEChERS extracts of horse feed samples spiked with pesticides, resulting in a total chromatographic cycle time of 5 minutes. 10 µL of each sample were injected onto a Hypersil GOLD™ PFP column (50 x 2.1 mm, 1.9 µm particle size) with a flow rate of 800 µL/min (see Figure 5). This resulted in peak widths of 3 to 6 seconds for the analytes of interest. For targeted analysis, 85 commonly occurring pesticides out of the list of spiked components were chosen, using built-in databases from ExactFinder software. This selection could be exported directly into an Exactive Plus inclusion mass list file used by the Exactive Plus method for the ddAIF scan triggering as described previously. No further optimization of the LC-MS system was needed.

FIGURE 4. The Exactive Plus MS with an Accela open autosampler and Accela 1250 UHPLC pump

FIGURE 5. LC gradient



Quantitative Analysis: Quantitative analysis was done on the described selection of 85 pesticides. The sequence measured contained a dilution series with five samples of spiked matrix ranging from 5 µg/kg to 150 µg/kg spike level. As to be expected, the majority of compounds eluted in little more than one minute, so already a great number of target components were co-eluting (see Figure 6), not to speak of matrix components. However, the extracted ion chromatograms of most target components were free from additional peaks, showing that the selectivity of a 5 ppm extraction window in combination with the resolving power of the mass spectrometer proves very high selectivity. A linear calibration curve could be achieved for nearly all target components (example shown in Figure 7), giving additional hint that no significant matrix interference was present.

FIGURE 6. Extracted chromatograms for a selection of compounds

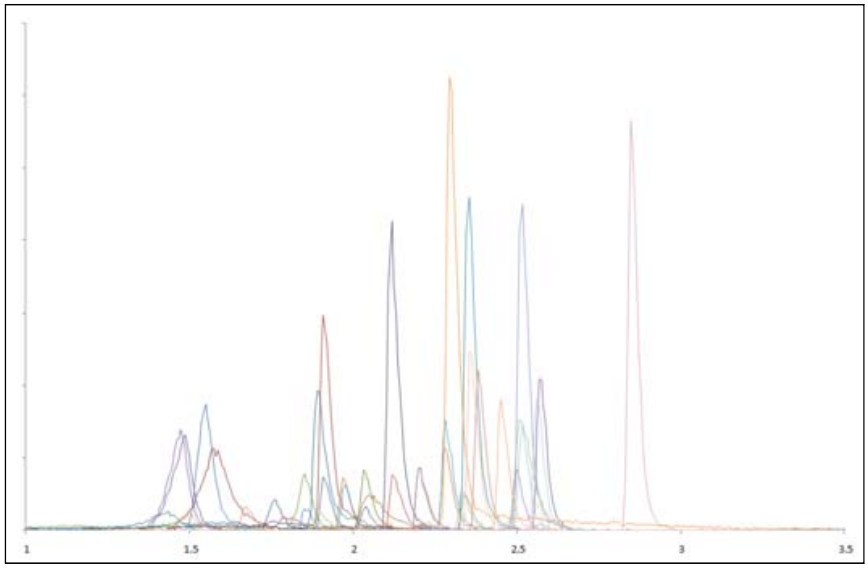
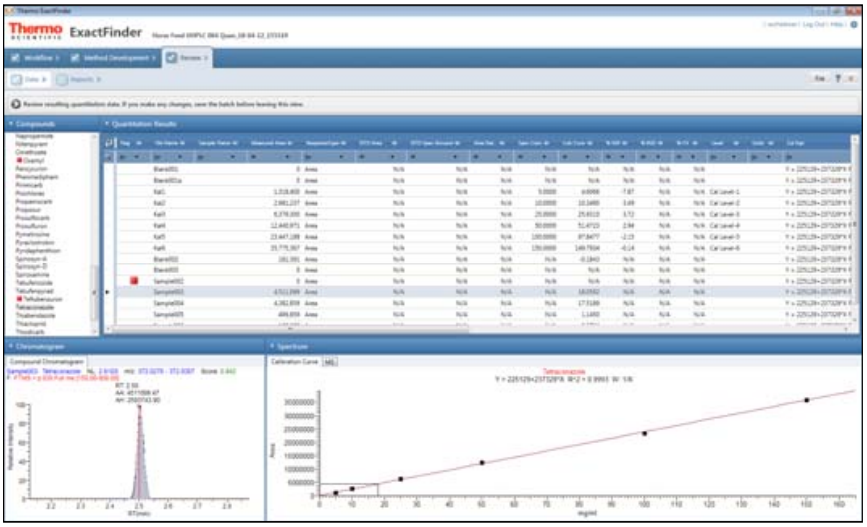


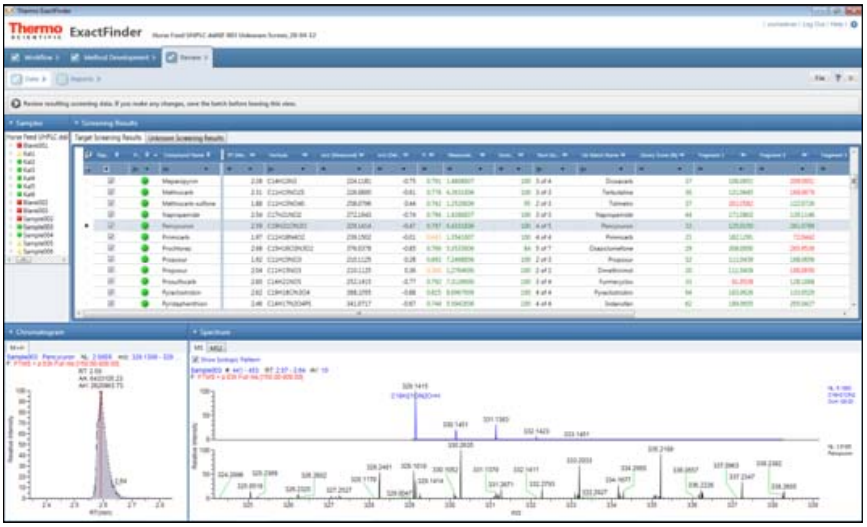
FIGURE 7. Quantitative Result Grid



Qualitative Analysis: Qualitative analysis was carried out as a combination of targeted analysis and general unknown screening. In this case, in a first stage a targeted analysis is carried out and in a second stage all peaks not identified in this targeted search are automatically forwarded to general unknown screening.

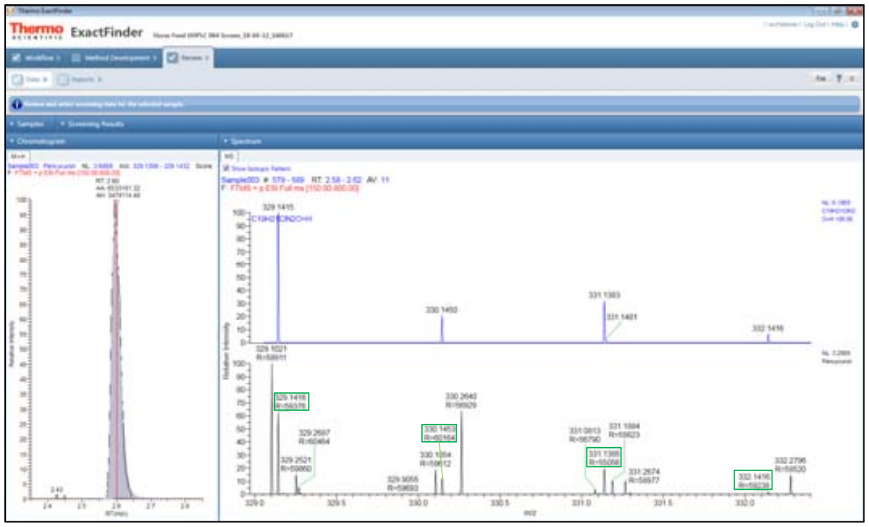
For the targeted search the same list of analytes as for quantitative analysis was applied. As confirmation criteria for targeted search retention time, isotopic pattern match, fragment search and library search were applied. The fragment information for the analytes of interest as well as the fragmentation spectra for the library search were taken from databases which are delivered with the ExactFinder software. Even at the lower end of the concentration range most components quantified could be easily confirmed on all four stages of confirmation (see Figure 8). The ExactFinder software provides easy and quick overview over the screening results with built-in reporting capabilities. It became clear very quickly that sufficient resolution is the key to success in full scan quantitation and screening. As shown in Figure 9, in complex samples like the ones analyzed here, most analyte signals are surrounded by numerous matrix signals. Only sufficient resolving power can ensure proper separation of analyte and matrix signals. This of course applies for the monoisotopic signals used for analysis as well as for the isotope signals used for confirmation. Looking at the fact that the peaks of interest show a resolution of close to 60,000, it becomes clear that significantly lower resolving power at this mass would lead to interference of these signals, causing significant mass shifts of the merged signals. As a consequence, this would lead to false negative results on the one hand, or it would require to widen up the extraction window, which would lower the selectivity of the analysis with the result of false positive results. Already the reduction of resolving power to 30,000 at this mass causes a shift of more than 2 ppm (results not shown).

FIGURE 8. Qualitative Result Grid



The general unknown screening carried out on the remaining peaks offers several options for automatic identification of the found peaks: database search, elemental composition determination based on isotopic pattern matching, library search and internet search. For the samples, roughly 15,000 components were detected which all went through the identification process. Database and library searches were carried out using built-in resources. Internet search was carried out using a selection of databases listed in the ChemSpider™ online search portal. A good number of additional contaminants could be identified, especially pesticides and a selection of Aflatoxins (results not shown).

FIGURE 9. Isotopic pattern match of Pencycuron. Green boxes mark the isotope signals surrounded by matrix signals



Conclusion

HR/AM analysis is a versatile method for residue analysis offering full quantitation capabilities in combination with unrestricted target and unknown screening options. Ultrahigh resolution delivered by the Orbitrap™ mass analyzer detection in the Exactive Plus mass spectrometer serves for reliability and selectivity comparable to established MS/MS techniques. Full UHPLC compatibility with uncompromised resolution and mass accuracy is shown with the new mass spectrometer.

References

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2. Improved analysis of biopharmaceutical samples using an MS-only Orbitrap mass spectrometer, Poster ThP28 #623

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Application Notebook

Drug Residues

Antibiotics

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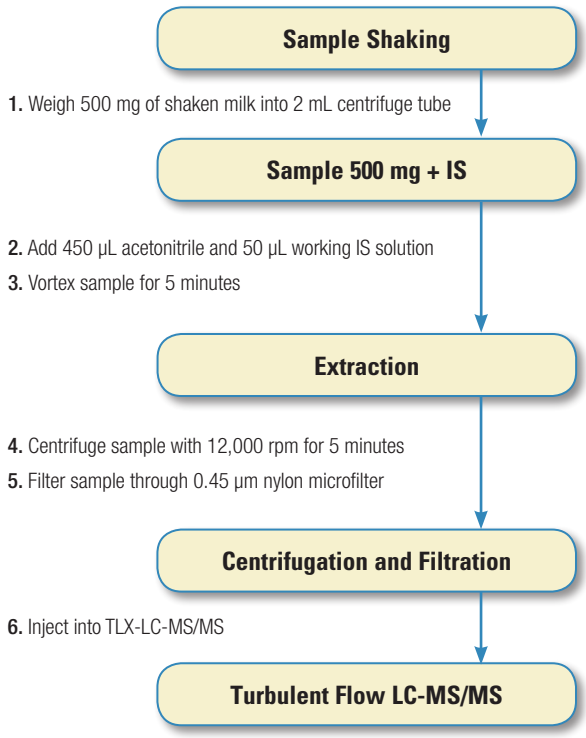
Multi-residue Automated Turbulent Flow Online LC-MS/MS Method for the Determination of Antibiotics in Milk

Katerina Bousova, Klaus Mittendorf, Thermo Fisher Scientific Food Safety Response Center, Dreieich, Germany

Method: 63551

Key Words
Transcend TLX, TSQ Quantum Access MAX, Antibiotics, Food Safety, Milk, TurboFlow Technology

1. Schematic of Method



2. Introduction

Antibiotics are a group of chemicals that are widely used in animal husbandry primarily for protection of animals from disease but also as growth promoters. The European Union (EU) has set maximum residue limits (MRL) for a variety of antibiotics in animal tissues, milk and eggs; suitable methods are required to be capable of detecting these residues at regulated levels. For the last decade laboratories have been using methods only for one class of antibiotics. However, an increasing number of multi-residue methods covering different classes of drugs are being developed as more efficient and cost-effective procedures.

For fast screening of antibiotics, microbiological or bioassay techniques are widely used. These techniques are not able to distinguish between the different types of antibiotics and provide only a semi-quantitative result for the total amount of drug residues. The big drawback is the incidence of false-negative or false-positive results because of low sensitivity and specificity. However, these screening assays are still very popular and widely used because of their cost-effectiveness and speed of analysis.

For quantitative analysis it is necessary to use instrumental techniques such as LC-MS/MS. This technique can also be used for screening, and provides much higher sensitivity and greater specificity. The use of LC-MS/MS for screening was described in a validated multi-residue screening method to monitor 58 antibiotics in milk[1].

Thermo SCIENTIFIC

This note describes a multi-residue confirmatory method for the quantitative determination of antibiotics in milk using turbulent flow chromatography coupled to LC-MS/MS. This method fulfills the increasing need for a cost-effective and fast method by employing Thermo Scientific TurboFlow technology (via the Thermo Scientific Transcend TLX) for online sample cleanup. This approach has already been applied in the development of a screening method for antibiotics in milk[2]. The method in this note is different due to the increased number of antibiotics detected as well as the inclusion of quantitative results.

3. Scope and Application

This online TLX-LC-MS/MS method can be applied to detect and quantify the presence of 36 compounds from 7 different classes of antibiotics (aminoglycosides, sulfonamides, macrolides, quinolones, tetracyclines, lincosamides and trimethoprim) in milk. This multi-residue method fulfills legislative requirements described in the EU Commission Decision 2002/657/EC[3].

4. Principle

This method uses turbulent flow chromatography for online cleanup of the sample. Sample concentration, cleanup and analytical separation are carried out in a single run using a TurboFlow™ column connected to an analytical LC column. Macromolecules are removed from the sample extract with high efficiency, while target analytes are retained on the column based on different chemical interactions. After application of a wash step, the trapped compounds are transferred onto the analytical LC column and separated conventionally. Before applying the sample extract onto the TurboFlow column, the sample is thoroughly mixed to evenly distribute the fat and then fortified with an internal standard, extracted with acetonitrile and centrifuged. Cleanup using the TLX system was optimized for maximum recovery of targeted compounds and minimal injection of co-extractives into the mass spectrometer. Identification of antibiotics is based on retention time, ion-ratios using multiple reaction monitoring (MRM) of characteristic transition ions, and quantification using matrix matched standards of one of the selected MRM ions.

5. Reagent List

		Part Number
5.1	Purified Water – Thermo Scientific Barnstead EASYpure II water system	D 13321
5.2	Methanol Fisher Scientific Optima, LC-MS grade	10767665
5.3	Water, LC-MS grade	10777404
5.4	Acetonitrile Optima® LC-MS grade	10001334
5.5	Isopropanol, HPLC grade	10674732
5.6	Acetone, HPLC grade	10131560
5.7	Formic acid, extra pure, >98%	10375990
5.8	Heptafluorobutyric acid, 99%	172800250
5.9	Ammonia, extra pure, 35%	10305170

6. Calibration Standards

6.1	Kanamycin	Sigma-Aldrich®
6.2	Amikacin	Sigma-Aldrich
6.3	Dihydrostreptomycin	Sigma-Aldrich
6.4	Streptomycin	Sigma-Aldrich
6.5	Lincomycin hydrochloride monohydrate	Sigma-Aldrich
6.6	Clindamycin hydrochloride	Sigma-Aldrich
6.7	Trimethoprim	Sigma-Aldrich
6.8	Josamycin	Sigma-Aldrich
6.9	Spiramycin	Sigma-Aldrich
6.10	Tilmicosin	Sigma-Aldrich
6.11	Tylosin tartrate	Sigma-Aldrich
6.12	Clarithromycin	Sigma-Aldrich
6.13	Erythromycin A dihydrate	Sigma-Aldrich
6.14	Oleandomycin phosphate dehydrate	Dr. Ehrenstorfer
6.15	Tylvalosin tartrate	FarmKemi®
6.16	Sulfadimethoxine	Sigma-Aldrich
6.17	Sulfadoxin	Sigma-Aldrich
6.18	Sulfaquinoxaline	Sigma-Aldrich
6.19	Sulfachlorpyridazine	Sigma-Aldrich
6.20	Sulfaclozine sodium	Dr. Ehrenstorfer
6.21	Oxytetracycline hydrochloride	Sigma-Aldrich
6.22	Doxycycline hyclate	Sigma-Aldrich
6.23	Marbofloxacin	Sigma-Aldrich
6.24	Ciprofloxacin	Sigma-Aldrich
6.25	Danofloxacin	Sigma-Aldrich
6.26	Enrofloxacin	Sigma-Aldrich
6.27	Difloxacin	Sigma-Aldrich
6.28	Oxolinic acid	Sigma-Aldrich
6.29	Flumequine	Sigma-Aldrich
6.30	Nalidixic acid	Sigma-Aldrich
6.31	Enoxacin	Sigma-Aldrich
6.32	Ofloxacin	Sigma-Aldrich
6.33	Lomefloxacin hydrochloride	Sigma-Aldrich
6.34	Norfloxacin	Sigma-Aldrich
6.35	Sarafloxacin hydrochloride trihydrate	Sigma-Aldrich
6.36	Cinoxacin	Sigma-Aldrich
Internal Standard		
6.37	Sulfaphenazole	Sigma-Aldrich

7. Standards Preparation

7.1 Stock Standard Solutions of Veterinary Drugs

Stock standard solutions (1000 µg/mL) are prepared individually by dissolving the analytes in methanol (lincosamides, macrolides, sulfonamides, tetracyclines and trimethoprim), in water (aminoglycosides) and in methanol with 2% 2M NH_4OH (quinolones). Solutions are stored at -20 °C.

7.2 Working Standard Solution

The working calibration standard solution containing 1000 µg/L is prepared by dilution of individual stock standard solutions with acetonitrile. Solution should be prepared fresh every time before using.

7.3 Stock Solution of Internal Standard

Stock standard solution of the internal standard (1000 µg/mL) is prepared by dilution of sulfaphenazole in methanol. Solution is stored at -20 °C.

7.4 Working Standard Solution of Internal Standard

The working standard solution of the internal standard (2000 µg/L) was prepared by dilution of stock standard solution (sulfaphenazole) with acetonitrile. Solution should be prepared fresh every time before using.

8. Aparatus

		Part Number
8.1	Turbulent flow chromatograph Transcend™ TLX-1 system	
8.2	Thermo Scientific TSQ Quantum Access MAX triple quadrupole mass spectrometer	
8.3	Fisher Science Education™ precision balance	02225102
8.4	Sartorius analytical balance	14557812
8.5	Barnstead™ EASYpure™ II water system	D 13321
8.6	Vortex shaker	14505141
8.7	Vortex universal cap	3205029
8.8	Accu-jet® pipettor	3140246
8.9	Thermo Scientific Heraeus Fresco 17 micro centrifuge	208590

9. Consumables

		Part Number
9.1	Thermo Scientific TurboFlow Cyclone P (50 × 0.5 mm) column	CH-953289
9.2	Thermo Scientific BetaSil phenyl-hexyl (50 × 2.1 mm, 3 µm) column	73003-052130
9.3	LC vials	3205111
9.4	LC caps	3151266
9.5	Thermo Scientific Pipette Finnpipette 100–1000 µL	3214535
9.6	Pipette Finnpipette™ 20–200 µL	3214534
9.7	Pipette Finnpipette 10–100 µL	3166472
9.8	Pipette Finnpipette 500–5000 µL	3166473
9.9	Pipette Finnpipette 1000–10,000 µL	3214536
9.10	Pipette holder	3651211
9.11	Pipette tips 0.5–250 µL, 500/box	3270399
9.12	Pipette tips 1–5 mL, 75/box	3270420
9.13	Pipette tips 100–1000 µL, 200/box	3270410
9.14	Pipette tips 20,000–10,000 µL, 40/box	3270425
9.15	Pipette Pasteur soda lime glass, 150 mm	FB50251
9.16	Pipette suction device	3120891
9.17	Spatula, 18/10 steel	3458179
9.18	Spatula, nylon	3047217
9.19	1 mL Single-use syringes	1066-4161
9.20	17 mm nylon syringe filter, 0.45 µm	F2513-1
9.21	Vial rack (2 mL)	12211001
9.22	Centrifuge plastic tube (2 mL)	3150968
9.23	Rack for 50, 15, 2 and 0.5 mL tubes	10321031
9.24	Pipette tips 20,000–10,000 µL, 40/box	3270425
9.25	Pipette Pasteur soda lime glass, 150 mm	FB50251

Glassware

		Part Number
9.26	Beaker, 50 mL	10527211
9.27	Beaker, 100 mL	10769541
9.28	Beaker, 25 mL	10683771
9.29	Volumetric flask, 25 mL	10107901
9.30	Volumetric flask, 10 mL	10406681
9.31	Volumetric flask, 5 mL	10770803
9.32	Volumetric flask, 100 mL	10675731
9.33	5 mL glass pipette	10179522

10. Procedure

10.1 Sample Preparation

The sample of milk is shaken vigorously by hand. The sample (500 mg) is then weighed into a 2 mL polypropylene tube. Working internal standard solution (50 µL) and acetonitrile (450 µL) are added to the sample. The sample is shaken for 5 min on the vortex and then centrifuged at 12000 rpm for 5 min for removal of protein. The supernatant is filtered through a nylon micro filter (0.45 µm pore size) directly into the LC vial and the sample is analyzed by TLX-LC-MS/MS.

10.2 The LC Conditions

LC analysis is performed on a Transcend TLX-1 System.

TurboFlow column: TurboFlow Cyclone P (50 × 0.5 mm)
Analytical column: BetaSil™ phenyl-hexyl (50 × 2.1 mm, 3 µm)
Total run time: 19 min
Mobile phases: A = 1mM heptafluorobutyric acid and 0.5% formic acid in water
B = 0.5% formic acid in acetonitrile/methanol (1/1)
C = 2% methanol in water
D = acetone/acetonitrile/isopropanol (20/40/40)

10.2.1 Injector Settings

Injector: Thermo Scientific Pal injector with 100 µL volume injection syringe
Tray temperature: 10 °C
Cleaning solvents for the autosampler:
Solvent 1: acetonitrile/water (20/80)
Solvent 2: acetone/acetonitrile/isopropanol – 20/40/40

- Pre clean with solvent 1 [steps]: 3
- Pre clean with solvent 2 [steps]: 3
- Pre clean with sample [steps]: 1
- Filling speed [µL/s]: 50
- Filling strokes [steps]: 1
- Injection port: LC Vlv1 (TX channel)
- Injection speed [µL/s]: 100
- Pre inject delay [ms]: 500
- Post inject delay [ms]: 500
- Post clean with solvent 1 [steps]: 5
- Post clean with solvent 2 [steps]: 5
- Valve clean with solvent 1 [steps]: 5
- Valve clean with solvent 2 [steps]: 5
- Injection volume: 25 µL

Sample concentration, cleanup and analytical separation are carried out in a single run using an automated online sample preparation system, which includes the Transcend TLX system and Thermo Scientific Aria operating software. First the sample is applied during the loading step by the loading pump onto the TurboFlow column. During the same step the macromolecules are removed, while the target analytes are retained on the TurboFlow column based on their different chemical interactions. In the next step, the trapped analytes are transferred with the help of an eluting pump, and an adequately strong solvent (eluent) in the loop onto the analytical LC column where the analytes are separated conventionally. While the separation on the analytical column is running, the loop is filled with the eluent, and the TurboFlow column is washed and conditioned to be ready for the injection of the next sample. The TLX and LC conditions are presented in Table 1.

The analytical column is conditioned during loading of the sample onto the TurboFlow column. The separation of the analytes on the analytical column is done by gradient (Table 1). To prevent the possibility of carry-over and cross contamination, the injection syringe as well as the injection valve are washed five times with cleaning solvent 1 (acetonitrile/water – 20/80) and cleaning solvent 2 (acetone/acetonitrile/isopropanol – 20/40/40) before and five times after injection.

Step			TurboFlow Column[a]					Cut-in Loop		Analytical LC Column[b]			
Description	**Start [min]**	**Time [s]**	**Flow [mL/min]**	**A%**	**B%**	**C%**	**D%**	**Tee**	**Loop**	**Flow [mL/min]**	**Step**	**A%**	**B%**
1. loading	0	60	1.5	–	–	100	–	–	out	0.3	Step	100	–
2. transferring	1	60	0.2	100	–	–	–	T	in	0.6	Step	100	–
3. washing	2	60	1.5	–	–	50	50	–	in	0.3	Step	100	–
4. washing	3	720	1.5	–	–	–	100	–	in	0.3	Ramp	5	95
5. filling loop	15	120	1.5	50	50	–	–	–	in	0.3	Step	5	95
6. equilibrating	17	120	1.5			100	–	–	out	0.3	Step	100	–

[a]mobile phases for the TurboFlow method:
A: 1 mM heptafluorobutyric acid + 0.5% formic acid in water,
B: 0.5% formic acid in acetonitrile / methanol – 1/1,
C: 2% methanol in water and
D: acetone/acetonitrile/isopropanol – 20/40/40

[b]mobile phases for the analytical method:
A: 1 mM heptafluorobutyric acid + 0.5% formic acid in water and
B: 0.5% formic acid in acetonitrile/methanol – 1/1

Table 1: Gradient program table for TurboFlow system coupled with an analytical column

10.3 Mass Spectrometric Conditions

Mass spectrometric analysis is carried out using a TSQ Quantum Access MAX™ triple quadrupole system. Data acquisition for quantification and confirmation are performed in MRM mode. All selected reaction monitoring (SRM) traces (parent, qualifier and quantifier ion) are individually tuned for each target analyte by direct injection of the individual working standard solution (10 mg/mL). Data acquisition and processing is performed using Thermo Scientific Xcalibur 2.1 software.

- Ionization mode: Electrospray (ESI)
- Scan type: SRM
- Polarity: positive ion mode
- Spray voltage [V]: 3500
- Ion sweep gas pressure [arb]: 0
- Vaporizer Temperature [°C]: 400
- Sheath gas pressure [arb]: 50
- Aux gas pressure [arb]: 10
- Capillary temperature [°C]: 370
- Collision gas pressure [mTorr]: 0
- Cycle time [s]: 0.6

Peak width: Q1/Q3 the full width of a peak at half its maximum height (FWHM) of 0.70 Da

The parameters for SRM analysis for targeted compounds and internal standards are displayed in the Table 2.

11. Calculations

11.1 Identification

Identification of the antibiotics is confirmed by the presence of transition ions (quantifier and qualifier) at retention times (± 2.5%) to the corresponding standards. In MRM mode, the measured peak area ratios for qualifier to quantifier ions should be in close agreement (according to EU Commission Decision 2002/657/EC) with the ratios of the standards, as shown in Table 3. The quantifier and qualifier ions were selected among the product ions produced by the fragmentation of the selected parent ion on the basis of the intensity. Representative chromatogram is shown in Figure 1.

11.2 Quantification

For quantification, internal standardization is used measuring peak area ratios for standards in matched matrixes. Sulfaphenazole is used as the internal standard for all target antibiotics. Calibration curves are plotted as the relative peak areas (analyte versus the corresponding standard) as a function of the compound concentration. The antibiotic concentration in the samples is determined from the equation:

$$c_a = \left(\frac{A_a}{A_{IS}} - b\right)/a$$

c_a – antibiotic concentration in µg/kg

A_a – peak area of the antibiotic

A_{IS} – peak area of internal standard

b – y-intercept

a – slope of the calibration curve.

12. Method Performance

The method was validated in-house according to the criteria specified in EU Commission Decision 2002/657/EC for a quantitative method. The validation parameters were determined by spiking blank milk at levels of 0.5, 1 and 1.5 times the MRL. For compounds without MRL, samples were spiked at 10, 50 and 100 µg/kg for clindamycin, macrolides and quinolones; at 100, 200 and 300 µg/kg for aminoglycosides; and 50, 100 and 150 µg/kg for doxycycline. The measured parameters were specificity, linear range, repeatability, accuracy, limit of detection (LOD), and limit of quantification (LOQ), decision limit (CCα), and detection capability (CCβ).

12.1 Samples and Quality Control Materials

For preparation of matrix-matched calibration standards and spiked samples for validation, skim milk with a fat content of 0.3% obtained from a local market was used. Before use, the milk was checked by repeated measurements to confirm that it was free of antibiotics. For determination of accuracy, a certified reference material ERM® – BB492 of partially skim milk containing a certified amount of oxytetracycline was used, obtained from the Institute for Reference Materials and Measurements (Geel, Belgium). The skim milk powder was reconstituted according to instructions.

12.2 Specificity

Using SRM, the specificity is confirmed based on the presence of the transition ions (quantifier and qualifier) at the correct retention times corresponding to those of the respective antibiotics. The measured peak area ratios of qualifier/quantifier are within the range defined in EU Commission Decision 2002/657/EC when compared to the standards (Table 3).

12.3 Linearity and Calibration Curve

The linearity of calibration curves is assessed over the range from 0-500 µg/kg for all target compounds. In all cases, the correlation coefficients of linear functions have to be >0.99. The calibration curves are created from 8 matrix-matched calibration standards that are injected in each batch in duplicate.

12.4 Precision

Precision (repeatability) of the method was determined using independently spiked blank samples at three different levels. In one day, the set of samples at three levels was measured with six repetitions. To determine between-day precision, two other sets at one level were measured with six repetitions over the next two days. The results for repeatability ranged from 4% to 28% (Table 4).

12.5 Accuracy

Method accuracy was determined using independently spiked blank samples at three different levels. Accuracy was evaluated by comparing found values with standard additions in spikes. Recovery values ranged between 78–120% (Table 5). Additionally, accuracy was established for oxytetracycline by analyzing the certified reference material ERM – BB492 which was partially skim milk powder. All measured concentrations of oxytetracycline were within the acceptable range (Table 6).

12.6 LOD and LOQ

LOD and LOQ are estimated following the IUPAC approach which consists of first analyzing the blank sample to establish noise levels and then estimating LODs and LOQs for signal/noise, 3 and 10 respectively. The values for milk are shown in Table 7, and in all cases, they are under the level of MRL for all analytes that have an assigned MRL.

12.7 Limit of Decision (CCα) and Limit of Capability (CCβ)

Both CCα and CCβ were established by the calibration curve procedure according to ISO 11843[4]. The blank material fortified at and below the MRL (for analytes with MRL) or at and above the lowest possible level (for analytes without MRL) in equidistant steps was used. The calculated values are shown in Table 7.

13. Conclusion

This in-house validated method enables quantification of 36 residues from seven different classes of antibiotics in milk. For all 36 compounds, only one extraction procedure was used although they come from different groups with widely varying polarities and solubilities. The use of turbulent flow chromatography combined with LC-MS/MS detection for analytical separation saves a significant amount of time in sample preparation and increases the sample throughput. The in-house validation results, according to IUPAC/AOAC harmonized protocol, reflected that this method is suitable for regulatory purposes. This method can be strongly recommended for use because it significantly speeds up sample analysis compared to traditional methods, is applicable for a large number of antibiotic residues and is convenient for regulatory purposes.

14. References

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4. ISO 11843: Capability of detection (1997)

Thermo Scientific Transcend TLX system coupled with the TSQ Quantum Access MAX triple quadrupole mass spectrometer

Analyte	Retention Time (min)	Molecular Weight	Precursor Ion	Quantifier Ion	Qualifier Ion	CE for Quantifier Ion (V)	CE for Qualifier Ion (V)	Tube Lens
Kanamycin	1.74	484.5	485.28	163.1	324.2	25	15	90
Amikacin	1.73	585.6	586.29	163.1	425.2	33	21	102
Dihydrostreptomycin	1.67	583.6	584.29	263.1	246.0	29	33	109
Streptomycin	1.66	581.6	582.26	263.1	221.1	30	40	141
Lincomycin	8.24	406.5	407.14	126.2	359.2	28	17	97
Clindamycin	10.75	425.0	425.14	126.2	377.2	28	18	86
Trimethoprim	8.84	290.3	291.1	230.1	261.1	23	24	93
Josamycin	12.55	828.0	828.43	174.0	109.1	30	34	18
Spiramycin	10.50	843.1	843.31	174.0	142.0	32	32	146
Tilmicosin	11.21	869.1	869.62	696.4	174.0	40	41	132
Tylosin	11.65	916.1	916.51	174.0	772.4	35	26	141
Clarithromycin	12.05	748.0	748.51	158.2	590.4	28	17	108
Erythromycin	11.40	733.9	734.46	576.4	158.0	17	28	103
Oleandomycin	11.13	687.8	688.44	544.4	158.1	14	25	106
Tylvalosin	13.25	1042.3	1042.64	109.1	174.0	41	37	133
Sulfadimethoxine	10.20	310.3	311.03	156.1	108.1	21	27	88
Sulfadoxin	9.17	310.3	311.04	156.0	108.1	18	26	88
Sulfaquinoxaline	10.40	300.3	301.04	156.0	92.2	17	28	92
Sulfachlorpyridazine	8.83	284.7	284.97	156.0	92.2	15	26	90
Sulfaclozine	10.14	284.7	284.96	92.2	108.1	29	26	87
Sulphafenazole	10.39	314.4	315.06	158.1	160.1	28	22	94
Oxytetracycline	8.94	460.4	461.11	426.1	337.0	18	29	93
Doxycycline	10.60	444.4	445.14	428.2	321.1	18	31	82
Marbofloxacin	8.90	362.4	363.11	72.3	320.1	22	14	97
Ciprofloxacin	9.17	331.3	332.08	288.1	314.1	18	22	89
Danofloxacin	9.26	357.4	358.11	340.1	314.2	24	16	99
Enrofloxacin	9.41	359.4	360.1	316.1	345.1	19	25	96
Difloxacin	9.90	399.4	400.1	356.1	299.0	19	28	98
Oxolinic acid	9.95	261.2	262.01	244.0	216.0	18	29	84
Flumequine	11.06	261.3	262.02	244.1	202.0	19	33	84
Nalidixic acid	10.77	232.2	233.04	215.1	187.1	15	25	77
Enoxacin	8.85	320.3	321.09	303.1	257.1	19	17	93
Ofloxacin	9.10	361.4	362.12	318.1	261.1	18	27	91
Lomefloxacin	9.34	351.3	352.1	265.1	308.1	23	15	100
Norfloxacin	9.07	319.3	320.07	276.1	302.1	16	22	94
Sarafloxacin	9.90	385.4	386.08	342.1	299.1	18	27	94
Cinoxacin	9.41	262.2	263.02	245.0	189.0	16	27	90

Table 2: LC-MS/MS parameters for selected reaction monitoring of analytes

Analyte	Ion Ratio (Std Mix)	Ion Ratio (Milk)
Kanamycin	0.74	0.64
Amikacin	0.65	0.61
Dihydrostreptomycin	0.26	0.26
Streptomycin	0.46	0.49
Lincomycin	0.24	0.30
Clindamycin	0.09	0.07
Trimethoprim	0.90	0.83
Josamycin	0.85	0.93
Spiramycin	0.26	0.23
Tilmicosin	1.19	0.72
Tylosin	0.20	0.22
Clarithromycin	0.48	0.57
Erythromycin	0.54	0.53
Oleandomycin	1.22	0.95
Tylvalosin	0.63	0.76
Sulfadimethoxine	0.11	0.14
Sulfadoxin	0.31	0.31
Sulfaquinoxaline	0.28	0.30
Sulfachlorpyridazine	0.27	0.29
Sulfaclozine	0.44	0.55
Sulphafenazole	0.89	0.88
Oxytetracycline	0.13	0.13
Doxycycline	0.04	0.05
Marbofloxacin	0.73	0.68
Ciprofloxacin	0.15	0.14
Danofloxacin	0.03	0.03
Enrofloxacin	0.53	0.48
Difloxacin	0.74	0.71
Oxolinic acid	0.05	0.06
Flumequine	0.22	0.28
Nalidixic acid	0.25	0.29
Enoxacin	0.02	0.01
Ofloxacin	0.73	0.79
Lomefloxacin	0.60	0.66
Norfloxacin	0.09	0.11
Sarafloxacin	0.28	0.34
Cinoxacin	0.30	0.35

Table 3: Ion ratios (Qual/Quant) in matrix and in standard mixture (the agreement between ion ratios should be within the permitted tolerance, which is defined in EU Commission Decision 2002/657/EC)

	RSD (%) – spiking level 2			Milk – RSD (%)		
Analyte	Day 1	Day 2	Day 3	Level 1 (µg/kg)	Level 2 (µg/kg)	Level 3 (µg/kg)
Kanamycin	17	12	21	24	17	10
Amikacin	13	11	23	23	13	16
Dihydrostreptomycin	14	21	22	10	14	6
Streptomycin	6	26	15	17	6	12
Lincomycin	16	19	15	17	16	9
Clindamycin	9	8	7	13	9	12
Trimethoprim	10	9	11	19	10	8
Josamycin	7	11	12	12	7	13
Spiramycin	15	6	7	21	15	6
Tilmicosin	4	6	6	10	4	12
Tylosin	4	9	7	12	4	9
Clarithromycin	8	12	8	28	8	12
Erythromycin	4	6	6	13	4	9
Oleandomycin	13	7	10	21	13	9
Tylvalosin	6	11	6	18	6	6
Sulfadimethoxine	14	9	4	9	14	6
Sulfadoxin	9	9	8	5	9	5
Sulfaquinoxaline	12	9	9	12	12	16
Sulfachlorpyridazine	11	14	16	24	11	5
Sulfaclozine	17	18	16	11	17	13
Oxytetracycline	26	27	17	28	26	15
Doxycycline	8	9	9	12	8	8
Marbofloxacin	9	17	15	15	9	11
Ciprofloxacin	6	9	10	8	6	10
Danofloxacin	11	18	23	18	11	11
Enrofloxacin	9	6	6	14	9	10
Difloxacin	11	10	18	16	11	5
Oxolinic acid	20	12	13	21	20	14
Flumequine	4	6	4	9	4	9
Nalidixic acid	10	12	6	10	10	10
Enoxacin	14	14	16	26	14	14
Ofloxacin	14	8	5	12	14	14
Lomefloxacin	19	14	22	8	19	11
Norfloxacin	10	15	11	13	10	14
Sarafloxacin	11	9	11	14	11	16
Cinoxacin	15	15	14	24	15	11

Table 4: Method intermediate precision as RSD (%) – 1 level – 3 sets with 6 replicates in 3 days and method repeatability expressed as RSD (%) and measured at 3 levels every time with 6 replicates

	Spiking levels			Milk – REC (%)		
Analyte	**Level 1 (µg/kg)**	**Level 2 (µg/kg)**	**Level 3 (µg/kg)**	**Level 1**	**Level 2**	**Level 3**
Kanamycin	75	150	225	104	78	102
Amikacin	100	200	300	90	101	111
Dihydrostreptomycin	100	200	300	111	104	111
Streptomycin	100	200	300	106	101	107
Lincomycin	75	150	225	74	81	96
Clindamycin	10	50	100	95	96	104
Trimethoprim	10	50	100	90	84	101
Josamycin	10	50	100	95	99	106
Spiramycin	100	200	300	84	102	94
Tilmicosin	25	50	75	98	95	101
Tylosin	25	50	75	103	95	100
Clarithromycin	10	50	100	98	108	94
Erythromycin	20	40	60	86	80	95
Oleandomycin	10	50	100	109	95	97
Tylvalosin	10	50	100	109	104	104
Sulfadimethoxine	50	100	150	93	97	104
Sulfadoxin	50	100	150	100	98	108
Sulfaquinoxaline	50	100	150	99	97	107
Sulfachlorpyridazine	50	100	150	96	99	109
Sulfaclozine	50	100	150	88	92	116
Oxytetracycline	50	100	150	108	98	105
Doxycycline	50	100	150	96	85	100
Marbofloxacin	37.5	75	112.5	102	98	120
Ciprofloxacin	50	100	150	94	85	106
Danofloxacin	15	30	45	94	80	95
Enrofloxacin	50	100	150	90	86	104
Difloxacin	10	50	100	102	99	99
Oxolinic acid	10	50	100	120	91	89
Flumequine	25	50	75	103	95	102
Nalidixic acid	10	50	100	100	90	98
Enoxacin	10	50	100	97	87	96
Ofloxacin	10	50	100	86	89	107
Lomefloxacin	10	50	100	87	81	99
Norfloxacin	10	50	100	95	90	103
Sarafloxacin	10	50	100	97	90	100
Cinoxacin	10	50	100	92	84	98

Table 5: Recoveries (%) for spiked samples of milk at 3 different spike levels (6 replicates)

Sample	Concentration [found] (µg/kg)
CRM 1	105
CRM 2	95
CRM 3	112
CRM 4	97

Table 6: Results of certified reference material – milk ERM-BB492 – oxytetracycline – c = 101 ± 11 µg/kg

Analyte	LOD (µg/kg)	LOQ (µg/kg)	MRL (µg/kg)	CCα (µg/kg)	CCβ (µg/kg)
Kanamycin	15.0	45.0	150	168	186
Amikacin	25.0	75.0	–	54	108
Dihydrostreptomycin	25.0	75.0	200	242	285
Streptomycin	25.0	75.0	200	231	261
Lincomycin	3.0	10.0	150	175	199
Clindamycin	0.3	1.0	–	8	16
Trimethoprim	1.5	5.0	–	6	12
Josamycin	0.3	1.0	–	11	23
Spiramycin	0.3	1.0	200	232	264
Tilmicosin	0.3	1.0	50	62	74
Tylosin	1.0	3.0	50	62	73
Clarithromycin	0.3	1.0	–	4	7
Erythromycin	0.3	1.0	40	56	72
Oleandomycin	0.3	1.0	–	7	13
Tylvalosin	0.3	1.0	–	8	17
Sulfadimethoxine	0.3	1.0	100[a]	116	131
Sulfadoxin	0.3	1.0	100[a]	117	133
Sulfaquinoxaline	1.5	5.0	100[a]	113	126
Sulfachlorpyridazine	15.0	45.0	100[a]	121	141
Sulfaclozine	15.0	45.0	100[a]	118	137
Oxytetracycline	15.0	45.0	100	117	134
Doxycycline	3.0	10.0	–	10	20
Marbofloxacin	1.5	5.0	75	85	96
Ciprofloxacin	0.3	1.0	100	115	129
Danofloxacin	0.3	1.0	30	36	42
Enrofloxacin	0.3	1.0	100	111	123
Difloxacin	0.3	1.0	–[b]	4	8
Oxolinic acid	0.3	1.0	–[b]	4	9
Flumequine	0.3	1.0	50	55	61
Nalidixic acid	0.3	1.0	–[c]	5	11
Enoxacin	0.3	1.0	–	5	11
Ofloxacin	0.3	1.0	–	6	12
Lomefloxacin	0.3	1.0	–	8	16
Norfloxacin	0.3	1.0	–	7	13
Sarafloxacin	0.3	1.0	–	5	11
Cinoxacin	1.5	5.0	–	13	27

Table 7: LOD and LOQ, MRL, CCα and CCβ for antibiotics in milk

[a] – *Expressed in form of sum-MRLs of all sulfonamides.*

[b] – *Banned for use in milk-producing animals.*

[c] – *No authorization in veterinary medicine.*

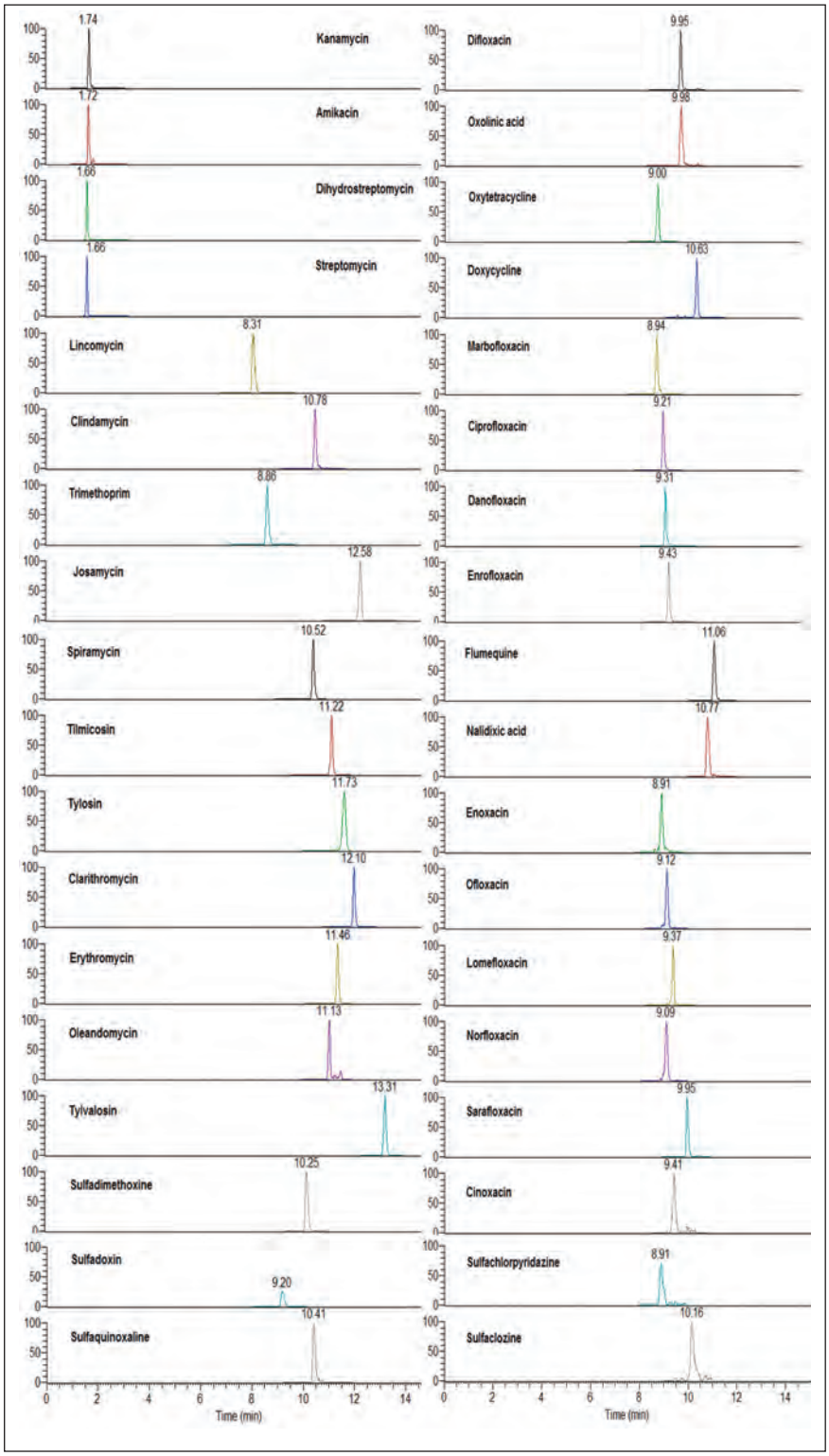

Figure 1: MRM chromatogram of all 36 antibiotics in spiked milk

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Application Note: 473

Measurement of Chloramphenicol in Honey Using Automated Sample Preparation with LC-MS/MS

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Key Words

- Aria TLX-1
- TurboFlow technology
- TSQ Vantage mass spectrometer
- Food safety

Introduction

Chloramphenicol (CAP) (Figure 1) is a bacteriostatic antimicrobial previously used in veterinary medicine. CAP has been found to be potentially carcinogenic, which makes it an unacceptable substance for use with any food-producing animals, including honey bees. The United States, Canada, and the European Union (EU), as well as many other countries, have completely banned the usage of CAP in the production of food. The EU has set a minimum required performance level (MRPL) for CAP in food of animal origin at a level of 0.3 µg/kg[1].

Currently sample preparation for the detection of CAP in honey by liquid chromatography-mass spectrometry (LC-MS/MS) involves complex offline extraction methods such as solid phase extraction, QuEChERS, or liquid/liquid extraction, all of which require additional sample concentration and reconstitution in an appropriate solvent. These sample preparation methods are time-consuming, often taking 2 hours or more per sample, and are more vulnerable to variability due to errors in manual preparation. To offer a high sensitivity (low ppbs) CAP detection method and timely, automated analysis of multiple samples, our approach is to use the Thermo Scientific Aria TLX-1 system powered by TurboFlow™ automated sample preparation technology coupled to the detection capabilities of a high-sensitivity Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer.

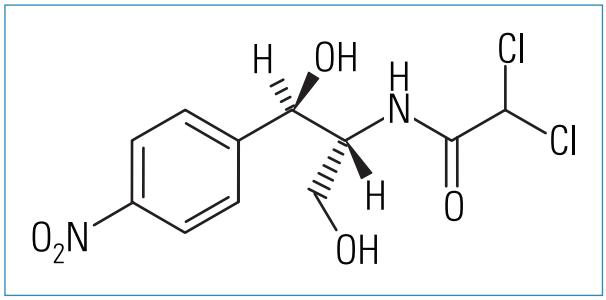

Figure 1: Chemical structure of chloramphenicol

Goal

Develop a quick, automated sample preparation, LC-MS/MS method for chloramphenicol (CAP) in honey by negative ion heated electrospray ionization (H-ESI) using a deuterated internal standard (CAP-d5).

Experimental

Sample Preparation

Organic wildflower honey used in this analysis for the preparation of blanks, QCs, and standards was obtained from a local supermarket. CAP was obtained from Sigma-Aldrich, US (Fluka) and CAP-d5 (100 µg/mL in acetonitrile) from Cambridge Isotope Labs, Inc. (Andover, MA, USA). A CAP working solution was prepared in 1:1 methanol/water at 100 ng/mL. The honey was diluted by adding 30 mL of purified water to 10 g of honey (1:3 w/v). CAP standards and QC standards were serially diluted to the target concentrations with 1:3 honey/water containing 250 pg/mL CAP-d5 as an internal standard. Target standard concentrations ranged from 0.024 µg/kg to 1.5 µg/kg. Four samples of honey obtained internationally and one sample obtained from a local grocery store were analyzed as "samples" and prepared by dissolving 5 g of honey in 15 mL of purified water. The internal standard was added to a final concentration of 250 pg/mL. The injection volume was 25 µL.

Method

The honey extract clean-up was accomplished using the Thermo Scientific TurboFlow method run on an Aria™ TLX-1 LC system using a TurboFlow Cyclone polymer-based extraction column. Simple sugars were un-retained and moved to waste during the loading step, while the analyte of interest was retained on the extraction column. This was followed by organic elution to a Thermo Scientific Hypersil GOLD end-capped silica-based C18 reversed-phase analytical column and gradient elution to a TSQ Vantage™ triple stage quadrupole MS with a H-ESI source. CAP precursor *m/z* 321 → 257, 152, and 194 high resolution selective reaction monitoring (H-SRM) transitions were monitored in the negative ionization mode. The 257 *m/z* product ion for CAP was used for quantitation and the 152 and 194 *m/z* product ions were used as confirmation. Precursor 326 *m/z* → 157 *m/z* and 262 *m/z* H-SRM transitions were monitored for CAP-d5. The total LC-MS/MS method run time was about 5 minutes.

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Aria TLX-1 System Parameters

Columns
Thermo Scientific TurboFlow Cyclone column (0.5 x 50 mm)
Thermo Scientific Hypersil GOLD (3 x 50 mm, 3 µm particle size)
The analytical column was kept at 30 °C

Mobile Phases

Loading Pump	
Mobile Phase A:	0.02% Acetic Acid (aq)
Mobile Phase B:	Methanol
Mobile Phase C:	1:1:1 Acetone/Acetonitrile/Isopropanol with 0.3% Formic Acid

Elution Pump	
Mobile Phase A:	0.02% Acetic Acid (aq)
Mobile Phase B:	Methanol

Mass Spectrometer Parameters

MS analysis was carried out on a TSQ Vantage™ triple stage quadrupole mass spectrometer. The MS conditions were as follows:

Ion Polarity:	Negative ion mode
Spray Voltage:	1000 V
Vaporizer Temperature:	526 °C
Capillary Temperature:	225 °C
Sheath Gas Pressure (N_2):	60 units
Auxiliary Gas Pressure (N_2):	35 units
Ion Sweep Gas Pressure (N_2):	0.500 units
Scan Type:	H-SRM
Chrom Filter Peak Width:	5.0 s
Collision Gas Pressure:	1.1 mTorr
Declustering Voltage:	0 V
Scan Width:	0.002 *m/z*
Scan Time:	0.200 s
Q1 Resolution:	0.200 Da FWHM
Q3 Resolution:	0.700 Da FWHM
S-Lens (*m/z* 321):	65 V
Collision Energy (*m/z* 321 > 257):	12 V

The entire experiment was controlled by Aria operating software 1.6.2. The data was processed with Thermo Scientific LCQUAN 2.5.6 quantitative software using Thermo Scientific Xcalibur 2.0.7 SP1 data system software.

Results and Discussion

Figure 2 shows comparison chromatography of CAP and CAP-d5 in 1:3 honey/water matrix pre-blank, at the lower limit of quantitation (LLOQ), the upper limit of quantitation (ULOQ), and a post-high standard blank. By comparing pre- and post- blanks, it is clearly indicated that the carryover level has been minimized by using TurboFlow technology. Matrix-matched calibration standards of CAP showed a linear response at greater than two orders of magnitude with $r^2 = 0.9944$ (Figure 3). All %CVs (n=3) were less than 19% for the LLOQ and less than 8% for all other points of the curve. Internal standard % relative standard deviation (RSD) was less than 9%. Chloramphenicol was not detected in any of the honey samples obtained internationally nor from the US. The calculation of actual concentrations of CAP in honey was based on a density of honey equal to 1.367 kg/L[2]. Signal suppression effects were examined by comparing the recovery of CAP and CAP-d5 in three neat (purified water) standards (0.19, 0.38, and 1.5 µg/kg) with their counterparts in 1:3 honey/water. The average recovery corrected by the internal standard was 80.9%, 96.0%, and 92.1% for 0.19, 0.38, and 1.5 µg/kg respectively.

Table 1 highlights current published results of detection methods for chloramphenicol in honey by LC-MS compared to the results of this study. Sample preparation in our study was between 7 and 24 times faster (estimated) than the three current alternative methods discussed. The LC-MS method run time was equal to or as much as four times faster. The limit of detection (LOD) was between 5.7 and 20 times lower than those that reported their LOD. Finally, the LLOQ was between 3.7 and 27 times lower.

Conclusion

A quick, automated online extraction, LC-MS/MS method has been developed here that is sensitive enough to detect 0.023 µg/kg (LOD) and quantify 0.047 µg/kg (LLOQ) of CAP in honey for screening purposes. This is significantly lower than the MRPL of 0.3 µg/kg (ppb) set by the EU. This method eliminates the need for time-consuming sample preparation procedures such as solid phase extraction, QuEChERS, and liquid-liquid extraction. Dilution with water to reduce sample viscosity is the only pretreatment required. The LC-MS/MS method run time is 5 minutes, and the sample throughput can be improved by multiplexing on an Aria TLX-4 system.

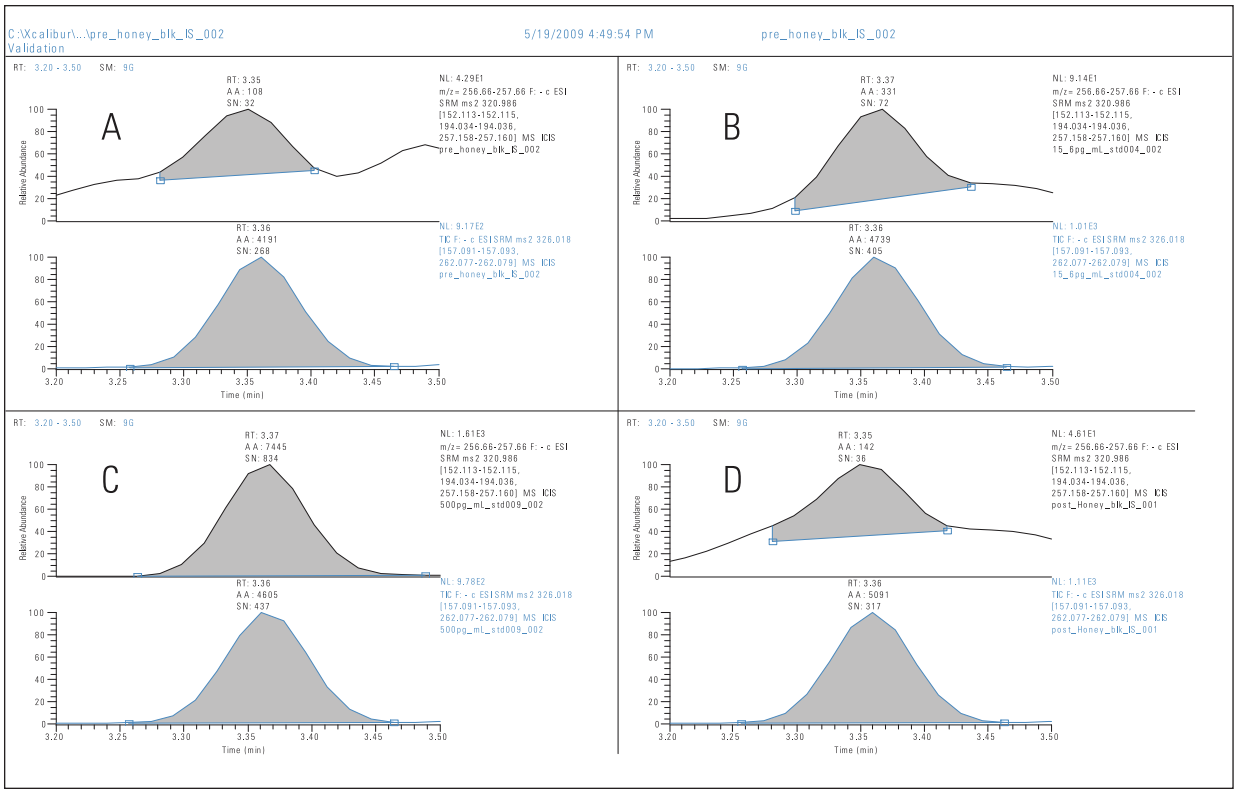

Figure 2: Chromatography comparison of CAP SRM *m/z* 257 transition (upper traces) and CAP-d5 (lower traces) in Pre-Blank Honey Matrix (panel A), at LLOQ of 0.047 µg/kg (panel B), at ULOQ of 1.5 µg/kg (panel C), and in Post-High Standard Blank (panel D)

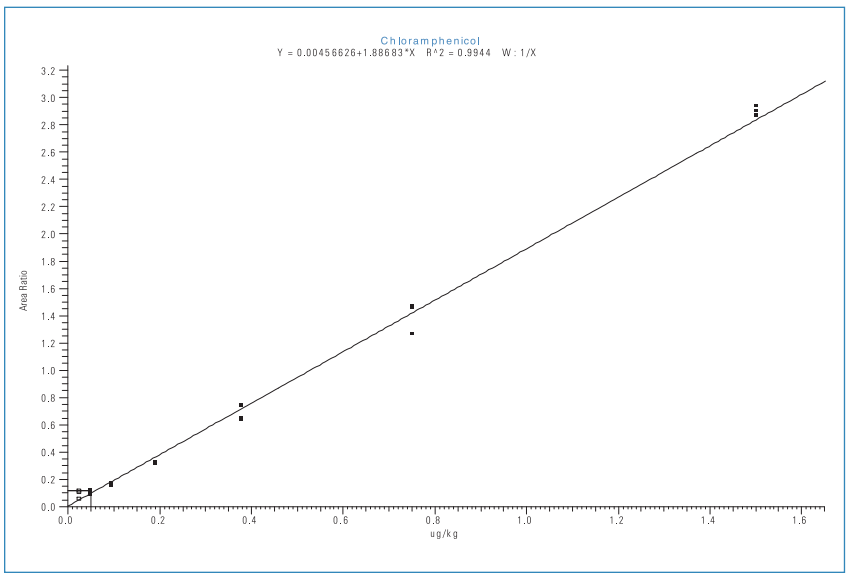

Figure 3: Linear regression curve of CAP in honey:water matrix standards based on area ratio with internal standard CAP-d5 (1/X weighting) showing linearity over two orders of magnitude using the TurboFlow method.

	TurboFlow Method (on-line)	SPE (off-line)	QuEChERS (off-line)	Liquid/Liquid Extraction (off-line)
Sample prep time (min)	5	120 (estimated)	35 (estimated)	60 (estimated)
LC/MS Method Runtime (min)	5	12	5 or 10	20
Sample Extraction	TurboFlow Cyclone column (0.5 X 50 mm) on-line LC extraction	J.T. Baker 500-mg Bakerbond C18 SPE	"Modified" QuEChERS	Hexane/Acetonitrile Extraction, Evaporation, and Redissolution
Analytical Column	Thermo Scientific Hypersil GOLD, 3 x 50 mm, 3 um	Macherey-Nagel Nucleosil 100-5 C18 HD column, 2 X 70 mm	100 mm × 4.6 mm RP-18e monolithic column (Merck USA) or a 4.6 mm × 250 mm, 5-µm particle, XDB conventional column (Agilent)	Phenomenex C18 Luna column, 2 X 150 mm, 5 µm
Injection volume (µL)	25	10	10	20
HPLC system	TLX-1	HP 1100 Binary pump	Agilent 1100 Binary pump	Agilent 1100 Binary pump
Detector	Thermo Scientific TSQ Vantage Triple Quadrupole MS	Micromass QuattroMicro Triple Quadrupole MS	ESI-MS (Not specified)	Applied Biosystems API 3000 Triple Quadrupole MS
LLOD (µg/kg)	0.024	0.2	Not specified.	0.11
LLOQ (µg/kg)	0.047	0.5	0.20	0.14
Reference	Data presented herein.	2004 by Ortelli et al. (3)	2006 by Pan et al. (4)	2007 by Rodziewicz et al. (5)

Table 1: Comparison of CAP detection in honey by TurboFlow method with current sample prep alternatives.

References and Acknowledgements

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Application Note: 464

Multi-class Antibiotic Screening of Honey Using Online Extraction with LC-MS/MS

Catherine Lafontaine, Yang Shi, Francois A. Espourteille, Thermo Fisher Scientific, Franklin, MA, USA

Key Words

- Aria TLX-1 System
- TSQ Quantum Ultra
- Food Testing
- TurboFlow Method

Introduction

Antibiotics are commonly used in bee hives to control bacterial disease in honey bees. Use of these antibiotics requires caution to prevent persistent residues from occurring in food-grade honey. If antibiotic residues are present in sufficient quantities, allergic reactions and bacterial resistance can develop.

Many countries now monitor antibiotic residues in honey. LC-MS/MS is currently a common analytical approach for the quantification of antibiotic contamination in honey. Sample preparation for LC-MS/MS analysis can be time and labor intensive, often involving pH modification, hydrolysis, liquid-liquid extraction, solid phase extraction, solvent evaporation, and pre-concentration. A quick, comprehensive, online screening liquid chromatography (LC) method using a Thermo Scientific Aria TLX system powered by Thermo Scientific TurboFlow technology has been developed here to monitor several classes of antibiotics.

Goal

To develop a broad, generic, automated LC-MS/MS method for screening multi-class antibiotics in honey.

Experimental

Method Information

Residues representative of several classes of antibiotics (macrolides, sulfonamides, aminoglycosides, and tetracyclines) were extracted from wildflower honey using buffer containing ethylenediaminetetraacetic acid (EDTA). The extract cleanup was accomplished using a TurboFlow™ method involving two TurboFlow columns placed in tandem, a mixed mode anion exchange column and a polar polymer-based column. Simple sugars were un-retained and moved to waste during the loading step while the analytes of interest were retained on the extraction column set. This was followed by organic elution to an end-capped silica-based mixed mode reversed phase analytical column (Thermo Scientific BETASIL Phenyl/Hexyl) and gradient elution to a Thermo Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer with a Heated Electrospray Ionization (H-ESI) source operating in positive selective reaction monitoring (SRM) mode. The total LC-MS/MS method run time was less than 18 minutes. Positive SRM transitions and other MS parameters for individual analytes are shown in Table 1.

Sample Preparation

A McIlvaine/0.1 M EDTA buffer was used as a 1:1 w:v (gram weight honey: milliliter volume buffer) diluent for wildflower honey, the testing matrix in this study.[1] A stock solution was prepared for sulfapyridine, sulfathiazole, tilmicosin, tylosin, oxytetracycline, and erythromycin in 3:1 methanol:water at 100 µg/mL. Additionally, one was prepared for doxycycline, demeclocycline, streptomycin, and dihydrostreptomycin in water at 100 µg/mL. These stocks were each spiked into 1:1 honey:buffer matrix and used as a spiking stock to make a set of calibration standards and quality controls (QCs). All blanks, standards, and QCs were prepared and analyzed in polypropylene vials. Injection volumes were 0.050 mL.

Aria™ TLX-1: TurboFlow Method Parameters

TurboFlow Cyclone MAX and TurboFlow Cyclone-P columns (0.5 × 50 mm), in-tandem
BETASIL Phenyl/Hexyl column, 100 × 3 mm, 3 µm
Aria operating system 1.6.2 software

Loading Pump Mobile Phases

Mobile Phase A: 1.0% formic acid in water
Mobile Phase B: 0.1% formic acid in acetonitrile
Mobile Phase C: 10 mM ammonium acetate in water, pH 9
Mobile Phase D: 50 mM ammonium acetate in methanol with 0.1% formic acid

Elution Pump Mobile Phases

Mobile Phase A: 1 mM NFPA*, 0.5% formic acid, 0.04% TFA** in water
Mobile Phase B: 0.5% formic acid, 0.04% TFA in 1:1 methanol:acetonitrile

**NFPA is nonafluoropentanoic acid.*

***TFA is trifluoroacetic acid.*

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TSQ Quantum Ultra™ Mass Spectrometer (MS) Parameters	
Ion Polarity:	Positive
Ionization Source:	H-ESI
Spray Voltage:	4000 V
Vaporizer Temperature:	400 °C
Capillary Temperature:	370 °C
Sheath Gas Pressure (N_2):	30 arb units
Auxiliary Gas Pressure (N_2):	60 arb units
Ion Sweep Gas Pressure (N_2):	0.0 arb units
Skimmer Offset:	5 V (for streptomycin), 0 V (for all others)
Collision Pressure:	1.2 mTorr
Chrom Filter Peak Width:	8.0 s
Scan Type:	SRM
Scan Time:	0.020 s
Scan Width:	0.100 *m/z*
Peak Width Q1 Da. (FWHM):	0.700
Peak Width Q3 Da. (FWHM):	0.700

Results and Discussion

Results were packaged using Thermo Scientific LCQUAN 2.5.6 data quantitation software and included subtraction of background due to the presence of a few endogenous analytes in the store-bought honey. Figure 1 shows a representative chromatogram of the 10 analytes at 100 ng/mL in 1:1 honey/buffer. Matrix-matched calibration standards showed linear response of two orders of magnitude ($r^2 > 0.99$) for all of the analytes investigated (Table 2). All %CVs (n=3) were less than 19% for the lower limit of quantifications (LLOQ) and less than 8% for all other points of the curves. Figure 2 shows an LCQUAN™ representative linear regression using oxytetracycline as an example. QC sample variability was determined by processing and analyzing three replicates of each of four QC samples (2, 50, 100, and 500 ng/mL). All % RSDs were lower than 7% (except for erythromycin which was below 15%). Data was not used for any QC level that fell below the analyte's determined LLOQ.

Structural Class	Analyte	Precursor Ion	Product Ions
Sulfonamides	Sulfapyridine	250.1	156.0 (Q), 108.1 (C), 92.1 (C)
	Sulfathiazole	256.1	156.1 (Q), 92.0 (C), 108.1 (C)
Tetracyclines	Doxycycline	445.3	154.0 (Q), 428.5 (C)
	Oxytetracycline	461.2	426.4
	Demeclocycline	465.2	448.4 (Q), 430.4 (C)
Aminoglycosides	Streptomycin	582.3	263.0 (Q), 246.0 (C), 203.9 (C), 221.0 (C)
	Dihydrostreptomycin	584.3	262.9 (Q), 245.9 (C)
Macrolides	Erythromycin	734.5	576.2
	Tilmicosin	869.6	696.3
	Tylosin	916.5	772.3

NOTE: (Q)=Quantification Ion; (C)=Confirmation Ion.

Table 1: The 10 analytes and their positive SRM transition ions

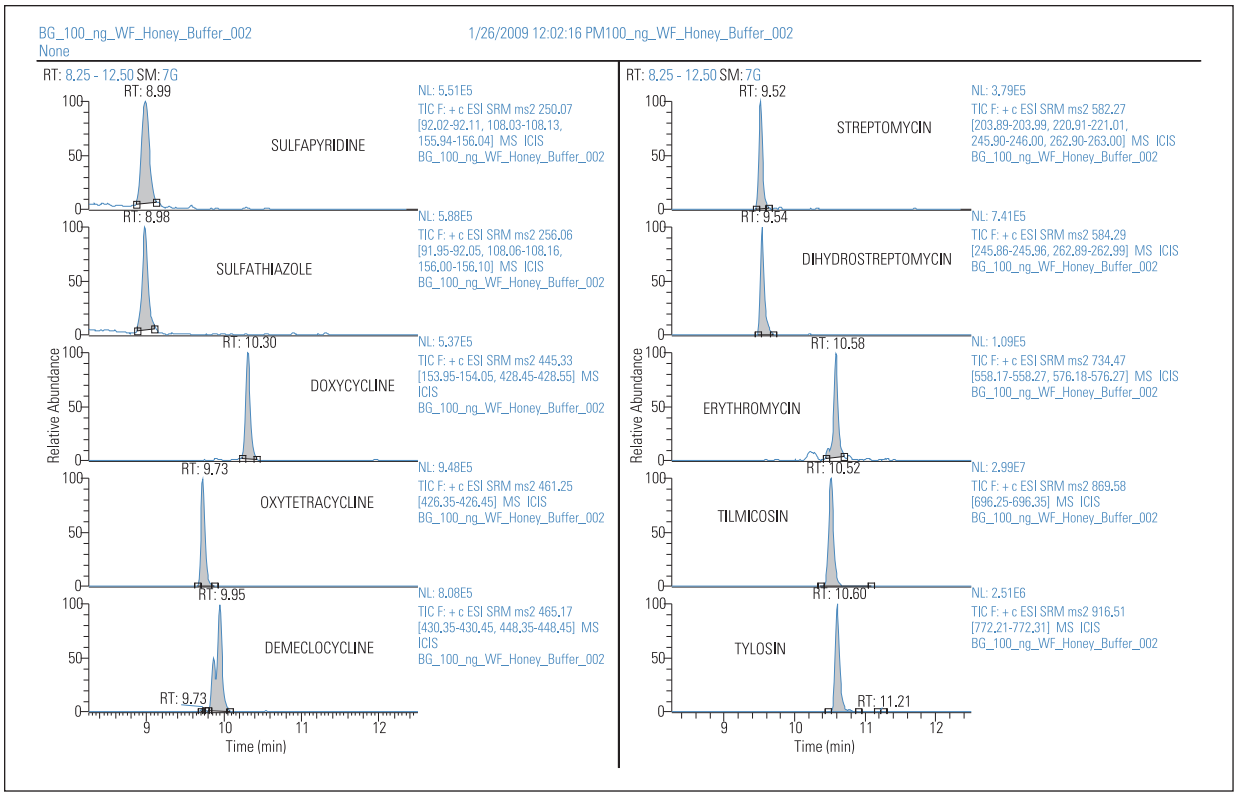

Figure 1: Example chromatogram of 100 ng/mL calibration standard in 1:1 honey/buffer

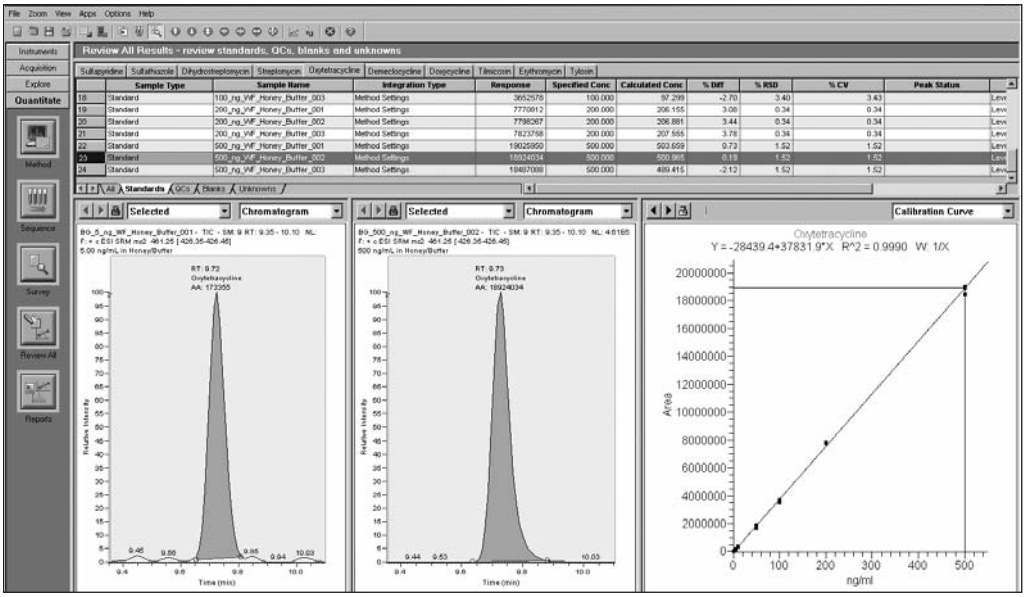

Figure 2: LCQUAN view of oxytetracycline calibration curve and LLOQ (left window) vs. ULOQ (right window) chromatograms

Analyte	R^2 (1/x weighting)	Dynamic Range* (ng/mL)**	Limit of Detection (ng/mL)	Percent Carryover (%)
Sulfapyridine	0.9980	50-500	10.0	8.95
Sulfathiazole	0.9988	50-500	10.0	5.46
Doxycycline	0.9990	10-500	5.0	10.80
Oxytetracycline	0.9990	5-500	2.0	11.70
Demeclocycline	0.9996	10-500	5.0	18.70
Streptomycin	0.9960	50-500	10.0	11.60
Dihydrostreptomycin	0.9980	50-500	10.0	6.47
Erythromycin	0.9877	50-500	10.0	1.16
Tilmicosin	0.9917	2-50	0.5	16.80
Tylosin	0.9958	10-100	5.0	13.70

**Based on analysis using 8 point standard curve (ng/mL): 0.500, 2.00, 5.00, 10.0, 50.0, 100, 200, & 500.*
***The level of carryover was included in the determination of dynamic range (kept to 20% or less).*

Table 2: Calibration curve statistics of the 10 analytes

Conclusion

During the honey quality monitoring process, it is always an analytical challenge to deal with a large number of antibiotics belonging to different classes. This often requires multiple LC-MS methods. In this study, a novel application was introduced using dual online TurboFlow extraction columns with different chemistries. The results reveal that this design facilitates the separation and quantification of all of the representative compounds in the complex honey matrix. Sample preparation time was minimal, requiring only the addition of a buffer to reduce sample viscosity. These factors enabled a broad screening for antibiotic contaminants to be performed quickly for a given sample, thus increasing sample throughput.

Additionally, multiplexing with an Aria TLX-4 system would further reduce total LC-MS/MS run time four-fold and enable screening of 12 samples per hour. Future work could involve screening a larger range of antibiotic and environmental contaminants and lowering detection limits for all analytes thus combining a screening method with accurate quantification.

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Application Note: 407

Simple and Rapid Analysis of Chloramphenicol in Milk by LC-MS/MS

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Key Words

- TSQ Quantum Access™
- Accela™ High Speed LC System
- Antibiotic
- Food Residue Analysis
- SRM

Introduction

Chloramphenicol (CAP) is a broad-spectrum antibiotic with historical veterinary uses in all major food-producing animals (see Figure 1 for structure). It has serious side effects on humans that may cause aplastic anemia, and the suspected carcinogen effect is also thought to be dose independent. Consequently, chloramphenicol has been banned for use in all food-producing animals by the European Union (EU), USA and Canada. A minimum required performance limit (MRPL) for chloramphenicol determination was recently set by the EU at 0.3 µg/kg (ppb) in all foods of animal origin, such as meat, seafood, egg, milk, honey, etc. However, residues of CAP at unacceptable levels continue to be found in food imports, as a result of illegal use in some countries to mask the poor hygiene conditions of animal-raising farm and to augment animal growth. The growing food safety concerns call for intensive surveillance of chloramphenicol in food products.

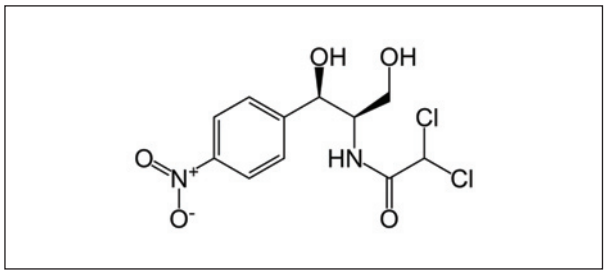


Figure 1: Structure of chloramphenicol

Analysis of residual of chloramphenicol in foodstuff is challenging because of the complicated sample matrices and stringent requirements of both low quantitation limit (<0.3 ppb) and method validation. The technique of liquid chromatography separation followed by tandem mass spectrometry detection, LC-MS/MS, is the technology of choice because of its sensitivity and specificity. A sample cleanup process is generally required to remove the sample matrix prior to the LC-MS/MS run. Typically, this involves the costly and labor-intensive solid phase extraction (SPE) and/or liquid-liquid extraction (LLE) procedures.

In this work, we report a simple sample preparation procedure involving only the acetonitrile protein precipitation and dilution to extract the CAP from milk, followed by a high-speed LC separation and detection by a triple quadrupole mass spectrometer operated in selected reaction monitoring (SRM) mode. The sample preparation is simple, fast, and inexpensive, and the method exceeds the sensitivity and specificity requirements for both screening and confirmatory assays. Validation according to the European Commission Decision 2002/657/EC has also been performed.

Goal

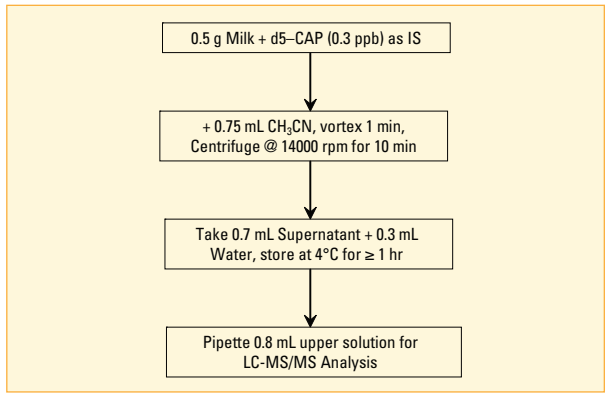
To develop a simple, rapid, and sensitive LC-MS/MS method for analyzing chloramphenicol in milk. The method should be suitable for both screening and confirmatory purposes.

Experimental Conditions

Sample Preparation

Standards and Regents: Chloramphenicol (98%) was purchased from Sigma-Aldrich (St. Louis, MO) and d5-chloramphenicol (100 µg/mL in acetonitrile) as internal standard from Cambridge Isotope Lab (Andover, MA). Regent grade water, acetonitrile and methanol were from Thermo Fisher Scientific (Pittsburgh, PA).

Procedures:



Chromatography Conditions

HPLC Module: Accela High Speed LC System (Thermo Scientific, San Jose, CA)
Column: Hypersil GOLD™ 50 mm × 2.1 mm and 1.9 µm particle size (Thermo Scientific, Bellefonte, PA)
Column Temperature: Ambient

Mobile Phase:	A: Methanol	B: Water
Gradient:	Time (min)	A%
	0.0-0.6	5%
	2.3	100%
	2.35-3.0	5%

Flow Rate: 500 µL/min
Injection Volume: 20 µL (with loop)

Mass Spectrometer Conditions

Mass Spectrometer: TSQ Quantum Access triple stage quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA)
Source: ESI-, 3000 V
Sheath Gas: 45 unit
Auxiliary Gas: 10 unit
Capillary Temperature: 300 °C
Source CID: -7 V
Q1 and Q3 Peak Width (FWHM): 0.7 Da
Scan Time: 0.1 s
Collision Gas: Ar (1.5 mTorr)
SRM Transitions: 3 SRMs for CAP, 1 SRM for d5-CAP (see Table 1)

	Precursor Ion	Product Ion (Collision Energy)
CAP (M^-H^-)	320.93	152 (17)* 257 (15) 194 (16)
d_5-CAP (M^-H^-)	326.93	157 (17)*

* Product ion used for quantitation

Table 1: SRM transitions for CAP and d5-CAP (IS)

Results and Discussion

Sample Preparation: A major goal for the method development in this study is to avoid using the labor intensive and time-consuming SPE or LLE procedures as in literatures. In curret work, the proteins from milk were removed with acetonitrile precipitation at ratio of 1.5:1 (v/v Acetonitrile:Milk), followed by dilution with water, which is necessary for gradient chromatographic separation. At such ratio, protein removal was not complete, trace amount of precipitates of proteins appeared after the sample was stored at 4 °C for some time. Thus, the supernatant was taken for LC-MS/MS analysis after the sample was stored at 4 °C for ≥ 1 hr.

Choice of Quantitation and Qualification Ions: Three product ions were chosen to give an Identification Points (IPs) of 5.5 to meet the requirement of ≥ 4.0 IPs by the Decision 2002/657/EC for confirmatory assay of the prohibited substances such as CAP. The *m/z* 152 was chosen as quantitation ion, the *m/z* 257 and 194 as confirmation ions, consisting with those reported in literatures.

The results of relative ion abundance measured at various concentrations are given Table 2. Both relative ion abundance ratios of 257/152 and 194/152 meet the requirements set by Decision 2002/657/EC.

Note that we found the 321>257 transition is more likely subjected to matrix interferences in many other cases of different matrices, thus if two SRM transitions need to be selected (4.0 IPs) for the method, 321>152 and 321>194 are preferred.

Method Performance: Figure 2 shows representative SRM chromatograms for a blank and 0.05 µg/kg spiked milk samples. As shown, with high-speed LC, each chromatographic run is only 3 min, allowing high throughput for screening assay. All three SRM traces for CAP at 0.05 µg/kg spiked samples can be well quantified. Note that the 0.05 µg/kg spiked in milk is equivalent to 0.46 pg injected on column by assuming a full recovery.

It should also be noted that with the high-speed LC separation of only 3 min for each chromatographic run, the CAP peak width (at 10% above baseline) is as narrow as 6 s. Under current MS acquisition conditions, there are 13-14 points across each peak, enough for maintaining a well-defined peak shape for accurate integration.

A representative calibration curve from standards prepared in milk is shown in Figure 3. Good linearity from 0.05 to 1.0 µg/kg with correlation coefficient of R^2= 0.9954 (Weighting factor W = 1/X) was obtained.

Table 3 shows excellent recovery and within-laboratory reproducibility of the method (at four different days).

Decision Limit (CCα) and Detection Capability (CCβ): According to Decision 2002/657/EC, the Decision Limit CCα is the minimum CAP concentration at which a sample is really non-compliant with an error probability of 1% (α = 0.01), and the Detection Capability (CCβ) is the minimum amount of CAP that can be quantified and confirmed with an error probability of 5% (β = 0.05).

Two methods can be used for calculating the CCα according to the Decision. One is to use the S/N ratio of 3:1 of blank samples, similar to those for estimation of limit of detection. The other is to use the intercept of calibration curve at low levels and the within-laboratory reproducibility. The former method does not work well for LC-MS/MS because the very low background (noise count ~0) of SRM chromatogram often yields unrealistically low values for CCα. Thus we use the latter approach by using cali-

CAP Spiked Level (µg/kg)	Relative Ion Abundance of 257/152			Relative Ion Abundance of 194/152		
	Mean *n*=6	%RSD *n*=6	Tolerance by Decision 2002/657/EC	Mean *n*=6	%RSD *n*=6	Tolerance by Decision 2002/657/EC
0.05	96%	16%	20%	26%	21%	25%
0.15	92%	7.6%		28%	25%	
0.30	93%	15%		31%	15%	
0.50	90%	3.4%		31%	17%	

Note: Relative ion abundance values were calculated by relative peak area ratios

Table 2: Relative ion abundances at various CAP concentrations in milk and the associated tolerances required by Decision 2002/657/EC

bration data of (0.05-0.15-0.30 µg/kg) to obtain the Y-intercept and its standard deviation, $SD_{Y\text{-intercept}}$,

$$CC\alpha = Y\text{-intercept} + 2.33 * SD_{Y\text{-intercept}}$$

Similarly, the CCβ can be calculated from CCα and the standard deviation of 20 measurement of samples spiked at CCα level. Here the latter term is approximated with the within-laboratory reproducibility data of 0.15 µg/kg spiking level, thus,

$$CC\beta = CC\alpha + 1.64 * SD_{0.15\ \mu g/kg}$$

Where $SD_{0.15\ \mu g/kg}$ is the within-laboratory reproducibility (in standard deviation) of the 0.15 µg/kg in Table 3. The calculated values of CCα and CCβ are 0.087 µg/kg and 0.12 µg/kg, respectively.

CAP Spiking Level (µg/kg)	Within-laboratory Reproducibility (n = 20)		
	Mean (%)	SD (µg/kg)	%RSD
0.05	97%	0.0065	14%
0.15	101%	0.020	13%
0.30	104%	0.037	11%
0.50	94%	0.042	8.0%

Table 3: Recovery and Reproducibility Data

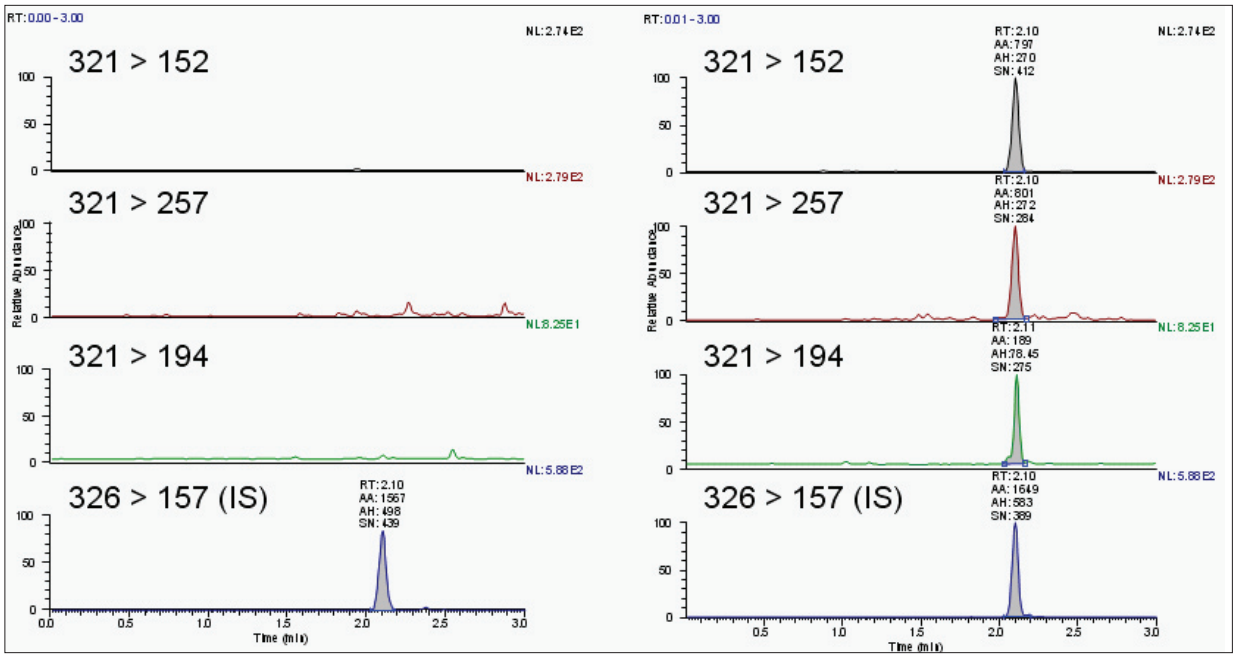


Figure 2: SRM chromatograms for milk blank and 0.050 µg/kg spiked milk samples

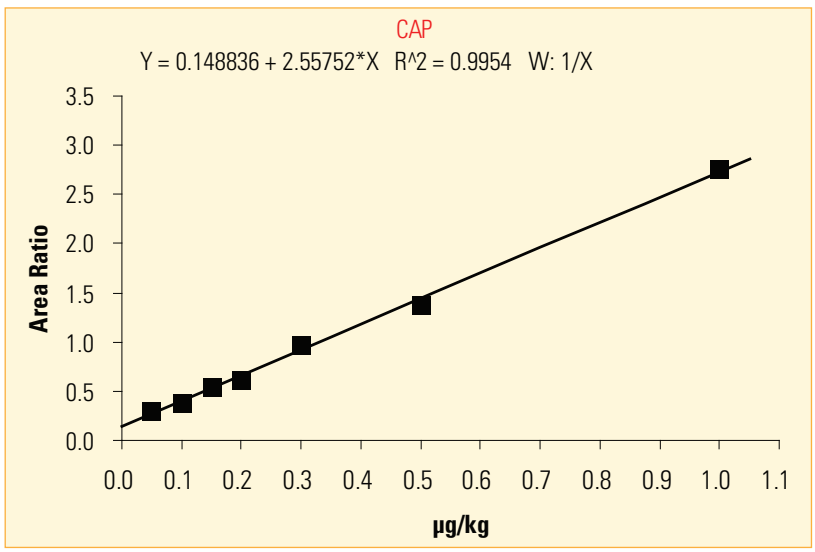


Figure 3: Calibration of CAP in milk

Conclusions

A simple, rapid and sensitive method for analysis of CAP in milk by LC-MS-MS has been developed and validated. The sample preparation by protein precipitation and dilution is very simple to perform and avoids the use of SPE or LLE. With the high-speed Accela LC coupled to a triple quadruple TSQ Quantum Access, each analytical run is as short as 3 min. The method can be used for the purposes of both high-throughput screening and rapid confirmatory assays.

For screening assay, the method can detect < 0.050 µg/kg CAP in milk. For confirmatory assay, the method validated according to Decision 2002/657/EC gives a CCα =0.087 µg/kg and CCβ = 0.12 µg/kg, both below the MRPL of 0.3 µg/kg.

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Application Note: 358

Highly Selective Detection and Identification of Nitrofuran Metabolites in Honey Using LC-MS/MS

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Key Words

- TSQ Quantum Discovery™
- LC-MS/MS
- Quantitative Analysis

Introduction

Nitrofurans are broad-spectrum antibiotics used to treat bees and other animals with bacterial infections. As a result of dosing bees with these antibiotics their metabolites are sometimes found in honey. Female rats given nitrofurans in both low and high doses have exhibited increased incidences of ovarian granulose cell tumors. In the same study, newborn mice showed an increased incidence of pulmonary papillary adenomas.[2] As a result, nitrofurans have been banned from use in food-producing animals in Australia (1993), the European Union (EU) (1995), The Philippines (2001), the United States (2002), Brazil (2002), Thailand (2002), and other countries.

Several studies have shown that animals rapidly metabolize nitrofurans within a few hours so detection has focused on the metabolites rather than the native drug.[3] The metabolites accumulate in tissue where they are stable and can be analyzed long after the nitrofurans have been administered. The EU has established a harmonized minimum required performance limit (MRPL) for the detection of residues of nitrofurans at one part per billion (ppb). Some European laboratories have been working to a detection limit of 0.3 ppb for several of the nitrofuran metabolites.[4] The EU recently tightened its inspection policy for food imports after nitrofuran residues were found in shrimp, fish, and poultry imports. This significantly reduced the volume of those imports.

As a result, food exporting countries are required to detect nitrofuran metabolites at very low levels. There are several challenges that must be overcome. The first is that honey, as well as other food products, provides a complex matrix which increases the difficulty of sample preparation. Second, efficient chromatography is critical in order to provide good separation of the various metabolites from each other and any contaminants that might be present. The third and most important requirement is a very high level of sensitivity and linearity in the mass spectrometer in order to achieve the required high levels of accuracy in quantifying the metabolites. This note describes LC-MS methods developed on the Thermo Scientific TSQ Quantum Discovery by the Food Science Laboratories and Eidomet in Argentina in cooperation with Thermo Fisher Scientific. The method exceeds all current detection limits as set by the EU.

Goal

To demonstrate the ability to accurately quantitate nitrofuran metabolites at levels as low as 0.3 ppb in a matrix consisting of honey using the TSQ Quantum Discovery.

Experimental Conditions

In this study, 2 grams of honey samples were treated with four nitrofuran metabolites, AOZ, AMOZ, SEM, and AHD.[5] An aliquot of honey was dissolved in 125 mM HCl and derivatized with 2-nitrobenzaldehyde and the mixture was shaken for 3 minutes. The slurry was then incubated at 37°C in a water bath for 17 hours. The mixture was then cooled to room temperature and neutralized by adding potassium phosphate to adjust the pH to approximately 7.0. Ethyl acetate was added to the slurry and it was hand shaken for 2 minutes and centrifuged for 15 minutes. The organic phase was collected into a tube, water added, and the mixture centrifuged. The supernatant was evaporated to dryness under a stream of nitrogen. The dry residue was reconstituted with water and injected into a filter cartridge. The residue was then washed with water and eluted with hexane, then analyzed by LC-MS/MS.

HPLC was performed on a Thermo Scientific Surveyor™ MS Pump with a Surveyor Autosampler from Thermo Fisher Scientifc. A 100 × 2.1 mm, 3 µm HPLC column was used. The mobile phase consisted of A (water containing 0.05% acetic acid) and B (methanol containing 0.05% acetic acid). The gradient program was as follows: 0-3.0 min. 90% A 10% B; 3.0-5.0 min. 85% A 15% B; 5.0 to 10.0 min. 75% A 25% B; 10.0-15.0 min. 70% A 30% B; 15.0-17 min. 65% A 35% B; 15-17 min. 65% A 35% B; 17.0-21.0 min. 60% A 40% B; and 21.0-25.0 min. 90% A 10% B.

Sample analysis was performed on a TSQ Quantum Discovery mass spectrometer. The 0-13.4 min segment eluted AMOZ and d5-AMOZ while the 13.4 to 25 min segment eluted AOZ, d4-AOZ, SEM, and AHD. Samples were analyzed using positive electrospray ionization (ESI) in SRM mode. The scan width was 0.002 *m/z* and the scan time was 0.1 second. A peak width of 0.7 FWHM was used in both Q1 and Q3. Argon was used as the collision gas at a pressure of 1.5 mTorr.

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Analyte	Precursor Ion	Product Ion	Collision Energy
AMOZ	335	262	15
	335	291	10
d5-AMOZ	340	296	10
AOZ	236	78	15
	236	134	8
d4-AOZ	240	134	8
SEM	209	166	6
	209	192	5
AHD	249	104	16
	249	134	7

Table 1: Transition reactions for MS/MS

The nitrofuran metabolites were quantified with five calibration standards at nominal concentrations of 0.63 ppb, 1.04 ppb, 2.09 ppb, 4.17 ppb, and 8.34 ppb. The area ratio of the analyte versus the quality control (QC) samples was plotted against the standard concentration ratio. The linearity of the MS response was determined by calculating the relative standard deviation (RSD) of the average of results from a series of injections at a single concentration. The method is generally considered to be validated if the RSD is less than 15%. The recovery ratio was also calculated by injecting a known amount of sample and comparing it with the calculated amount delivered by the detector.

Results and Discussion

Tables 2 through 5 report the results obtained with the standard and QC samples in honey for each metabolite. Note that for clarity purposes, all areas reported in the tables are divided by 1000. The concentrations of the QC samples were calculated by comparing the area to the standards. Then the relative standard deviation for each set of QC samples was calculated. The RSD for AOZ ranged from 6.7% at 0.3 ppb to 2.7% at 4 ppb. The RSD for AMOZ ranged from 3.60% at 0.3 ppb to 2.50% at 4 ppb. The RSD for AHD ranged from 9.0% at 0.3 ppb to 2.9% at 4 ppb. The RSD for SEM ranged from 8.3% at 0.3 ppb to 4.2% at 4 ppb.

Equation	
Y = 0.5424 X + 0.0973	R^2 = 0.9998

IDENT. LEVEL	NOMINAL CONC. (ppb)	AREA AOZ	AREA ISTD (d4-AOZ)	AREA RATIO
Std 0,6 ppb	0.63	217.3	559	0.39
Std 1 ppb	1.04	344.1	524.3	0.66
Std 2 ppb	2.09	671.2	532.3	1.26
Std 4 ppb	4.17	1286.3	567.2	2.27
Std 8 ppb	8.34	2601.2	580.6	4.48

IDENT. LEVEL	AREA AOZ	AREA ISTD (d4-AOZ)	AREA RATI	SPECIFIED CONC. (ppb)	CALCULATED CONC. (ppb)	Diff %	RSD %	RECOVERY %
QC-4ppb	1609.6	356.5	4.52	4.172	4.204	0.77%		100.8
QC-4ppb	1728.1	381.1	4.53	4.172	4.222	1.20%		101.2
QC-4ppb	1849.5	392.6	4.71	4.172	4.253	1.94%	2.7%	101.9
QC-4ppb	1743.8	378.7	4.60	4.172	4.155	-0.41%		99.6
QC-4ppb	1919.4	389.7	4.93	4.172	4.451	6.69%		106.7
QC-2ppb	864.7	372.9	2.32	2.086	2.111	1.20%		101.2
QC-2ppb	912.9	370.1	2.47	2.086	2.252	7.96%		108.0
QC-2ppb	789.1	358.8	2.20	2.086	1.938	-7.09%	5.5%	92.9
QC-2ppb	924.2	377.7	2.45	2.086	2.166	3.84%		103.8
QC-2ppb	912.2	375.5	2.43	2.086	2.150	3.07%		103.1
QC-1ppb	436.6	356.7	1.22	1.043	1.068	2.40%		102.4
QC-1ppb	466.2	390.9	1.19	1.043	1.038	-0.48%		99.5
QC-1ppb	431.3	359.3	1.20	1.043	1.017	-2.49%	4.7%	97.5
QC-1ppb	477.7	358.6	1.33	1.043	1.138	9.11%		109.1
QC-1ppb	451.5	346.4	1.30	1.043	1.112	6.62%		106.6
QC-0.5ppb	258.3	376.1	0.69	0.521	0.556	6.72%		106.7
QC-0.5ppb	271.1	388.1	0.70	0.521	0.567	8.83%		108.8
QC-0.5ppb	260.2	366.4	0.71	0.521	0.565	8.45%	7.4%	108.4
QC-0.5ppb	228.8	372.2	0.61	0.521	0.477	-8.45%		91.6
QC-0.5ppb	249.0	380.6	0.65	0.521	0.513	-1.54%		98.5
QC-0.3ppb	162.6	357.3	0.46	0.313	0.335	7.03%		107.0
QC-0.3ppb	146.9	369.5	0.40	0.313	0.280	-10.54%		89.5
QC-0.3ppb	145.5	341.0	0.43	0.313	0.304	-2.88%	6.7%	97.1
QC-0.3ppb	171.5	412.6	0.42	0.313	0.293	-6.39%		93.6
QC-0.3ppb	156.0	369.4	0.42	0.313	0.300	-4.15%		95.8

Table 2: AOZ data

Equation	
Y = 0.6123 X + 0.0413	R^2 = 1.0000

LEVEL	CONC. (ppb)	AMOZ	ISTD (d5-AMOZ)	AREA RATIO
Std 0,6 ppb	0.6	760.3	1799.0	0.40
Std 1 ppb	1.0	1320.8	1936.8	0.69
Std 2 ppb	2.0	2811.8	2170.0	1.37
Std 4 ppb	4.0	5582.3	2166.6	2.68
Std 8 ppb	8.0	11265.8	2231.2	5.32

IDENT. LEVEL	AREA AMOZ	AREA ISTD (d5-AMOZ)	AREA RATIO	SPECIFIED CONC. (ppb)	CALCULATED CONC. (ppb)	Diff %	RSD %	RECOVERY %
QC-4ppb	8814.0	1700.1	5.18	4.000	4.200	5.00%		105.0
QC-4ppb	6866.9	1376.6	4.99	4.000	4.040	1.00%		101.0
QC-4ppb	9431.2	1894.0	4.98	4.000	3.938	-1.55%	2.50%	98.5
QC-4ppb	7438.6	1464.6	5.08	4.000	4.017	0.43%		100.4
QC-4ppb	7235.0	1439.7	5.03	4.000	3.974	-0.65%		99.4
QC-2ppb	3117.9	1246.4	2.50	2.000	2.000	0.00%		100.0
QC-2ppb	3951.3	1511.5	2.61	2.000	2.100	5.00%		105.0
QC-2ppb	3617.4	1477.9	2.45	2.000	1.914	-4.30%	3.45%	95.7
QC-2ppb	3214.0	1263.0	2.54	2.000	1.991	-0.45%		99.6
QC-2ppb	4164.8	1662.7	2.50	2.000	1.959	-2.05%		98.0
QC-1ppb	1742.6	1357.0	1.28	1.000	1.010	1.00%		101.0
QC-1ppb	2178.9	1687.2	1.29	1.000	1.020	2.00%		102.0
QC-1ppb	1864.6	1476.0	1.26	1.000	0.967	-3.30%	2.90%	96.7
QC-1ppb	2307.9	1853.7	1.25	1.000	0.952	-4.80%		95.2
QC-1ppb	2281.8	1781.8	1.28	1.000	0.981	-1.90%		98.1
QC-0.5ppb	1153.4	1710.4	0.67	0.500	0.517	3.40%		103.4
QC-0.5ppb	850.2	1345.0	0.63	0.500	0.482	-3.60%		96.4
QC-0.5ppb	1067.5	1602.7	0.67	0.500	0.489	-2.20%	4.63%	97.8
QC-0.5ppb	830.8	1331.6	0.62	0.500	0.455	-9.00%		91.0
QC-0.5ppb	1067.2	1640.8	0.65	0.500	0.477	-4.60%		95.4
QC-0.3ppb	548.5	1289.1	0.43	0.300	0.314	4.67%		104.7
QC-0.3ppb	485.0	1139.9	0.43	0.300	0.314	4.67%		104.7
QC-0.3ppb	559.5	1309.3	0.43	0.300	0.298	-0.67%	3.60%	99.3
QC-0.3ppb	727.5	1719.1	0.42	0.300	0.295	-1.67%		98.3
QC-0.3ppb	529.3	1264.3	0.42	0.300	0.291	-3.00%		97.0

Table 3: AMOZ data

Equation	
Y = 0.1396 X + 0.0174	R^2 = 0.9955

IDENT. LEVEL	NOMINAL CONC. (ppb)	AREA AHD	AREA ISTD (d4-AOZ)	AREA RATIO
Std 0,6 ppb	0.61	70.9	559.0	0.127
Std 1 ppb	1.02	112.2	524.3	0.210
Std 2 ppb	2.03	200.3	532.3	0.380
Std 4 ppb	4.06	416.4	567.2	0.730
Std 8 ppb	8.13	797.2	580.6	1.370

IDENT. LEVEL	AREA AHD	AREA ISTD (d4-AOZ)	AREA RATIO	SPECIFIED CONC. (ppb)	CALCULATED CONC. (ppb)	Diff %	RSD %	RECOVERY %
QC-4ppb	436.6	356.5	1.22	4.063	3.585	-11.76%		88.2
QC-4ppb	461.5	381.1	1.21	4.063	3.543	-12.80%		87.2
QC-4ppb	397.0	392.6	1.01	4.063	3.559	-12.40%	2.9%	87.6
QC-4ppb	408.3	378.7	1.08	4.063	3.799	-6.50%		93.5
QC-4ppb	402.6	389.7	1.03	4.063	3.638	-10.46%		89.5
QC-2ppb	222.6	372.9	0.60	2.034	1.683	-17.26%		82.7
QC-2ppb	224.4	370.1	0.61	2.034	1.711	-15.88%		84.1
QC-2ppb	179.1	358.8	0.50	2.034	1.726	-15.14%	2.6%	84.9
QC-2ppb	194.8	377.7	0.52	2.034	1.785	-12.24%		87.8
QC-2ppb	181.8	375.5	0.48	2.034	1.672	-17.80%		82.2
QC-1ppb	123.0	356.7	0.34	1.017	0.919	-9.64%		90.4
QC-1ppb	126.8	390.9	0.32	1.017	0.857	-15.73%		84.3
QC-1ppb	100.6	359.3	0.28	1.017	0.941	-7.47%	5.5%	92.5
QC-1ppb	90.6	358.6	0.25	1.017	0.843	-17.11%		82.9
QC-1ppb	98.3	346.4	0.28	1.017	0.954	-6.19%		93.8
QC-0.5ppb	78.7	376.1	0.21	0.508	0.509	0.20%		100.2
QC-0.5ppb	70.6	388.1	0.18	0.508	0.425	-16.34%		83.7
QC-0.5ppb	57.7	366.4	0.16	0.508	0.502	-1.18%	9.2%	98.8
QC-0.5ppb	50.8	372.2	0.14	0.508	0.427	-15.94%		84.1
QC-0.5ppb	52.6	380.6	0.14	0.508	0.433	-14.76%		85.2
QC-0.3ppb	44.5	357.3	0.12	0.305	0.251	-17.70%		82.3
QC-0.3ppb	45.8	369.5	0.12	0.305	0.249	-18.36%		81.6
QC-0.3ppb	35.4	341.0	0.10	0.305	0.309	1.31%	9.0%	101.3
QC-0.3ppb	39.6	412.6	0.10	0.305	0.281	-7.87%		92.1
QC-0.3ppb	35.1	369.4	0.10	0.305	0.278	-8.85%		91.1

Table 4: AHD data

Equation	
Y = 0.1396 X + 0.0174	R^2 = 0.9955

IDENT. LEVEL	NOMINAL CONC. (ppb)	AREA AHD	AREA ISTD (d4-AOZ)	AREA RATIO
Std 0,6 ppb	0.61	70.9	559.0	0.127
Std 1 ppb	1.02	112.2	524.3	0.210
Std 2 ppb	2.03	200.3	532.3	0.380
Std 4 ppb	4.06	416.4	567.2	0.730
Std 8 ppb	8.13	797.2	580.6	1.370

IDENT. LEVEL	AREA AHD	AREA ISTD (d4-AOZ)	AREA RATIO	SPECIFIED CONC. (ppb)	CALCULATED CONC. (ppb)	Diff %	RSD %	RECOVERY %
QC-4ppb	436.6	356.5	1.22	4.063	3.585	-11.76%		88.2
QC-4ppb	461.5	381.1	1.21	4.063	3.543	-12.80%		87.2
QC-4ppb	397.0	392.6	1.01	4.063	3.559	-12.40%	2.9%	87.6
QC-4ppb	408.3	378.7	1.08	4.063	3.799	-6.50%		93.5
QC-4ppb	402.6	389.7	1.03	4.063	3.638	-10.46%		89.5
QC-2ppb	222.6	372.9	0.60	2.034	1.683	-17.26%		82.7
QC-2ppb	224.4	370.1	0.61	2.034	1.711	-15.88%		84.1
QC-2ppb	179.1	358.8	0.50	2.034	1.726	-15.14%	2.6%	84.9
QC-2ppb	194.8	377.7	0.52	2.034	1.785	-12.24%		87.8
QC-2ppb	181.8	375.5	0.48	2.034	1.672	-17.80%		82.2
QC-1ppb	123.0	356.7	0.34	1.017	0.919	-9.64%		90.4
QC-1ppb	126.8	390.9	0.32	1.017	0.857	-15.73%		84.3
QC-1ppb	100.6	359.3	0.28	1.017	0.941	-7.47%	5.5%	92.5
QC-1ppb	90.6	358.6	0.25	1.017	0.843	-17.11%		82.9
QC-1ppb	98.3	346.4	0.28	1.017	0.954	-6.19%		93.8
QC-0.5ppb	78.7	376.1	0.21	0.508	0.509	0.20%		100.2
QC-0.5ppb	70.6	388.1	0.18	0.508	0.425	-16.34%		83.7
QC-0.5ppb	57.7	366.4	0.16	0.508	0.502	-1.18%	9.2%	98.8
QC-0.5ppb	50.8	372.2	0.14	0.508	0.427	-15.94%		84.1
QC-0.5ppb	52.6	380.6	0.14	0.508	0.433	-14.76%		85.2
QC-0.3ppb	44.5	357.3	0.12	0.305	0.251	-17.70%		82.3
QC-0.3ppb	45.8	369.5	0.12	0.305	0.249	-18.36%		81.6
QC-0.3ppb	35.4	341.0	0.10	0.305	0.309	1.31%	9.0%	101.3
QC-0.3ppb	39.6	412.6	0.10	0.305	0.281	-7.87%		92.1
QC-0.3ppb	35.1	369.4	0.10	0.305	0.278	-8.85%		91.1

Table 5: SEM data

Figure 1 shows the chromatograms of a negative and a positive unknown sample set in which the AOZ metabolite is clearly identified at the 0.6 ppb level. Table 6 summarizes the average method results for the four metabolites over multiple sample sets. The limits of detection (LODs) and limits of quantification (LOQs) are reported and the data shows good accuracy at the LOQ levels for all the metabolites. The LODs and LOQs achieved on the four nitrofuran metabolites are all at sub ppb levels.

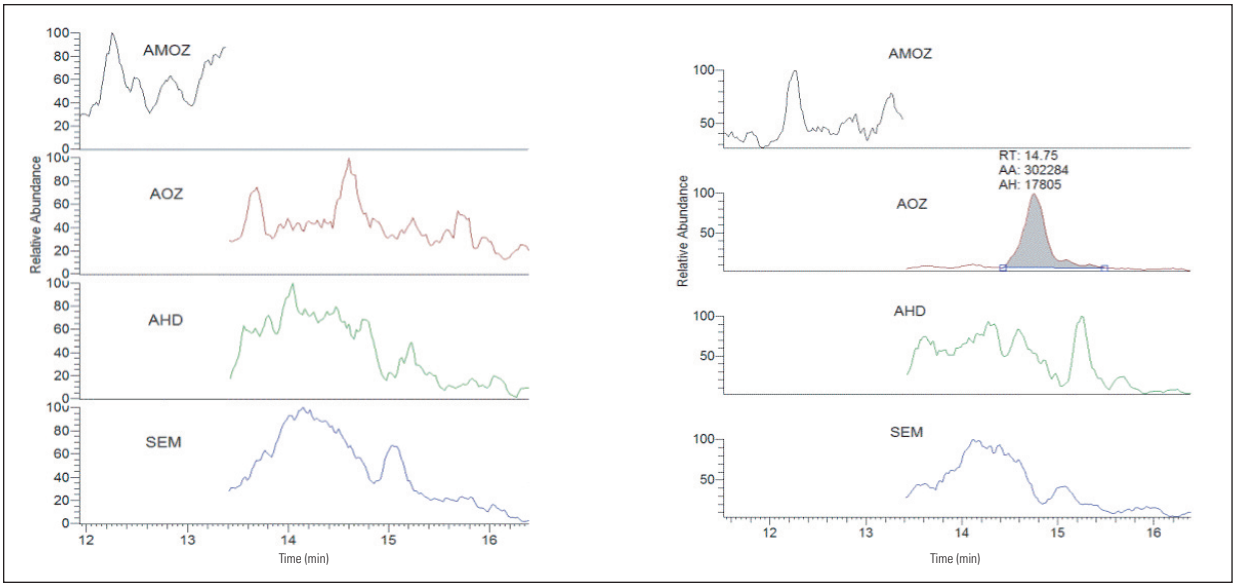

Figure 1: Identification of AOZ metabolite in honey at 0.6 ppb level

ANALYTE	MATRIX	LOD (ppb)	LOQ (ppb)	ANALYTICAL RANGE (ppb)	% RECOVERY	RANGE % REC	CV %	INCERTITUDE %
AMOZ	Honey	0.04	0.09	0,04-4,0	99.3	88,8-109.8	3.5	7.1
AHD	Honey	0.06	0.16	0,06-4,063	88.5	71.2-105.8	6.5	13.0
AOZ	Honey	0.06	0.14	0,06-4,172	101.2	84.2-118.5	5.6	11.3
SEM	Honey	0.08	0.18	0,08-4,064	94.3	70.6-117.8	8.3	16.6

Table 6: Summary of Method Results

Conclusion

An LC-MS/MS assay to detect and identify nitrofuran metabolites was developed using the TSQ Discovery. The extraction method appears to be extremely robust and reliable with good recovery efficiency (better than 80%), allowing unambiguous routine identification and quantification of all nitrofuran metabolites in honey. The LC-MS/MS-based method described here provides high speed, excellent sensitivity, and specificity of detection. The assay demonstrated the ability to easily meet the 0.3 ppb limit of quantitation that is required by the most stringent current requirements of food monitoring applications operating under FDA and EC regulations.

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Application Note: 465

Analysis of (Fluoro)quinolones in Honey with Online Sample Extraction and LC-MS/MS

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Key Words

- TurboFlow Technology
- Aria TLX-1
- TSQ Quantum Ultra
- Food Safety

Introduction

The global food market has become more competitive and equally cost responsive. The need for analytical procedures that permit high sample throughput as well as higher sensitivity allied to good reproducibility is growing by the day.[1,2,3] A method using automated online extraction with tandem mass spectrometry is presented as an alternative to the commonly used, time-consuming solid-phase extraction (SPE) method.

Quinolones, including fluoroquinolones, are a group of synthetic antibacterial compounds used in the treatment of several diseases. There has been a significant and progressive increase in the use of quinolones in animal production, which has led to their residual presence in food. In the European Union, the maximum residue limits (MRLs) for several of these compounds are defined for different food matrices of animal origin, but not for honey.[4] Furthermore, the presence of these compounds is an indication of unsafe practices of food production and deficient methods in the production of honey.

The complexity of the matrix plays a fundamental role on the adoption of the method of analysis. Thermo Scientific TurboFlow technology enables the reduction of sample preparation as well as the elimination of interferences from complex matrices such as honey.

Goal

To develop a sensitive and reproducible liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the quantitation of 12 fluoroquinolones and 4 quinolones in honey using automated extraction by TurboFlow™ technology.

Experimental

Sample Preparation

To a sample of 1 g of honey, 1 mL of water was added and the mixture was homogenized. The sample was then filtered directly to the HPLC vial using a 0.22 µm polyethersulfone membrane syringe filter.

Different concentration levels were achieved by spiking the sample with different concentration levels of standard stock solution.

The total sample preparation time was 40 minutes for 12 samples.

TurboFlow Method Conditions:

System:	Thermo Scientific Aria TLX-1 controlled by Aria™ software (Figure 1)
Online Extraction:	TurboFlow Cyclone 50 x 0.5 mm
Mobile Phase A:	0.1 % formic acid in water
Mobile Phase B:	0.1 % formic acid in acetonitrile
Mobile Phase C:	10 mM ammonium formate in water
Mobile Phase D:	acetonitrile/isopropanol/acetone (4:3:3 v/v/v)
Injection Volume:	90 µL

HPLC conditions:

Analytical Column:	Thermo Scientific Hypersil GOLD 2.1 x 50 mm, 3 µm column at 40° C
Solvent A:	0.5 % formic acid in water
Solvent B:	0.5 % formic acid in methanol/acetonitrile (1:1 v/v)

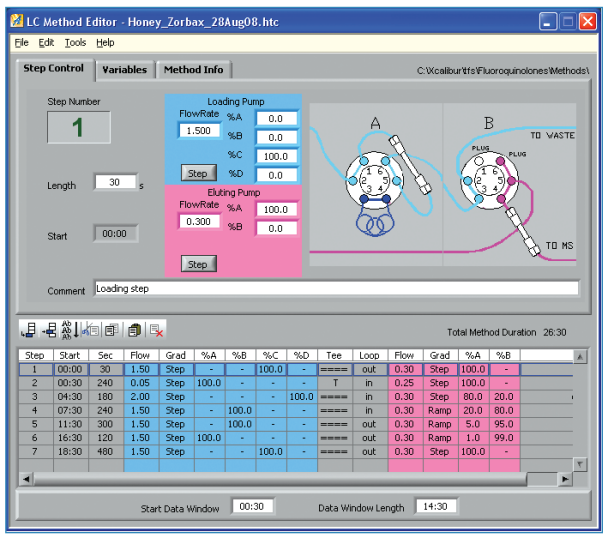

Figure 1: Aria software with LC Method Editor

MS Conditions

MS analysis was carried out on a Thermo Scientific TSQ Quantum Ultra AM triple stage quadrupole mass spectrometer. The MS conditions were as follows:

Ion Source Polarity:	Positive Ion Mode
Spray Voltage:	3000 V
Vaporizer Temperature:	350 °C
Sheath Gas Pressure (N_2):	40 units
Auxiliary Gas Pressure (N_2):	35 units
Capillary Temperature:	325 °C
Collision Gas (Ar):	1.5 mTorr
Q1/Q3 Peak Resolution:	0.7 u (unit mass resolution)
Scan Time:	0.025 s
Scan Width:	0.010 m/z
Data Acquisition Mode:	SRM

The optimization of Selective Reaction Monitoring (SRM) parameters was performed by direct infusion of standards in the positive electrospray ionization mode. Collision induced dissociation (CID) mass spectra were recorded for each analyte and the optimum collision energies were obtained for the selected ion transitions. Table 1 summarizes these parameters and Figure 2 displays the MS method controlled by Thermo Scientific Xcalibur software.

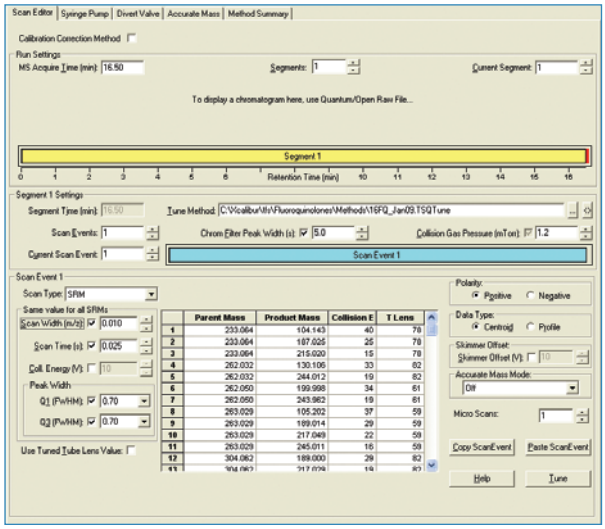

Figure 2: MS method showing the SRM transitions and other conditions

Table 1: Selected ion transitions (*m/z*), collision energy (CE) and tube lens voltages (TL) for studied compounds

Analyte	Precursor Ion (*m/z*)	Product Ion (*m/z*)	CE (V)	TL (V)
1. Nalidixic Acid	233.064	104.143	40	78
		215.020	15	78
		187.025	25	78
2. Oxolinic Acid	262.032	130.106	33	82
		244.012	19	82
3. Flumequine	262.050	199.998	34	61
		243.962	19	61
4. Cinoxacin	263.029	105.202	37	59
		189.014	29	59
		217.049	22	59
		245.011	16	59
5. Pipemidic Acid	304.062	189.000	29	82
		217.029	19	82
		286.075	20	82
6. Norfloxacin	320.096	276.058	17	70
		302.055	21	70
7. Enoxacin	321.083	206.012	29	65
		302.981	21	65
8. Ciprofloxacin	323.100	231.024	36	74
		314.018	22	74
9. Lomefloxacin	352.104	265.010	23	78
		308.067	17	78
10. Danofloxacin	358.120	82.215	39	75
		314.097	18	75
		340.089	24	75
11. Enrofloxacin	360.128	245.025	26	72
		315.958	19	72
12. Ofloxacin	362.107	261.041	27	109
		318.055	19	109
13. Marbofloxacin	363.066	70.067	34	66
		72.073	22	66
		276.064	14	66
		320.022	14	66
14. Fleroxacin	370.094	269.023	27	112
		326.061	19	112
15. Sarafloxacin	386.095	298.979	28	105
		342.078	18	105
		367.878	22	105
16. Difloxacin	400.107	299.009	29	75
		356.017	20	75

Results and Discussion

The analysis of food samples normally requires long preparation times due to the complexity of the matrices. The Thermo Scientific Aria TLX-1 system powered by TurboFlow technology enables reduction of the sample preparation time. It took only 40 minutes to prepare the batch of samples for LC-MS/MS analysis, instead of an average time of 6 hours when using Solid Phase Extraction (SPE). Even when dealing with complex matrices, such as honey, the use of the TLX-1 system enables the elimination of possible interferences and creates less noisy chromatograms (Figure 3).

The results of a high-throughput, rapid, sensitive and linear method for the determination of 16 quinolones, including 12 fluoroquinolones, by LC-MS/MS using TurboFlow technology are presented (Table 2). The Limit of Detection (LOD) was calculated by using the statistical definition LOD = $Y_B + 3S_B$, where Y_B is the blank signal and S_B is the standard deviation of the blank.

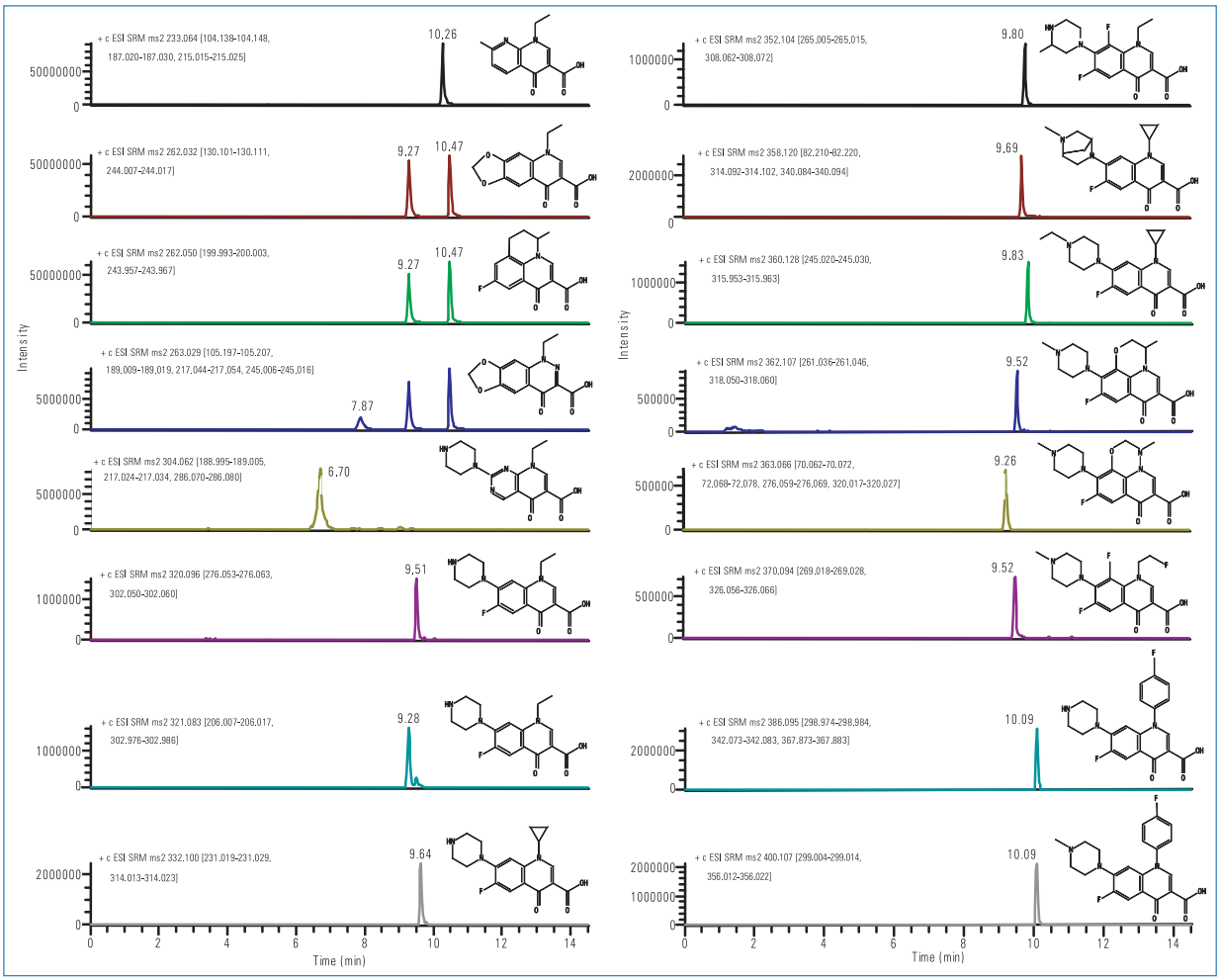

Figure 3: Representative SRM chromatogram (20 µg/kg) showing the selected ion transitions and retention times for the studied analyte

Table 2: Linearity, sensitivity and precision of the method

Analyte	Range (µg/kg)	LOD (µg/kg)	RSD (%)	R2 (1/X)
1	1-50	0.8	1.3 - 4.7	0.9943
2	1-50	1.4	0.3 - 10.6	0.9909
3	1-100	0.9	1.7 - 8.9	0.9902
4	2-100	2.0	4.3 - 7.7	0.9918
5	1-100	0.9	1.5 - 10.1	0.9964
6	1-100	2.3	2.7 - 11.5	0.9925
7	1-100	1.9	2.1 - 11.7	0.9928
8	1-100	1.4	2.4 - 11.6	0.9967
9	1-100	0.5	0.2 - 13.7	0.9954
10	1-100	1.1	2.3 - 13.6	0.9961
11	1-100	0.8	1.5 - 16.9	0.9907
12	2-100	1.3	2.1 - 11.5	0.9945
13	1-100	2.6	2.4 - 13.9	0.9939
14	1-50	1.5	6.0 - 16.8	0.9903
15	1-100	1.1	1.1 - 11.2	0.9966
16	1-100	0.8	1.9 - 10.4	0.9947

The method proved to be linear in the range studied. Three replicates were used for each point of the calibration levels, which, in addition to the relative standard deviation values, demonstrate the precision of the method.

Conclusion

A rapid, sensitive and reliable method for the quantitation of 16 quinolones, including 12 fluoroquinolones, was developed using a TurboFlow method in combination with a TSQ Quantum Ultra™ mass spectrometer. The use of TurboFlow technology enables a significant reduction of the sample preparation time. For 12 samples the preparation time was reduced from 5 hours to 40 minutes. Preliminary trials indicate this online extraction coupled with a TSQ Quantum Ultra is an excellent total solution for the quantification of a large number of compounds in food samples.

References and Acknowledgements

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AN63070_E 06/09S

Application Note: 475

High-throughput Screening Method for Multiple Classes of Antibiotics in Milk Using Automated Sample Preparation and LC-MS/MS

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Key Words

- TurboFlow Technology
- Aria TLX-2
- Food Safety
- TSQ Quantum Ultra

Introduction

Veterinary drugs are widely used to prevent the outbreak of disease in livestock and are commonly administered as feed additives or in drinking water. In addition, veterinary drugs are given to treat diseases, for drying-off purposes, or to prevent losses during transportation. Many countries, such as the United States and those in the European Union, have set maximum residue limits (MRLs) for different food products of animal origin. Japan has also set MRLs for compounds identified on the Japanese Positive List. Recently, China has defined some new national standards to monitor banned antibiotics in foods.

As a response to this, new methods are being developed for the determination of these compounds in a cost-effective way. By using the Thermo Scientific Aria TLX system powered by TurboFlow™ technology a drastic reduction in sample preparation time can be achieved while minimizing matrix interferences. LC-MS/MS is a powerful tool in food analysis, especially when combined with automated sample preparation that reduces matrix interferences. In addition, minimizing sample handling improves the performance characteristics of the method, such as recovery, repeatability, and reproducibility. However, most analytical techniques developed for quantitative analysis of antibiotic residues in food have been based on off-line methods involving solid phase extraction (SPE) or liquid-liquid extraction (LLE) followed by LC-MS[1,2]. Only recently, methods employing automated sample preparation have been reported, but usually for a specific class of compounds, rather than a multi-class method[3]. We propose a quick, high-throughput, sensitive screening method for the determination of different classes of antibiotics in milk samples.

Goal

To develop a high-throughput, sensitive and precise screening method, with minimal sample preparation, for the determination of multi-class antibiotic residues in milk samples by LC-MS/MS.

Experimental

Sample Preparation

Sample preparation involved protein precipitation, by mixing 100 µL of milk products with 900 µL of a solution of 50 mM ammonium acetate in acetonitrile (50%) and water (50%) with 7.5 mM Na_2EDTA. After centrifuging the mixtures at 10,000 rpm for 10 minutes, the supernatants were collected and injected into the Aria™ TLX LC-MS system. The sample preparation took approximately 15 minutes to complete.

TurboFlow Method Conditions:

System:	Aria TLX-2
On-line Extraction:	Thermo Scientific TurboFlow Cyclone 0.5 x 50 mm and Cyclone P 0.5 x 50 mm columns connected in tandem
Mobile Phase A:	0.10% Formic acid and 0.05% Trifluoroacetic acid in water
Mobile Phase B:	Methanol
Mobile Phase C:	Isopropanol/Acetone (50:50)
Mobile Phase D:	2.0% Acetonitrile and 0.1% ammonium hydroxide in water
Injection Volume:	50 µL

HPLC conditions:

Analytical Column:	Thermo Scientific BETASIL Phenyl/Hexyl column 3.0 x 50, 3 µm at 50 °C maintained by a Thermo Scientific HOT POCKET column heater.
Solvent A:	0.10% Formic acid and 0.01 % Trifluoroacetic acid in water
Solvent B:	Methanol

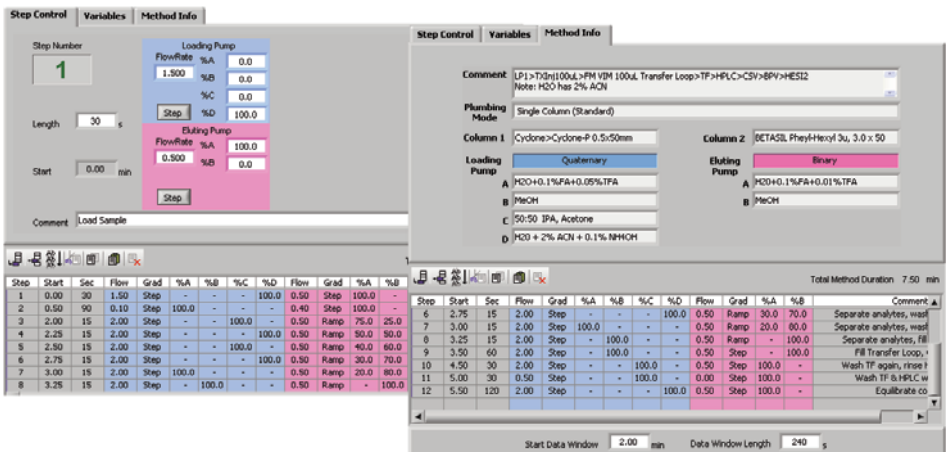

Figure 1: Aria OS provides easy-to-use software for setting up TurboFlow methods.

MS Conditions

MS analysis was carried out on a Thermo Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer equipped with a heated electrospray ionization probe. The MS conditions were as follows:

Ion Source Polarity	Positive Ion Mode
Spray Voltage	3500 V
Vaporizer Temperature	475 °C
Sheath Gas Pressure (N_2)	50 units
Auxiliary Gas Pressure (N_2)	25 units
Ion Sweep Gas Pressure	2 units
Capillary Temperature	250 °C
Collision Gas (Ar)	1.5 mTorr
Q1/Q3 Peak Resolution	0.7 u (unit mass resolution)
Scan Time	0.100 s
Scan Width	0.020 m/z
Data Acquisition Mode	SRM

The optimization of Selective Reaction Monitoring (SRM) parameters was performed by direct infusion of standards using positive electrospray ionization (ESI). Collision induced dissociation (CID) mass spectra were recorded for each analyte and the optimum collision energies obtained for the selected ion transitions. Table 1 summarizes these parameters.

Table 1: Selected ion transitions *(m/z)* and collision energy (CE) for studied compounds.

Analyte	Precursor Ion *(m/z)*	Product Ion *(m/z)*	CE (V)
1. Albendazole	266.1	234.0	15
		191.0	31
2. Sulphamethazine	279.1	124.2	14
		108.0	16
3. Phenylbutazone	309.2	211.3	16
		188.3	15
4. Difloxacin	400.1	356.1	20
		299.1	27
5. Spiramycin	422.0	174.0	35
		350.5	12
6. Tetracycline	445.5	410.0	17
		427.0	6
7. Oxytetracycline	461.2	426.0	19
		201.0	36
8. Salinomycine Na	773.4	265.4	50
		432.0	44

Results and Discussion

Liquid chromatography coupled to atmospheric pressure ionization tandem mass spectrometry is currently the method of choice for the quantitative determination of antibiotics in food matrices. The advantages of this technique include high specificity, sensitivity, and throughput. Representative SRM chromatograms of a neat standard, whole milk, and fat-free milk sample containing 100 ppb of the veterinary drugs are shown in Figure 2.

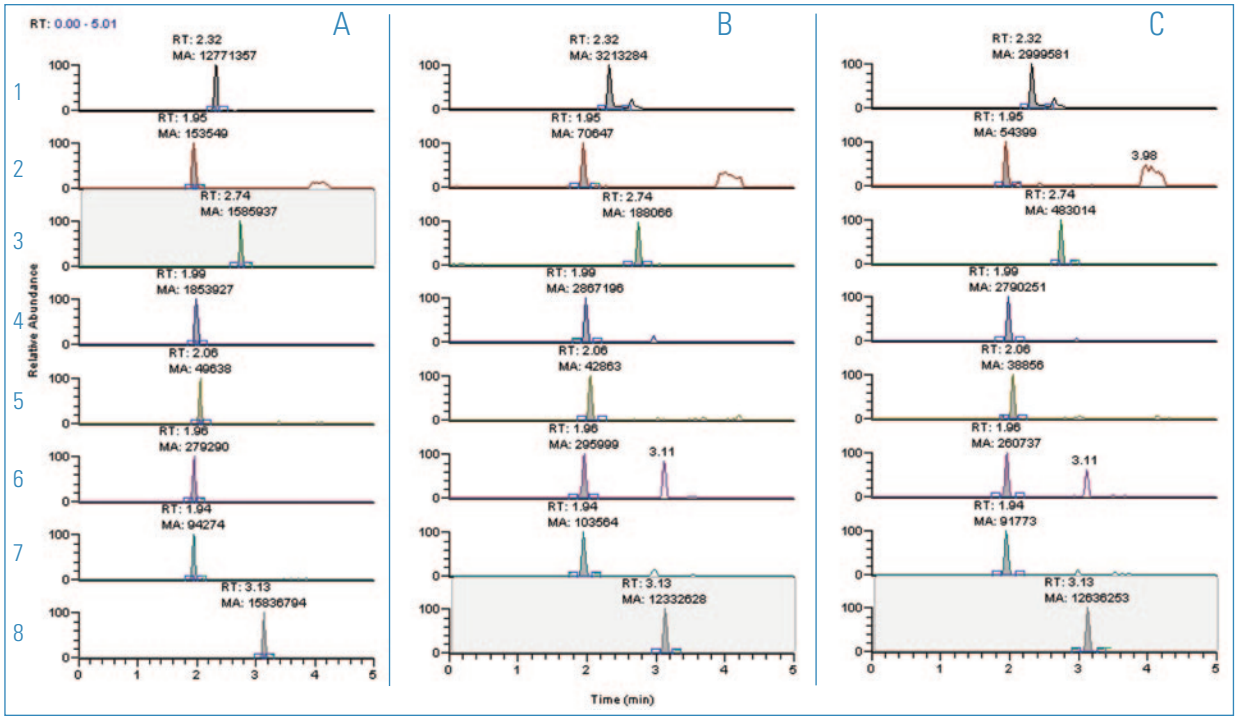

Figure 2: Representative SRM chromatograms of a neat standard (A) and milk samples (B-whole fat milk; C-low fat milk) containing antibiotics at 100 ppb level. 1-albendazole; 2-sulphamethazine; 3-phenylbutazone; 4-difloxacin; 5-spiramycin; 6-tetracycline; 7-oxytetracycline; 8-salinomycine Na

Table 2 presents linearity and precision data for the range of concentration studied in three types of commercially available milk samples. The analysis of a blank sample showed no major interferences present (Figure 3). The method proved to be linear in the studied range as well as reproducible (n=3) and precise. However, the amount of fat present in the sample seemed to influence the precision of the method for difloxacin and sulphamethazine at the highest level of the fortification (n<3).

Table 2: Linearity (r^2), precision (RSD %) for the different fortification levels when studying various fat content milk samples (Brand A)

Milk Samples – Brand A		Non-Fat				Low-Fat (2%)				Whole Fat			
Fortification Levels		50	100	250	500	50	100	250	500	50	100	250	500
Albendazole	r^2	0.9984				0.9967				0.9928			
	(RSD %)	1.6	1.7	1.7	2.7	6.3	3.2	3.6	4.2	2.6	6.2	1.2	2.9
Sulphamethazine	r^2	0.9964				0.9908				0.9970			
	(RSD %)	2.4	7.2	4.9	2.4	6.6	14.5	5.2	5.6	8.9	1.2	5.1	n/a*
Phenylbutazone	r^2	0.9947				0.9922				0.9963			
	(RSD %)	2.9	3.3	0.8	2.6	8.1	4.1	4.9	3.1	0.6	0.9	0.7	0.3
Difloxacin	r^2	0.9958				0.9907				0.9968			
	(RSD %)	12.2	4.3	6.0	2.4	10.8	4.6	2.7	5.5	2.6	6.1	5.1	n/a*
Spiramycin	r^2	0.9920				0.9740				0.9951			
	(RSD %)	11.1	11.8	8.4	4.1	10.9	4.0	10.0	9.4	13.3	6.5	5.2	0.2
Tetracycline	r^2	0.9923				0.9948				0.9903			
	(RSD %)	6.2	6.4	5.4	3.7	7.3	4.8	5.9	4.5	4.1	9.5	6.5	5.5
Oxytetracycline	r^2	0.9947				0.9922				0.9663			
	(RSD %)	2.9	3.3	0.8	2.6	8.1	4.1	4.9	3.1	0.6	0.9	0.7	0.3
Salinomycine Na	r^2	0.9993				0.9966				0.9984			
	(RSD %)	1.2	0.7	1.2	1.5	1.5	0.8	3.1	0.4	2.8	3.1	1.3	1.2

*n/a: n<3

A study evaluating the matrix effect was performed because it is well known that molecules originating from the sample matrix that co-elute with the compounds of interest can interfere with the ionization, causing either suppression or enhancement of the signal. The response areas of the neat standards were compared with the spiked milk samples for the 100 ppb level, for two different brands. Table 3 shows the relative response (%) as well as carry-over values and limits of detection (LOD). Carry-over was determined by injecting the higher calibration level standard (500 ppb) in triplicate, followed by a blank, and was found to be minimal.

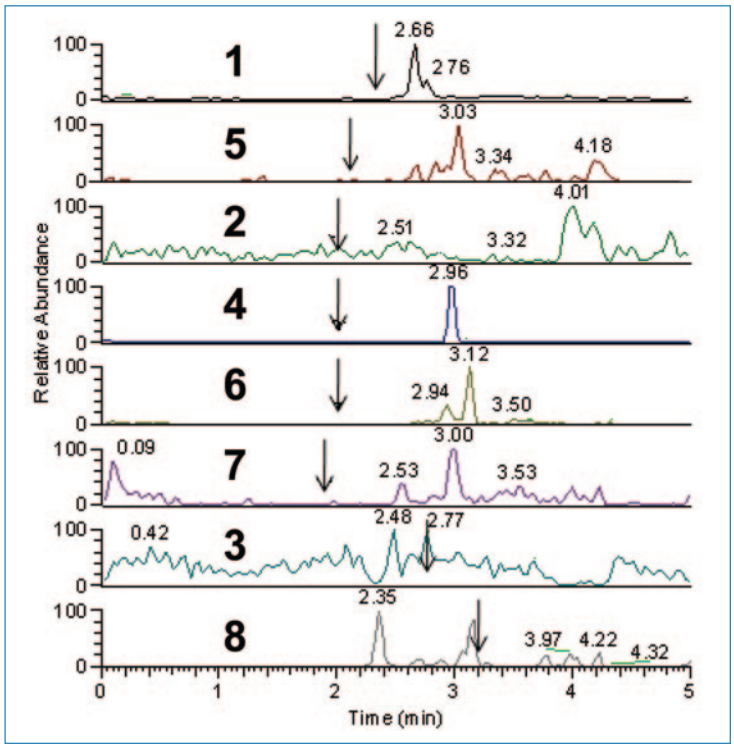

Figure 3: Representative SRM chromatogram of a blank whole milk sample. The arrows indicate the expected retention time for each of the analytes. 1-albendazole; 2-sulphamethazine; 3-phenylbutazone; 4-difloxacin; 5-spiramycin; 6-tetracycline; 7-oxytetracycline; 8-salinomycine Na

Table 3: Relative Response (%) found when running the method for two milk brands commercially available in the US market. The different milk samples were spiked with 100 ppb of stock solution and the peak areas compared with neat standards. Limits of detection of the method were calculated by linear regression analysis of the matrix matched calibration curve. Carry Over was minimal.

Analyte	Relative Response[1] (%) Brand A	Brand B	LOD (ppb)	Carry-over[2] (%)
Albendazole	- 82	- 85	0.4	1.2
Sulphamethazine	- 57	- 59	1.6	0
Phenylbutazone	- 69	- 25	1.9	0
Difloxacin	70	40	1.7	0.6
Spiramycin	- 28	- 37	5.2	0
Tetracycline	8	8	2.4	0
Oxytetracycline	31	- 5	3.0	0
Salinomycine Na	- 19	- 31	0.7	0.2

[1]*Relative Response (%) = (Area milk/Area Standard -1) x 100*
[2]*Carry-over (%) = (Area blank/Area standard) x 100*

Albendazole showed the strongest suppression because the signal was less than 20% than that of a neat standard while difloxacin showed signal enhancement indicating that the matrix is probably not completely removed. On the other hand, with two exceptions, the matrix effects seem to be similar for both brands of milk. While some matrix effects remain, the study showed that accurate quantitative data can be obtained because the method is linear in the concentration range of 50 to 500 µg/L as well as reproducible and precise (RSD <15%). Limits of detection ranged from 0.4 to 5.2 µg/L, which is well under most MRL values for veterinary drugs in milk. The use of an internal standard would compensate for the matrix effects.

The method was tested by screening a batch of real milk samples. The proposed method proved to be able to detect all the compounds presumably present in the sample.

Table 4: Screening of real milk samples

Sample	Preliminary results	Aria TLX coupled to TSQ Quantum Ultra™
01	Negative	Negative
02	Negative	Negative
03	Negative	Negative
04	Negative	Negative
05	Negative	Negative
06	Negative	Negative
07	Negative	Negative
08	Negative	Negative
09	Negative	Negative
10	Negative	Oxytetracycline 5ppb
11	Oxytetracycline 200 ppb	Oxytetracycline 1 ppm Tetracycline 5 ppb
12	Sulphamethazine 200 ppb	Sulphamethazine 200 ppb

Conclusion

This application note presents a new online LC-MS method for the simultaneous screening of different classes of antibiotics in milk. This method proved to be quick, sensitive, and reproducible. It can be successfully applied for the quantitative determination of several classes of antibiotics in milk samples. Accurate quantitative measurement of these compounds subjected to residual matrix interferences could be accomplished by using a suitable internal standard.

The automated TurboFlow LC-MS/MS method significantly improves the laboratory throughput by significantly minimizing the necessary sample preparation while still allowing limits of detection of low ppb levels.

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AN63161_E 09/09S

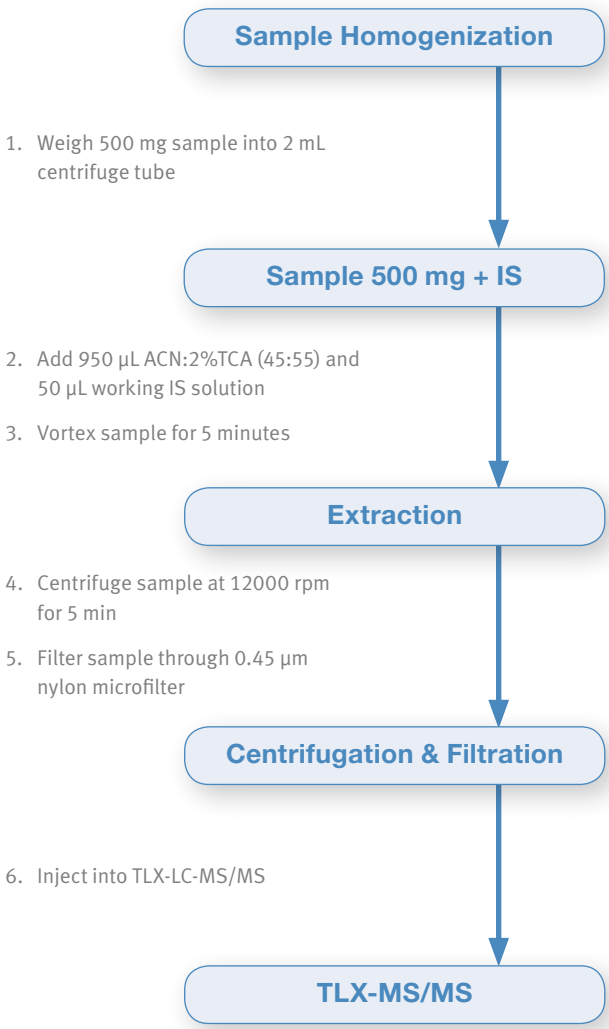
Automated Online Multi-Residue LC-MS/MS Method for the Determination of Antibiotics in Chicken Meat

Katerina Bousova, Klaus Mittendorf, Thermo Fisher Scientific Food Safety Response Center, Dreieich, Germany

Method 63646

Key Words
Antibiotics, Transcend TLX, TurboFlow Technology, TSQ Quantum Access MAX, Chicken meat, Food Safety

1. Schematic of Method



2. Introduction

Throughout the world, antibiotics are widely used for veterinary purposes to treat diseased animals, prevent diseases and promote growth. Due to inappropriate or excessive usage of antibiotics, residues of these compounds can be found in food and food products of animal origin. The use of antibiotics cannot be avoided; however, it is necessary to ensure the safety of food and food products for human consumption. For this reason, the European Commission has established maximum residue limits (MRLs) for antibiotics in animal tissue, milk and eggs in Council Regulation 2377/90/EC.[1] To detect and quantify antibiotics for regulatory purposes, laboratories need to utilize suitable analytical methods.

With the number of samples to be checked for the presence of antibiotic residues increasing, the need for multi-analyte methods that can efficiently handle high throughputs is growing as well. Generally, methods used for monitoring antibiotic residues can be classified in two groups: screening and confirmatory.

For fast *screening* of antibiotic residues, an immunoassay, microbiological assay or biosensor technique is typically used. Among the benefits are short analysis time, high sensitivity and selectivity for immunoassays, simplicity and automation. However, the disadvantages include the incidence of false-negative or false-positive results, the inability to distinguish between the different types of antibiotics and the possibility to provide only a semi-quantitative result for the total amount of drug residue.

The *confirmatory* quantitative methods are typically based on liquid chromatography coupled to mass spectrometry (LC/MS). This technique can also be used for screening and provides much higher sensitivity and greater specificity. The use of LC-MS/MS for screening was reported in a multi-residue semi-quantitative screening method for 39 drug residues covering eight drug classes in veal muscle.[2]

Thermo SCIENTIFIC

This note describes a multi-residue, confirmatory method for the quantitative determination of antibiotics in chicken meat using the Thermo Scientific Transcend TLX system coupled to an LC-MS/MS. This method was developed on the basis of previous work concerning a confirmatory method for antibiotics in milk[3]. The Transcend TLX™ system powered by TurboFlow™ technology was used for online sample cleanup instead of lengthy offline solid-phase extraction (SPE). Combining the number of target compounds with the high sample throughput, this approach fulfills the demand for a fast and cost-effective multi-analyte method.

3. Scope and Application

This online TLX-MS/MS method can be applied to detect and quantify the presence of 36 compounds from seven different classes of antibiotics (aminoglycosides, sulfonamides, macrolides, quinolones, tetracyclines, lincosamides and trimethoprim) in chicken meat. This multi-residue method fulfills legislative requirements described in the EU Commission Decision 2002/657/EC[4].

4. Principle

The Transcend TLX system uses TurboFlow technology for online sample cleanup. Sample concentration, cleanup and analytical separation are carried out in a single run using a TurboFlow column connected to an analytical LC column. Macromolecules are removed from the sample extract with high efficiency, while target analytes are retained on the column based on different chemical interactions. After a wash step, the trapped compounds are transferred onto the analytical LC column and separated conventionally. Before introducing the sample extract onto the TurboFlow column, the sample is thoroughly homogenized and fortified with an internal standard, extracted with a solvent mixture of acetonitrile (ACN):2% trichloroacetic acid (TCA) (45:55) and centrifuged. Cleanup using the TLX system is optimized for maximum recovery of targeted compounds and minimal injection of co-extractives into the mass spectrometer. Identification of antibiotics is based on retention time, ion ratios using multiple reaction monitoring (MRM) of characteristic transition ions, and quantification using matrix-matched standards of one of the selected MRM ions.

5. Reagent List

5.1	Purified water, Thermo Scientific Barnstead Easypure II water system
5.2	Methanol, Optima, LC-MS grade
5.3	Water, LC-MS grade
5.4	Acetonitrile, Optima, LC-MS grade
5.5	Isopropanol, HPLC grade
5.6	Acetone, HPLC grade
5.7	Formic acid, extra pure, >98%
5.8	Heptafluorobutyric acid, 99%
5.9	Ammonia, extra pure, 35%
5.10	Trichloroacetic acid, extra pure, 99%

6. Calibration Standards

6.1 Standards

6.1.1	Chlortetracycline	Sigma-Aldrich®
6.1.2	Clarithromycin	Sigma-Aldrich
6.1.3	Clindamycin hydrochloride	Sigma-Aldrich
6.1.4	Cinoxacin	Sigma-Aldrich
6.1.5	Ciprofloxacin	Sigma-Aldrich
6.1.6	Danofloxacin	Sigma-Aldrich
6.1.7	Doxycycline hyclate	Sigma-Aldrich
6.1.8	Difloxacin	Sigma-Aldrich
6.1.9	Enoxacin	Sigma-Aldrich
6.1.10	Enrofloxacin	Sigma-Aldrich
6.1.11	Flumequine	Sigma-Aldrich
6.1.12	Josamycin	Sigma-Aldrich
6.1.13	Kanamycin	Sigma-Aldrich
6.1.14	Lincomycin hydrochloride monohydrate	Sigma-Aldrich
6.1.15	Lomefloxacin hydrochloride	Sigma-Aldrich
6.1.16	Marbofloxacin	Sigma-Aldrich
6.1.17	Nalidixic acid	Sigma-Aldrich
6.1.18	Neomycin	Sigma-Aldrich
6.1.19	Norfloxacin	Sigma-Aldrich
6.1.20	Ofloxacin	Sigma-Aldrich
6.1.21	Oleandomycin phosphate dehydrate	Dr. Ehrenstorfer
6.1.22	Oxolinic acid	Sigma-Aldrich
6.1.23	Oxytetracycline hydrochloride	Sigma-Aldrich
6.1.24	Sarafloxacin hydrochloride trihydrate	Sigma-Aldrich
6.1.25	Spiramycin	Sigma-Aldrich
6.1.26	Sulfadimethoxine	Sigma-Aldrich
6.1.27	Sulfadoxin	Sigma-Aldrich
6.1 28	Sulfaquinoxaline	Sigma-Aldrich
6.1.29	Sulfachlorpyridazine	Sigma-Aldrich
6.1.30	Sulfaclozine sodium	Dr. Ehrenstorfer
6.1.31	Sulfamethoxazole	Sigma-Aldrich
6.1.32	Tetracycline	Sigma-Aldrich
6.1.33	Tilmicosin	Sigma-Aldrich
6.1.34	Trimethoprim	Sigma-Aldrich
6.1.35	Tylosin tartrate	Sigma-Aldrich
6.1.36	Tylvalosin tartrate	FarmKemi

6.2 Internal Standard

6.2.1	Sulfaphenazole	Sigma-Aldrich

7. Standards Preparation

7.1 Stock standard solutions of veterinary drugs

Stock standard solutions (1000 µg/mL) are prepared individually by dissolving the analytes in methanol (lincosamides, macrolides, sulfonamides, tetracyclines and trimethoprim), in water (aminoglycosides) and in methanol with 2% 2M NH_4OH (quinolones). Solutions are stored at -20° C.

7.2 Working standard solution

The working 1000 µg/L calibration standard solution is prepared by dilution of individual stock standard solutions with acetonitrile. The solution should be prepared fresh each time before using.

7.3 Stock solution of internal standard

Stock solution of the internal standard (1000 µg/mL) is prepared by dilution of sulfaphenazole in methanol. Solution is stored at -20 °C.

7.4 Working standard solution of internal standard

The working solution of the internal standard (2000 µg/L) was prepared by dilution of stock standard solution (sulfaphenazole) with acetonitrile. Solution should be prepared fresh each time before using.

8. Apparatus

8.1	Transcend TLX-1 system
8.2	Thermo Scientific TSQ Quantum Access MAX triple quadrupole mass spectrometer
8.3	Fisher Science Education precision balance
8.4	Sartorius® analytical balance *(Sartorius GmbH, Germany)*
8.5	Barnstead™ Easypure™ II water system
8.6	Vortex shaker
8.7	Vortex universal cap
8.8	Waring® laboratory blender *(Waring Laboratory Science, USA)*
8.9	BRAND™ accu-jet® pipettor *(BRAND GmBh + Co. KG, Germany)*
8.10	Thermo Scientific Heraeus Fresco 17 microcentrifuge

9. Consumables

9.1	Thermo Scientific TurboFlow Cyclone P (50 × 0.5 mm) column
9.2	Thermo Scientific BetaSil Phenyl/Hexyl (50 × 2.1 mm, 3 µm) column
9.3	LC vials
9.4	LC caps
9.5	Thermo Scientific Finnpipette 100 – 1000 µL pipette
9.6	Finnpipette™ 20 – 200 µL pipette
9.7	Finnpipette 10 – 100 µL pipette
9.8	Finnpipette 500 – 5000 µL pipette
9.9	Finnpipette 1000 – 10 000 µL pipette
9.10	Pipette holder
9.11	Pipette tips 0.5–250 µL, 500/box
9.12	Pipette tips 1–5 mL, 75/box
9.13	Pipette tips 100–1000 µL, 200/box
9.14	Pipette tips 20000–10000 µL, 40/box
9.15	Pipette, Pasteur, soda lime glass 150 mm
9.16	Pipette suction device
9.17	Spatula, 18/10 steel
9.18	Spatula, nylon
9.19	Single-use syringes, 1 mL
9.20	Nylon syringe filter 0.45 µm, 17 mm
9.21	Vial rack (2 mL)
9.22	Centrifuge plastic tube (2 mL)
9.23	Rack for 50, 15, 2 and 0.5 mL tubes
9.24	Pipette tips 20000–10000 µL, 40/box

Glassware

9.25	Beaker, 50 mL
9.26	Beaker, 100 mL
9.27	Beaker, 25 mL
9.28	Volumetric flask, 25 mL
9.29	Volumetric flask, 10 mL
9.30	Volumetric flask, 5 mL
9.31	Volumetric flask, 100 mL
9.32	Glass pipette, 5 mL

10. Procedure

10.1 Sample Preparation

Approximately 150 g of the chicken sample is homogenized in a Waring laboratory blender for five minutes. Then, 500 mg is weighed into a 2 mL polypropylene tube. Working internal standard solution (50 µL) and solvent mixture ACN:2% TCA (45:55) (450 µL) are added to the sample. The sample is shaken for five minutes on the vortex and then centrifuged at 12000 rpm for five minutes. The supernatant is filtered through a nylon microfilter (0.45 µm pore size) directly into the LC vial and the sample is analyzed by TLX-MS/MS.

10.2 LC Conditions

LC analysis is performed on a Transcend TLX-1 system.

TurboFlow column:	TurboFlow Cyclone P (50 × 0.5 mm)
Analytical column:	BetaSil™ Phenyl/Hexyl (50 × 2.1 mm, 3 µm particle size)
Total run time:	19 minutes
Mobile phases:	A = 1 mM heptafluorobutyric acid and 0.5% formic acid in water B = 0.5% formic acid in acetonitrile/methanol (1/1) C = 2% methanol in water D = acetone/acetonitrile/isopropanol (20/40/40)

10.3 Injector Settings

Injector:	Thermo Scientific PAL injector with 100 µL volume injection syringe
Tray temperature:	10 °C
Cleaning solvents for the autosampler:	
Solvent 1:	Acetonitrile/water (20/80)
Solvent 2:	Acetone/acetonitrile/isopropanol - (20/40/40)
Pre-clean with solvent 1 [steps]:	3
Pre-clean with solvent 2 [steps]:	3
Pre-clean with sample [steps]:	1
Filling speed [µL/s]:	50
Filling strokes [steps]:	1
Injection port:	LC Vlv1 (TX channel)
Injection speed [µL/s]:	100
Pre-inject delay [ms]:	500
Post-inject delay [ms]:	500
Post-clean with solvent 1 [steps]:	5
Post-clean with solvent 2 [steps]:	5
Valve clean with solvent 1 [steps]:	5
Valve clean with solvent 2 [steps]:	5
Injection volume:	35 µL

Sample concentration, cleanup and analytical separation are carried out in a single run using an automated online sample preparation system, which includes the Transcend TLX system and Thermo Scientific Aria operating software. The sample is injected during the loading step by the loading pump and autosampler onto the TurboFlow column. During this step, macromolecules are removed while the target analytes are retained on the TurboFlow column based on their different chemical interactions. In the next step, the trapped analytes are transferred with the eluting pump, and an adequately strong solvent (eluent) in the loop onto the analytical LC column where the analytes are separated conventionally. While the separation on the analytical column is running, the loop is filled with the eluent and the TurboFlow column is washed and conditioned to be ready for the injection of the next sample. The TLX and LC conditions are set up in Aria™ software and presented in Table 1.

The analytical column is conditioned during the loading of the sample onto the TurboFlow column. The separation of the analytes on the analytical column is done by gradient (Table 1). To prevent the possibility of carryover and cross contamination, the injection syringe as well as the injection valve are washed with cleaning solvent 1 (acetonitrile/water - 20/80) and cleaning solvent 2 (acetone/acetonitrile/isopropanol - 20/40/40), five times before and five times after each injection.

Table 1. Gradient program table for TurboFlow system coupled with an analytical column

Step			TurboFlow column[a]					Cut-in loop		Analytical LC column[b]			
Description	Start [min]	Time [s]	Flow [mL/min]	A%	B%	C%	D%	Tee	Loop	Flow [mL/min]	Step	A%	B%
1.loading	0	60	1.5	-	-	100	-	-----	out	0.3	Step	100	-
2.transffering	1	60	0.2	100	-	-	-	T	in	0.6	Step	100	-
3.washing	2	60	1.5	-	-	50	50	-----	in	0.3	Step	100	-
4.washing	3	720	1.5	-	-	-	100	-----	in	0.3	Ramp	5	95
5.filling loop	15	120	1.5	50	50	-	-	-----	in	0.3	Step	5	95
6.equilibrating	17	120	1.5			100	-	-----	out	0.3	Step	100	-

[a]Mobile phases for the TurboFlow method:

A: 1mM heptafluorobutyric acid + 0.5% formic acid in water

B: 0.5% formic acid in acetonitrile/methanol – 1/1

C: 2% methanol in water

D: acetone/acetonitrile/isopropanol - 20/40/40

[b]Mobile phases for the analytical method:

A: 1mM heptafluorobutyric acid + 0.5% formic acid in water

B: 0.5% formic acid in acetonitrile/ methanol – 1/1

10.4 Mass Spectrometric Conditions

Mass spectrometric analysis is carried out using a TSQ Quantum Access MAX™ triple quadrupole system. Data acquisition for quantification and confirmation are performed in selected reaction monitoring (SRM) mode. All SRM traces (parent, qualifier and quantifier ion) are individually tuned for each target analyte by direct injection of the individual working standard solution (10 mg/mL). Data acquisition and processing is performed using Thermo Scientific Xcalibur 2.1 software.

Ionization mode:	Heated Electrospray (HESI)
Scan type:	SRM
Polarity:	Positive ion mode
Spray voltage [V]:	3500
Ion sweep gas pressure [arb]:	0
Vaporizer temperature [°C]:	400
Sheath gas pressure [arb]:	50
Aux gas pressure [arb]:	10
Capillary temperature [°C]:	370
Collision gas pressure [mTorr]:	0
Cycle time [s]:	0.6
Peak width:	Q1/Q3 the full width of a peak at half its maximum height (FWHM) of 0.70 Da

The parameters for SRM analysis for targeted compounds and internal standards are displayed in the Table 2.

11. Calculations

11.1 Identification

Identification of the antibiotics is indicated by the presence of transition ions (quantifier and qualifier) measured in SRM mode corresponding to the retention times (± 2.5%) of appropriate standards. In SRM mode, the measured peak area ratios for qualifier to quantifier ions should be in close agreement (according to EU Commission Decision 2002/657/EC) with those ratios of the standards, as shown in Table 3. The quantifier and qualifier ions were selected among the product ions produced by the fragmentation of the selected parent ion on the basis of the intensity and selectivity. A representative chromatogram is shown in Figure 1.

11.2 Quantification

For quantification, internal standardization is used to measure peak area ratios for matrix matched standards. Sulfaphenazole is used as the internal standard for all target antibiotics. Calibration curves are plotted as the relative peak areas (analyte versus the corresponding standard) as a function of the compound concentration. The antibiotic concentration in the samples is determined from the equation:

$$c_a = \frac{\frac{A_a}{A_{IS}} - b}{a}$$

c_a = antibiotic concentration in µg/kg

A_a = peak area of the antibiotic

A_{IS} = peak area of internal standard

b = y-intercept

a = slope of the calibration curve

12. Method Performance

The method was validated in-house according to the criteria for a quantitative method specified in EU Commission Decision 2002/657/EC[4]. The validation parameters were determined by spiking blank chicken meat at levels of 0.5, 1 and 1.5 times the MRL. For compounds without an MRL for chicken meat, samples were spiked at 10, 20 and 30 µg/kg for clindamycin, josamycin, clarithromycin, oleandomycin, tylvalosin, marbofloxacin, nalidixic acid, enoxacin, ofloxacin, lomefloxacin, norfloxacin, sarafloxacin and cinoxacin. The measured parameters were specificity, linear range, repeatability, accuracy, limit of detection (LOD), limit of quantification (LOQ), decision limit (CCα) and detection capability (CCβ).

12.1 Samples and Quality Control Materials

For preparation of matrix-matched calibration standards and spiked samples for validation, chicken meat was obtained from a local market and checked by repeated measurements to confirm that it was free of antibiotics. For determination of accuracy, a Food Analysis Performance Assessment Scheme (FAPAS®) test material T02174QC of fish muscle containing a certified amount of ciprofloxacin, which was obtained from the Food and Environmental Research Agency (York, United Kingdom) was used.

12.2 Selectivity

Using SRM, the specificity is confirmed based on the presence of the transition ions (quantifier and qualifier) at the correct retention times corresponding to those of the respective antibiotics. The measured peak area ratios of qualifier/quantifier are within the range defined in EU Commission Decision 2002/657/EC[4] when compared to the standards (Table 3).

12.3 Linearity & Calibration Curve

The linearity of calibration curves was assessed over the range from 0–400 µg/kg for all target compounds. In all cases, the correlation coefficients of linear functions had to be >0.99. The calibration curves were created from nine matrix-matched calibration standards, which were injected into each batch in duplicate.

12.4 Precision

Precision (repeatability) of the method was determined using independently spiked, blank samples at three different levels. In one day, the set of samples at three levels was measured with six repetitions. To determine between-day precision, two other sets at one level were measured with six repetitions over the next two days. The results for repeatability ranged from 4%–27% (Table 4).

12.5 Accuracy

Method accuracy was determined using independently spiked blank samples at three different levels. Accuracy was evaluated by comparing found values with standard additions in spikes. Recovery values ranged between 71%–120% (Table 5). Additionally, accuracy was established for ciprofloxacin by analyzing the certified reference material T02174QC, which was fish muscle. All measured concentrations of ciprofloxacin were within the acceptable range (Table 6).

12.6 LOD and LOQ

LOD and LOQ were estimated to be 3 and 10, respectively, by following the IUPAC approach of first analyzing the blank sample to establish noise levels and then estimating LODs and LOQs for signal/noise. The values for chicken meat are shown in Table 7 and, in all cases, they are under the level of MRL for all analytes that have an assigned MRL.

12.7 CCα and CCβ

Both CCα and CCβ were established by the calibration curve procedure according to ISO 11843[5]. The blank material fortified at and below the maximum residue limit (for analytes with MRL) or at and above the lowest possible level (for analytes without MRL) in equidistant steps was used. The calculated values are shown in Table 7.

13. Conclusion

This in-house validated method enables quantification of 36 residues from seven different classes of antibiotics in chicken meat. Although the 36 compounds come from different groups with widely varying polarities and solubilities, only one extraction procedure was used. The use of the Transcend TLX system and TurboFlow technology combined with TSQ triple quadrupole mass spectrometry detection for analytical separation saves a significant amount of time in sample preparation and increases sample throughput. Additionally, by using the Transcend TLX system very clean sample extracts enter the mass spectrometer so routine maintenance on the system, such as cleaning the ion source, is not required as often as with analytical methods in which the sample extracts are not cleaned but only diluted. The method was validated and fulfilled the requirements of the EU Commission Decision 2002/675/EC[4]; therefore, it can be recommended for enforcement of the legislation limits. Using this method, the control laboratory can measure up to 40 samples of chicken meat a day including sample preparation and measurement by instrument.

14. References

1. European Commission. 1994. Council Regulation (EEC) No. 2377/90 of 26 June 1990: laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin, amending regulation no. 1430/94 of 22 June 1994. Off J Eur Comm. L156:23.
2. Martos, P.A.; Jayasundara, F.; Dolbeer, F.; Dolbeer, J.; Jin, W.; Spilsbury, L.; Mitchell, M.; Varilla, C.; Shurmer, B. Multiclass, Multiresidue Drug Analysis, Including Aminoglycosides, in Animal Tissue Using Liquid Chromatography Coupled to Tandem Mass Spectrometry. *J. Agric. Food Chem.* **2010**, 58, 5932-5944.
3. Bousova, K.; Mittendorf, K. Multi-residue automated Turbulent Flow™ on-line LC-MS/MS method for the determination antibiotics in milk. Method number: 63551. Thermo Fisher Scientific, **2012**.
4. EU Commission Decision 2002/657/EC. Off. J. Eur. Commun. L221/8 (2002).
5. ISO 11843: Capability of detection (1997).

Table 2. LC-MS/MS parameters for selected reaction monitoring of analytes

Analyte	Molecular Weight	Precursor Ion	Quantifier Ion	CE for Quantifier Ion (V)	Qualifier Ion	CE for Qualifier Ion (V)	Tube Lens (V)
Kanamycin	484.5	485.28	163.10	25	324.15	15	90
Neomycin	614.6	615.34	161.03	29	163.11	31	101
Lincomycin	406.5	407.14	126.17	28	359.16	17	97
Clindamycin	425.0	425.14	126.17	28	377.17	18	86
Trimethoprim	290.3	291.10	230.10	23	261.09	24	93
Josamycin	828.0	828.43	173.99	30	109.10	34	18
Spiramycin	843.1	843.31	173.95	32	142.02	32	146
Tilmicosin	869.1	869.62	696.41	40	174.00	41	132
Tylosin	916.1	916.51	174.00	35	772.44	26	141
Clarithromycin	748.0	748.51	158.15	28	590.37	17	108
Oleandomycin	687.8	688.44	544.35	14	158.05	25	106
Tylvalosin	1042.3	1042.64	109.06	41	173.95	37	133
Sulfadimethoxine	310.3	311.03	156.06	21	108.13	27	88
Sulfamethoxazole	253.2	254.03	156.05	15	92.18	27	96
Sulfadoxin	310.3	311.04	156.04	18	108.14	26	88
Sulfaquinoxaline	300.3	301.04	156.02	17	92.16	28	92
Sulfachlorpyridazine	284.7	284.97	156.03	15	92.18	26	90
Sulfaclozine	284.7	284.96	92.16	29	108.12	26	87
Sulphafenazole (IS)	314.4	315.06	158.10	28	160.10	22	94
Oxytetracycline	460.4	461.11	426.10	18	426.10	18	93
Tetracycline	444.4	445.16	410.14	18	427.15	11	99
Chlortetracycline	478.8	479.09	444.08	22	462.11	16	98
Doxycycline	444.4	445.14	428.15	18	321.05	31	82
Marbofloxacin	362.4	363.11	72.30	22	320.06	14	97
Ciprofloxacin	331.3	332.08	314.10	18	288.11	22	89
Danofloxacin	357.4	358.11	340.14	24	314.15	16	99
Enrofloxacin	359.4	360.10	316.13	19	342.14	22	96
Difloxacin	399.4	400.10	382.11	21	356.1	19	98
Oxolinic acid	261.2	262.01	244.04	18	216.02	29	84
Flumequine	261.3	262.02	244.05	19	202.03	33	84
Nalidixic acid	232.2	233.04	215.08	15	187.05	25	77
Enoxacin	320.3	321.09	303.09	19	257.10	17	93
Ofloxacin	361.4	362.12	318.14	18	261.08	27	91
Lomefloxacin	351.3	352.10	265.09	23	308.13	15	100
Norfloxacin	319.3	320.07	302.08	22	276.1	16	94
Sarafloxacin	385.4	386.08	368.10	23	342.11	18	94
Cinoxacin	262.2	263.02	245.02	16	217.04	22	90

CE: Collision Energy

Table 3. Ion ratios (Qual/Quant) in matrix and in standard mixture (the agreement between ion ratios should be within the permitted tolerance, which is defined in EU Commission Decision 2002/657/EC)

Analyte	Ion Ratio (Std Mix)	Ion Ratio (Chicken Meat)	Difference (%)
Kanamycin	0.53	0.50	6.0
Neomycin	0.95	0.94	1.1
Lincomycin	0.09	0.09	0.0
Clindamycin	0.05	0.04	25.0
Trimethoprim	0.76	0.70	8.6
Josamycin	0.90	0.91	1.1
Spiramycin	0.17	0.21	19.0
Tilmicosin	0.88	0.88	0.0
Tylosin	0.23	0.23	0.0
Clarithromycin	0.62	0.61	1.6
Oleandomycin	0.65	0.71	8.5
Tylvalosin	0.55	0.50	10.0
Sulfadimethoxine	0.60	0.56	7.1
Sulfamethoxazole	0.31	0.30	3.3
Sulfadoxin	0.46	0.58	20.7
Sulfaquinoxaline	0.24	0.27	11.1
Sulfachlorpyridazine	0.44	0.46	4.3
Sulfaclozine	0.20	0.29	31.0
Sulfaphenazole (IS)	0.76	0.75	1.3
Oxytetracycline	0.13	0.10	30.0
Tetracycline	0.80	0.84	4.8
Chlortetracycline	0.48	0.42	14.3
Doxycycline	0.03	0.05	40.0
Marbofloxacin	0.79	0.61	29.5
Ciprofloxacin	0.13	0.14	7.1
Danofloxacin	0.06	0.03	50.0
Enrofloxacin	0.58	0.62	6.5
Difloxacin	0.58	0.69	15.9
Oxolinic acid	0.06	0.08	25.0
Flumequine	0.44	0.42	4.8
Nalidixic acid	0.30	0.32	6.3
Enoxacin	0.02	0.03	33.3
Ofloxacin	0.70	0.70	0.0
Lomefloxacin	0.58	0.64	9.4
Norfloxacin	0.05	0.09	44.4
Sarafloxacin	0.18	0.25	28.0
Cinoxacin	0.28	0.30	6.7

Table 4. Method intermediate precision as RSD (%) – 1 level – 3 sets with 6 replicates in 3 days and method repeatability expressed as RSD (%) and measured at 3 levels every time with 6 replicates Table 4. Method intermediate precision as RSD (%) – 1 level – 3 sets with 6 replicates in 3 days and method repeatability expressed as RSD (%) and measured at 3 levels every time with 6 replicates

Analyte	RSD (%) – spiking level 2			Chicken meat – RSD (%)		
	Day 1	Day 2	Day 3	Level 1 (µg/kg)	Level 2 (µg/kg)	Level 3 (µg/kg)
Kanamycin	19	18	26	19	25	21
Neomycin	23	28	18	23	18	19
Lincomycin	4	13	6	4	10	10
Clindamycin	6	9	12	6	3	10
Trimethoprim	9	9	10	9	7	9
Josamycin	9	6	11	9	6	21
Spiramycin	8	31	10	8	10	21
Tilmicosin	7	7	6	7	7	9
Tylosin	9	16	4	9	7	19
Clarithromycin	11	14	5	11	6	12
Oleandomycin	13	24	15	13	10	10
Tylvalosin	15	17	11	15	6	16
Sulfadimethoxine	3	3	8	3	5	10
Sulfamethoxazole	7	8	3	7	10	5
Sulfadoxin	14	11	12	14	9	6
Sulfaquinoxaline	17	15	34	17	21	5
Sulfachlorpyridazine	8	10	17	8	8	13
Sulfaclozine	14	8	7	14	14	10
Oxytetracycline	27	6	16	27	13	11
Tetracycline	10	9	11	10	12	10
Chlortetracycline	13	4	17	13	19	12
Doxycycline	7	6	13	7	8	9
Marbofloxacin	9	15	19	9	12	18
Ciprofloxacin	10	3	12	10	8	8
Danofloxacin	5	3	7	5	3	9
Enrofloxacin	11	5	10	11	7	6
Difloxacin	4	4	9	4	8	10
Oxolinic acid	4	5	7	4	5	7
Flumequine	6	3	10	6	7	9
Nalidixic acid	6	9	9	6	6	8
Enoxacin	17	8	8	17	14	22
Ofloxacin	9	11	12	9	20	15
Lomefloxacin	27	18	16	27	19	16
Norfloxacin	11	8	10	11	7	16
Sarafloxacin	24	10	16	24	22	6
Cinoxacin	16	13	14	16	19	12

Table 5. Recoveries (%) for spiked samples of chicken meat at 3 different spike levels (6 replicates)

Analyte	Spiking levels			Chicken meat - REC (%)		
	Level 1 (µg/kg)	Level 2 (µg/kg)	Level 3 (µg/kg)	Level 1	Level 2	Level 3
Kanamycin	50	100	150	119	109	120
Neomycin	250	500	750	84	71	83
Lincomycin	50	100	150	104	94	102
Clindamycin	10	20	30	111	115	104
Trimethoprim	25	50	75	99	91	83
Josamycin	10	20	30	102	91	95
Spiramycin	100	200	300	108	102	92
Tilmicosin	37.5	75	112.5	115	105	102
Tylosin	50	100	150	86	84	82
Clarithromycin	10	20	30	101	105	98
Oleandomycin	10	20	30	116	93	92
Tylvalosin	10	20	30	91	101	99
Sulfadimethoxine	50	100	150	101	97	91
Sulfamethoxazole	50	100	150	113	108	96
Sulfadoxin	50	100	150	101	104	98
Sulfaquinoxaline	50	100	150	100	94	99
Sulfachlorpyridazine	50	100	150	109	102	94
Sulfaclozine	50	100	150	118	110	106
Oxytetracycline	50	100	150	115	109	114
Tetracycline	50	100	150	102	94	94
Chlortetracycline	50	100	150	96	85	87
Doxycycline	50	100	150	117	98	95
Marbofloxacin	10	20	30	104	105	106
Ciprofloxacin	50	100	150	101	114	103
Danofloxacin	100	200	300	116	108	109
Enrofloxacin	50	100	150	112	108	103
Difloxacin	150	300	450	106	105	102
Oxolinic acid	50	100	150	109	100	95
Flumequine	200	400	600	108	94	88
Nalidixic acid	10	20	30	118	103	99
Enoxacin	10	20	30	99	103	88
Ofloxacin	10	20	30	101	92	89
Lomefloxacin	10	20	30	98	100	94
Norfloxacin	10	20	30	101	112	101
Sarafloxacin	10	20	30	105	99	90
Cinoxacin	10	20	30	102	94	91

Table 6. Results of FAPAS quality control test material – fish muscle T02174QC – ciprofloxacin – c = 113 ± 50 µg/kg

Sample	concentration [found] (µg/kg)
Sample 1	90
Sample 2	103
Sample 3	107

Table 7. Limit of detection and quantification (LOD and LOQ), maximum residual limit (MRL) and limit of decision (CCα) and limit of capability (CCβ) for antibiotics in chicken meat

Analyte	MRL (µg/kg)	ccα (µg/kg)	ccβ (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)
Kanamycin	100	121	143	10.0	25.0
Neomycin	500	602	704	40.0	120.0
Lincomycin	100	110	119	3.0	10.0
Clindamycin	-	1	2	0.3	1.0
Trimethoprim	50	57	64	1.0	3.0
Josamycin	-	2	4	0.3	1.0
Spiramycin	200	223	247	0.3	1.0
Tilmicosin	75	80	85	0.3	1.0
Tylosin	100	107	114	1.0	3.0
Clarithromycin	-	3	5	0.3	1.0
Oleandomycin	-	2	4	0.3	1.0
Tylvalosin	-	3	6	0.3	1.0
Sulfadimethoxine	100[a]	110	120	0.3	1.0
Sulfamethoxazole	100[a]	119	137	1.5	5.0
Sulfadoxin	100[a]	108	116	0.3	1.0
Sulfaquinoxaline	100[a]	111	122	0.3	1.0
Sulfachlorpyridazine	100[a]	111	122	10.0	25.0
Sulfaclozine	100[a]	116	132	3.0	10.0
Oxytetracycline	100	112	125	3.0	10.0
Tetracycline	100	115	130	3.0	10.0
Chlortetracycline	100	112	124	5.0	15.0
Doxycycline	100	110	120	1.0	3.0
Marbofloxacin	-	4	8	1.5	5.0
Ciprofloxacin	100[b]	104	109	0.3	1.0
Danofloxacin	200	217	233	0.3	1.0
Enrofloxacin	100[b]	108	116	0.3	1.0
Difloxacin	300	334	369	0.3	1.0
Oxolinic acid	100	109	118	0.3	1.0
Flumequine	400	438	476	0.3	1.0
Nalidixic acid	-	1	2	0.3	1.0
Enoxacin	-	2	5	0.3	1.0
Ofloxacin	-	2	4	0.3	1.0
Lomefloxacin	-	3	5	0.3	1.0
Norfloxacin	-	4	8	0.3	1.0
Sarafloxacin	-	3	5	0.3	1.0
Cinoxacin	-	3	5	1.0	3.0

[a] Expressed in form of sum-MRLs of all sulfonamides.

[b] Expressed in form of sum-MRLs of Enrofloxacine and its metabolite (Ciprofloxacine).

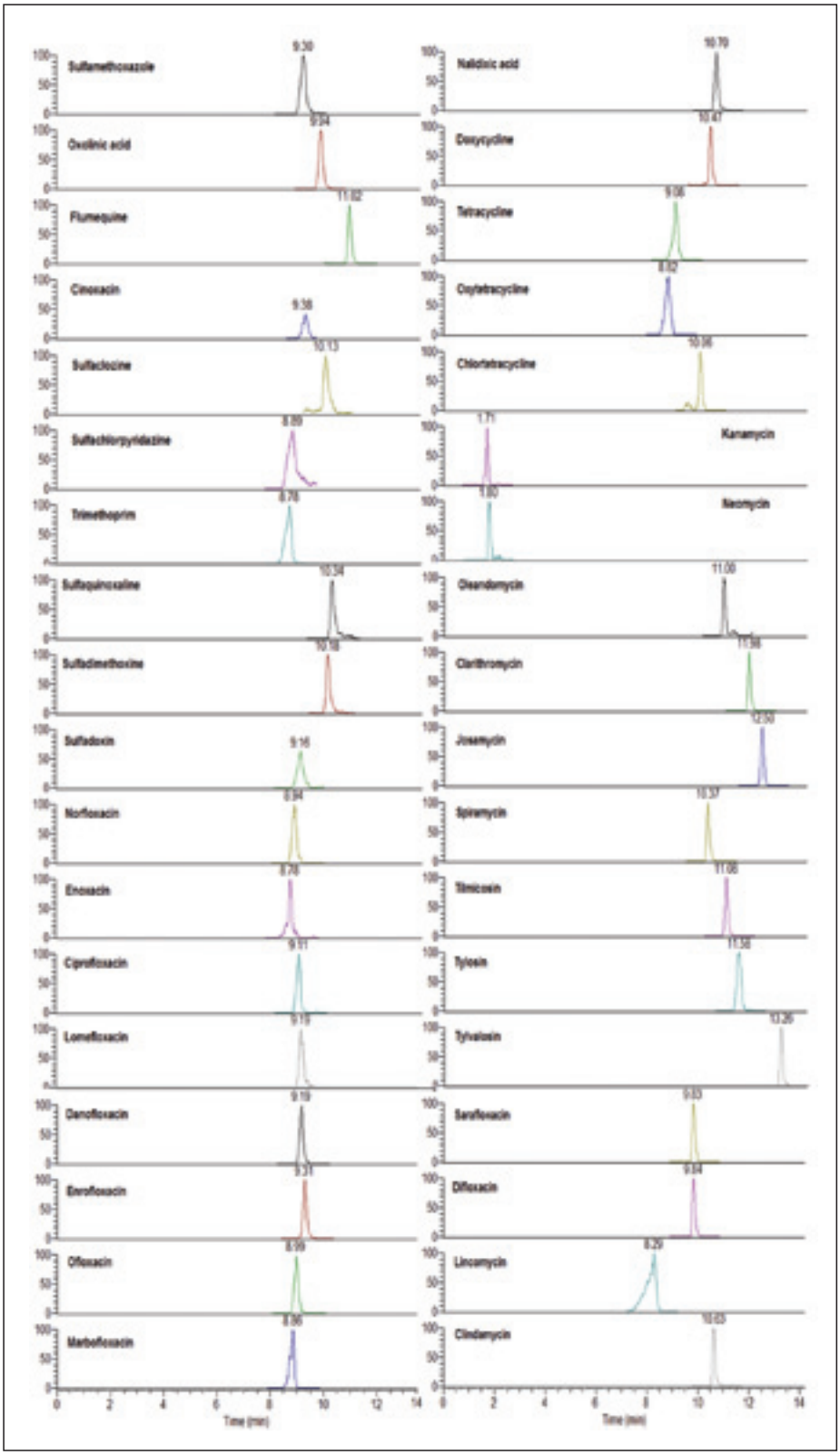


Figure 1. MRM chromatogram of chicken meat sample fortified with 36 antibiotics

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Quantitative Analysis of Ractopamine in Beef using Automated Online Sample Preparation with Liquid Chromatography-Tandem Mass Spectrometry

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Application Note 63554

Key Words
Transcend TLX-1, TurboFlow Technology, TSQ Quantum Access MAX, Ractopamine, Beef

Goal

To develop a rapid and sensitive automated online sample preparation LC-MS/MS method to determine ractopamine in beef.

Introduction

Ractopamine is a phenethanolamine member of the family of β2-adrenergic receptor agonists (β-agonists). It has been widely used as a veterinary additive drug in livestock production to promote leanness in meat, accelerate average daily weight gain and improve feed efficiency[1]. Recently, there have been growing concerns about the safety of meat containing ractopamine residues due to its potential health risks for humans[2]. Over 150 countries including China and the European Union (EU) have banned the use of ractopamine in animal feeds, but in other countries such as the US and Japan, ractopamine use is allowed. The maximum residue limit (MRL) of ractopamine has been set at 30 ppb for beef and 50 ppb for pork in the US and 10 ppb for beef muscle by Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA)[3]. The EU also proposed 10 ppb as the minimum required performance limit (MRPL)[4].

Ractopamine has been a specific trade concern since a number of shipments of US beef containing trace level contamination were rejected by the Taiwanese government as early as 2007. Therefore, it is essential to develop a sensitive, reliable and effective analytical method for quantitative measurement of ractopamine in samples of animal meat and organs. A number of methods have been reported to monitor ractopamine residue in meat-producing animals using a combination of chromatographic techniques with mass spectrometry, including gas chromatography/mass spectrometry (GC/MS)[5] and liquid chromatography/mass spectrometry (LC/MS)[4,6]. These approaches usually require complicated off-line sample clean-up procedures, primarily based on solid phase extraction (SPE), which can be very time-consuming, labor-intensive and are vulnerable to variability due to errors in manual preparation.

Thermo Scientific TurboFlow technology has been widely used as an automated online sample extraction technique in the food testing industry[7] and was recently applied to β-agonists in urine[8]. Sample extracts are directly injected onto a narrow diameter TurboFlow column, minimizing lengthy offline sample preparation steps. High linear velocities inside the column force large molecules to quickly flow through to waste while small molecules are retained. In this application note, we describe a simple and rapid method using TurboFlow™ technology and tandem MS for the quantitative analysis of ractopamine in beef.

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Experimental

Reagents/Matrix

Ractopamine hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO, USA) and its isotope labeled internal standard (IS), ractopamine-d_6 hydrochloride, was obtained from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada). All other chemicals and solvents were purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA). Ground beef was purchased from a local supermarket.

Sample Preparation

Four replicates of 5 grams each of homogenized ground beef were weighed into separate 50-mL conical polypropylene centrifuge tubes. Each 5 g sample was first extracted with 20 mL of extraction solvent consisting of 0.2 % formic acid in 80:20 methanol/H_2O by vortexing for 2 minutes followed by centrifugation at 5,700 RCF and 15 °C for 10 minutes. The resultant replicate supernatants were decanted and combined into a glass bottle. A second 10-mL solvent extraction was performed as above and the supernatants combined with the first extracts. The extract was then filtered through a 0.45 µm syringe filter and the resulting solution was used to make matrix calibrators and quality control (QC) samples. The overall sample preparation time is approximately 30 min. Twenty milliliters each of two solvents (ground beef extract and extraction solvent) were spiked with an IS stock solution of ractopamine-d_6 (1 µg/mL) to a final concentration of 5.0 ng/mL. The calibration concentration levels, were 0.00 (blank sample), 0.06, 0.12, 0.30, 0.60, 1.2, 3.0, 6.0, 30.0, 60.0 and 120.0 ng/g.

LC/MS conditions

LC/MS analysis was performed using a Thermo Scientific Transcend TLX-1 system powered by TurboFlow technology coupled to a Thermo Scientific TSQ Quantum Access MAX triple stage quadrupole mass spectrometer. Heated electrospray ionization source or HESI was selected for its sensitivity and ruggedness. As required in Commission Decision 2002/657/EC[9], one quantifier and two confirmation ions were selected. The precursor and product ion fragments of ractopamine and its IS were detected using positive ionization selective reaction monitoring (+SRM) as listed in Table 1.

TurboFlow Method Parameters	
System:	Transcend TLX-1 system
Column:	TurboFlow Cyclone-P, 0.5 x 50 mm
Injection Volume:	25 µL
Solvent A:	0.1 % Formic Acid in Water
Solvent B:	0.1 % Formic Acid in Methanol
Solvent C:	0.01M Ammonium Hydroxide in Water
Solvent D:	1:1:1 Acetonitrile (ACN): Isopropanol: Acetone (v:v:v)

HPLC Method Parameters	
Analytical Column:	Accucore C18, 3 x 50 mm, 2.6 µm
Solvent A:	0.1 % Formic Acid in Water
Solvent B:	0.1 % Formic Acid in ACN
Solvent C:	100 % Isopropanol

Mass Spectrometer Parameters	
Mass spectrometer:	TSQ Quantum Access MAX
Ionization Source:	Heated electrospray ionization II (HESI II)
Ion Polarity:	Positive ion mode
Spray Voltage:	2.5 KV
Sheath Gas Pressure (N_2):	60 arbitrary units
Auxiliary Gas Pressure (N_2):	30 arbitrary units
Vaporizer Temperature:	350 °C
Capillary Temperature:	300 °C
Tube Lens Voltage:	110 V
Collision Gas Pressure (Ar):	1.5 mTorr
Q1 Resolution:	*m/z* 0.7 (full width at half maximum)
Q3 Resolution:	*m/z* 0.7

Figure 1 illustrates the schematic diagram of a typical TurboFlow online sample extraction system. The LC method schematic view in Thermo Scientific Aria OS software is shown in Figure 2. The entire experiment was controlled by Aria OS version 1.6.3.

Table 1. The +SRM transitions for ractopamine and its internal standard as run on the mass spectrometer operating with a HESI source

Analyte	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Collision Energy (CE)	Tube Lens
Ractopamine	302.141	164.074 (Q)	15	83
		107.122	29	
		121.089	23	
Ractopamine-d_6	308.170	168.094 (Q)	15	85
		107.137	30	
		121.090	22	

Q: Quantifier

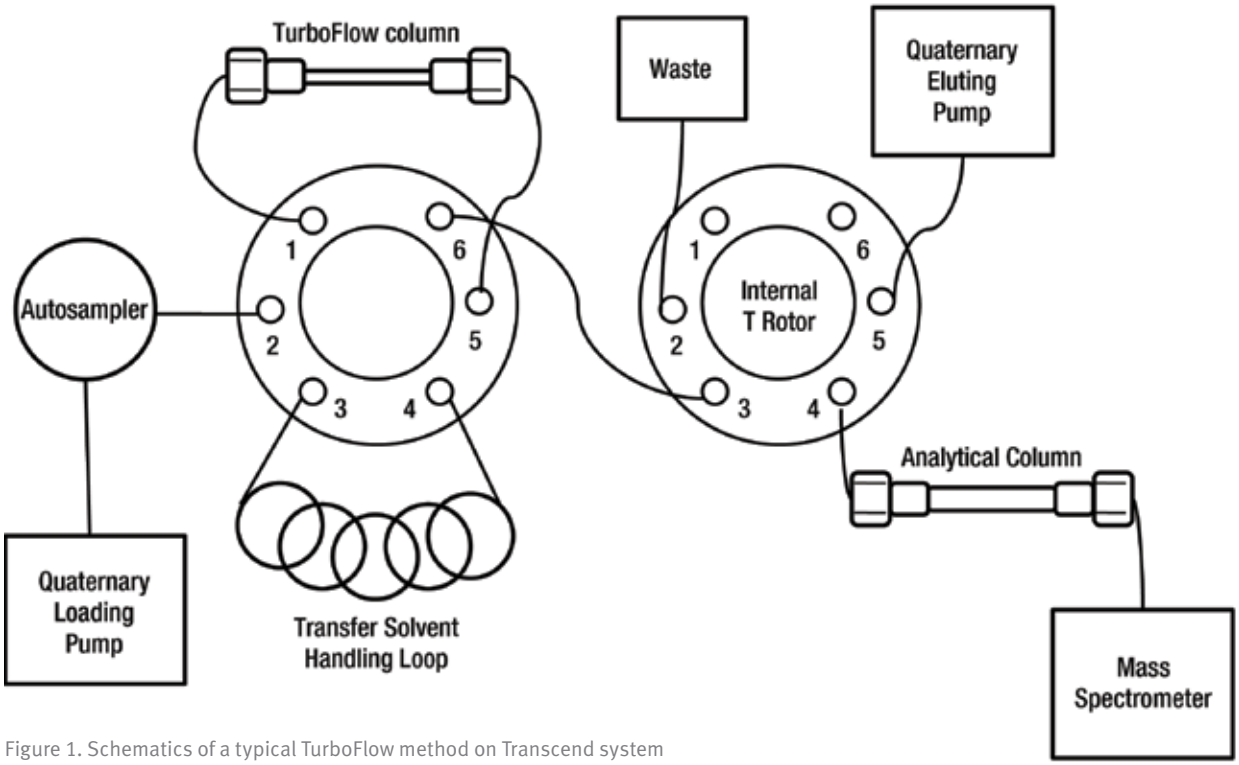

Figure 1. Schematics of a typical TurboFlow method on Transcend system

Step	Start	Sec	Flow	Grad	%A	%B	%C	%D	Tee	Loop	Flow	Grad	%A	%B	%C	%D
1	0.00	30	1.50	Step	100.0	-	-	-	====	out	0.70	Step	100.0	-	-	-
2	0.50	10	0.20	Step	100.0	-	-	-	====	out	0.70	Step	100.0	-	-	-
3	0.67	60	0.20	Step	100.0	-	-	-	T	in	1.30	Step	100.0	-	-	-
4	1.67	30	1.50	Step	-	-	-	100.0	====	in	0.70	Step	40.0	60.0	-	-
5	2.17	30	1.50	Step	-	-	100.0	-	====	out	0.70	Ramp	-	100.0	-	-
6	2.67	10	1.50	Step	-	-	-	100.0	====	in	0.70	Step	-	100.0	-	-
7	2.83	30	1.50	Step	-	-	-	100.0	====	out	1.00	Step	-	-	100.0	-
8	3.33	60	1.50	Step	90.0	10.0	-	-	====	in	0.70	Step	-	100.0	-	-

Start Data Window 0.02 min Data Window Length 5.05 min

Figure 2. TurboFlow method schematic diagram as viewed in Aria OS software

Results and discussion

Limit of detection and quantitation

In the present study, in addition to a double blank sample (processed matrix sample without IS) and a zero sample (processed matrix sample with IS), ten calibration concentration levels ranging from 0.06 to 120 ng/g were used. Good linearity was observed over the entire tested range for 25 µL injections except at the two lowest concentration levels. As shown in Figure 3, the correlation coefficient obtained using weighted (1/x) linear regression analysis of standard curves was greater than 0.99 for beef. For the concentration range studied, limits of quantitation (LOQ) was estimated from triplicate injections (coefficient of variation CV < 20%) of standard solutions at a concentration level corresponding to a minimum signal-to-noise (S/N) ratio of 10. The limit of quantification of the current protocol was 0.30 ng/g in beef. Figure 4 shows the extracted ion chromatogram of ractopamine at LOQ in beef.

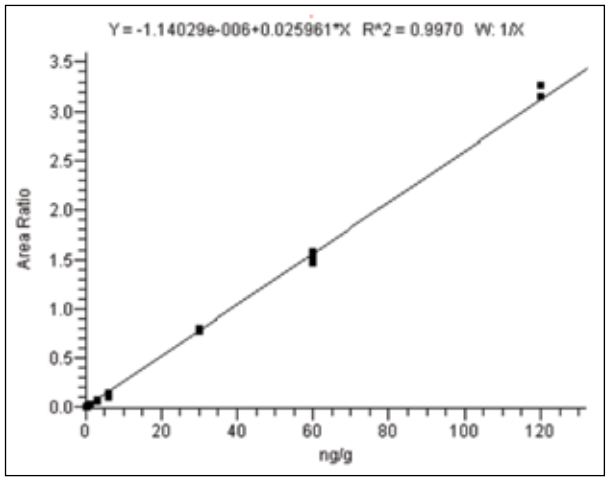

Figure 3. Linear regression curve of ractopamine standards based on area ratio with internal standard ractopamine-d_6

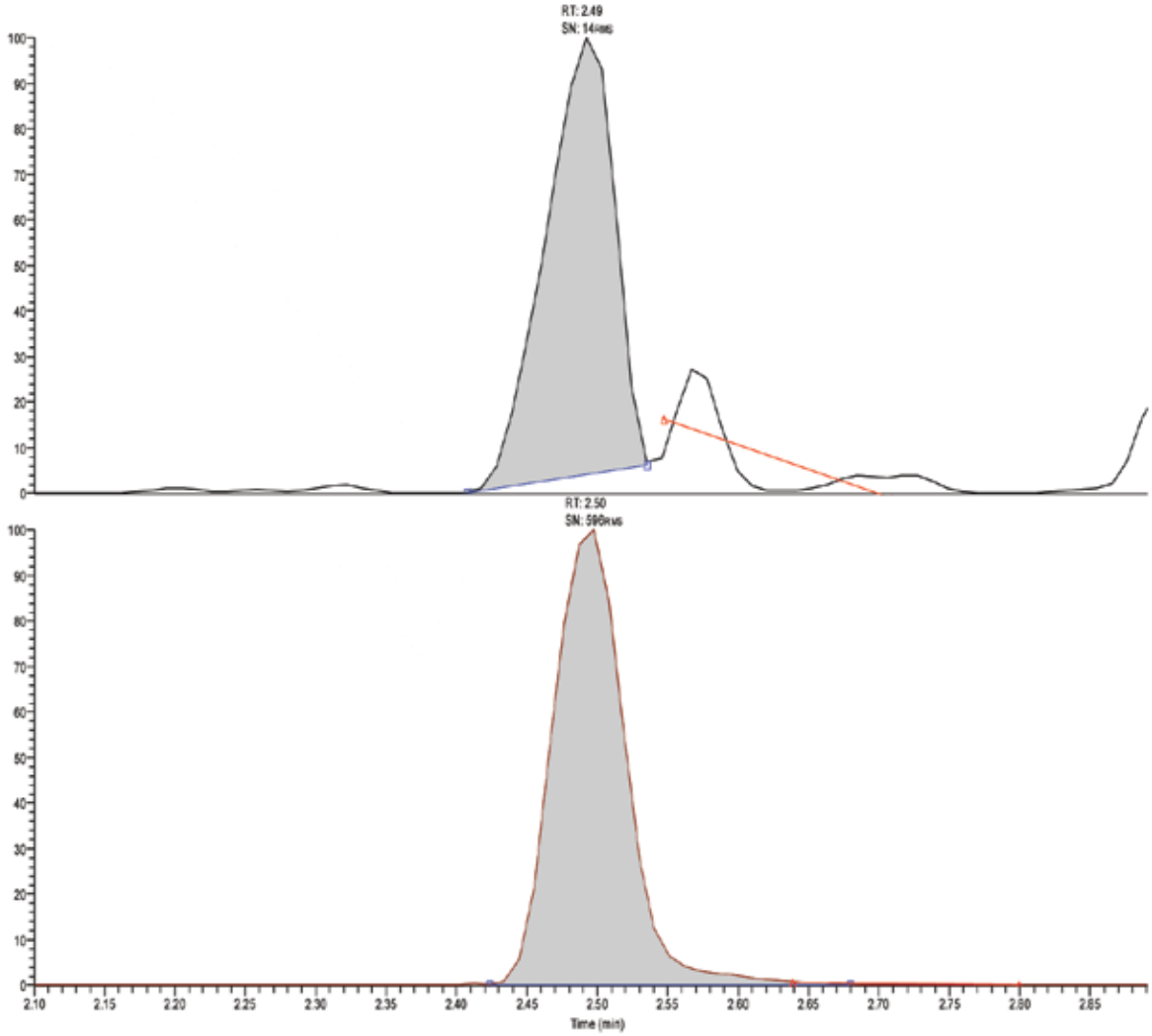

Figure 4. Extracted ion chromatography of ractopamine SRM *m/z* 164 transition at LOQ (upper trace) and ractopamine-d_6 at 5.0 ng/mL in matrix (lower trace)

Accuracy and precision

Method accuracy and precision were assessed using blank matrix spiked at four concentration levels injected in six replicates. Samples were spiked at 0.6, 3.0, 6.0 and 60.0 ng/g concentration levels. Between-run accuracy and precision was expressed as mean of the overall accuracy and precision data from four individual runs over three consecutive days. The accuracy is reported as percent of the nominal spiked known concentration value and the precision is expressed as the relative standard deviation (%RSD). Table 2 summarizes the data obtained from the method validation study. The within-run and between-run accuracy varied from 84.0 to 99.6 and 82.8 to 99.5 % of nominal concentrations, respectively, while within-run and between-run precision ranged from 2.6 to 11.2 %RSD and 2.9 to 14.0 %RSD, respectively. These values are all within the requirements (15% for both accuracy and precision) determined by various regulatory agencies[9,10].

Table 2. Results of the method validation

Ractopamine Spike level (ng/g)	Within-run Accuracy (Run 1) (%)	Between-run Accuracy (%)	Within-run Precision (Run 1) (%RSD)	Between-run Precision (%RSD)	Mean Recovery (%) (n=6)
0.6	94.9	97.3	11.2	14.0	86.3
3.0	89.9	85.7	8.1	8.9	91.7
6.0	84.0	82.8	7.2	7.7	85.5
60.0	99.6	99.5	2.6	2.9	103.8
Internal Standard			3.2	5.1	

Recovery

The recovery study was performed on beef matrix fortified with ractopamine at 0.6, 3.0, 6.0, and 60.0 ng/g. The recovery was assessed by comparing the detector response of a post-extracted spiked sample with that determined from a spiked neat standard sample at the same concentration. Addition of isotope labeled IS compensated for matrix interference during LC/MS analysis. Recovery values of 70–125% are deemed acceptable. As shown in Table 2, recoveries ranged from 85 to 104% in beef. Method performance data show the feasibility and reliability of using this approach for the determination of ractopamine in meat matrices.

Carryover

The level of carryover was determined by evaluating the analyte peak area in a matrix double blank injected immediately after the highest calibration matrix standard. Peak area should not be greater than 20% of the LOQ. To minimize autosampler cross-contamination, the injector and syringe were washed with 0.1% formic acid in 2% ACN and 0.1% formic acid in ACN three times each between injections. There is relatively low affinity between ractopamine and hydrophobic alkyl phases. Carryover associated with the TurboFlow column was inhibited by washing the column with over 150 column volume of 1:1:1 ACN isopropanol acetone (v:v:v) after the analytes were transferred onto the analytical column. The analytical column was washed with 1.5 column volume isopropanol after the gradient completion. A blank sample was also injected after each standard or sample to minimize the possible impact of carryover on accuracy and precision. As a result, no measurable carryover peak was detected.

Conclusion

A quick, rugged, sensitive and automated online LC/MS method was developed to measure ractopamine in animal meat matrices. The TurboFlow method eliminates the need for time-consuming sample preparation procedures such as offline SPE, allowing for higher throughput and better reproducibility. The method quantitation limit has been determined to be 0.30 ng/g, which is significantly lower than the limits set by the US government and MRPL proposed by the EU. Owing to the large volume and/or multiple injection capability of Transcend TLX system[11], the quantitation limit could be further lowered if necessary.

The relevant data window of this method represents only a part of total run time, thus sample throughput can be doubled or even quadrupled by multiplexing across a two or four channel Transcend TLX system. As a result, up to 40 samples per hour can be analyzed. There is also a potential to add other β-agonists, such as clenbuterol, into the same method.

Acknowledgements

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Validated Method for the Determination of Phenylbutazone in Horse Meat with LC-MS/MS

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Method 63799

Key Words
TSQ Vantage, UltiMate 3000, TraceFinder 3.0, Horse Meat, In-house Validation, Phenylbutazone

1. Schematic of Method

Sampling and Internal Standardization

1. Weigh 2 g of homogenized horse meat to 15 mL centrifuge tube; add IS (20 ppb).

Enzymatic Hydrolysis

2. Add 4 mL acetate buffer (pH 4.5) and 50 µL β-glucuronidase.
3. Vortex sample and incubate for 1 hr at 37 °C.

Extraction

4. Cool sample and add 10 mL ACN; shake it vigorously.
5. Centrifuge sample at 4500 rpm for 5 min, collect supernatant.
6. Repeat extraction with 5 mL, centrifuge, and combine supernatants.

Defatting

7. Pass supernatant through Thermo Scientific™ HyperSep™ Silica (2 g) cartridge.

Clean up

8. Blow down extract to ~ 4 mL and apply onto C18 SPE cartridge.
9. Elute analyte with 4 mL ACN and evaporate to dryness.
10. Redissolve samples in 500 µL mobile phase, filter (0.2 µm PTFE).

LC-MS/MS

2. Introduction

Phenylbutazone (bute, PBT) is one of the most widely used (non-steroidal) anti-inflammatory (NSAiD) and painkiller drugs for dogs and horses. Bute was used as a treatment for rheumatoid arthritis and gout in humans as well, but its use was discontinued due to health concerns. It was found to cause different blood disorders (discrasia, anemia, leuko- and thrombocytopenia) and in some cases allergic or severe toxic reactions.

Horse meat is still widely used for human consumption in some European countries. For those animals marked for human consumption, a limited number of medicines can be administered. Any horse meat products that contain substances that are not on a prescribed list (like PBT) must be permanently excluded from the food chain. Despite this regulation, bute continues to be found in 2–5% of slaughtered horse samples, indicating non-compliance with existing meat tracking systems or that feed containing bute was eaten by a horse for which

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it not was intended. As a response to the possible human health threat from the discovery of horse meat in beef products in some European countries, the Standing Committee of the Food Chain and Animal Health (SCoFCAH) of the European Commission has required member states to test representative samples of beef meat products to see if they contain horse meat and to detect possible illegal residues like PBT.

A newly developed LC-MS/MS method for the determination of PBT in horse meat with LC-MS/MS is described here. The method was in-house validated and its applicability for routine testing according to the European legislation requirements is critically reviewed.[1]

3. Scope and Application

This method can be applied to horse and beef meat samples at a limit of quantification (LOQ) below 5 µg/kg, which is the action limit used by the European Commission for monitoring purposes. The method has been validated for the determination of phenylbutazone in horse meat.

4. Principle

This liquid-phase chromatographic method is based on offline sample preparation and triple quadrupole mass spectrometric detection. Due to the fact that a high amount (>90%) of PBT is bound to carrier proteins, samples were enzymatically hydrolyzed prior to extraction with acetonitrile.[2] The centrifuged extracts were collected and defatted on silica cartridge. The volume of the collected effluent was reduced prior to application onto C18 SPE cartridges for clean-up. The eluate was collected and evaporated under a nitrogen stream and, after reconstitution in initial mobile phase and filtration, the samples were directly injected into the LC-MS/MS system.

5. Reagent List

		Part Number
5.1	Purified Water	Obtained from Thermo Scientific™ Barnstead™ EASYpure™ II water system
5.2	Methanol, Fisher Chemical™ Optima™, LC-MS grade	10767665
5.3	Water, Optima, LC-MS grade	10505904
5.4	Acetonitrile, Optima, LC-MS grade	10001334
5.5	Formic acid, extra pure, >98%	10375990
5.6	Sodium acetate anhydrous	10103243
5.7	Acetic acid, HPLC grade	10060000
5.8	Ascorbic acid	Sigma-Aldrich®
5.9	β-glucuronidase from *Helix pomatia*	Sigma-Aldrich

6. Calibration Standards

6.1	Phenylbutazone	Sigma-Aldrich
6.2	Internal standard: Phenylbutazone – diphenyl $^{13}C_{12}$	Sigma-Aldrich

7. Standards Preparation

7.1 Stock Standard Solutions

Stock standard solutions (1000 µg/mL) were prepared individually. Phenylbutazone was prepared by weighing 25 mg of standard and dissolving in 25 mL of methanol. Phenylbutazone – diphenyl $^{13}C_{12}$ (IS) was prepared by weighing of 5 mg of standard and dissolving in 5 mL of methanol. Solutions were stored at -20 °C and were stable for 12 months.

7.2 Working Standard Solutions

The working standard solutions containing 10 µg/mL were prepared individually by dilution of individual stock standard solutions with methanol. Solutions were stored at -20 °C and were stable for 6 months.

7.3 Spiking Standard Solutions

Spiking standard solution (200 µg/L) of phenylbutazone was prepared by dilution of working standard solution with methanol. Spiking internal standard solution (1000 µg/L) was prepared by dilution of working standard solution with methanol. All standard spiking solutions were stored at -20 °C and were stable for 3 months.

8. Apparatus

		Part Number
8.1	Thermo Scientific™ Dionex™ UltiMate™ 3000 HPLC system	
8.2	Thermo Scientific™ TSQ Vantage™ triple quadrupole mass spectrometer	
8.3	Fisher precision balance	10145222
8.4	Sartorius analytical balance	1056-4833
8.5	EASYpure II water system	10010682
8.6	Vortex shaker	1013-2562
8.7	Vortex universal cap	1014-2902
8.8	BrandTech® accu-jet® pipettor	1047-5062
8.9	Centrifuge, Thermo Scientific™ Heraeus™ Multifuge™ X3R	10667815
8.10	Thermo Scientific 16 port SPE vacuum manifold	11390731
8.11	Heating Bath B-491, Büchi®	1069-5793
8.12	Thermo Scientific™ Orion™ 2 Star, pH meter	1015-5714
8.13	Horizontal shaker 501 digital, IKA® Werke	1053-6011
8.14	Waring® laboratory blender	10221962
8.15	Evaporator EVMT-130-32-16	1027-9991

9. Consumables

		Part Number
9.1	Thermo Scientific™ Accucore™ C18 (50 × 2.1 mm, 2.6 µm) HPLC column	17126-052130
9.2	Centrifuge tubes, PP disposable 50 mL	05-539-8
9.3	Glass tubes, 15 mL	FB12170
9.4	LC vials	3205111
9.5	LC caps	3151266
9.6	Thermo Scientific™ Finnpipette™ Pipette 100–1000 µL	3214535
9.7	Pipette Finnpipette 20–200 µL	3214534
9.8	Pipette Finnpipette 10–100 µL	3166472
9.9	Pipette Finnpipette 500–5000 µL	3166473
9.10	Pipette Finnpipette 1000–10000 µL	3214536
9.11	Pipette holder	651211
9.12	Pipette tips 0.5–250 µL, 500/box	3270399
9.13	Pipette tips 1–5 mL, 75/box	3270420
9.14	Pipette tips 100–1000 µL, 200/box	3270410
9.15	Pipette tips 20000–10000 µL, 40/box	3270425
9.16	Pipette Pasteur soda lime glass 150 mm	FB50251
9.17	Pipette suction device	3120891
9.18	PTFE syringe filter 0.2 µm	F2513-4
9.19	SPE cartridges, HyperSep Silica (2 g, 15 mL)	60108-710
9.20	SPE cartridges, HyperSep C18 (0.5 g, 6 mL)	60108-305
9.21	Spatula, 18/10 steel	3458179
9.22	Spatula, nylon	3047217
9.23	Tube rack (15 mL)	1034-3461
9.24	Tube rack (50 mL)	1024-1861
9.25	1 mL disposable syringes	1066-4161
9.26	Vial rack (2 mL)	12211001

Glassware

		Part Number
9.27	Beaker, 50 mL	10527211
9.28	Beaker, 100 mL	10769541
9.29	Beaker, 25 mL	10683771
9.30	Volumetric flask, 25 mL	10107901
9.31	Volumetric flask, 10 mL	10406681
9.32	Volumetric flask, 5 mL	10770803
9.33	Volumetric flask, 100 mL	10675731

10. Procedure

10.1 Sample Preparation[3]

10.1.1

50 g of horse meat was homogenized with laboratory blender.

10.1.2

2 g of homogenized meat was weighed into 50 mL polypropylene centrifuge tube. 40 µL of IS spiking solution was added. Sample was mixed by vortex and let to stay for 10 min.

10.1.3

4 mL of acetate buffer (2.7 g of sodium acetate and 0.17 g of ascorbic acid were diluted in 100 mL of H_2O, the pH was adjusted to 4.5 with acetic acid) and 50 µL of β-glucuronidase were added. The sample was vortexed and incubated for 1 h at 37 °C in a water bath.

10.1.4

After cooling the sample, 10 mL of acetonitrile (ACN) was added and the sample was shaken by the horizontal shaker for 5 min and consequently centrifuged (5 °C, 5000 × g, 5 min). The supernatant was transferred into clean 50 mL centrifuge tube. A second extraction procedure was performed by addition of 5 mL of ACN, shaking for 5 min and centrifugation (5 °C, 5000 × g, 5 min).

10.1.5

The combined extracts (ca. 19 mL) were passed through the SPE HyperSep Silica (2 g, 15 mL) cartridge and collected entirely in a glass tube. The cartridge was washed with 4 mL of mixture ACN:H_2O (75:25, v/v) and the portion was added to the eluate.

10.1.6

The eluate was evaporated by nitrogen flow to approximately 4 mL at 50 °C.

10.1.7

The extract was loaded onto the C18 cartridge preconditioned with 2 mL of methanol and 2 mL of 0.02 M ascorbic acid. The glass tube was washed with 2 × 3 mL portions of 0.02 M ascorbic acid and both portions were loaded on the column. The cartridge was washed with 2 mL of 0.02 M ascorbic acid, 2 mL of H_2O and 2 mL of mixture ACN:H_2O (30:70, v/v). The analytes were eluted from the cartridge with 4 mL of ACN and evaporated to dryness by nitrogen stream at 50 °C.

10.1.8

The extract was reconstituted in 0.5 mL mixture of ACN: 0.1% formic acid (50:50, v/v) and filtered through the syringe filter (PTFE, 0.2 µm) directly to the vial.

10.2 LC Conditions

LC analysis was performed on an UltiMate 3000 UHPLC system equipped with Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System and Thermo Scientific™ TraceFinder™ software version 3.0 for system control, acquisition, and data evaluation.

The LC conditions were as follows:

Analytical column:	Accucore C18 (50 × 2.1 mm, 2.6 µm)
Total run time:	7.5 min
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	Acetonitrile
Gradient:	Table 1
Injection volume:	10 µL
Column temperature:	Ambient

Table 1. Gradient program of the UHPLC method

Time [min]	A%	B%	Flow rate [µl/min]
0	50	50	350
5	0	100	350
5.5	0	100	350
5.6	50	50	350
7.5	50	50	350

10.2.1 Injector Settings

The injector settings were as follows:

Injector:	UltiMate 3000 autosampler
Injection mode:	Normal
Cleaning solvent:	MeOH
Draw speed [µL/s]:	2
Pre clean with solvent 2 [steps]:	3
Draw delay [ms]:	500
Dispense speed [µL/s]:	5
Dispense delay [ms]:	200
Dispense to waste speed [µL/s]:	5
Sample height [mm]:	0.2
Inject wash:	Both
Wash volume [µL]:	100
Wash speed [µL/s]:	5
Loop wash factor:	2

10.3 Mass Spectrometric Conditions

Mass spectrometric analysis was carried out using a TSQ Vantage triple quadrupole system. Data acquisition for quantification and confirmation was performed in the selected reaction monitoring mode (SRM). All SRM traces (parent, qualifier, and quantifier ion) were individually tuned for each target analyte by direct injection of the individual working standard solution (100 ng/mL). Data acquisition and processing was performed using TraceFinder 3.0 software.

The MS conditions were as follows:

Ionization mode:	Heated Electrospray (HESI)
Scan type:	Selected reaction monitoring (SRM)
Polarity:	Negative ion mode
Spray voltage [V]:	2500
Ion sweep gas pressure [arb]:	0
Vaporizer temperature [°C]:	320
Sheath gas pressure [arb]:	20
Aux Gas Pressure [arb]:	10
Capillary temperature [°C]:	310
Collision gas pressure [mTorr]:	1.5
Cycle time [s]:	0.3
Peak width:	Q1/Q3 the full width of a peak at half its maximum height (FWHM) of 0.70 Da

The parameters for SRM analysis for target compound and internal standard are displayed in Table 2.

11. Calculations

11.1 Identification

Identification of phenylbutazone and phenylbutazone – diphenyl $^{13}C_{12}$ was confirmed by the presence of transition ions (quantifier and qualifier) at retention times (+/-2.5%) to the corresponding standards. In multiple reaction monitoring (MRM) mode the measured peak area ratios for qualifier to quantifier ion should be in close agreement (according to Commission Decision 2002/657/EC) with those of the standards as shown in Table 2. The quantifier and qualifier ion were selected among the product ions produced by the fragmentation of the selected parent ion on the basis of the intensity.

11.2 Quantification

For quantification internal standardization was used, measuring peak area ratios for standards in matched matrixes. Phenylbutazone – diphenyl $^{13}C_{12}$ was used as the internal standard for phenylbutazone. The calibration curve was plotted as the relative peak area (phenylbutazone versus the phenylbutazone – diphenyl $^{13}C_{12}$) as a function of the compound concentration. The phenylbutazone concentration in the samples was determined from the equation:

$$c_{PBT} = \left(\frac{A_{PBT}}{A_{IS}} - b \right)$$

c_{PBT} – phenylbutazone concentration in µg/kg

A_{PBT} – peak area of the phenylbutazone

A_{IS} – peak area of internal standard

b – y-intercept

a – slope of the calibration curve

12. Method Performance

The method was in-house validated according to the criteria specified in European Commission Decision 2002/675/EC for a quantitative method.[1] The validation parameters were determined by spiking phenylbutazone-free horse meat at levels of 2.5, 5, and 7.5 µg/kg. The measured parameters were specificity, linear range, repeatability, accuracy, limit of detection and quantification (LOD and LOQ), and limit of decision and capability (CCα and CCβ). Data were evaluated by TraceFinder 3.0 software. A screenshot of result overview is presented in Figure 1.

12.1 Samples and Quality Control Materials

For preparation of matrix-matched calibration samples and spiking of samples for validation, horse meat was obtained from a local butcher. The meat was analyzed by repeated measurements to confirm that it was free of phenylbutazone.

For determination of accuracy, a former proficiency test sample FAPAS – 02157 of bovine muscle test material from the Food and Environmental Research Agency (York, UK) was analyzed.

12.2 Specificity

Using SRM the specificity was confirmed based on the presence of the transition ions (quantifier and qualifier) at the correct retention times corresponding to those of the phenylbutazone and phenylbutazone – diphenyl $^{13}C_{12}$. The measured peak area ratios of qualifier/quantifier were in the range (defined in Commission Decision 2002/657/EC) comparing to the standards (Table 2).[1]

12.3 Linearity and Calibration Curve

The linearity of calibration curves was assessed over the range from 0–30 µg/kg for phenylbutazone. The correlation coefficient of linear function was 0.9941. The calibration curve was created from five matrix-matched calibration standards (0, 2.5, 5, 7.5, 10 µg/kg) which were injected in each batch in triplicate.

12.4 Precision

Precision (repeatability) of the method was determined using independently spiked blank horse meat at three different levels (2.5, 5, and 7.5 µg/kg) (within day repeatability) with % RSD of 5.2, 2.0, and 6.3 respectively. In one day the set of three levels with six repetitions was measured. For the determination of the repeatability (day-to day-repeatability) two other sets at one level (5 µg/kg) with six repetitions were measured over the next two days with a % RSD of 9.8. The results are summarized in Table 3 and Table 4. The LC-MS/MS chromatogram of phenylbutazone spiked and extracted in horse meat at 2.5 mg/kg is shown in Figure 2.

12.5 Accuracy

Method accuracy was determined using independently spiked blank samples at three different levels. Accuracy was evaluated by comparison of found values with standard addition in spikes. Recovery values ranged between 95.6–103.9% (Table 3). Additionally, accuracy was established by analyzing a former proficiency test sample FAPAS – 02157 of bovine muscle test material in duplicate. All measured concentrations of phenylbutazone were within the acceptable satisfactory range (Table 5). The LC-MS/MS chromatogram of phenylbutazone detected in reference material is presented in Figure 3.

12.6 Matrix Effect

The matrix effect was investigated by comparison of calibration results in solvent and in matrix. The Youden plot of both calibration series was applied. The slope of the fitted linear resulted in y = 0.9821x, which represents less than 20% deviation from the idealistic y = x value, indicating no matrix effect for the investigated matrix (Figure 4).

12.7 LOD and LOQ

Limits of detection and quantification were estimated following the IUPAC approach, which consisted of analyzing the blank sample to establish noise level and then estimating LOD and LOQ for signal/noise, 3 and 10 respectively. LOQ was found to be at 2.0 µg/kg and LOD at 0.8 µg/kg.

12.8 CCα and CCβ

Both CCα and CCβ were established by the calibration curve procedure according to ISO 118434 guideline.[4] The blank material fortified at and above the lowest possible level (for analytes without MRL) in equidistant steps was used. The calculated values were 1.0 µg/kg for CCα and 1.29 µg/kg for CCβ (Table 3).

12.9 Robustness

The robustness of the method was tested by the analysis of samples with another HPLC-MS/MS system and the sample preparation carried out by another chemist. All results were comparable to the validated data presented in this document.

13. Conclusion

The reported in-house validated method enables quantification of PBT residues from horse meat matrix. With the applied sample preparation method, effective deconjugation and prevention of early degradation of target compound with high recovery and good repeatability was achieved. The sample pretreatment procedure requires two clean-up steps, which ensures clean meat extracts with no matrix effect and enables high sensitive quantification. In-house validation of the method was carried out according to the current European legislation recommendations. Taking into account the current rapidly growing interests in phenylbutazone measurement we have proven that this method is fit for purpose and can be applied for routine testing analysis.

14. References

1. Commission Decision 2002/657/EC. Off. J. Eur. Commun. L221/8 (2002).
2. http://www.merckvetmanual.com/mvm/index.jsp?cfile=htm/bc/191606.htm.
3. Jedziniak, P.; Szprengier-Juzkiewicz, T.; Olejnik, M.; Zmudzki, J. Determination of non-steroidal anti-inflammatory drugs residues in animal muscles by liquid chromatography-tandem mass spectrometry. *Anal. Chim. Acta*. **2010**, *672*, 85-92.
4. ISO 11843: Capability of detection (1997).

15. Annex

Tables and Figures

Table 2. MS/MS parameters for selected reaction monitoring of PBT and PBT-IS and ion ratios (Qual/Quant) in matrix and in standard mixture (the agreement between ion ratios should be in the permitted tolerance, which is defined in the Commission Decision 2002/657/EC)

Analyte	Rt Time (min)	Molecular Weight	Precursor Ion $[M-H]^+$	Quantifier Ion (CE)	Qualifier Ion (CE)	Tube Lens	Ion Ratio (Solvent)	Ion Ratio (Matrix)	Difference In Ion Ratio (%)
PBT	1.67	308.37	307.10	279.10 (21)	130.50 (24)	95	60.7	63.2	4.2
PBT-IS	1.67	320.29	319.17	291.30 (20)	136.82 (24)	95	59.6	61.3	2.9

CE = Collision energy (eV)

Table 3. Validation results: Method linearity, method recovery (%), and method repeatability expressed as RSD (%) for spiked samples of horse meat at three different spike levels with six replicates, limit of detection and quantification (LOD and LOQ), and limit of decision and capability (CCα and CCβ)

Parameter		Phenylbutazone
Linearity	Slope	0.0425
	Intercept	-0.0056
	R^2	0.9941
Spiking Levels (µg/kg)	I.	2.5
	II.	5.0
	III.	7.5
Recovery (%)	I.	103.9
	II.	97.5
	III.	95.6
Repeatability (%)	I.	5.2
	II.	2.0
	III.	6.3
LOD (µg/kg)		0.80
LOQ (µg/kg)		2.00
CCα (µg/kg)		1.00
CCβ (µg/kg)		1.29

Table 4. Method precision expressed as RSD (%) – at one level – with three sets with six replicates

Parameter		PBT
Spiking Level (µg/kg)	II.	5.0
Identification (t_r)	Repeatability (%)	0.5
	Intermediate precision (%)	0.9
Quantification (peak area)	Within day repeatability (%)	2.0
	Day to day repeatability (%)	9.8

Table 5. Results of certified reference material – Fapas 02157 (bovine muscle) – phenylbutazone – assigned value 19.3 ± 8.4 µg/kg

Sample	Found Concentration (µg/kg)
CRM 1	14.6
CRM 2	15.8

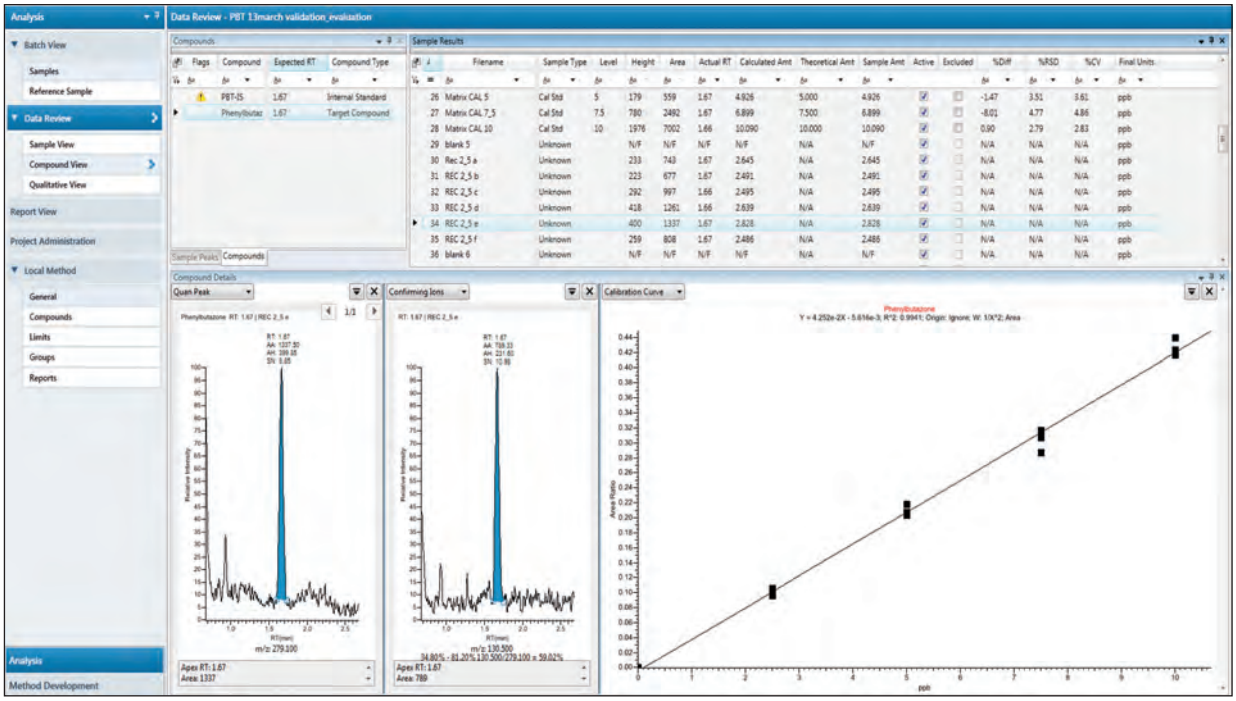

Figure 1. Screenshot data evaluation overview with TraceFinder 3.0 software

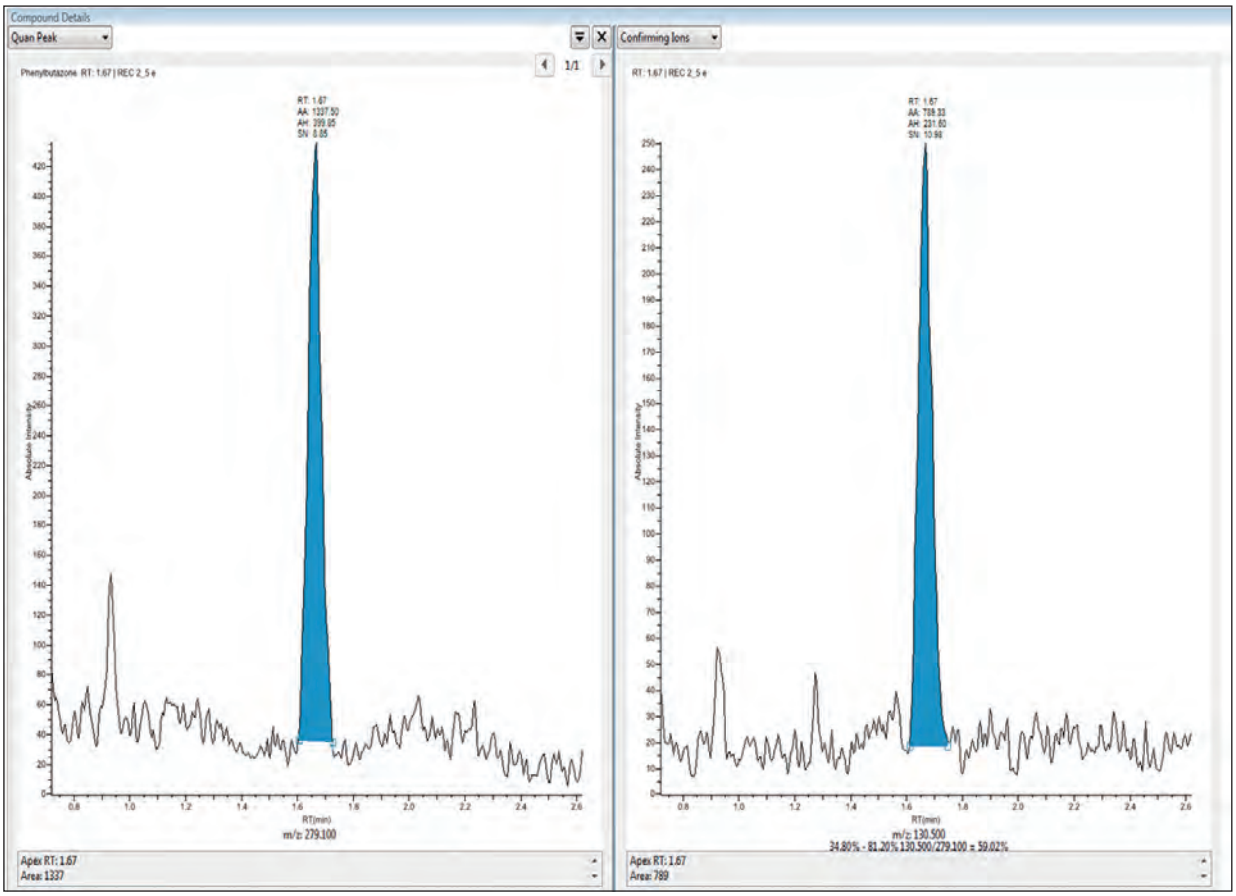

Figure 2. LC-MS/MS chromatogram of phenylbutazone in horse meat at 2.5 µg/kg. Left: quantifier ion *m/z* 279.1, right: qualifier ion *m/z* 130.5 at 1.67 min.

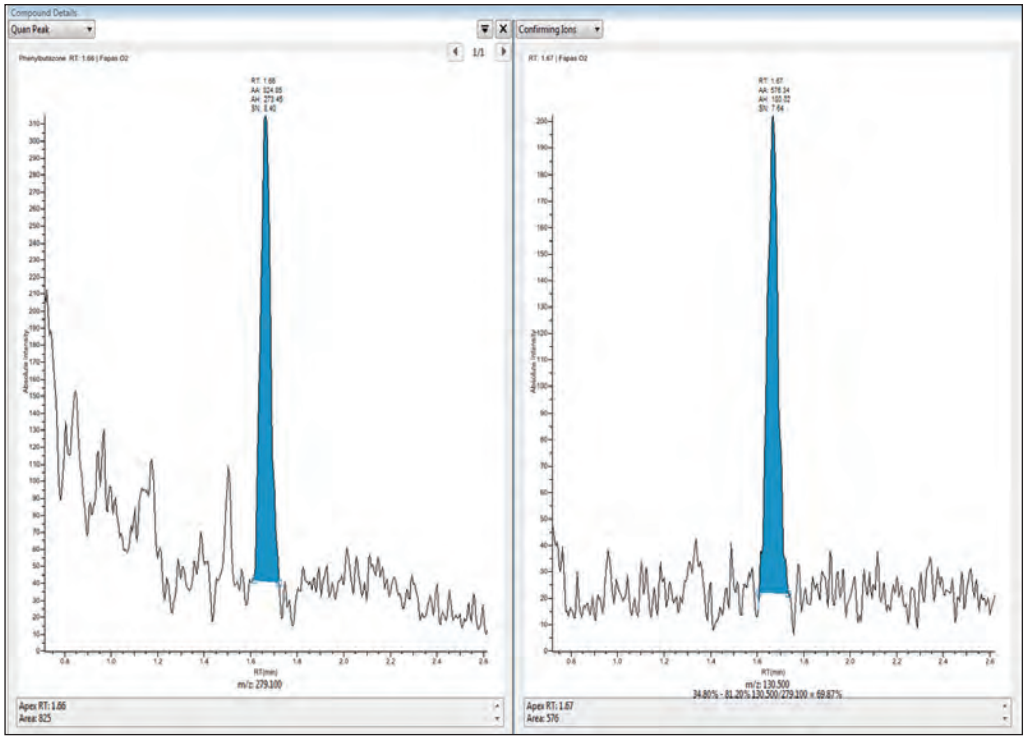

Figure 3. LC-MS/MS chromatogram of phenylbutazone in beef meat quality control reference material found concentration 15.8 µg/kg (assigned value 19.3 µg/Kg). Left: quantifier ion *m/z* 279.1, right: qualifier ion *m/z* 130.5 at 1.67 min.

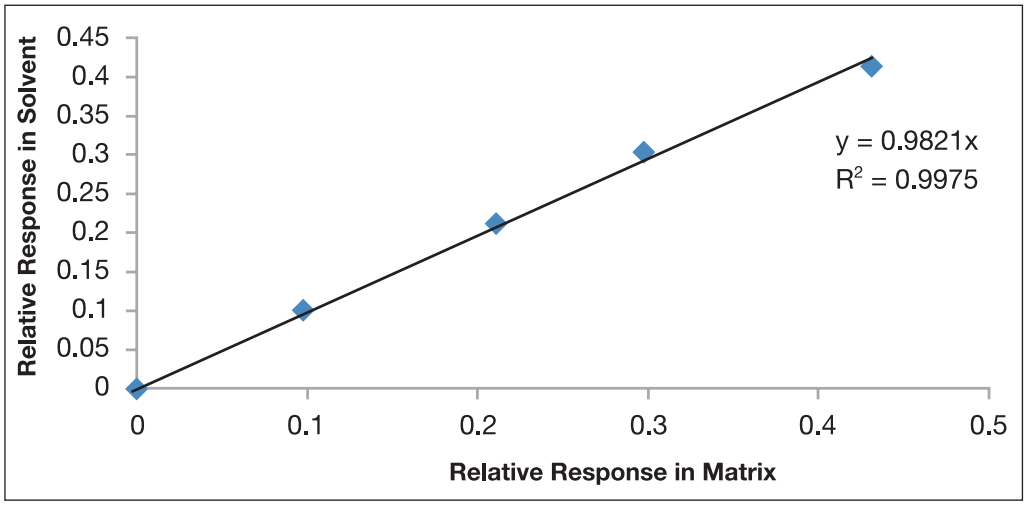

Figure 4. Matrix effect study. Plot of relative responses of calibration levels in solvent versus in horse meat.

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Application Note: 481

Study of β-agonist Residues in Animal-derived Foods by LC-MS/MS

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Key Words

- Food residue analysis
- Veterinary drugs
- TSQ Quantum
- SRM (Selected Reaction Monitoring)

Introduction

Beta-adrenoceptor agonists (β-agonists) are bronchodilators that open air passages by relaxing the tightened muscles surrounding the bronchial tubes. These drugs are commonly prescribed to treat pulmonary diseases such as asthma, emphysema, and bronchitis. Additionally, at higher doses β-agonist drugs have anabolic effects and are very attractive as growth promoters and repartitioning agents in the livestock industry. Due to the potential health risks, β-agonists have been banned for use as growth promoters in livestock in the European Union.[1] Regulatory agencies require highly sensitive and specific analysis methods to ensure that β-agonist residues are not present in animal-derived foods.

In this application note, a sensitive LC-MS/MS method has been developed to detect β-agonist drugs in animal-derived foods including liver, pork, milk, and eggs. Ten β-agonists were identified: cimaterol, clenbuterol, clorprenaline hydrochloride, fenoterol, pentubutolol, propranolol, ractopamine, salbutamol, terbutaline, and tulobuterol.

Goal

To develop a sensitive and reproducible LC-MS/MS method to detect ten β-agonists drugs in animal-derived foods.

Experimental Conditions

Sample Preparation

A 2 g animal-derived tissue sample was weighed into a 50 mL centrifuge tube and 10 mL of 0.2 mol/L ammonium acetate (pH 5.2) was added. Then, 40 µL of β-glucosidase/arylsulfatase was added to digest the β-agonist drug residue. The mixture was vortexed for 3 minutes and incubated in a 37 °C water bath for 16 hours in the dark.

After incubation, the hydrolyzed sample was cooled to room temperature, vortexed for 3 minutes, and then centrifuged at 10,000 rpm for 10 minutes. The supernatant was transferred to another 50 mL centrifuge tube and 1 mL of 1 mol/L perchloric acid was added. The solution was centrifuged at 4000 rpm for 10 minutes. The supernatant was loaded to the solid phase extraction (SPE) column, which was previously conditioned with 5 mL of methanol. After drying, the analytes were eluted with 5 mL of 5% ammonium hydroxide methanol solution and evaporated to dryness under nitrogen at 40 °C. The residues were reconstituted in 1.0 mL of 20% acetonitrile aqueous solution. The resulting solutions were vortexed for 1 minute and then centrifuged at 10,000 rpm for 10 minutes. The upper clear solutions were transferred to another sample vial for LC-MS/MS analysis.

LC

HPLC analysis was performed using the Thermo Scientific Surveyor HPLC system. Each 10 µL of sample was injected onto a Thermo Scientific Hypersil GOLD column (150 mm x 2.1 mm, 5 µm). A gradient LC method used mobile phases A (5 mM ammonium acetate) and B (methanol) at a flow rate of 250 µL/min. Table 1 illustrates the gradient LC method.

Table 1. Gradient details

Retention Time (min)	A (%)	B (%)
0	90	10
0.5	90	10
5	10	90
10	10	90
10.1	90	10
12	90	10

MS

MS analysis was carried out on a Thermo Scientific TSQ Quantum triple stage quadrupole mass spectrometer with an electrospray ionization (ESI) probe. The MS conditions were as follows:

Ion source polarity:	Positive ion mode
Spray voltage:	4500 V
Sheath gas pressure (N_2):	30 units
Auxiliary gas pressure (N_2):	5 units
Capillary temperature:	350 °C
Collision gas pressure (Ar):	1.5 mTorr

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The SRM transitions that were monitored are summarized in Table 2.

Table 2. SRM transitions

Drug	Parent Ion *(m/z)*	Product Ions *(m/z)* [Collision Energy (V)]
Cimaterol	220	143 [22], 202 [10], 160 [18]
Clenbuterol	277	132 [27], 168 [26], 203 [16], 259 [8]
Clorprenaline hydrochloride	214.1	119.1 [27], 154 [17]
Fenoterol	304.1	107.1 [30], 135.1 [18]
Pentubutolol	292.2	201 [21], 236.2 [16]
Propranolol	260.2	183 [18], 116.2 [18]
Ractopamine	302	121 [20], 164 [14], 284 [8]
Salbutamol	240	148 [18], 166 [14], 222 [11]
Terbutaline	226	107 [31], 152 [35], 152 [15]
Tulobuterol	228.1	119 [28], 154 [16], 172 [14]

Results and Discussion

Figure 1 displays the SRM chromatograms for the ten β-agonists at 1 ng/mL. The limits of quantitation (LOQ) for clenbuterol, clorprenaline hydrochloride, fenoterol, pentubutolol, propranolol, ractopamine, salbutamol, and tulobuterol are 0.1 µg/kg in the liver, pork, milk, and egg samples. The LOQs for cimaterol and terbutaline are 0.5 µg/kg.

The extraction recovery rates of the β-agonist drugs are between 75% and 120%, which achieve the minimum detection requirements. Thus, the qualification method is accurate and reproducible.

Conclusion

This LC-MS/MS method is able to detect β-agonists drugs in animal-derived foods. The method yielded high recovery rates and enabled the accurate quantification of the residues. The sensitivity, extraction recovery, and reproducibility of this method meet international regulation and detection requirements.

References

1. European Community, Council Directive 96/22/EC concerning the prohibition on the use in stockfarming of certain substances having a hormonal or thyrostatic action and of ß-agonists, (Official Journal of the European Communities Official Journal L 125, 23 May **1996**, pp. 3-9).

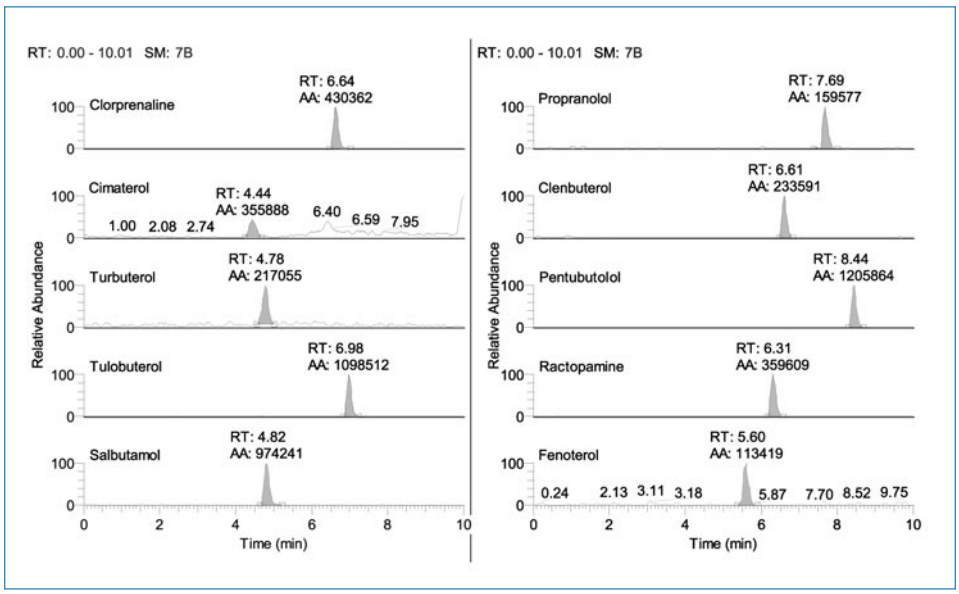

Figure 1. SRM chromatograms for the ten β-agonists at 1 ng/mL

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AN62969_E 10/09S

Application Note: 480

Detection of Six Zeranol Residues in Animal-derived Food by HPLC-MS/MS

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Key Words

- Food residue analysis
- Veterinary drugs
- TSQ Quantum
- SRM (Selective Reaction Monitoring)

Introduction

Zeranol is a non-steroidal estrogenic growth stimulator that is widely used in food-producing animals in the United States and other countries. A synthetic derivative of the mycotoxin zearalenone, zeranol is a potential endocrine disrupter that may have adverse effects on humans, such as birth defects and reproductive disorders, because of its hormone-like properties.[1] In addition, zeranol residues in animal-derived food may increase human breast cancer risks by acting like estrogen in the body and stimulating estrogen-modulated genes that promote carcinogenesis.[2] The use of zeranol in the livestock industry may also cause secondary pollution and environmental contamination of drinking water and foods.

Because of the potential health concerns, the use of zeranol and other anabolic growth promoters in food animals is banned in the member states of the European Union (EU)[3], China[4], and other countries. Therefore, highly sensitive and specific methods with low levels of detection are needed to analyze zeranol residues in edible tissues. Here an LC-MS/MS method is established to detect zeranol (α-zearalanol) and its main metabolites, β-zearalanol and zearalanone, and the mycotoxin zearalenone and its metabolites, α-zearalenol and β-zearalenol, in animal-derived foods, such as pork, beef, lamb, chicken liver, milk, and eggs.

Goal

To develop an effective LC-MS/MS method to detect six zeranol residues in animal-derived foods.

Experimental Conditions

Sample Preparation

A 5 g tissue sample, taken from about 500 g muscle or liver, 10 eggs, or 500 mL milk, was weighed into a 50 mL centrifuge tube and 10 mL acetonitrile was added. The sample was vortexed for 1 minute and then centrifuged at 5000 rpm for 10 minutes. The supernatant was transferred into another 50 mL centrifuge tube. The sample was extracted again with 10 mL acetonitrile. The two supernatants were combined and evaporated to dryness at 50 °C. The residue was dissolved in 3.0 mL 0.1 M NaOH and the pH was adjusted to 11.0. The solution was loaded to the MCX cartridges SPE column. The SPE column was conditioned with 2 mL of both methanol and water. After drying, the analytes were eluted with 3 mL 5% formic acid methanol solution and evaporated to dryness under nitrogen at 50 °C. The residues were reconstituted in 1.0 mL 20% acetonitrile aqueous solution. The resulting solutions were vortexed for 1 minute and then centrifuged at 10,000 rpm for 10 min. The upper clear solutions were transferred to another sample vial for LC-MS/MS analysis.

LC

HPLC analysis was performed using the Thermo Scientific Surveyor HPLC system. Each 10 µL sample was injected onto a Thermo Scientific Hypersil GOLD 150 mm x 2.1 mm, 5 µm column. A gradient LC method used mobile phases A (water with 0.1% formic acid) and B (acetonitrile) at a flow rate of 250 µL/min. Table 1 illustrates the gradient LC method.

Table 1. Gradient details

Retention Time (min)	A (%)	B (%)
0	70	30
5	70	30
8	10	90
12	10	90
12.1	70	30
14	70	30

MS

MS analysis was carried out on a Thermo Scientific TSQ Quantum triple stage quadrupole mass spectrometer with an electrospray ionization (ESI) probe. The MS conditions were as follows:

Ion source polarity:	Negative ion mode
Spray voltage:	3500 V
Sheath gas pressure (N_2):	40 units
Auxiliary gas pressure (N_2):	8 units
Capillary temperature:	350 °C
Collision gas pressure (Ar):	1.5 mTorr

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The SRM transitions that were monitored are summarized in Table 2.

Table 2. SRM transitions

Drug	Parent Ion (m/z)	Product Ions (m/z) [Collision Energy (V)]
α-zearalanol	321	277 [24], 303 [24]
β-zearalanol	321	277 [24], 303 [24]
α-zearalenol	319	205 [22], 275 [24], 301 [26]
β-zearalenol	319	205 [22], 275 [24], 301 [26]
Zearalanone	319	205 [22], 275 [24], 301 [26]
Zearalenone	317	175 [26], 273 [20]

Results and Discussion

Figure 1 displays the SRM chromatograms for the six zeranol residues. The limits of detection (LOD) for all six zeranol residues in animal-derived foods are 0.1 µg/kg. The limits of quantitation (LOQ) of these residues are 1.0 µg/kg. These LOQs easily meet the specified MRLs of the European Union and China of 2 µg/kg in meat and 10 µg/kg in liver.[5]

The extraction recovery of zeranol is between 65% and 115% and achieves the minimum detection requirements. Thus, the qualification method is accurate and reproducible.

Conclusion

The LC-MS/MS method described here is able to detect zeranol residues from animal-derived foods, such as pork, beef, lamb, chicken liver, milk, and eggs. The method yielded high recovery rates and enabled the accurate quantification of the residues. The sensitivity, extraction recovery, and reproducibility of this method meet international regulation and detection requirements.

References

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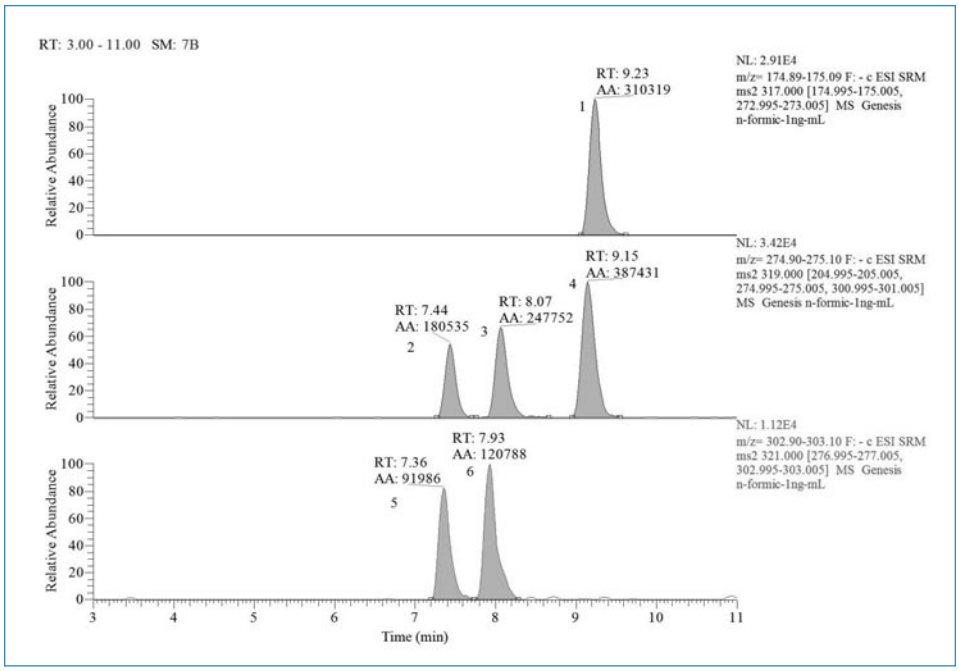

Figure 1. SRM chromatograms for the six zeranol residues. The LODs for all six zeranol residues in animal-derived foods are 0.1 µg/kg.

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AN62968_E 10/09S

Application Note: 482

Detection of Glucocorticoid Residues in Animal-derived Food by HPLC-MS/MS

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Key Words

- Food residue analysis
- Veterinary drugs
- TSQ Quantum
- SRM (Selective Reaction Monitoring)

Introduction

Glucocorticoids are a class of steroid hormones, produced by the adrenal cortex, that affect metabolism and have immunosuppressive and anti-inflammatory properties. The common chemical structure of these compounds is shown in Figure 1. The chemical structures of eight glucocorticoid drugs are listed in Table 1.

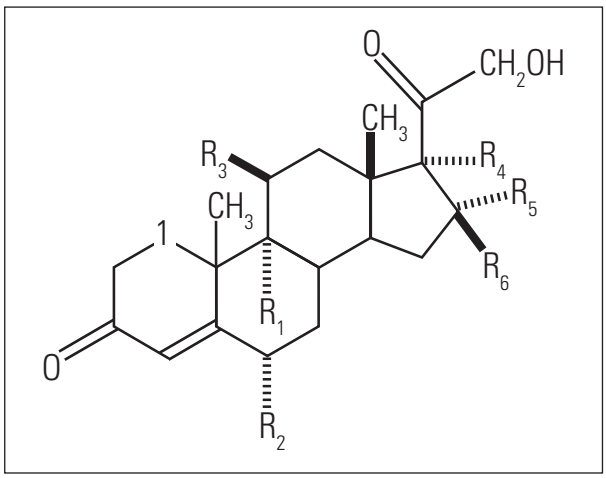

Figure 1. Common chemical structure of glucocorticoids

Table 1. Glucocorticoid drugs and their functional groups

	R_1	R_2	R_3	R_4	R_5	R_6
Dexamethasone	-F	-H	-OH	-OH	$-CH_3$	-H
Betamethasone	-F	-H	-OH	-OH	-H	$-CH_3$
Prednisone	-H	-H	=O	-OH	-H	-H
Prednisolone	-H	-H	-OH	-OH	-H	-H
Methylprednisolone	-H	$-CH_3$	-OH	-OH	-H	-H
Beclomethasone	-Cl	-H	-OH	-OH	-H	$-CH_3$
Hydrocortisone	-H	-H	-OH	-OH	-H	-H
Fludrocortisone	-F	-H	-OH	-OH	-H	-H

Synthetic glucocorticoids are often administered to livestock to treat inflammatory diseases, disorders of the musculoskeletal system, and other diseases common to farm animals. They can also be used to improve feed intake and stimulate growth in livestock. However, glucocorticoid residues in animal-derived food may be harmful to humans. Therefore, the use of certain substances with a hormonal or thyrostatic action as growth-promoting additives has been banned by the European Commission.[1] Dexamethasone, betamethasone, prednisolone, and methylprednisolone are the only glucocorticoids permitted for therapeutic purposes in livestock. Maximum residue levels (MRLs) have been established for these compounds in tissues and in milk intended for human consumption by the EC[2-5] and other countries. Table 2 lists the MRLs for glucocorticoids that were set by the European Union. In China, the Institute for the Control of Agrochemicals, Ministry of Agriculture, has established MRLs of 0.75 µg/kg for dexamethasone in the muscles, livers, and kidneys of cattle, pigs, and horses and 10 µg/kg for hydrocortisone in milk.

Table 2. European Union MRLs for glucocorticoids

Substance	Animal Species	MRL	Target Tissues
Dexamethasone	Cattle	0.3 µg/kg	Milk
	Cattle, Pig, Horse	0.75 µg/kg	Muscle
		2.0 µg/kg	Liver
		0.75 µg/kg	Kidney
Betamethasone	Cattle	0.75 µg/kg	Muscle
		2.0 µg/kg	Liver
		0.75 µg/kg	Kidney
		0.3 µg/kg	Milk
	Pig	0.75 µg/kg	Muscle
		2.0 µg/kg	Liver
		0.75 µg/kg	Kidney
Prednisolone	Cattle	4 µg/kg	Muscle
		4 µg/kg	Fat
		10 µg/kg	Liver
		10 µg/kg	Kidney
		6 µg/kg	Milk
Methylprednisolone	Cattle	10 µg/kg	Muscle
		10 µg/kg	Fat
		10 µg/kg	Liver
		10 µg/kg	Kidney

Goal

To develop an effective LC-MS/MS method to detect eight gluococorticoid residues in the liver and muscles of pig, cattle, and lamb, as well as in chicken, eggs and milk.

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Experimental Conditions

Sample Preparation

A 2 g tissue sample was weighed into a 50 mL centrifuge tube and 15 mL of ethyl acetate was added. The sample was vortexed for 3 minutes and then centrifuged at 4000 rpm for 10 minutes. The supernatant was transferred into another 50 mL centrifuge tube. The sample was extracted again with 10 mL sodium hydroxide (0.1 M) and 15 mL ethyl acetate. The two supernatants were combined and evaporated to dryness at 40 °C. The residue was dissolved in 1.0 mL of ethyl acetate and 5.0 mL of n-hexane before being loaded to the silica extraction cartridges (SPE column). The SPE column was previously conditioned with 5 mL of n-hexane. The column was washed with 5 mL of n-hexane. After drying, the analytes were eluted with 5 mL n-hexane:acetone (40:60, v/v) and evaporated to dryness under nitrogen at 40 °C. The residues were reconstituted in 1.0 mL of 20% acetonitrile aqueous solution. The resulting solutions were vortexed for 1 minute and then centrifuged at 10,000 rpm for 10 minutes. The upper clear solutions were transferred to another sample vial for LC-MS/MS analysis.

The glucocorticoid standards were obtained from Sigma (Sigma Chemical Company, St. Louis, MO). All other chemicals were HPLC grade.

LC

HPLC analysis was performed using the Thermo Scientific Surveyor HPLC system. Each 10 µL of sample was injected into a Thermo Scientific Hypersil GOLD column (150 x 2.1 mm, 5 µm). A gradient LC method was used and mobile phases A (water with 0.1% formic acid) and B (acetonitrile) were at a flow rate of 250 µL/min together. Table 3 illustrates the gradient LC method.

Table 3. Gradient details

Retention Time (min)	A (%)	B (%)
0	70	30
18	40	60
23	40	60
23.1	70	30
28	70	30

MS

MS analysis was performed on a Thermo Scientific TSQ Quantum triple stage quadrupole mass spectrometer with an electrospray ionization (ESI) probe. The MS conditions were as follows:

Ion source polarity:	Negative ion mode
Spray voltage:	3500 V
Sheath gas pressure (N_2):	40 units
Auxiliary gas pressure (N_2):	5 units
Capillary temperature:	350 °C
Collision gas pressure (Ar):	1.5 mTorr
Q1/Q3 Peak Resolution:	0.7 FWHM

The SRM transitions that were monitored are summarized in Table 4.

Table 4. SRM transitions

	SRM Transitions (*m/z*)	
Drug	Quantitative	Qualitative
Prednisone	403.0 → 326.9	403.0 → 357.2
Prednisolone	405.0 → 329.0	405.0 → 358.8
Hydrocortisone	407.1 → 331.1	407.1 → 361.1
Methylprednisolone	419.0 → 342.9	419.0 → 373.1
Dexamethasone	437.0 → 361.0	437.0 → 391.5
Betamethasone	437.0 → 361.0	437.0 → 391.5
Beclomethasone	453.0 → 406.9	453.0 → 376.8
Fludrocortisone acetate	467.1 → 420.8	467.1 → 349.2

Results and Discussion

Figure 2 displays the SRM chromatograms for the eight glucocorticoids. The limit of quantitation (LOQ) for prednisone, prednisolone, dexamethasone, betamethasone, and methylprednisolone in milk is 0.1 µg/L. The LOQ of these residues in muscle, eggs and livers is 0.2 µg/kg. For fludrocortisone acetate and beclomethasone, the LOQ is 0.2 µg/L in milk, and 0.2 µg/kg in eggs, and livers. For hydrocortisone, the LOQ in milk is 0.2 µg/L and in eggs and livers is 0.5 µg/kg. These LOQs easily meet the specified MRLs of the European Union and China.

The extraction recovery of glucocorticoids from the muscle and livers of cattle, pigs, and lamb; chicken; eggs; and milk is between 60% and 110%. This achieves the minimum detection requirements. Thus, the qualification method is accurate and reproducible.

The method validation data is summarized in Table 5. Linearity of the method was assumed because the R^2 values were greater than 0.99 for the linear regression equations (1/x weighted).

Table 5. Linearity and dynamic range

Drug	Dynamic Range (µg/L, µg/kg)	Equation	R^2
Prednisone	0.2 ~ 50	y = 106952 x + 5288	0.9992
Prednisolone	0.2 ~ 50	y = 100276 x - 2420.7	0.9954
Hydrocortisone	0.8 ~ 200	y = 85500 x + 6127.5	0.9947
Methylprednisolone	0.2 ~ 50	y = 98154 x - 5291.5	0.9995
Dexamethasone	0.2 ~ 50	y = 106501 x - 8596.1	0.9991
Betamethasone	0.2 ~ 50	y = 116052 x - 17056	0.9986
Beclomethasone	0.4 ~ 100	y = 52195 x - 6621.2	0.9911
Fludrocortisone acetate	0.4 ~ 100	y = 33779 x - 2193.9	0.9992

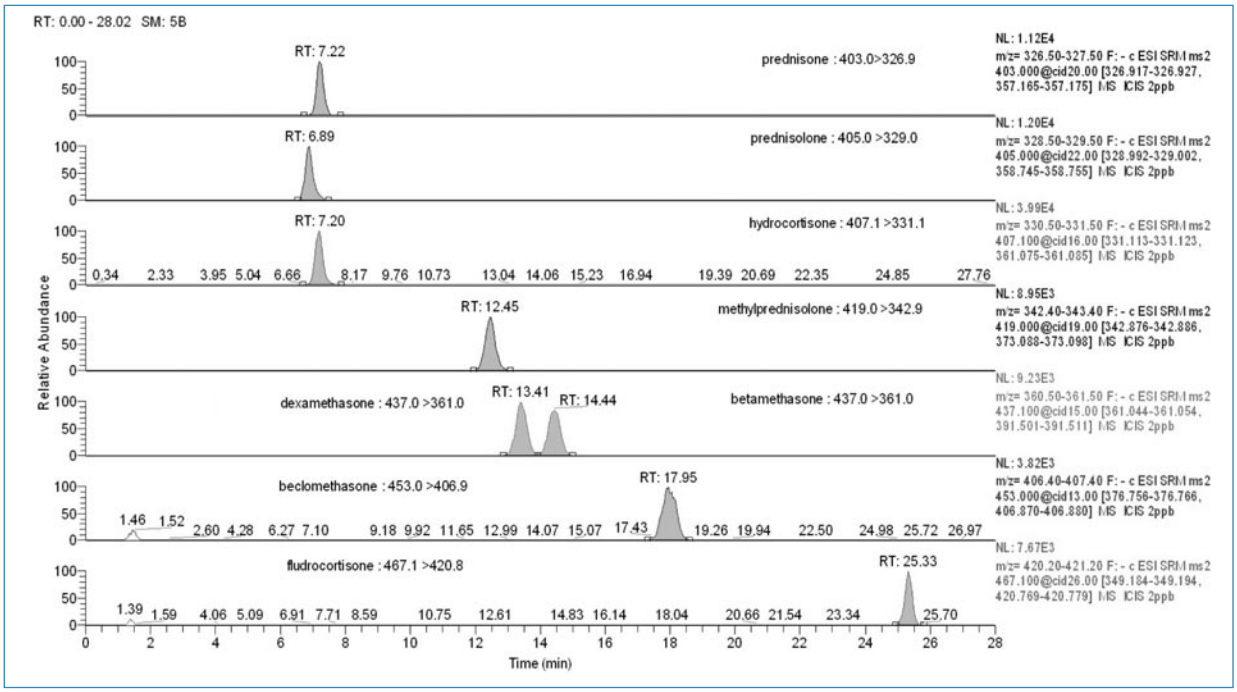

Figure 2. SRM chromatograms for the eight glucocorticoids.

Conclusion

The LC-MS/MS method described above is able to detect glucocorticoid residues with high recoveries and enable their accurate quantification. The sensitivity, extraction recovery, and reproducibility of this method meet international regulation and detection requirements.

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Application Notebook

Mycotoxins

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Targeted Screening of Fungal and Plant Metabolites in Wheat, Corn, and Animal Feed Using Automated Online Sample Preparation Coupled to Orbitrap LC-MS

Ebru Ates, Michal Godula, and Klaus Mittendorf
Food Safety Response Center, Dreieich, Germany

Method 63747

1. Schematic of Method

1. Weigh 5 g of homogenized sample into a 50 mL bottle.

Homogenized Sample, 5 g

2. Add 20 mL of extraction solvent (water 0.1% FA/ACN (43:57)) and shake for 45 min.

Extraction

3. Filter sample through 0.2 µm nylon microfilter.

Filtration

4. Place the vial in autosampler of TLX-LC-HRMS.

TLX-HRMS

Data Analysis

2. Introduction

Mycotoxins are secondary metabolites produced by fungal infection of agricultural crops in the field, during harvest, drying, or subsequent storage. Mycotoxins are very stable compounds that cannot be readily destroyed by heating or during food processing, although there can be reductions in levels during milling of grains, for example. Approximately 400 mycotoxins are known today, but only a few of them are regulated by legislation.[1–3] Besides the detection of the mycotoxins, it is also important to analyze their biosynthetic precursors, degradation products, and related masked forms, which are indicative of fungal contamination of food and feed. On the other hand, plants themselves can produce toxins as secondary metabolites, such as pyrrolizidine or ergot alkaloids.

It is a big challenge to analyze all these toxins with a single method, as most of the compounds are not commercially available as analytical standards. The only approach that can be employed is to perform targeted screening using databases of accurate masses, aimed at searching in full scan spectra. High-resolution mass spectrometry has the capability of acquiring mass spectrometric data with very high resolving power, in case of Thermo Scientific™ Orbitrap™ mass analyzers typically >140,000 (FWHM) and with a mass accuracy of <3 ppm. This enables the separation of compounds with similar accurate masses and helps to distinguish the target compound from matrix interferences. This method is an extension of a previously validated method for the quantification of *fusarium* mycotoxins (DON, T2, HT2, FB_1, FB_2, and ZON) in corn, wheat, and animal feed.[4] It can be applied, for targeted screening of 21 fungal and plant metabolites with automated online sample cleanup utilizing a Thermo Scientific™ Transcend™ system coupled to a Thermo Scientific™ Exactive™ high-resolution mass spectrometer. This method has been validated according to current legislation.[5,6] Full scan data processing was performed using Thermo Scientific™ ExactFinder™

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software enabling targeted screening of toxins. The criteria for compound identification using ExactFinder software is based on detection of accurate mass at a resolving power of 100,000 (FWHM) at *m/z* 200 with a minimum of one fragment ion at the correct retention time with a mass deviation <5ppm and retention time tolerance of ±2.5% for compound confirmation.[3] As this method is intended for screening, no further optimization of peak shapes was performed for the additional 16 compounds.

3. Scope

Extracted samples of corn, wheat, and animal feed can be injected directly into an automated online clean-up system coupled to a high-resolution mass spectrometer. This method also enables rapid targeted screening for possible fungal metabolites employing data analysis with ExactFinder software.

4. Principle

This method uses Thermo Scientific™ TurboFlow™ technology for online cleanup of the sample. Finely ground and homogenous sample (5 g) is extracted for 45 min with a mixture of water 0.1% formic acid (FA)/acetonitrile (ACN) (43:57). After filtration with a 0.2 µm nylon filter into an LC-vial, the sample is injected in the Transcend TLX-1 system, an online chromatography–reversed phase chromatography clean-up system coupled with high-resolution mass spectrometric (HRMS) detection. Data analysis is performed with ExactFinder software using a fungal metabolite database in positive and negative ionization mode. Criteria for compound confirmation and identification are defined.

5. Reagent List

5.1 Acetonitrile Optima, for LC-MS

5.2 Water Optima grade, for LC-MS

5.3 Methanol Optima grade, for LC-MS

5.4 Formic acid (FA), LC-MS grade

5.5 Thermo Scientific™ Pierce™ LTQ™ ESI positive ion calibration solution

5.6 Pierce LTQ ESI negative ion calibration solution

6. Standards

6.1	Aflatoxin B_1 (AFB_1)	Sigma-Aldrich®
6.2	Aflatoxin B_2 (AFB_2)	Sigma-Aldrich
6.3	Aflatoxin G_1 (AFG_1)	Sigma-Aldrich
6.4	Aflatoxin G_2 (AFG_2)	Sigma-Aldrich
6.5	Apicidin	Sigma-Aldrich
6.6	Deoxynivalenol (DON)	Sigma-Aldrich
6.7	Ergocornine	Römer Labs®
6.8	Fumagillin	Sigma-Aldrich
6.9	Fumonisin B_1 (FB_1)	Sigma-Aldrich
6.10	Fumonisin B_2 (FB_2)	Sigma-Aldrich
6.11	Fusarenone X	Sigma-Aldrich
6.12	HT-2 toxin (HT2)	Sigma-Aldrich
6.13	Malformin A	Sigma-Aldrich
6.14	Monocrotaline	Römer Labs
6.15	Ochratoxin A (OTA)	Sigma-Aldrich
6.16	p-Anisaldehyde	Sigma-Aldrich
6.17	Retrorsine	Römer Labs
6.18	Sterigmatocystin	Sigma-Aldrich
6.19	T-2 toxin (T2)	Sigma-Aldrich
6.20	Tenuazonic acid	Sigma-Aldrich
6.21	Zearalenone (ZON)	Sigma-Aldrich

7. Standard Preparation

Stock standard solutions of mycotoxins (100 µg/mL) are prepared individually by dissolving in methanol. Solutions are stored at –20° C.

8. Apparatus

8.1 Transcend TLX 1 system

8.2 Exactive mass spectrometer

8.3 Column oven, HotDog 5090 (Prolab GmbH, Switzerland)

8.4 Fisher Scientific™ precision balance

8.5 Sartorius® analytical balance (Sartorius GmbH, Switzerland)

8.6 Thermo Scientific™ Barnstead™ EASYpure™ II water

8.7 Elmasonic® S 40 (H) ultrasonic bath, (ELMA® Hans Schmidbauer GmbH & Co. KG, Germany)

8.8 Vortex shaker

8.9 Vortex standard cap

8.10 IKA® HS 501, digital Shaker (IKA-Werke GmbH & Co. KG, Germany)

9. Consumables

9.1 Thermo Scientific™ Hypersil GOLD™, 50 × 4.6 mm, 5 µm

9.2 Thermo Scientific™ TurboFlow™ Cyclone™ MCX column, 50 × 0.5 mm

9.3 LC vials

9.4 LC vial caps

9.5 Thermo Scientific™ Finnpipette™ 10–100 µL

9.6 Finnpipette 100–1000 µL

9.7 Finnpipette 500–5000 µL

9.8 Pipette holder

9.9 Pipette Pasteur soda lime glass 150 mm

9.10 Pipette suction device

9.11 Pipette tips 0.5–250 µL, 500/box

9.12 Pipette tips 1–5 mL, 75/box

9.13 Pipette tips 100–1000 µL, 200/box

9.14 Disposable plastic syringe, 1 mL

9.15 Nylon filter 0.2 µm

10. Glassware

10.1 Beaker, 25 mL

10.2 Volumetric flask, 10 mL

10.3 Volumetric flask, 100 mL

10.4 Volumetric flask, 1000 mL

10.5 Amber bottle 50 mL

Table 1. Gradient program table in Aria software for TurboFlow Method coupled with an analytical column

Step			Loading Pump				Cut-in loop		Eluting Pump			
Step	Start [min]	Time [s]	Flow [mL/min]	Grad	A [%]	B [%]	Tee	Loop	Flow [mL/min]	Grad	A [%]	B [%]
1. Loading	0	90	1.5	Step	100	0	===	Out	0.5	Step	99	1
2. Transferring	1:30	1	0.3	Step	85	15	T	In	0.2	Step	99	1
3. Transferring/HPLC	1:31	59	0.3	Step	85	15	T	In	0.2	Ramp	80	20
4. Washing/HPLC	2:30	360	1.5	Step	85	15	===	In	0.6	Ramp	0	100
5. Washing/HPLC	8:30	130	1.5	Step	100	0	===	In	0.6	Step	0	100
6. Washing/HPLC	10:40	160	1.5	Step	0	100	===	In	0.6	Step	0	100
7. Loop filling/equilibrating	13:20	120	1.5	Step	10	90	===	In	0.5	Step	99	1
8. Equilibrating	15:20	160	1.5	Step	100	0	===	Out	0.5	Step	99	1

11. Procedure

11.1 Chemical Preparation

The extraction solvent is prepared by mixing 1000 mL of acetonitrile with 750 mL of water containing 0.1% FA.

11.2 Sample Preparation and Spiking

As no blank materials were available, a number of samples of corn, wheat, and animal feed were analyzed to test whether they could be used as blank material for spiking purposes. These samples, with trace levels (below LOD) of target mycotoxins, were used as blank materials for the method validation. Spiking was performed at two different levels (250 and 500 µg/kg) with solutions of standards.

To prepare the spiked sample, 500 g of matrix is homogenized by a laboratory blender and ground to a fine powder using a mortar and pestle. A sample of 5 g (±0.01 g) is weighed and put into a 50 mL amber flask and spiked with the appropriate amount of standard. Spiked samples are stored for 30 min in the dark for equilibration of the spike. After the addition of 20 mL of extraction solvent, bottles are closed and shaken for 45 min in the laboratory shaker. Samples are filtered through a nylon filter (0.2 µm) and injected into the TLX-HRMS system.

12. TLX-LC conditions

LC Conditions	
TurboFlow column:	Cyclone MCX, 50 × 0.5 mm
Analytical column:	Hypersil GOLD, 50 × 4.6 mm, 5 µm
Total run time:	18 min
Mobile phase:	A: Water (0.1% formic acid)
	B: Methanol (0.1% formic acid)

The autosampler sample tray temperature is kept at 10 °C. Sample injection volume is 10 µL with a 100 µL injection syringe. The injection syringe is rinsed as described in the injector settings. The gradient program is presented in Table 1. Mobile phase composition in loading- and eluting- pump is A) water (0.1% FA) and B) methanol (0.1% FA). Total run time for TLX cleanup and separation on the analytical column is 18 min.

Injector Settings	
Injector:	CTC Analytics (CTC Analytics AG, Switzerland) with 100 µL injection syringe volume
Wash solvents for the autosampler	
Wash 1:	Methanol
Wash 2:	5% Methanol
Pre-clean syringe with wash 1: ×2	
Clean injector (TX) with wash 1: ×2	
Get sample (SEQ Tray: SEQ. Index): SEQ. Volume	
Inject sample (Syringe content) to TX	
Clean syringe with wash 1: ×7	
Clean injector (TX) with wash 1: ×7	
Clean syringe with wash 2: ×7	
Clean injector (TX) with wash 2: ×7	
Injection volume:	10 µL
Tray temperature:	10 °C
Column oven:	40 °C

13. Mass Spectrometric Conditions

MS analysis is carried out using an Exactive Orbitrap high-resolution benchtop mass spectrometer controlled by Thermo Scientific™ Aria™ MX software version 1.1. Data acquisition and processing is performed using Thermo Scientific™ Xcalibur™ software version 2.1. The Exactive MS was calibrated in positive and negative mode every 48 hours.

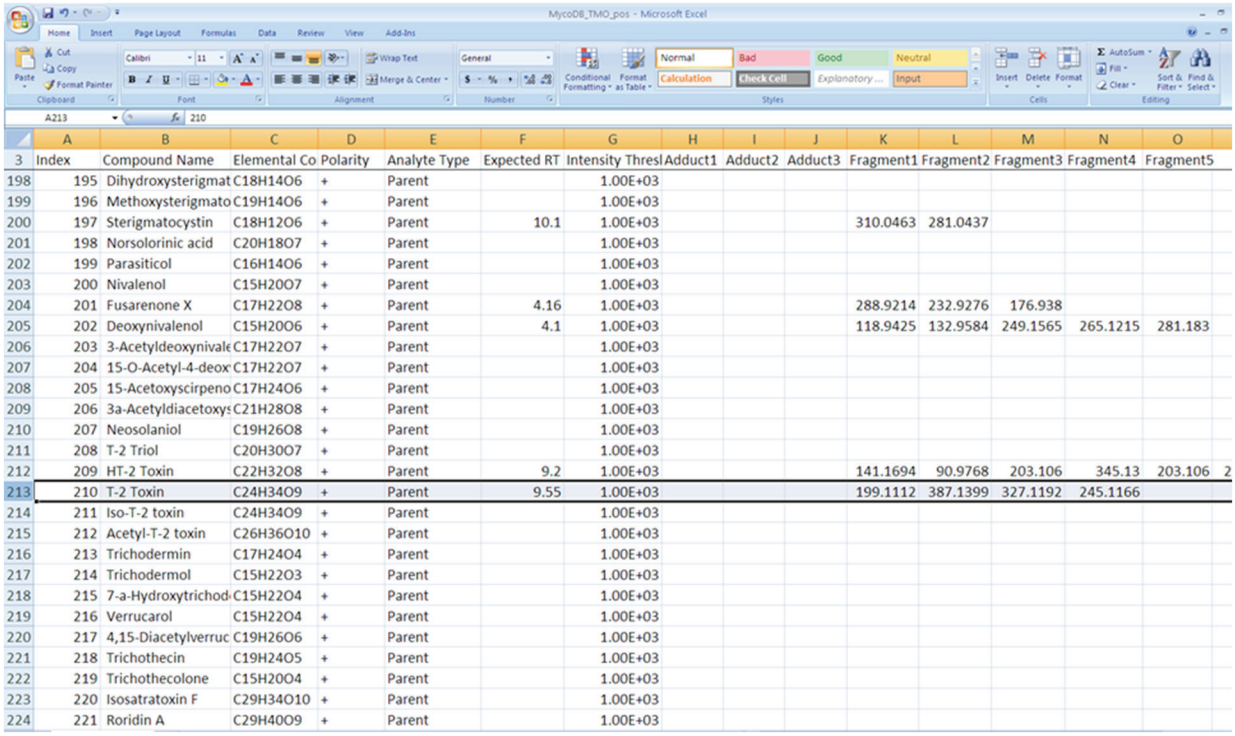
Mass Spectrometer Conditions	
Ionization:	Heated electrospray (HESI II)
Polarity:	Positive/negative switching mode
Sheath gas flow rate:	60 arb
Aux gas flow rate:	20 arb
Spray Voltage:	3.60 kV
Capillary temperature:	260 °C
Capillary voltage:	60 V
Tube lens voltage:	120 V
Skimmer voltage:	25 V
Heater temperature:	250 °C
Scan mode:	Full scan
Scan range:	100–900 *m/z*
Microscans:	1
Resolution:	100,000 (FWHM) at *m/z* 200
AGC target:	1e6
Scan events:	Full scan positive mode *m/z* 100–900 Full scan negative mode *m/z* 100–900 HCD fragmentation in positive mode *m/z* 50–500 HCF fragmentation in negative mode *m/z* 50–500
Collision energy:	35 eV

14. Database

A database containing more than 600 plant and fungal metabolites and other fungal metabolites comprising their empirical formula, exact mass, polarity, fragment ions (max. 5), and retention time is maintained as an Excel® spreadsheet and converted to a comma separated values (.csv) file (Figure 1). The .csv file is uploaded to the ExactFinder as a compound database which is saved as a .cdb file. The .cdb file is modified by addition of adduct ions of $[M+H]^+$ and $[M+Na]^+$ (adduct ions can be defined already in the .csv file as well) in positive mode and $[M-H]^+$ in negative mode. Additional adducts that can be chosen from the software are $[M+K]^+$ and $[M+NH_4]^+$. The isotopic pattern match can be defined as an additional identification or confirmation criteria. Two .cdb files are saved, one for data processing in ESI positive mode and one for data processing in ESI negative mode. The sequence is processed once with the database in negative mode and once in positive mode. The database was created based on the work of Senyuva et al.[7], Nielsen and Smedsgaard[8], Mol et al.[9], Cole and Cole[10], and an internal Thermo Scientific database.

14.1 Confirmation and Identification of Toxins

Compound identification criteria by processing the data with the .cdb file database are set to be the accurate mass with a mass tolerance of <5 ppm and a peak threshold of 20,000 units (defined in method development settings screening method in ExactFinder software). Identified compounds are shown as yellow flag in the software. Compound confirmation is deemed as having been achieved with the additional detection of a minimum of one fragment ion at the corresponding retention time with a time tolerance of ±2.5%. Confirmed hits are marked with a green flag in the software. An example of data evaluation is demonstrated with T-2 toxin in Figures 2 and 3. In Figure 2, a screen shot of processed data is shown. On the upper window the targeted screening results can be found with information about compound, accurate mass (theoretical and found), mass deviation in ppm, retention time (defined and found), intensity, and fragment ions (green is found, red is not found). On the left hand side there is a list of sequence samples with additional information about compound identification. In the window below chromatogram (left) and spectrum (right) of selected compound can be seen.



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
3	Index	Compound Name	Elemental Co	Polarity	Analyte Type	Expected RT	Intensity Thresl	Adduct1	Adduct2	Adduct3	Fragment1	Fragment2	Fragment3	Fragment4	Fragment5
198	195	Dihydroxysterigmat	C18H14O6	+	Parent		1.00E+03								
199	196	Methoxysterigmato	C19H14O6	+	Parent		1.00E+03								
200	197	Sterigmatocystin	C18H12O6	+	Parent	10.1	1.00E+03				310.0463	281.0437			
201	198	Norsolorinic acid	C20H18O7	+	Parent		1.00E+03								
202	199	Parasiticol	C16H14O6	+	Parent		1.00E+03								
203	200	Nivalenol	C15H20O7	+	Parent		1.00E+03								
204	201	Fusarenone X	C17H22O8	+	Parent	4.16	1.00E+03				288.9214	232.9276	176.938		
205	202	Deoxynivalenol	C15H20O6	+	Parent	4.1	1.00E+03				118.9425	132.9584	249.1565	265.1215	281.183
206	203	3-Acetyldeoxyniva	C17H22O7	+	Parent		1.00E+03								
207	204	15-O-Acetyl-4-deox	C17H22O7	+	Parent		1.00E+03								
208	205	15-Acetoxyscirpeno	C17H24O6	+	Parent		1.00E+03								
209	206	3a-Acetyldiacetoxys	C21H28O8	+	Parent		1.00E+03								
210	207	Neosolaniol	C19H26O8	+	Parent		1.00E+03								
211	208	T-2 Triol	C20H30O7	+	Parent		1.00E+03								
212	209	HT-2 Toxin	C22H32O8	+	Parent	9.2	1.00E+03				141.1694	90.9768	203.106	345.13	203.106
213	210	T-2 Toxin	C24H34O9	+	Parent	9.55	1.00E+03				199.1112	387.1399	327.1192	245.1166	
214	211	Iso-T-2 toxin	C24H34O9	+	Parent		1.00E+03								
215	212	Acetyl-T-2 toxin	C26H36O10	+	Parent		1.00E+03								
216	213	Trichodermin	C17H24O4	+	Parent		1.00E+03								
217	214	Trichodermol	C15H22O3	+	Parent		1.00E+03								
218	215	7-a-Hydroxytrichod	C15H22O4	+	Parent		1.00E+03								
219	216	Verrucarol	C15H22O4	+	Parent		1.00E+03								
220	217	4,15-Diacetylverruc	C19H26O6	+	Parent		1.00E+03								
221	218	Trichothecin	C19H24O5	+	Parent		1.00E+03								
222	219	Trichothecolone	C15H20O4	+	Parent		1.00E+03								
223	220	Isosatratoxin F	C29H34O10	+	Parent		1.00E+03								
224	221	Roridin A	C29H40O9	+	Parent		1.00E+03								

Figure 1. Database template in Excel converted to an .csv file

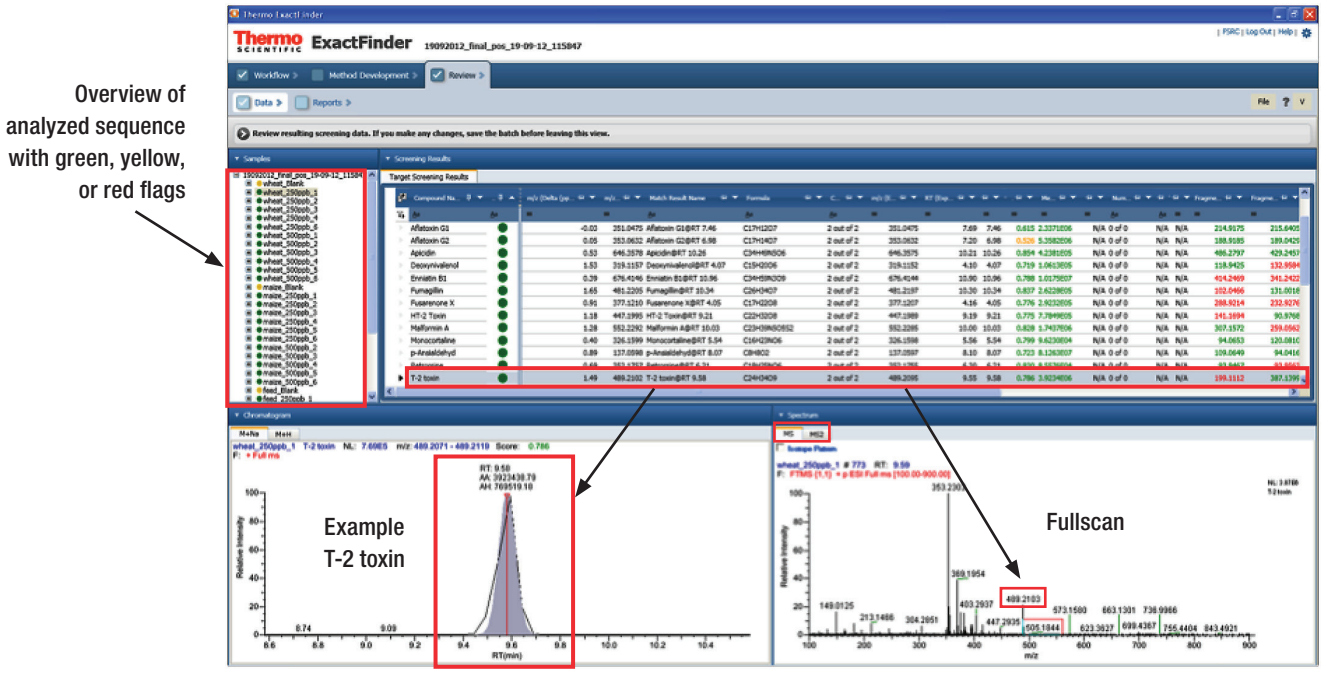


Figure 2. Accurate mass confirmation of T-2 toxin in wheat 250 µg/kg sample in ESI_{pos} mode

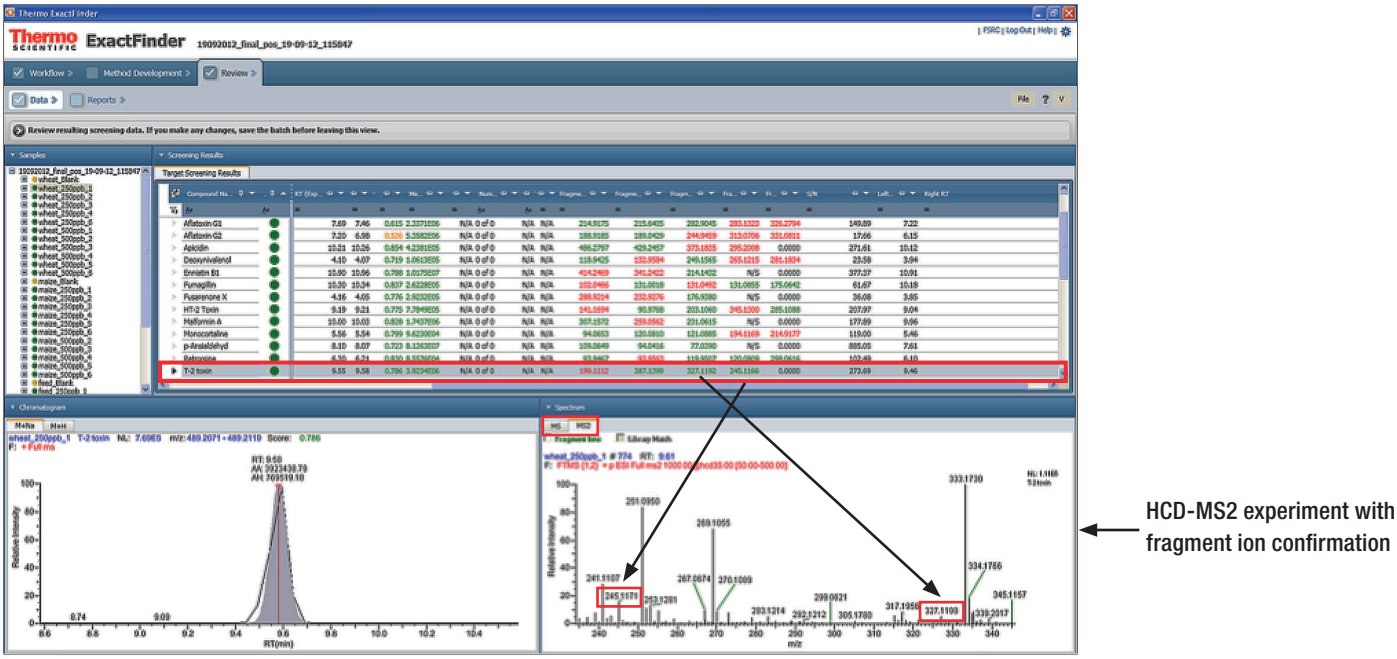


Figure 3. HCD fragment ion confirmation of T-2 toxin in wheat 250 µg/kg sample in ESI_{pos} mode

Figure 3 documents how additional information about fragment ions of T-2 toxin from the HCD experiment can be provided (bottom right).

14.2 Not Detected Compounds

All peaks that cannot be confirmed or identified by attempting to match against reference compounds in the database are marked with red flags and defined as not found.

15. Method Validation

15.1 Specificity

Method specificity is based on the detection of ions with a mass accuracy <5 ppm.[2] Detected ions, mass deviation from theoretical value, and fragment ions of 21 targeted fungal and plant metabolites are listed in Table 2.

15.2 Quality Control Materials

Six samples of certified reference materials have been prepared according to the section "Sample Preparation and Spiking" to determine the accuracy of compound identification and confirmation by ExactFinder software.

Table 2. Theoretical and found accurate masses in standards in methanol and fragment ions detected by HCD fragmentation

Mycotoxins	Molecular Formula	Adduct	Found Molecular Mass in Wheat [*m/z*] (Δ ppm)	Found Molecular Mass in Corn [*m/z*] (Δ ppm)	Found Molecular Mass in Feed [*m/z*] (Δ ppm)	RT in Wheat [min]	RT in Corn [min]	RT in Feed [min]	Fragment Ion 1 [*m/z*]	Fragment Ion 2 [*m/z*]	Fragment Ion 3 [*m/z*]	eV HCD
Apicidin (ESIpos)	$C_{34}H_{49}N_5O_6$	Na^+	646.3576 (+0.22)	646.3585 (+1.6)	646.3581 (+0.87)	10.29	10.24	10.27	429.2457	373.1835		35
Apicidin (ESIneg)	$C_{34}H_{49}N_5O_6$	$-H^+$	622.3618 (+1.21)	622.3615 (+0.82)	622.3619 (+1.4)	10.28	10.25	10.25	462.2748	252.1350		35
AFB_1	$C_{17}H_{12}O_6$	Na^+	335.0530 (+1.23)	335.0530 (+1.2)	335.0531 (+1.38)	8.31	8.18	8.25	197.0118	175.0638		35
AFB_2	$C_{17}H_{14}O_6$	Na^+	337.0681 (-0.33)	337.0684 (+0.35)	337.0688 (+1.53)	7.91	7.8	7.88	259.0603	314.6734		35
AFG_1	$C_{17}H_{12}O_7$	Na^+	351.0474 (-0.23)	351.0477 (+0.42)	351.0481 (+1.54)	7.8	7.76	7.88	215.6405			35
AFG_2	$C_{17}H_{14}O_7$	Na^+	353.0631 (-0.25)	353.0636 (+1.13)	353.0638 (+1.66)	7.6	7.4	7.62	188.9185	331.0811	313.0706	35
DON	$C_{15}H_{20}O_6$	Na^+	319.1154 (+0.6)	319.1160 (+2.39)	319.1157 (+1.68)	4.32	4.06	4.15	249.1565	265.1215	281.1834	35
Ergocornine	$C_{31}H_{39}N_5O_5$	H^+	562.3033 (+1.68)	562.3035 (+1.93)	562.3041 (+2.96)	7.84	7.8	7.6	266.9992	351.0471		35
Fumagillin	$C_{26}H_{34}O_7$	Na^+	481.2204 (+1.47)	481.2205 (+1.71)	481.2204 (+1.59)	10.37	10.33	10.36	102.0466	131.0018		35
FB1	$C_{34}H_{59}NO_{15}$	H^+	722.3973 (+2.18)	722.3973 (+2.17)	722.3980 (+3.17)	8.64	8.62	8.69	352.3198	334.0913		35
FB2	$C_{34}H_{59}NO_{14}$	H^+	706.4020 (+1.62)	706.4025 (+2.39)	706.4030 (+3.01)	9.27	9.22	9.27	336.3253	318.3147		35
Fusarenone X	$C_{17}H_{22}O_8$	Na^+	377.1208 (+0.33)	377.1213 (+1.65)	377.1214 (+1.95)	4.0	4.1	4.13	176.9380	232.9276	288.9214	35
HT-2	$C_{22}H_{32}O_8$	Na^+	447.1996 (+1.46)	447.1999 (+2.13)	447.2000 (+2.44)	9.24	9.2	9.23	203.1060	285.1088		35
Malformin A (ESIpos)	$C_{23}H_{39}N_5O_5S_2$	Na^+	552.2293 (+1.46)	552.2295 (+1.91)	552.2295 (+1.92)	10.08	10.07	10.06	307.1572	231.0615		35
Malformin A (ESIneg)	$C_{23}H_{39}N_5O_5S_2$	$-H^+$	528.2324 (+0.86)	528.2324 (+0.74)	528.2326 (+1.13)	9.98	10.09	10.25	141.0658	221.1543		35
Monocrotaline	$C_{16}H_{23}NO_6$	H^+	326.1599 (+0.19)	326.1601 (+0.85)	326.1601 (+1.0)	5.58	5.55	5.57	94.0653	120.0810	194.1169	35
OTA	$C_{20}H_{18}NO_6Cl$	Na^+	426.0721 (+1.34)	426.0722 (+1.66)	426.0724 (+2.23)	9.95	9.9	9.94	260.9917	239.0100		35
p-Anisaldehyde	$C_8H_8O_2$	H^+	137.0598 (+0.74)	137.0599 (+1.12)	137.0600 (+1.9)	8.15	8.08	8.11	109.0649	94.0416	77.0390	35
Retrorsine	$C_{18}H_{25}NO_6$	H^+	352.1756 (+0.33)	352.1758 (+0.94)	352.1760 (+1.54)	6.28	6.23	6.26	93.9467	119.9507	299.0616	35
Sterigmatocystin	$C_{18}H_{12}O_6$	Na^+	347.0532 (+1.66)	347.0534 (+2.32)	347.0533 (+2.0)	10.13	10.11	10.1	281.0437	310.0463		35
T-2	$C_{24}H_{34}O_9$	Na^+	489.2102 (+1.39)	489.2103 (+1.62)	489.2105 (2.06)	9.61	9.59	9.6	199.1112	387.1399	327.1192	35
Tenuazonic acid	$C_{10}H_{15}NO_3$	H^+	198.1129 (+2.09)	198.1130 (+2.77)	198.1131 (+3.09)	8.95	8.86	8.93	124.9913	149.0448		35
ZON (ESI neg)	$C_{18}H_{22}O_5$	$-H^+$	317.1395 (+0.31)	317.1395 (+0.27)	317.1397 (+0.66)	9.98	9.97	9.96	131.0490	175.0391		35

16. Results and Discussion

16.1 Compound Confirmation, Identification, and Not Detected Compounds by ExactFinder Software

Samples of corn, wheat, and animal feed were spiked with fungal metabolite standards at two concentration levels (250 and 500 µg/kg). Each level in each matrix was prepared in six replicates.

Identification of 21 targeted metabolites was sought by processing with the ExactFinder software. Compound confirmation or identification was based on previously defined criteria (see the sections "Confirmation and Identification of Toxins" and "Not Detected Compounds"). Evaluation of % hits of confirmed, identified, and not found mycotoxins is illustrated graphically in Figure 4 and summarized in Table 3.

Evaluation of targeted screening of 21 fungal and plant metabolites shows an average confirmed/identified rate of 98% in corn, 97% in wheat, and 100% in animal feed. The overall results (Table 3) show 99% identified or confirmed with 1% of not found hits. In wheat, few not found hits (3%) have been found for OTA, fumagillin, ergocornine, DON, and FB1. This can be explained by chromatographic problems such as poor peak shape or matrix interferences.

Figure 4. Graphical illustration of % hits of compound confirmation (green), identification (yellow), and not found (red) in corn, wheat, and animal feed at two concentration levels (250 and 500 µg/kg)

Table 3. Evaluation of total confirmed, identified, and not found hits by ExactFinder software

Total Number of Analyzed Samples	Confirmed	Identified	Not Found
756	673	73	10
100%	89%	10%	1%

16.2 Analysis of Quality Control Materials

Quality control materials were analyzed for the determination of compound confirmation (green), identification (yellow), or not found (red) hits. The results are listed in Table 4. Most of the compounds have been confirmed by the software. HT-2 in sample T2280 has only been identified because of the low signal of the present fragment ion. Yellow hits in the ergot alkaloid sample can be explained by the missing information in the database about retention time and fragment ions.

Table 4. Results of quality control materials

QC Material	Matrix	Target Analyte (Assigned Value µg/kg)	Found
FAPAS T2280	Oat flour	T-2 (220)	
		HT-2 (89)	
FAPAS T2268	Breakfast cereal	DON (618)	
Römer labs 3020		Ergot alkaloids (331–1349)	Ergosine, Ergocornine
			Ergometrine, Ergometrinine, Ergosinine, Ergotamine, Ergotaminine, α-Ergocryptine, α-Ergocryptinine
FAPAS T2273	Corn	ZON (44)	
FAPAS T2275	Corn	FB1 (501)	
		FB2 (369)	
FAPAS T2276	Feed	ZON (129)	

17. Conclusion

This method documents a fast screening method for the detection of fungal metabolites in corn, wheat, and animal feed. Two sets of samples were prepared for each matrix at 250 and 500 µg/kg spiking level. The extracted samples were injected to the Transcend TLX-1 system for automated sample preparation clean up and analyzed with HRAM. Compound identification was based on the detection of a peak with minimum threshold of 20,000 and accurate mass with <5 ppm mass deviation. Compounds were confirmed by additional detection of minimum one fragment ion at the specific retention time. Data processing with ExactFinder software has proved to be an effective tool with 99% of compounds identified and confirmed and 1% not found. The false positive rate was 0%. This method is in compliance with the guidelines of the validation of the screening method in which a reliable method is defined to have a false-compliant rate of <5%.[6] Additional confirmation of accurate compound confirmation and identification was given by the analysis of certified quality control materials.

18. References

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Application Note: 52146

High-Throughput Food Safety Control Employing Real Time Ionization (DART) Coupled to Orbitrap High-Resolution Mass Spectrometry

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Key Words
- Exactive
- DART
- Melamine
- Mycotoxins

Introduction

In recent years, substantial developments have taken place in the field of mass spectrometry, enabling the introduction of a number of novel ambient desorption ionization techniques[1] such as direct analysis in real time (DART®),[2] desorption electrospray ionization (DESI),[3] surface desorption atmospheric pressure chemical ionization (DAPCI)[4] and atmospheric solids analysis probe (ASAP™).[5] These novel ion sources are characterized by remarkably high throughput of analyses which can be carried out under ambient conditions without (chromatographic) separation of sample components prior to desorption/ionization or the need for complicated and time demanding sample pre-treatment procedures. The DART technology employed in this study relies upon fundamental principles of atmospheric pressure chemical ionization (APCI). Excited-state helium atoms produce reactive species for analyte ionization.[2] Numerous applications of the DART ion source coupled to various types of mass spectrometers have been reported.[6-16] DART found its use in many areas of analytical chemistry as a tool for rapid qualitative analysis of numerous compounds. Due to the relatively high signal fluctuation of ion intensities obtained by repeated DART measurements, an internal standard usually has to be employed for compensation during quantitative analysis. However, implementation of Vapur® gas ion separator and automatic sampling systems were reported to significantly improve the repeatability for some analytes.[15]

Due to the absence of separation, the whole sample is introduced into a mass spectrometer. This unavoidably leads to a significant number of spectral interferences. In order to correctly determine the masses of relevant compounds and potential unknowns in the case of fingerprinting analysis, it is essential to separate them from the matrix ions. A mass spectrometer based on Orbitrap technology routinely achieves the mass resolving power of up to 100,000 FWHM (full width half maximum) while maintaining excellent mass accuracy of < 5 ppm, without the use of internal mass correction.[17] Those features make it an ideal tool to complement DART ionization for the analysis of complex samples.

This application note shows the possibilities using the DART ion source coupled with the ultra high-resolution Thermo Scientific Exactive mass spectrometer for rapid detection and quantitation of a wide range of food contamin - ants like mycotoxins and food adulterants (melamine).

Experimental

DART-Exactive MS

DART-Exactive MS system used in this study consisted of a new commercial model of DART ion source (DART-SVP) with a 12 Dip-It™ tip scanner autosampler coupled to the Exactive™ benchtop mass spectrometer – see Figure 1. Vapur interface was employed to hyphenate the ion source and the mass spectrometer, low vacuum in the interface chamber

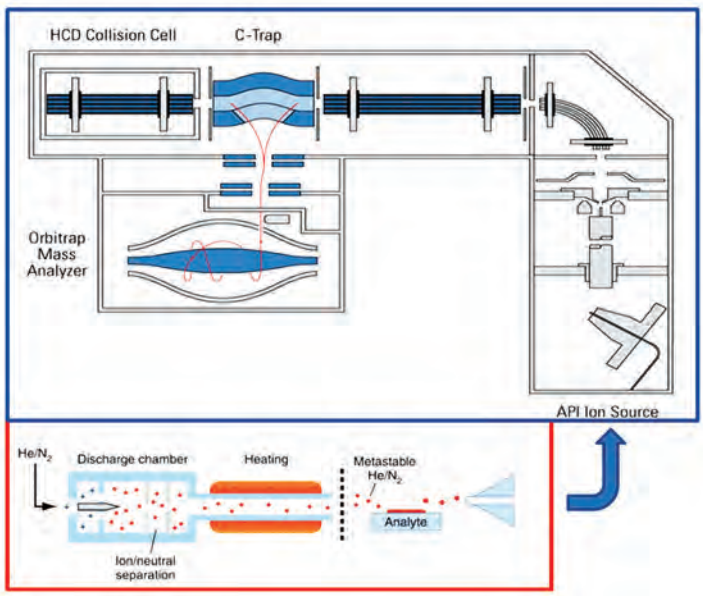

Figure 1: Schematics of a DART-Exactive system (source: *www.ionsense.com*). DART ionization source (bottom), Exactive MS (top).

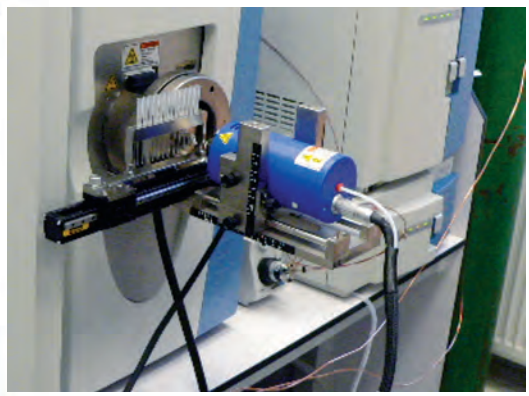

Figure 2: The DART ionization source coupled to Exactive MS

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was maintained by a membrane pump (Vacuubrand, Wertheim, Germany). The use of Vapur gas ion separator during DART ionization was essential in order to maintain stable vacuum within the operating pressure limits of the Exactive instrument. Vapur interface also improved transport efficiency of ions from the sampling area to the atmospheric-pressure interface inlet of the mass spectrometer, thus enhancing both sensitivity and reproducibility of the measurement. The distance between the exit of the DART gun and the ceramic transfer tube of the Vapur was set to 10 mm, the gap between the ceramic tube and the inlet to the heated capillary of the Exactive was 2 mm.

DART-MS instrument was operated either in positive or negative ionization mode; optimized settings of the system parameters were as follows: *(i)* DART positive ionization: helium flow: 2.5 L min^{-1}; gas temperature: 350 °C; discharge needle voltage: -5000 V; grid electrode: +200 V. *(ii)* DART ionization negative ionization: helium flow: 2.5 L min^{-1}; gas temperature: 350 °C; discharge needle voltage: -5000 V; grid electrode: +350 V. *(iii)* Mass spectrometric detection: capillary voltage: ±55 V; tube lens voltage: ±130 V; capillary temperature: 250 °C. Sheath, auxiliary and sweep gases were disabled during DART-MS analysis. The acquisition rate was set according to desired resolving power of the Exactive mass analyzer, and was 10 spectra s^{-1} at 10,000 FWHM (full width at half maximum), 4 spectra s^{-1} at 25,000 FWHM and 2 spectra s^{-1} at 50,000 FWHM. In all cases, the mass resolving power was calculated for *m/z* 200.

Semi-automatic analysis of liquid samples was carried out with the use of 12 Dip-It tip scanner autosampler. Dip-It tips were inserted into a holder and immersed in sample extracts placed in deepwell micro-plate (Life Systems Design, Merenschwand, Switzerland). The holder was mounted on the body of the autosampler. Subsequently, the Dip-It tips automatically moved at a constant speed of 0.5 mm s^{-1} through the helium gas beam in perpendicular direction to the axis leading from DART gun exit to the mass spectrometers inlet. Using the above moving speed, the time of desorption from the surface of each tip was 9 s; total run time of 12 analyses was approx. 4.2 min. To enable and/or enhance ionization of certain analytes, 2 mL autosampler vial containing dopant solution was placed in the distance of 20 mm from the DART gun exit. Aqueous solution of ammonia (25%, *w/w*, Penta, Chrudim, Czech Republic) and neat dichloromethane (Scharlau, Barcelona, Spain) were used as dopants in positive and negative ionization mode, respectively.

Mycotoxin Analysis

Chemicals and standards

Standards of 3-acetyldeoxynivalenol (3-ADON), deoxynivalenol (DON), deoxynivalenol-3-glucoside (DON-3-Glc), fusarenon-X (FUS-X), nivalenol (NIV), HT-2 toxin (HT-2), T-2 toxin (T-2), diacetoxyscirpenol (DAS), aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin B2 (AFG2), ochratoxin A (OTA), fumonisin B1 (FB1), fumonisin B2 (FB2), sterigmatocystin, zearalenone (ZEA), $^{13}C_{15}$-deoxynivalenol ($^{13}C_{15}$-DON), $^{13}C_{15}$-nivalenol ($^{13}C_{15}$-NIV) and $^{13}C_{18}$-zearalenone ($^{13}C_{18}$-ZEA) were supplied by Biopure (Tulln, Austria). Standards of deepoxy-deoxynivalenol (deepoxy-DON), altenuene, alternariol, alternariolmethylether (alternariol-met), ergocornine, ergocrystine and ergosine were obtained from Sigma-Aldrich (Steinheim, Germany).

Composite standard was prepared in acetonitrile containing each of analytes (isotope-labeled compounds not included) at concentration level of 5000 ng mL^{-1} and further diluted to obtain solvent standards at 500 ng mL^{-1}. Individual solvent solutions of ^{13}C-labeled internal standards were prepared at 5000 µg mL^{-1} in acetonitrile. Matrix-matched standards in the concentration range 10 to 1000 ng mL^{-1} (corresponding to 50 to 5000 µg kg^{-1} in matrix) were obtained by spiking of blank wheat and maize extracts (prepared by procedures described below), additionally, isotopically labelled compounds were added at level 100 ng mL^{-1} (500 µg kg^{-1} in matrix).

Acetonitrile and methanol, both of HPLC-grade, were supplied by Merck (Darmstadt, Germany). Pure water was obtained from Milli-Q® purification system. Anhydrous magnesium sulphate, sodium chloride and ammonium formate (≥ 99% purity), were from Sigma-Aldrich. Primary secondary amine (PSA) sorbent was obtained from Varian (Harbor City, CA, USA), formic acid (≥ 98% purity) was from AppliChem GmbH (Darmstadt, Germany).

Samples and sample preparation

Modified QuEChERS procedure[18] was employed to extract target analytes from the examined matrices (wheat, maize and millet). 2 g of homogenized sample were weighed into a 50 mL polypropylene (PP) centrifuge tube, 7.5 mL of deionized water and 10 mL of acetonitrile were added. Vigorous shaking of the mixture (4 min) was followed by the addition of 4 g $MgSO_4$, 1 g NaCl, further shaking for 3 min and centrifugation (5 min, 10,000 rpm, 20 °C). 4 mL aliquot of the upper organic phase was transferred into a 15 mL PP tube containing 200 mg of PSA and 600 mg $MgSO_4$ and shaken for 3 min to perform solid phase extraction (SPE) clean-up of the extract. After centrifugation (3 min, 10,000 rpm, 20 °C), approx. 600 µL were taken for DART-Exactive MS analysis.

Analysis of Melamine

Chemicals and standards

Solid standard of melamine (MEL, ≥ 99.0%) was supplied by Sigma-Aldrich; isotopically labeled $^{13}C_3$-melamine ($^{13}C_3$-MEL, ≥ 98.0%) was from Witega (Berlin, Germany). Individual stock solutions of MEL and $^{13}C_3$-MEL were prepared at 1000 µg mL^{-1} in water. By further dilution, aqueous solutions at 100 and 10 µg mL^{-1} were obtained and used for preparation of matrix matched standards and spiking experiments. Matrix-matched calibration was prepared by spiking of blank raw milk, standards containing MEL in the range from 25 to 2500 ppb and fixed amount of $^{13}C_3$-MEL at 250 ppb were obtained this way. Water used in this study was purified with the use of Milli-Q purification system.

Samples and sample preparation

Raw milk samples were analyzed without any pre-treatment. Prior to DART-MS analyses, blank milk was spiked with MEL at 100 and 500 ppb and with $^{13}C_3$-MEL at 250 ppb. Additionally real-life samples (n = 2) representing contaminated powdered milk were, according to producers instructions, reconstituted in water (1:10, *w/v*), spiked with $^{13}C_3$-MEL and subjected to instrumental analysis.

Results and Discussion

DART-Exactive Analysis of Mycotoxins

The efficiency and practical applicability of DART technology for ionization of aflatoxins, fusarium toxins, alternaria toxins, ochratoxins, ergot alkaloids, and sterigmatocystin (analytes possessing relatively largely differing physico-chemical properties) was evaluated in this part of the study. For this purpose, solvent standards containing respective mycotoxin at level 500 ng mL^{-1} were analyzed. Various settings (100 – 400 °C) of ionization gas temperature and grid electrode voltage were tested in order to obtain best sensitivity and best efficiency of analyte's thermo-desorption. As shown below, most mycotoxins could be transferred into gaseous phase at temperature 350 °C which was found as an optimal compromise between signal intensity and analytes' thermal desorption speed. While the use of lower grid voltage (200 V) in positive ionization mode enabled approx. 50% intensity increase compared to 350 V setting, 350 V potential was optimal for analytes ionized in negative mode. It was also found that ionization of some mycotoxins is improved by introducing dopant vapours (dichloromethane or ammonia) into the region between the ion source exit and Vapur interface ceramic tube inlet.

The list of ions generated by DART, when analyzing mycotoxin standard solutions, is provided in Table 1. As it can be seen, most of the examined mycotoxins could be effectively ionized in positive or negative ion mode, either as pseudomolecular ions or forming charged adducts supposing dichloromethane or ammonia vapors were present in the ionization region. Relatively poor ionization efficiencies were obtained for aflatoxins where electrospray ionization (ESI)[19, 20] was documented to be option for their control at ultra trace levels which are of regulatory interest.

				Detected Ions		
Compound	**Elemental Formula**	**Exact MW**	**Ionization Mode**	**Ion**	**Elemental Composition**	**Exact Mass**
ADON	$C_{17}H_{22}O_7$	338.1360	Negative	$[M+Cl]^-$	$C_{17}H_{22}O_7Cl$	373.1049
DON	$C_{15}H_{20}O_6$	296,1254	Negative	$[M+Cl]^-$	$C_{15}H_{20}O_6Cl$	331.0943
Deepoxy-DON	$C_{15}H_{20}O_5$	280,1305	Negative	$[M+Cl]^-$	$C_{15}H_{20}O_5Cl$	315.0993
FUS-X	$C_{17}H_{22}O_8$	354,1309	Negative	$[M+Cl]^-$	$C_{17}H_{22}O_8Cl$	389.0998
NIV	$C_{15}H_{20}O_7$	312,1204	Negative	$[M+Cl]^-$	$C_{15}H_{20}O_7Cl$	347.0903
ZEA	$C_{18}H_{22}O_5$	318,1462	Negative	$[M-H]^-$	$C_{18}H_{21}O_5$	317.1394
				$[M+Cl]^-$	$C_{18}H_{22}O_5Cl$	353.1150
HT-2	$C_{22}H_{32}O_8$	424,2092	Positive	$[M+NH_4]^+$	$C_{22}H_{36}NO_8$	442.2435
T-2	$C_{24}H_{34}O_9$	466,2197	Positive	$[M+NH_4]^+$	$C_{24}H_{38}NO_9$	484.2541
DAS	$C_{19}H_{26}O_7$	366,1673	Positive	$[M+H]^+$	$C_{19}H_{27}O_7$	367.1750
				$[M+NH_4]^+$	$C_{19}H_{30}NO_7$	384.2017
Altenuene	$C_{15}H_{16}O_6$	292,0941	Negative	$[M-H]^-$	$C_{15}H_{15}O_6$	291.0874
				$[M+Cl]^-$	$C_{15}H_{16}O_6Cl$	327.0630
Alternariol	$C_{14}H_{10}O_5$	258,0523	Negative	$[M-H]^-$	$C_{14}H_9O_5$	257.0455
				$[M+Cl]^-$	$C_{14}H_{10}O_5Cl$	293.0211
Alternariol-met	$C_{15}H_{12}O_5$	272,0679	Negative	$[M-H]^-$	$C_{15}H_{11}O_5$	271.0612
				$[M+Cl]^-$	$C_{15}H_{12}O_5Cl$	307.0368
AFB1	$C_{17}H_{12}O_6$	312,0628	Positive	$[M+H]^+$	$C_{17}H_{13}O_6$	313.0712
AFB2	$C_{17}H_{14}O_6$	314,0785	Positive	$[M+H]^+$	$C_{17}H15O_6$	315.0868
AFG1	$C_{17}H_{12}O_7$	328,0578	Positive	$[M+H]^+$	$C_{17}H_{13}O_7$	329.0661
AFG2	$C_{17}H_{14}O_7$	330,0734	Positive	$[M+H]^+$	$C_{17}H_{15}O_7$	331.0712
Sterigmatocystin	$C_{18}H_{12}O_6$	324,0628	Positive	$[M+H]^+$	$C_{18}H_{13}O_6$	325.0707
OTA	$C_{20}H_{18}ClNO_6$	403,0817	n.d.	n.d.	–	–
FB1	$C_{34}H_{59}NO_{15}$	721,3879	n.d.	n.d.	–	–
FB2	$C_{34}H_{59}NO_{14}$	705,3930	n.d.	n.d.	–	–
Ergocornine	$C_{31}H_{39}N_5O_5$	561,2946	n.d.	n.d.	–	–
Ergocristine	$C_{39}H_{39}N_5O_5$	657,2946	n.d.	n.d.	–	–
Ergosine	$C_{30}H_{37}N_5O_5$	547,2789	n.d.	n.d.	–	–

n.d. - signal not detected

Table 1: Overview of most intensive mycotoxins ions detected under optimized DART-Exactive MS conditions in solvent standard (500 ng mL^{-1})

Material Description	Analyte (Assigned/Certified Value)	DART-Exactive MS External Calibration/Isotope Dilution	
		Mean (µg kg^{-1})	RSD (%)[a]
CRM, maize flour	DON (474 ± 30 µg kg^{-1})	459/486	9.0/5.9
CRM, wheat flour	DON (2800 ± 200 µg kg^{-1})	2608/2819	6.7/5.4
CRM, ground millet	ZEA (648 ± 140 µg kg^{-1})	583/613	7.5/6.0
CRM, maize flour	ZEA (60 ± 9 µg kg^{-1})	< LCL[b]	– / –

a Relative standard deviation (RSD) calculated from 3 analyses.
b The concentration of analyte was below LCL of the method.

Table 2: Trueness of data obtained by DART-Exactive MS analysis of certified reference materials

No ions were obtained under tested conditions for a few other mycotoxins, such as ochratoxin A, fumonisins or ergocornine, ergocrystine and ergosine. These compounds are rather polar, and especially in case of fumonisins and ergot alkaloids, have relatively high molecular weight (MW). Both of these properties are associated with low volatility that hampers the transfer of such analytes into gaseous phase. To facilitate and/or enhance DART ionization of troublesome mycotoxins, derivatization of polar functional groups, which enables avoiding hydrogen bonding, may represent a conceivable strategy.[21]

Quantitative Analysis

For quantitative purposes, the most abundant ions yielded by respective mycotoxins (see Table 1) were used and narrow isolation window of 4 ppm was employed to extract ion records (chronograms) of target analytes with high selectivity. The quantitative parameters of the method for DON and ZEA, demonstrated by analysis of available certified reference materials containing incurred *Fusarium* toxins, are presented in Table 2. For evaluation of repeatability, peak areas were preferred since they were shown to give better results compared to calculations based on peak heights. Typical RSDs for cereals spiked by mycotoxins at 500 µg mL^{-1} level ranged from 8.1 to 14.3%. Further decrease of RSDs (4.7–8.7%) and improved linearity of calibration plots compared to external calibration, was obtained when isotopically labelled internal standards were employed for compensation of absolute signal fluctuation. In case of regulated mycotoxins (DON, ZEA) DART-MS method lowest calibration levels allowed a reliable control of maximum limits established for tested matrices.[22] The recoveries of all target mycotoxins at both tested spiking levels 150 and 500 µg kg^{-1} were in the range 82–120% when external calibration based on matrix matched standards was employed for quantification. Regarding the requirements for performance characteristics in analysis of regulated analytes,[23] these were reliably met for both target toxins.

Melamine Analysis

Under experimental conditions, both MEL and $^{13}C_3$-MEL were detected as $[M+H]^+$ ions in positive DART ionization mode. The efficiency of ionization was comparable for both compounds. Very good mass accuracy, with mass error less than 3 ppm was achievable with Exactive mass analyzer (operated under mass resolving power setting 50,000 FWHM) within all measurements in this study; analyte confirmation based on elemental composition estimation could be performed. As shown in Figure 3, abundant spectral interference observed at *m/z* 127.04 was detected both in blank and spiked samples (in contrast to solvent standards). At mass resolving power ~3,500 FWHM obtained by time-of-flight mass analyzer it was not possible to resolve signals of analyte and interference. Especially at low concentration levels of MEL, the signal of analyte was completely overlapped by the interference making its detection impossible. On the other hand, employing high mass resolving power of DART-Exactive MS, reliable detection of MEL in milk, was feasible even at low concentration levels. Using

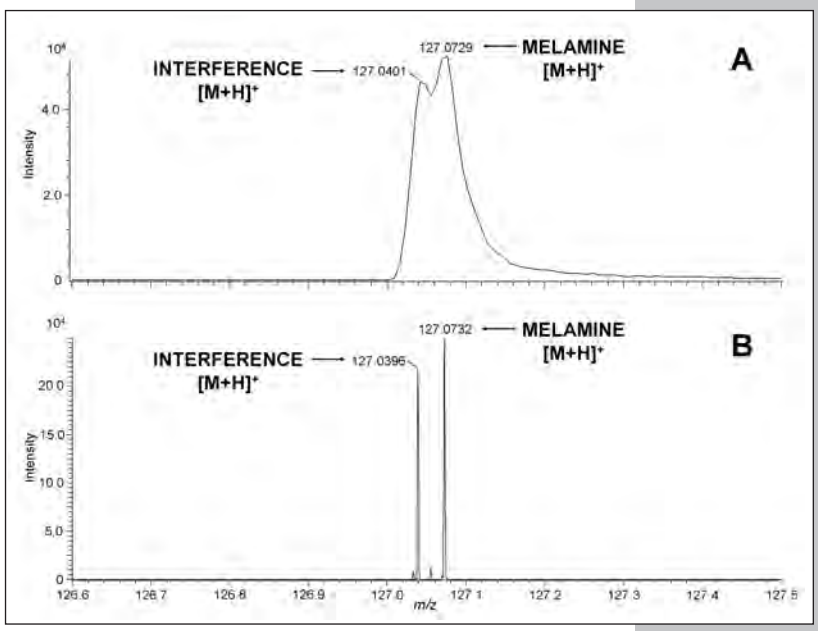

Figure 3: The improvement of mass separation by applying high mass resolution during analysis of melamine in milk sample at 2.5 mg/kg.
(A) DART-TOFMS (mass resolving power 3,500 FWHM);
(B) DART-Exactive MS (mass resolving mass power 50,000 FWHM).

Sample	DART-Exactive MS		LC-MS/MS	
	Mean (ppb)	RSD (%)	Mean (ppb)	RSD (%)
Milk powder 1	501	8.2	530	5.1
Milk powder 1	2496	6.4	2612	2.1

Table 3: Concentration of MEL in real-life samples as measured by DART-Exactive MS and LC-MS/MS

accurate mass of interference ion, elemental composition $C_6H_7O_3$ was estimated. This value corresponds to protonated molecule of hydroxymethyl furfural (HMF) which is typically formed during thermal processing of sugars containing foods.

The detectability of the method was characterized as lowest calibration level (LCL). Generally used limit of detection could not be calculated due to absence of noise in obtained records. LCL for melamine in milk was 25 ppb. For quantification purpose, isotope dilution technique was used. Figure 4 shows record of calibration standards analysis of which can be completed within 4.2 min (duplicate of each standard), Figure 5 documents acceptable linearity obtained for calibration curve in the range 25 to 2500 ppb ($R^2 \geq 0.99$). The LCL for melamine in milk was determined at 25 ppb and recoveries calculated at 100 and 500 ppb were in the range 98–119% and 101–109%. Repeatability at 100 ppb (n = 5) was 7.2%.

The results of real life samples analyses were compared to those obtained by validated LC–MS/MS method. Good agreement between respective values was observed (see Table 3).

Conclusions

The results presented in this application note demonstrate the feasibility of DART ionization source in combination with Exactive mass spectrometer for the rapid detection and quantification of various food contaminants, including set of priority mycotoxins and melamine selected as an example. Comparable trueness of generated results was achieved by applying isotope dilution-based quantification and matrix-matched calibration to compensate for signal suppression and other matrix effects that unavoidably occur during direct analysis of real matrix samples.

The major advantages of the combination of ambient ionization technique with Exactive mass spectrometry are the simplicity of operation, day-to-day robustness and broad application range. In addition, ultra high-resolution provided by Exactive mass analyzer helps to solve some of the problems caused by isobaric interferences from matrix components. The choice of ultra high-resolution mass spectrometer such as Exactive is one of the key requirements when considering the application of DART ionization as a reliable tool in the food laboratory.

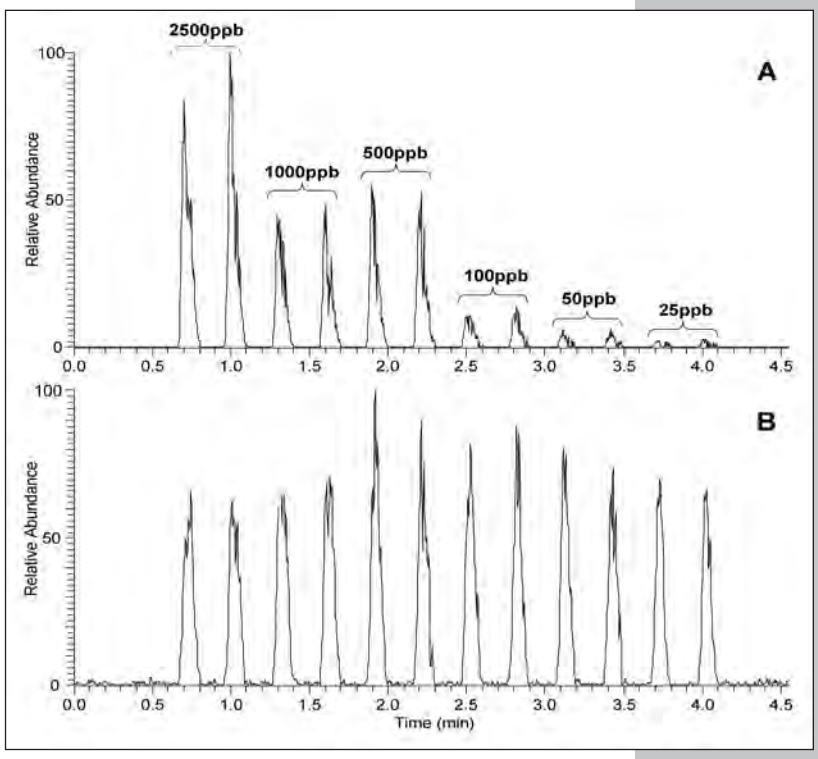

Figure 4: DART-Exactive MS record of milk spiked with MEL in the range 25 to 2500 ppb. (A) MEL (m/z 127.0726 ± 3 ppm); (B) $^{13}C_3$-MEL (m/z 130.0827 ± 3 ppm).

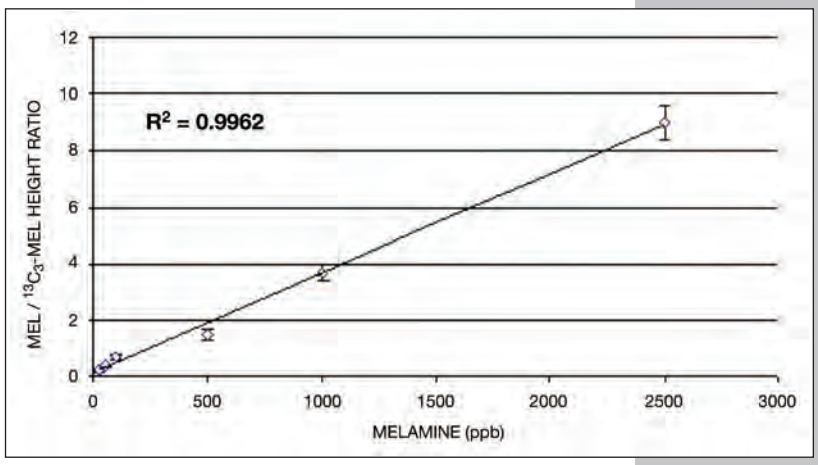

Figure 5: Calibration curve of melamine obtained by DART-Exactive MS analysis of matrix-matched standards constructed by plotting analyte-to-internal standard peak height ratio. Error bars are standard deviation (n = 3).

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Application Note: 523

Detection of Mycotoxins in Corn Meal Extract Using Automated Online Sample Preparation with LC-MS/MS

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Thermo Fisher Scientific, Franklin, MA

Key Words

- Transcend TLX-1
- TurboFlow Technology
- TSQ Vantage
- Food Safety

Introduction

Since the discovery of aflatoxin in 1960, mycotoxin research has received considerable attention. Mycotoxins are a group of naturally occurring toxic substances produced by certain molds, which can contaminate food and feed. The inhalation or absorption of mycotoxins into the body may cause harm, including kidney or liver damage, cancer, or even death in man or animals.[1] From a food safety perspective, the aflatoxins, ochratoxin A, patulin, fumonisins, trichothecenes, and zearalenone are the mycotoxins of major concern.

Many countries now monitor mycotoxin levels in food and feed products. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is currently a common analytical approach for the quantification of mycotoxin contamination.[2] Sample preparation for LC-MS/MS analysis can be time and labor intensive, often involving pH modification, solid phase or immunoaffinity column clean-up extraction, multi-step extract clean-up, and pre-concentration.[3] The strict regulation published by the European Union in 1999 asking for lower detection limits and higher method reliability presented a new analytical challenge.[4]

In this study we describe an easy, comprehensive, LC-MS/MS method using a Thermo Scientific Transcend TLX-1 system powered by Thermo Scientific TurboFlow technology to analyze multiple mycotoxin residues in corn meal extract. Figure 1 illustrates a typical Transcend™ TLX-1 system with the Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer.

Goal

Develop a rapid and sensitive automated, online sample preparation LC-MS/MS method to detect and quantify multiple mycotoxins in corn meal extract resulting in a shorter assay time and increased throughput.

Experimental

The matrix standard curve

Five grams of corn meal purchased from a local grocery store were extracted using 25 mL of 70% methanol in water followed by 60 minutes of ultra-sonication. The extract sat overnight at room temperature. The resulting solution was then centrifuged at 6000 RPM for 20 minutes. The supernatant was used to prepare the matrix calibrators and QC samples. Each milliliter of supernatant corresponds to 0.2 g solid corn meal powder as the unit of conversion.

Figure 1. Thermo Scientific Transcend TLX system with TSQ Vantage triple quadrupole mass spectrometer

The stock mix solution of analytes was prepared in methanol. Table 1 lists selected reaction monitoring (SRM) transitions and stock concentrations for individual analytes. Eight mycotoxins were analyzed under positive electrospray ionization (ESI) mode. The remaining three compounds, deoxynivalenol (DON), nivalenol (NIV), and 3-acetyl-DON (3-AcDON), were analyzed under negative electrospray ionization (ESI) mode.

Table 1. Analytes list

Compounds	Parent *(m/z)*	Primary *(m/z)*	Secondary *(m/z)*	Stock concentration (µg/mL)
Aflatoxins B1	313	241	285	0.050
Aflatoxins B2	315	259	287	0.015
Aflatoxins G1	329	243	283	0.050
Aflatoxins G2	331	245	275	0.015
Zearalenone (ZEA)	319	187	185	10.000
Ochratoxin A (OTA)	404	239	221	1.000
Fumonisins B1 (FB1)	722	334	352	2.500
Fumonisins B2 (FB2)	706	336	318	2.500
Deoxynivalenol (DON)	295	138	265	20.000
Nivalenol (NIV)	311	281	205	20.000
3-Acetyl-DON (3-AcDON)	337	307	173	20.000

LC/MS Methods using positive ESI mode (Method A):

TurboFlow™ Method Parameters

Column:	TurboFlow Cyclone-P 0.5 x 50 mm
Injection Volume:	10 µL
Solvent A:	10 mM ammonium acetate in water
Solvent B:	0.1% formic acid in acetonitrile (ACN)
Solvent C:	1:1:1 ACN: isopropanol: acetone (v:v:v) with 0.3% formic acid

HPLC Method Parameters

Analytical Column:	Thermo Scientific Hypersil GOLD 2.1 x 100 mm, 1.9 µm
Solvent A:	0.1% formic acid in water
Solvent B:	0.1% formic acid in ACN

Mass Spectrometer Parameters

MS:	TSQ Vantage™ triple stage quadrupole mass spectrometer
MS Ionization Source:	Heated Electrospray Ionization (H-ESI)
Spray Voltage:	5 KV
Sheath Gas Pressure (N_2):	50 arbitrary units
Auxiliary Gas Pressure (N_2):	20 arbitrary units
Vaporizer Temperature:	209 °C
Capillary Temperature:	270 °C
Collision Gas Pressure:	1.5 mTorr

LC/MS Methods using negative ESI mode (Method B):

TurboFlow Method Parameter

Column:	Research column A 0.5 x 50 mm
Injection Volume:	10 µL
Solvent A:	water
Solvent B:	methanol
Solvent C:	0.1% ammonium hydroxide
Solvent C:	45:45:10 ACN: isopropanol: acetone (v:v:v)

HPLC Method Parameters

Analytical Column:	Hypersil GOLD™ 2.1 x 50 mm, 1.9 µm
Solvent A:	0.1% formic acid in water
Solvent B:	0.1% formic acid in ACN

Mass Spectrometer Parameters

MS:	TSQ Vantage triple stage quadrupole mass spectrometer
MS Ionization Source:	H-ESI
Spray Voltage:	4.5 kV
Sheath Gas Pressure (N_2):	50 arbitrary units
Auxiliary Gas Pressure (N_2):	20 arbitrary units
Vaporizer Temperature:	250 °C
Capillary Temperature:	270 °C
Collision Gas Pressure:	1.5 mTorr

The LC method views from Thermo Scientific Aria Operating Software are shown in Figures 2 and 3.

Step	Start	Sec	Flow	Grad	%A	%B	%C	%D	Tee	Loop	Flow	Grad	%A	%B
1	0.00	45	2.00	Step	100.0	-	-	-	====	out	0.30	Step	98.0	2.0
2	0.75	5	0.10	Step	100.0	-	-	-	====	out	0.30	Step	98.0	2.0
3	0.83	120	0.10	Step	100.0	-	-	-	T	in	0.30	Step	98.0	2.0
4	2.83	5	2.00	Step	100.0	-	-	-	====	out	0.30	Step	98.0	2.0
5	2.92	90	2.00	Step	-	-	100.0	-	====	out	0.30	Ramp	60.0	40.0
6	4.42	220	2.00	Step	-	100.0	-	-	====	out	0.30	Ramp	30.0	70.0
7	8.08	220	2.00	Step	-	-	100.0	-	====	out	0.30	Ramp	2.0	98.0
8	11.75	45	2.00	Step	-	100.0	-	-	====	in	0.30	Step	2.0	98.0
9	12.50	180	2.00	Step	100.0	-	-	-	====	out	0.30	Step	98.0	2.0

Figure 2. Method A view in Aria OS software

Step	Start	Sec	Flow	Grad	%A	%B	%C	%D	Tee	Loop	Flow	Grad	%A	%B
1	00:00	45	1.50	Step	100.0	-	-	-	====	out	0.30	Step	98.0	2.0
2	00:45	60	0.07	Step	100.0	-	-	-	T	in	0.50	Step	98.0	2.0
3	01:45	45	1.50	Step	-	100.0	-	-	====	out	0.30	Ramp	20.0	80.0
4	02:30	45	1.50	Step	-	100.0	-	-	====	out	0.30	Ramp	10.0	90.0
5	03:15	45	1.50	Step	-	50.0	50.0	-	====	out	0.30	Ramp	2.0	98.0
6	04:00	30	1.50	Step	-	-	-	100.0	====	out	0.30	Step	2.0	98.0
7	04:30	15	1.50	Step	-	100.0	-	-	====	out	0.30	Step	2.0	98.0
8	04:45	30	1.50	Step	-	5.0	95.0	-	====	in	0.30	Step	98.0	2.0
9	05:15	150	1.50	Step	100.0	-	-	-	====	out	0.30	Step	98.0	2.0

Figure 3. Method B view in Aria OS software

Results and Discussion

Figure 4 shows the comparison of chromatograms of eight analytes at 1:100 dilutions in methanol and corn meal extract, indicating excellent chromatographic separation in both solvent standard and matrix. Matrix-matched calibration standards showed linear response of two orders of magnitude ($r^2 > 0.99$) for six of them (Table 2). Significant signal enhancement was observed for FB1 and FB2 due to matrix-induced ionization variability, which was previously reported by other researchers.[5] In future work, the isotope-labeled internal standard might be used to compensate for the matrix interference.

Because DON, NIV, and 3-AcDON have a better signal response under negative ionization mode, a separate LC-MS/MS method was developed. Figure 5 shows the chromatograms of DON, NIV, and 3-AcDON identified at 100 ng/mL fortified in the corn meal extract.

Figure 6 presents the linear fit calibration curves for DON and NIV, indicating excellent linear fits over the dynamic range. Table 3 summarizes detection, quantitation limits, and standard curve linearity for three analytes analyzed in negative ion mode. For all analytes, the quantitation limits obtained using the present methodology comply with the maximum levels in foods defined by European Union.[6] To the best of our knowledge, this is the first application of its type to detect these three compounds using an automated online sample preparation technique coupled to tandem mass spectrometry.

In addition, a lower limit of quantitation (LOQ) could be achieved by increasing sample injection volume since TurboFlow columns can handle larger injections (up to a few hundred microliters) while regular HPLC or UHPLC columns can not.

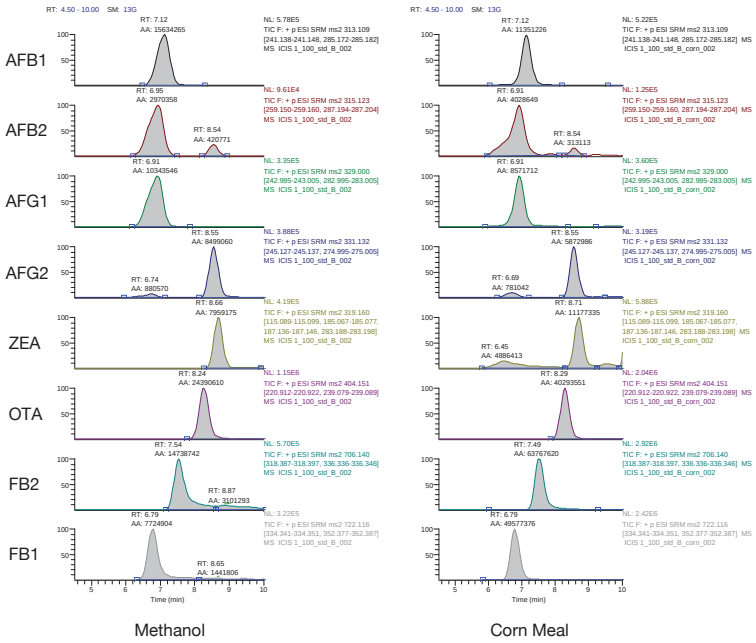

Figure 4. Comparison of chromatograms of 8 SRM analytes in methanol and corn flour extract (1:100 dilution of stock mixture)

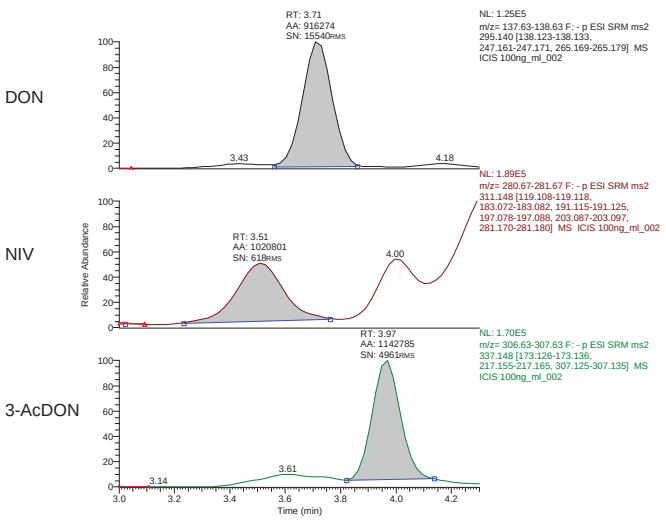

Figure 5 Selected chromatograms of DON, NIV, and 3-AcDON detected at 100 ng/mL fortified in the corn meal extract

Table 2. Limit quantitation (LOQ) and standard curve linearity (r^2) for analytes detected in positive ion mode

Compounds	LOQ (ng/g)	r^2
B1	0.50	0.9956
G1	0.50	0.9910
OTA	5.00	0.9937
ZEA	50.00	0.9955
FB1	12.50	0.9984
FB2	12.50	0.9965

Table 3. LOQ and standard curve linearity for analytes detected in negative ion mode

Compounds	LOQ (ng/g)	r^2
Deoxynivalenol (DON)	25.00	0.9934
Nivalenol (NIV)	25.00	0.9933
3-Acetyl-DON (3-AcDON)	25.00	0.9925

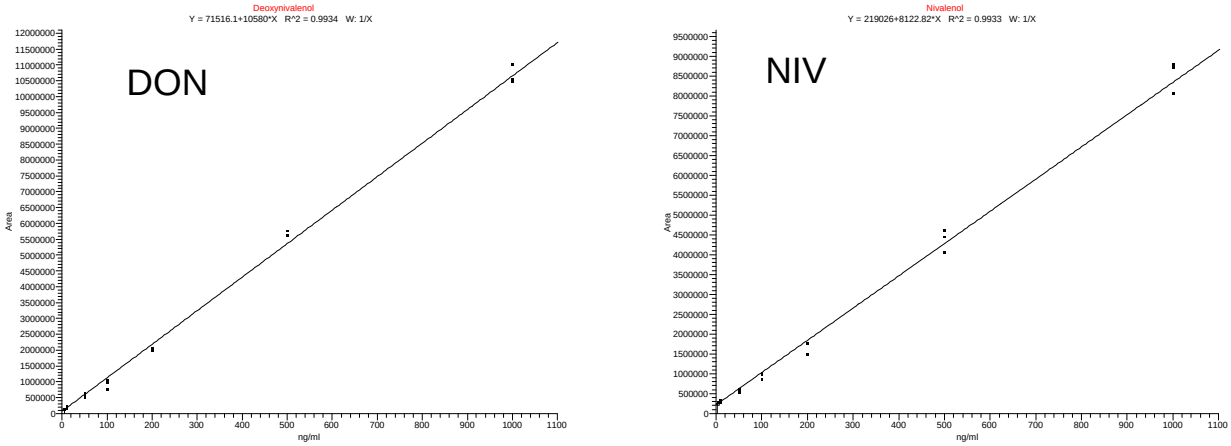

Figure 6. Calibration curves for DON and NIV

Conclusion

Developing a rapid and sensitive quantitative method is always a major goal for mycotoxins analysis.[7] Two quick, automated online sample preparation LC-MS/MS methods have been developed that are sensitive enough to detect mycotoxins in corn meal extract. By eliminating manual sample preparation, the reliability of this methodology was improved significantly. The sample throughput could be improved by multiplexing the two methods on different LC channels using a Transcend TLX-2 (or TLX-4) system. Future work will focus on the application of this methodology on various food matrices and references.

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Multi-mycotoxin Screening and Quantitation Using UHPLC, High Resolution and Accurate Mass

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Key Words

- Exactive LC-MS
- Beer
- Cereals
- Mycotoxins

Introduction

Mycotoxins are the toxic secondary metabolites produced by many species of microscopic filamentary fungi occurring on field cereals, including barley. The most abundant fungal genera affecting the malting barley are *Alternaria, Aspergillus, Penicillium* and *Fusarium*, which simultaneously showed relatively high-producing potential for a wide range of mycotoxins.[1] In addition to the relatively common micro mycetes mentioned above, *Claviceps purpurea* which causes ergot disease, belongs to numerous barley pathogens.

Although the carry-over of aflatoxins, ochratoxin A, zearalenone, fumonisins, and ergot alkaloids from malted grains into beer was documented, the main research in this area focused on deoxynivalenol, the most frequent Fusarium mycotoxin.[2,3] In recent years, the presence of deoxynivalenol's main metabolite, deoxynivalenol-3-glucoside, has been reported at relatively high levels in malt and beer. This fact was further confirmed in the follow-up study, in which both deoxynivalenol and its glucoside were identified as the main contaminants of beers retailed on the European market.[4] As beer is a significant dietary constituent to a large portion of the population, control of mycotoxins in this commodity is very important. For this purpose, reliable analytical methods for fast and effective monitoring of mycotoxins during the beer production chain are needed.

There is a trend toward the simplification of sample preparation procedures as much as possible. Full spectral data acquisition techniques are also preferred because of their ease of usage, along with the possibility of retrospective archived data mining. Until now, the most common full spectral mass-spectrometric approach has been the time-of-flight technology (TOF-MS), with typical resolving power of approx. 12,500 FWHM (full width half maximum). However, in complex food matrices such as beer, this rather limited mass resolving power leads to the risk of inaccurate mass measurements caused by unresolved background matrix interferences.[5,6] Mass spectrometry systems based on the Thermo Scientific Orbitrap technology routinely achieve mass resolving power of up to 100,000 FWHM and maintain excellent mass accuracy up to <5 ppm without the use of internal mass correction.[7]

The aim of this study was to introduce a multi-mycotoxin method for analysis of 32 mycotoxins in beer based on very simple sample preparation and ultra high performance liquid chromatography coupled with full spectral Orbitrap™ MS detection.

Mycotoxin standards of (i) Fusarium toxins, major conjugate and other products of transformation (nivalenol, deoxynivalenol, deoxynivalenol-3-glucoside, deepoxydeoxy - nivalenol, fusarenon-X, neosolaniol, 3-acetyldeoxynivalenol, diacetoxyscirpenol, HT-2 toxin, T-2 toxin, verrucarol, zearalenone, α-zearalenole, β-zearalenole); (ii) aflatoxins (aflatoxin G1, aflatoxin G2, aflatoxin B1, aflatoxin B2), (iii) sterigmatocystin; and (iv) ochratoxins (ochratoxin A, and ochratoxin α) were purchased from Biopure (Tulln, Austria), standards of (v) alternaria toxins (altenuene, alternariol, and alternariol-methylether) were obtained from Sigma-Aldrich (Taufkirchen, Germany), and standards of (vi) ergot alkaloids (ergosine, ergocornine, ergocryptine, ergocristine) were provided by The Czech Agricultural and Food Inspection Authority. The purity of standards was declared in the range 96–98.9%. Solid standards of nivalenol, deoxynivalenol, fusarenon-X, neosolaniol, 3-acetyldeoxynivalenol, T-2 toxin, verrucarol, zearalenone, α-zearalenole, β-zearalenole, sterigmatocystin, ochratoxin A, altenuene, alternariol and alternariol-methylether were dissolved in acetonitrile. Liquid standards of deepoxyde-oxynivalenol, diacetoxyscirpenol, HT-2 toxin, alfa-zear-alenole, beta-zearalenole, ochratoxin α, and ergot alkaloids were supplied in acetonitrile, and deoxynivalenol-3-glucoside was delivered in acetonitrile:water (1:1, v/v) solution. All of the standards were stored at -20 °C. For spiking experiments and calibration purposes, a composite working standard solution in acetonitrile (1000 µg L^{-1}) was prepared. All of the standards were brought to room temperature before use. The organic solvents acetonitrile and methanol (HPLC grade) were obtained from Sigma-Aldrich (Taufkirchen, Germany). Ultra-pure water was produced by Milli-Q system (Millipore Corporation, Bedford, MA, USA).

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Sample Preparation

The aliquot of 4 mL of beer sample in PTFE cuvette was degassed in the ultrasonic bath, and after addition of 16 mL acetonitrile, the content was vigorously shaken for approximately 1 min. The dark colored matrix precipitated under these conditions and was then separated by centrifugation (10 min, 11,000 rpm). In the next step, the 5 mL aliquot of the supernatant was evaporated to dryness and reconstituted in 1 mL of methanol:water (50:50, v/v). To avoid obstruction of the UHPLC system, microfiltration was performed prior to injection (centrifugation through the 0.2 µm microfilter, (PVDF Zentrifugenfilter, Alltech, USA)).

To control potential losses due to partition between precipitate and aqueous phase, aliquots of ^{13C}-labelled deoxynivalenol and ^{13C}-labelled zearalenone standard solution were added as the surrogates prior to processing (^{13C}-deoxynivalenol and ^{13C}-zearalenone for correction of more and less polar analytes, respectively).

Instrument Setup and Conditions

The Thermo Scientific Accela UHPLC system was used for the separation of target analytes. Detection was carried out using a Thermo Scientific Exactive benchtop single stage mass spectrometer, powered by Orbitrap technology and operated in full scan mode at different resolution settings. The use of internal mass axis calibration (lock mass) was not necessary. Conditions used are summarized in Table 1. The capillary and tube lens were set for ±45 and ±115 V respectively.

For the mass accuracy estimation, the mass at the apex of the chromatographic peak, obtained as the extracted ion chromatogram, was used. The calculated (exact) masses of quantification ions are summarized in Table 2.

Results and Discussion

Considering the current trend of analyzing for multiple food contaminants while maintaining high throughput and simplified sample preparation, direct analysis of a liquid sample may seem like the preferred option. However, in this case, direct injection of the matrix directly on the chromatographic column was not feasible because of its very high complexity. Direct injection also provided poor detectability of target analytes due to high matrix interference. In addition to this limitation, direct injection also lowered the analytical column lifetime and rapidly contaminated the ion source. Because of the complex properties of the 32 mycotoxins and their metabolites, neither adsorption nor immunoaffinity chromatography represented a feasible sample preparative strategy. The only simple approach to eliminating at least part of the matrix components, while keeping target analytes in solution, was by reducing the polarity of beer sample by addition of water-miscible solvent – acetonitrile.

It should be noted, that until now, most published studies concerned with determination of multiple mycotoxins in a single analysis used electrospray source ionization (ESI). However, the detection limits obtained by ESI were still rather poor for several Fusarium toxins, particularly for DON and its conjugate. Due to the importance of reliable analysis of these very common natural beer contaminants, the capability of atmospheric pressure chemical ionization (APCI) was evaluated. The optimal flow rate of mobile phase was determined to be 5 mL min^{-1} and the vaporizer temperature was set to 250 °C. Under APCI conditions, the enhancement in detectability of Fusarium toxins was as high as 1200% of the value achievable by ESI.

UHPLC Conditions		**MS Conditions (APCI)**	
Column	Hypersil GOLD aQ, 100 mm × 2.1 mm i.d., 1.9 µm	Sheath Gas	35 units
Mobile phase A	5 mM NH_4COOH in water	Auxiliary Gas	10 units
Mobile phase B	Methanol	Capillary Temperature	250 °C
Flow Rate	500 µL/min	Vaporizer Temperature	250 °C
Column Temperature	40 °C	Capillary Voltage	+60/-50 V
Injection Volume	5 µL	Discharge Current	5 µA
Gradient Elution Program		Scan Range	100-1000 *m/z*
0.0 min	5% B	Resolution Settings	10,000
6.0 min	50% B	(FWHM)	25,000
10.0 min	95% B		50,000
15.0 min	95% B		100,000
15.1 min	5% B		
18.0 min	5% B		

Table 1: Accela™ UHPLC/Exactive MS settings

Recommended Thermo Fisher Scientific Supplies

- Hypersil GOLD aQ, *p/n 25302-102130*, Thermo Scientific
- Methanol Optima LC/MS Grade, *p/n A456-212*, Fisher Scientific
- Acetonitrile Optima LC/MS Grade, *p/n A955-212*, Fisher Scientific
- Water, *p/n W6-212*, Fisher Scientific
- Ammonium Formate, *p/n A666-500*, Fisher Scientific
- Fisherbrand™ Higher-Speed Easy Reader Plastic Centrifuge Tubes, *p/n 06-443-19*, Fisher Scientific

Analyte	Retention Time (min)	Elemental Formula	Molecular Weight Da	Exact Mass $[M+H]^+$ *m/z*	Exact Mass $[M+NH_4]^+$ *m/z*	Exact Mass $[M-H]^-$ *m/z*	Exact Mass $[M+HCOO]^-$ *m/z*
Nivalenol	2.4	$C_{15}H_{20}O_7$	312.1209				357.1191
Deoxynivalenol	3.3	$C15H_{20}O_6$	296.1260				341.1242
Deoxynivalenol-3-glucoside	3.4	$C_{21}H_{30}O_{11}$	458.1788				503.1770
Deepoxydeoxynivalenol	4.5	$C_{15}H_{20}O_5$	280.1311				325.1293
Fusarenon-X	4.5	$C_{17}H_{22}O_8$	354.1315				399.1297
Neosolaniol	4.9	$C_{19}H_{26}O_8$	382.1628		400.1966		
Verrucarol	5.2	$C_{15}H_{22}O_4$	266.1518		284.1856		
3-acetyldeoxynivalenol	5.7	$C_{17}H_{22}O_7$	338.1366				383.1348
Ochratoxin α	5.7	$C_{11}H_9ClO_5$	256.0139			255.0061	
Aflatoxin G2	6.5	$C_{17}H_{14}O_7$	330.0740	331.0812			
Aflatoxin G1	6.8	$C_{17}H_{12}O_7$	328.0583	329.0656			
Altenuene	7.1	$C_{15}H_{16}O_6$	292.0947				337.0924
Aflatoxin B2	7.2	$C_{17}H_{14}O_6$	314.0790	315.0863			
Aflatoxin B1	7.5	$C_{17}H_{12}O_6$	312.0634	313.0707			
Diacetoxyscirpenol	7.6	$C_{19}H_{26}O_7$	366.1779		384.2017		
Ochratoxin A	8.5	$C_{20}H_{18}ClNO_6$	403.0823	404.0901			
Alternariol	8.7	$C_{14}H_{10}O_5$	258.0528			257.045	
HT-2 Toxin	8.7	$C_{22}H_{32}O_8$	424.2097		442.2435		
β-zearalenol	9.2	$C_{18}H_{24}O_5$	320.1624			319.1546	
T-2 Toxin	9.6	$C_{24}H_{34}O_9$	466.2203		484.2541		
α-zearalenol	9.9	$C_{18}H_{24}O_5$	320.1624			319.1546	
Ergosin	10.2	$C_{30}H_{37}N_5O_5$	547.2795	548.2867			
Zearalenone	10.2	$C_{18}H_{22}O_5$	318.1467			317.1394	
Sterigmatocystin	10.6	$C_{18}H_{12}O_6$	324.0634	325.0712			
Alternariol-methylether	10.7	$C_{15}H_{12}O_5$	272.0685			271.0607	
Ergocornine	10.7	$C_{31}H_{39}N_5O_5$	561.2951	562.3024			
Ergosinine	11.8	$C_{30}H_{37}N_5O_5$	547.2795	548.2867			
Ergocryptine	11.1	$C_{32}H_{41}N_5O_5$	575.3108	576.3180			
Ergocristine	11.2	$C_{35}H_{39}N_5O_5$	609.2951	610.3024			
Ergocorninine	11.8	$C_{31}H_{39}N_5O_5$	561.2951	562.3024			
Ergocryptinine	12.1	$C_{32}H_{41}N_5O_5$	575.3108	576.3180			
Ergocristinine	12.3	$C_{35}H_{39}N_5O_5$	609.2951	610.3024			

Table 2: Overview of the most intensive ions used for quantification by the Exactive

The lone exception was ochratoxin A, which showed better ionization efficiency under the electrospray conditions, APCI was chosen for use because it provided significant improvement of detection limits for most of the tested analytes. The extracted ion chromatograms of individual mycotoxins shown in Figure 1 document very good and fast separation achieved on the Accela™ UHPLC system.

In a routine trace analysis, both high mass resolving power and high mass accuracy play an important role in the unbiased identification and reliable quantification of target analytes.[5] Figure 2 illustrates the benefits of high resolving power setting on the discrimination of isobaric interferences. The importance of optimal choice of extraction window width is demonstrated here mainly for the use of lower mass resolution. While the use of a wide mass window typically results in worsened selectivity, using a narrow mass window presents a risk of removing some analytes from the chromatogram.

As demonstrated in Figure 3, the risk of false negative results occurs, especially for low intensity ions. While 50 µg L^{-1} of deoxynivalenol-3-glucoside was still detectable at the mass resolving power setting of 10,000 FWHM, almost no signal was detected by the same mass resolution at level 5 µg L^{-1}. At resolving power of 25,000 FWHM, the peak shape was improved. When the resolving power of 50,000 and/or 100,000 FWHM was enabled, optimal peak shape of deoxynivalenol-3-glucoside at 5 µg L^{-1} was obtained. As demonstrated, the higher resolving power, the better mass accuracy of deoxynivalenol-3-glucoside is obtained.

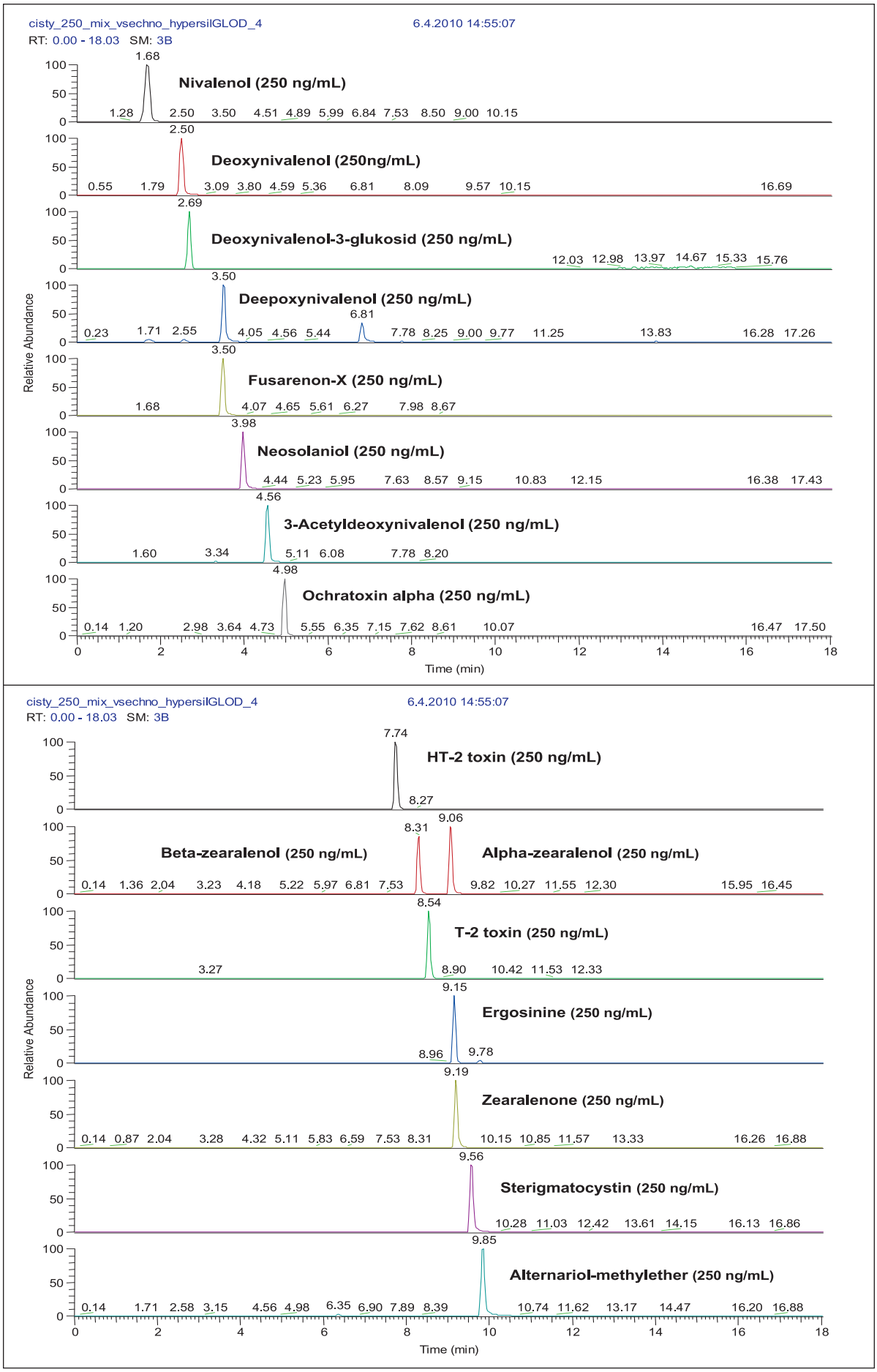

Figure 1: Extracted ion chromatograms of analyzed mycotoxins

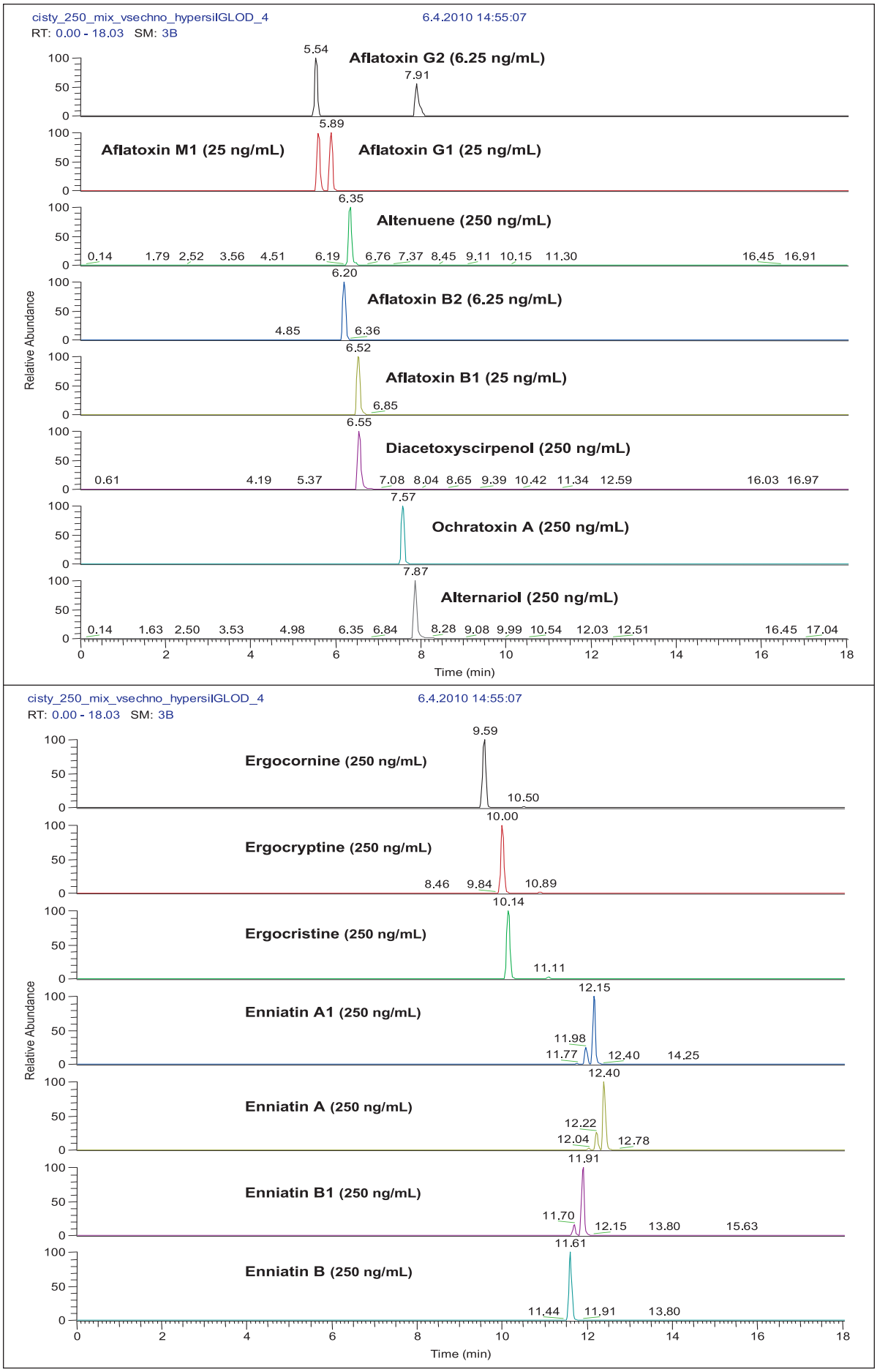

Figure 1 Continued: Extracted ion chromatograms of analyzed mycotoxins

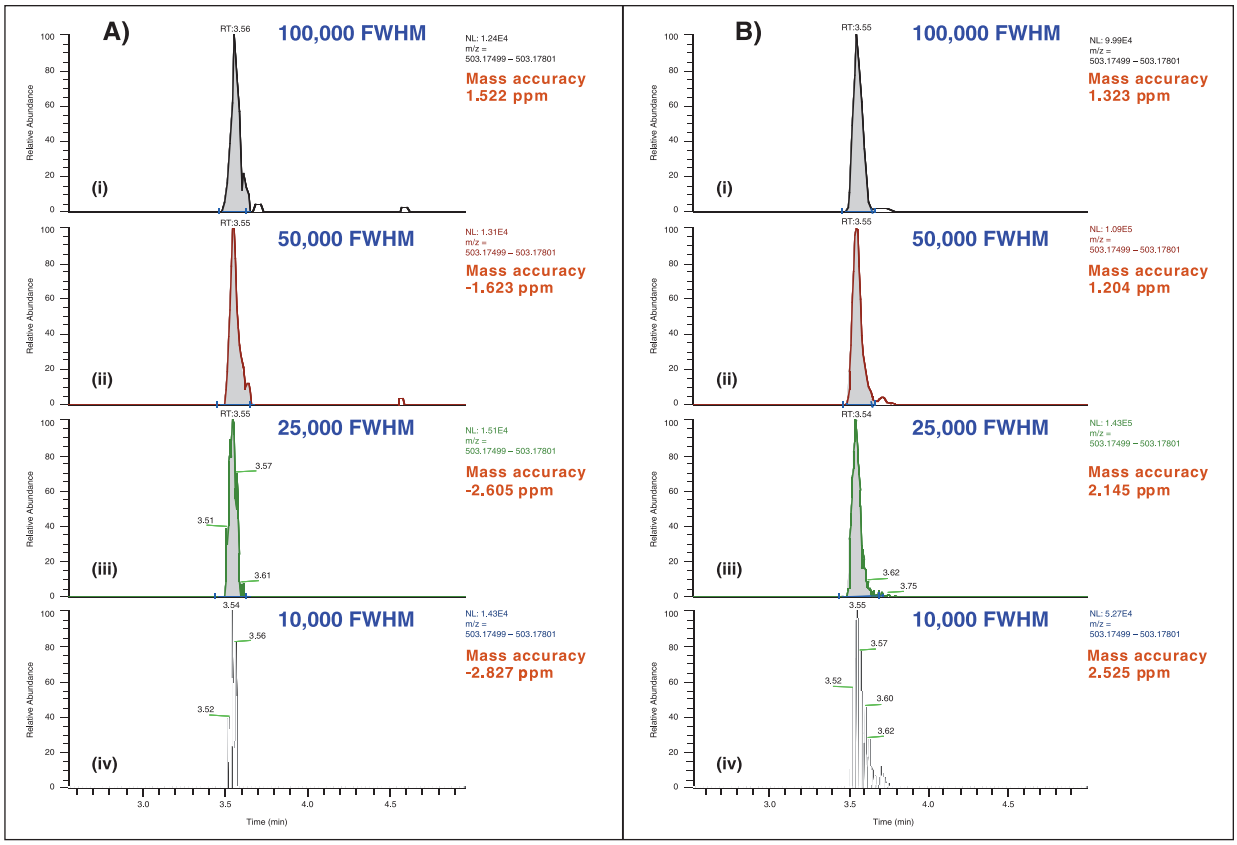

Figure 2: Extracted ion chromatograms of deoxynivalenol-3-glucoside in beer when performing four different resolving power settings (10,000; 25,000; 50,000; and 100,000 FWHM), mass extraction window ±3 ppm. The spiking levels were 5 µg L^{-1} (A) and 50 µg L^{-1} (B).

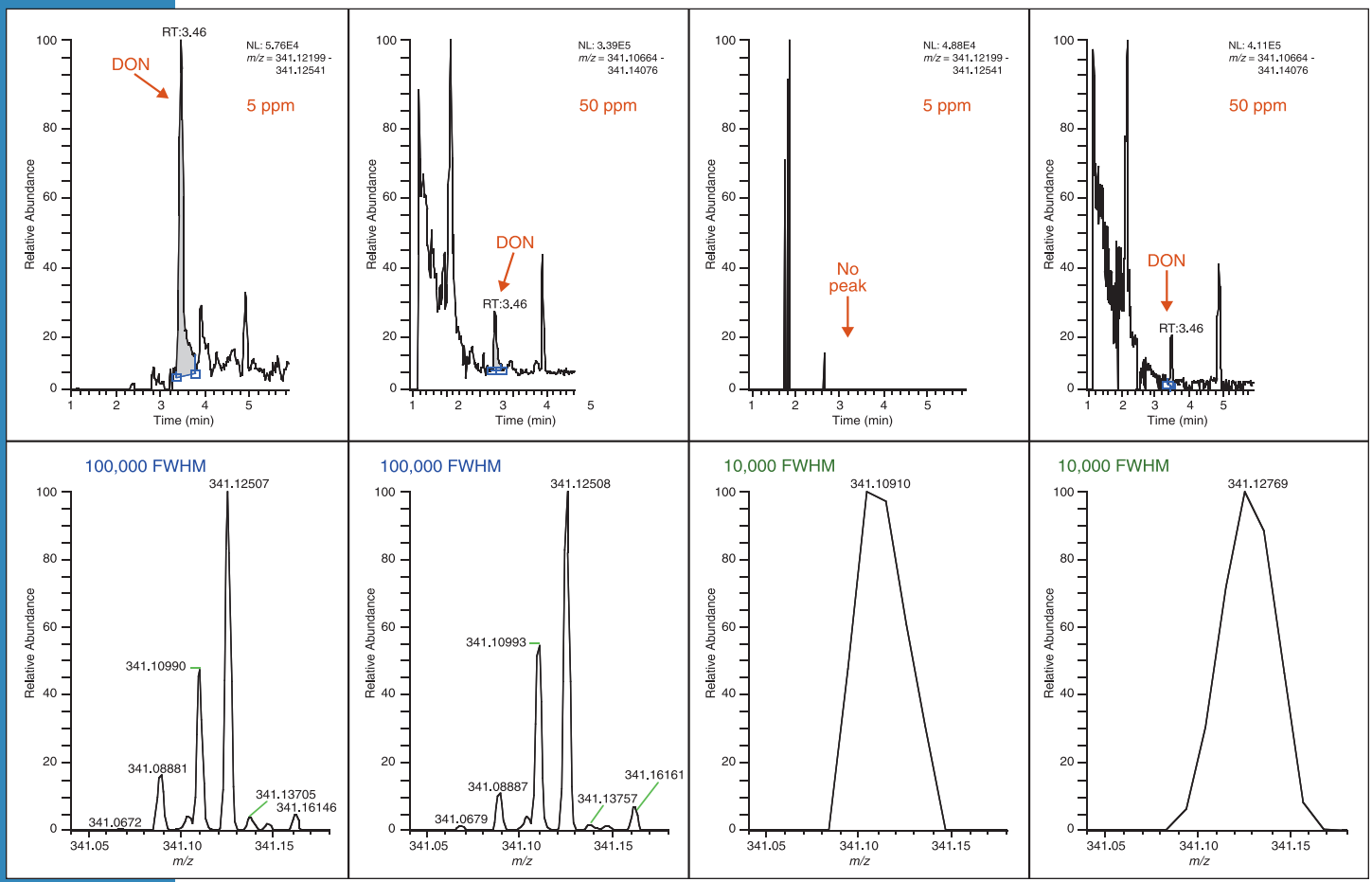

Figure 3: Extracted ion chromatograms and the mass spectra of deoxynivalenol in beer (10 µg L^{-1}) when performing two different resolving power settings (10,000 and 100,000 FWHM) and two different mass extraction windows (±5 and ±50 ppm).

Method Validation

The optimized multi-mycotoxin UHPLC-MS method was thoroughly validated. Prior to analysis of spiked samples, the extent of matrix effects was investigated in order to determine the quantification strategy. For this purpose, two calibration sets were prepared: (i) standards net solvent; (ii) matrix-matched standards. In both cases, the concentration of target mycotoxins was in the range 0.5–250 µg L^{-1}. Although the signal suppression/enhancement (SSE) range was not too broad (63–112%) matrix-matched calibration standards were used.

An important issue to address is calculating an equivalent to limit of quantification (LOQ). Tandem mass spectrometry's classical definition of LOQs based on signal to noise ratio (typically S/N > 6) is not always applicable in high resolution MS because a chemical noise is, in fact, absent in the chromatogram. Due to that fact, lowest calibration levels (LCL) were determined to be the most suitable option. The LCLs of analytes in our study were experimentally established as the lowest concentrations of matrix-matched standards repeatedly identified over time. The relative standard deviations of measurement calculated from nine repeated injections ranged between 11–28% (see Table 3). While these lowest calibration levels for 91% of analytes were at 1-10 µg L^{-1} level, a relatively high LCL level was found for ochratoxin A, which showed much better ionization under electrospray conditions (less than 5 µg L^{-1}).

Mycotoxin	LCL Pure Standard (µg L^{-1})	LCL Matrix-matched Standard (µg L^{-1})	Recovery %			RSD (%) at the Spiking Level 10 µg L^{-1} [1]	RSD (%) at the LCL Level[2]	SSE (%)[3]
			Spike 10 µg L^{-1}	Spike 30 µg L^{-1}	Spike 60 µg L^{-1}			
Nivalenol	2	6	107	97	103	8.9	19	92
Deoxynivalenol	2	3	104	112	99	4.9	24	112
Deoxynivalenol-3-glucoside	2	2	96	103	100	4.3	23	92
Deepoxydeoxynivalenol	4	15	102	116	104	7.2	19	94
Fusarenon-X	2	4	105	113	119	10.3	16	75
Neosolaniol	2	2	99	111	112	10.5	14	93
Verrucarol	3	4	98	99	101	8.4	18	84
3-acetyldeoxynivalenol	4	8	103	96	102	13.7	24	86
Ochratoxin α	4	31	102	98	108	9.8	21	67
Aflatoxin G2	1	2	103	106	99	10.9	25	65
Aflatoxin G1	1	4	117	94	107	8.9	19	63
Altenuene	0.5	1	119	120	113	8.4	22	93
Aflatoxin B2	0.5	1	111	106	104	5.5	12	91
Aflatoxin B1	0.5	2	107	90	92	5.2	13	105
Diacetoxyscirpenol	0.5	1	116	113	124	7.4	17	94
Ochratoxin A[4]	60	60	105	96	97	9.15[5]	26	84
Alternariol	0.5	2	101	107	98	8.5	16	76
HT-2 Toxin	2	4	117	116	104	6.9	19	87
β-zearalenol	1	2	111	92	98	9.1	11	85
T-2 Toxin	1	2	99	119	105	7.9	17	88
α-zearalenol	1	1	114	107	97	8.9	16	84
Ergosin	1	3	111	109	106	12.9	26	78
Zearalenone	1	1	106	117	105	9.4	19	91
Sterigmatocystin	0.5	0.5	118	98	110	11.6	16	107
Alternariol-methylether	1	1	114	109	113	9.1	14	88
Ergocornine	1	2	115	121	102	9.6	20	81
Ergosinine	1	2	98	114	102	8.4	12	91
Ergocryptine	1	2	103	111	101	14.9	23	101
Ergocristine	2	8	95	112	94	6.1	24	81
Ergocorninine	1	2	114	124	104	11.7	15	95
Ergocryptinine	1	2	88	113	101	11.4	26	97
Ergocristinine	2	8	104	119	99	9.1	28	103

Table 3: Validation data for the developed UHPLC-Orbitrap-MS method

1. RSD at the spiking level 10 µg L^{-1} was calculated from 6 spikes
2. RSD at the LCL level was calculated from 11 repeated injections of the particular matrix-matched standard
3. SSE (%) = matrix-matched calibration slope/solvent calibration slope * 100; SSE value of 100% means no effect of matrix on the ion signal
4. The spiking levels of ochratoxin A were 80, 100, and 120 µg L^{-1}
5. The RSD of ochratoxin A was determined at the spiking level of 100 µg L^{-1}

The linearity of the new method was tested for solvent as well as matrix-matched calibration curve constructed in the ranges LCL to 250 µg L^{-1}. The majority of analytes showed linearity in the range 0.9960–0.9999 (R^2). The recoveries of analytes tested at levels 10, 30, and 60 µg L^{-1} ranged from 92–124%, with no losses of analytes during the sample preparation occurred (Table 3).

Conclusion

The UHPLC-MS technology represents the most interesting alternative equivalent to tandem mass spectrometry with the possibility of retrospective data mining. Our UHPLC-MS operated in APCI mode enables rapid determination of trace levels of multiple mycotoxins occurring in complex beer samples. At the highest resolving power setting, 100,000 FWHM, the mass error up to 5 ppm (without the use of internal mass correction) enables the use of a very narrow mass extracting window, ±5 ppm, for the routine work, which significantly improves the selectivity of detection.

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Determination of *Fusarium* Mycotoxins in Wheat, Maize and Animal Feed Using an Online TurboFlow and Orbitrap LC/MS Method

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Method 63559

Key Words
Mycotoxins, food safety, Transcend system, Exactive, TurboFlow technology, Orbitrap, method validation

1. Schematic of Method

1. Weigh 5 g of homogenized sample into a 50 mL bottle.

Homogenized sample, 5 g

2. Add 20 mL of extraction solvent (water 0.1% FA/ACN (43:57)) and shake for 45 minutes.

Extraction

3. Filter sample through 0.2 µm nylon microfilter.

Filtration

4. Place the vial in autosampler of TLX-HRMS.

TurboFlow - Orbitrap LC/MS

2. Introduction

Mycotoxins are secondary metabolites produced by fungal infection of agricultural crops while in the field or during harvest, drying or subsequent storage. These compounds are very stable and cannot be readily destroyed by heating or food processing, although some processes, such as the milling of grains, can reduce the level in the end product. Good Agricultural Practices (GAP) are important to ensure that fungal infection is minimized and food and feed are produced with the lowest levels of mycotoxins achievable. Approximately 400 mycotoxins are known, but only a few are regulated by legislation.

The general approach to the analysis of mycotoxins involves liquid extraction, solid-phase extraction (SPE) or immunoaffinity-column (IAC) cleanup followed by HPLC with fluorescence detection or LC-MS (LC-MS/MS). The requirement for high sensitivity and the problems of matrix interferences either necessitate a lengthy cleanup process or require the use of high-specificity detection such as LC-MS/MS. However, even though direct analysis is possible without cleanup, dirty extracts can result in ion suppression and the need for frequent cleaning of the instrument source in LC-MS/MS.

Thermo Scientific TurboFlow technology is an online, automated sample cleanup and pre-concentration technique that enables the direct injection of food extracts that saves time by eliminating manual sample preparation. High-resolution mass spectrometry enables the determination of accurate masses with < 5ppm mass accuracy. Additional compound identification is given by fragmentation ions produced in a higher collision dissociation cell (HCD fragments).

Thermo SCIENTIFIC

A method using the Thermo Scientific Transcend TLX system with TurboFlow™ technology, which combines chromatography with high-resolution mass spectrometry, was developed and validated in house for the determination of deoxynivalenol, zearalenone, T-2 toxin, HT-2 toxin, fumonisins B_1 and B_2 in maize (corn), wheat and animal feed. Linearity range, limit of detection (LOD), limit of quantification (LOQ), recovery, repeatability, intermediate precision and accuracy were established by analysis using certified reference materials and the performance of successful proficiency testing.

3. Scope

The TurboFlow method based on online sample cleanup and high-resolution mass spectrometric detection can be applied to the determination of *Fusarium* mycotoxins (deoxynivalenol, zearalenone, T-2 toxin, HT-2 toxin and fumonisins B_1 and B_2) below the legislative limits[1-3] in maize, wheat and animal feed. The method replaces cleanup techniques involving numerous manual sample preparation steps, such as the purification of sample extracts using immunoaffinity cleanup cartridges.

4. Principle

This method uses TurboFlow technology for online cleanup of the sample. Finely ground and homogenous sample (5 g) is extracted for 45 minutes with a mixture of water containing 0.1% formic acid (FA)/acetonitrile (ACN) (43:57). After filtration with a 0.2 µm nylon filter into an LC-vial, the sample is injected into the Transcend TLX™ system, an online chromatography–reversed phase chromatography system (TLX-LC) coupled with high-resolution mass spectrometric (HRMS) detection on a Thermo Scientific Exactive Orbitrap mass spectrometer. TurboFlow technology serves as a novel sample preparation technique in food analysis due to its special flow profile, size exclusion, and reversed phase column chemistry. This enables very effective separation of matrix and target compounds, resulting in relatively clean sample extracts. Identification of mycotoxins is based on accurate mass determination at a resolution power of 100,000 and additional HCD fragments with mass deviation below 5 ppm.

5. Reagent List

5.1	Acetonitrile, Fisher Chemical Optima grade, for LC-MS	
5.2	Water, Optima™ grade, for LC-MS	
5.3	Methanol, Optima grade, for LC-MS	
5.4	Formic acid (FA)	
5.5	Thermo Scientific Pierce LTQ ESI positive ion calibration solution	
5.6	Thermo Scientific Pierce LTQ ESI negative ion calibration solution	

6. Calibration Standards

6.1	Deoxynivaneol (DON)	Sigma-Aldrich®
6.2	Zearalenone (ZON)	Sigma-Aldrich
6.3	T-2 toxin (T-2)	Sigma-Aldrich
6.4	HT-2 toxin (HT-2)	Sigma-Aldrich
6.5	Fumonisin B1 (FB1)	Sigma-Aldrich
6.6	Fumonisin B2 (FB2)	Sigma-Aldrich

7. Standard Preparation

7.1 Stock standard solutions of mycotoxins

Stock standard solutions (10 µg/mL) are prepared individually by dissolving the analytes in methanol. Solutions are stored at -20 °C. The standard stock solution is used for spiking, as different spiking levels are required for method validation of each mycotoxin.

8. Apparatus

8.1	Transcend TLX-1 system
8.2	Exactive™ Orbitrap™ mass spectrometer
8.3	Column oven, HotDog 5090 *(Prolab GmbH, Switzerland)*
8.4	Precision balance
8.5	Sartorius® analytical balance *(Sartorius GmbH, Germany)*
8.6	Thermo Scientific Barnstead Easypure II water
8.7	Elmasonic® S 40 (H) ultrasonic bath, *(ELMA® Hans Schmidbauer GmbH & Co. KG, Germany)*
8.8	Vortex shaker
8.9	Vortex standard cap
8.10	IKA® HS 501 digital shaker *(IKA-Werke GmbH & Co. KG, Germany)*

9. Consumables

9.1	Thermo Scientific Hypersil GOLD column, 50 x 4.6 mm, 5 µm particle size
9.2	TurboFlow Cyclone MCX column, 0.5 x 50 mm
9.3	LC vials
9.4	LC vial caps
9.5	Thermo Scientific Finnpipette 10–100 µL pipette
9.6	Finnpipette™ 100–1000 µL pipette
9.7	Finnpipette 500–5000 µL pipette
9.8	Pipette holder
9.9	Fisherbrand Pasteur Pipet, soda lime glass, 150 mm
9.10	Pipette suction device
9.11	Pipette tips 0.5–250 µL, 500/box
9.12	Pipette tips 1–5 mL, 75/box
9.13	Pipette tips 100–1000 µL, 200/box
9.14	Disposable plastic syringe, 1 mL
9.15	Nylon filter 0.2 µm

10. Glassware

10.1	Beaker, 25 mL
10.2	Volumetric flask, 10 mL
10.3	Volumetric flask, 100 mL
10.4	Volumetric flask, 1000 mL
10.5	Amber bottle, 50 mL

11. Procedure

11.1 Chemical preparation

Extraction solvent is prepared by mixing 1000 mL acetonitrile with 750 mL of water containing 0.1% FA.

11.2 Sample preparation and spiking

As no blank certified reference materials are available, a number of samples of maize, wheat and animal feed are analyzed to be used as blank material for spiking purposes. These samples, with trace levels (below LOD) of target mycotoxins, are used as blank materials for the validation study. Spiking is performed at three different levels with mycotoxin standard solutions.

To prepare the spiked sample, 500 g of matrix is homogenized by a laboratory blender and ground to a fine powder using a mortar and pestle. A sample of 5 g (±0.01 g) is weighed, put into a 50 mL amber flask and spiked with the appropriate amount of mycotoxin standard. Spiked samples are stored for 30 minutes in the dark for equilibration of mycotoxins. After the addition of 20 mL of extraction solvent, the bottles are closed and shaken for 45 minutes in the laboratory shaker. Samples are filtered through a nylon filter (0.2 µm) and injected into the TLX-HRMS system.

12. TLX-LC Conditions

TurboFlow methods are performed on a Transcend TLX-1 system. The LC conditions are as follows:

TurboFlow column:	TurboFlow Cyclone MCX, 0.5 x 50 mm
Analytical column:	Hypersil GOLD™, 50 x 4.6 mm, 5 µm particle size
Mobile phases:	A: Water (0.1 % formic acid) C: Methanol (0.1 % formic acid)
Total run time:	18 minutes

The autosampler sample holder temperature is kept at 10 °C. Sample injection volume is 10 µL with a 100 µL injection syringe. The injection syringe is rinsed as described in the injector settings. The gradient program is presented in Table 1.

Table 1. Gradient program table in Thermo Scientific Aria software for TurboFlow Method coupled with an analytical column

Step			Loading Pump[a]					Cut-in Loop		Eluting Pump[b]				
Step	Start (min)	Time (s)	Flow (mL/min)	Grad	A (%)	B (%)	C (%)	Tee	Loop	Flow (mL/min)	Grad	A (%)	B (%)	C (%)
1. Loading	0	90	1.5	Step	100	0	0	===	Out	0.45	Step	99	0	1
2. Transferring	1.30	1	0.3	Step	85	0	15	T	In	0.2	Step	99	0	1
3. Transferring/ HPLC	1.31	59	0.3	Step	85	0	15	T	In	0.2	Ramp	80	0	20
4. Washing/ HPLC	2.30	360	1.5	Step	85	0	15	===	In	0.6	Ramp	0	0	100
5. Washing/ HPLC	8.30	130	1.5	Step	100	0	0	===	In	0.6	Step	0	0	100
6. Washing/ HPLC	10.40	160	1.5	Step	0	0	100	===	In	0.6	Step	0	0	100
7. Loop filling/ equilibrating	13.20	120	1.5	Step	10	0	90	===	In	0.5	Step	99	0	1
8. Equilibrating	15.20	160	1.5	Step	100	0	0	===	Out	0.5	Step	99	0	1

[a]Mobile phases for the TurboFlow method:
A: Water (0.1% FA)
C: Methanol (0.1 % FA)

[b]Mobile phases for the analytical method:
A: Water (0.1% FA)
C: Methanol (0.1 % FA)

The injector settings are as follows:

Injector:	CTC injector *(CTC Analytics AG, Switzerland)* with 100 µL injection syringe volume
Wash solvents for the autosampler:	
Wash 1:	Methanol
Wash 2:	5% Methanol in water
Pre-clean syringe with wash 1 [steps]:	2
Clean injector (TX) with wash 1 [steps]:	2
Get sample (SEQ Tray: SEQ Index):	SEQ Volume

After injecting sample (syringe content) to TX:

Clean syringe with wash 1 [steps]:	7
Clean injector (TX) with wash 1 [steps]:	7
Clean syringe with wash 2 [steps]:	7
Clean injector (TX) with wash 2 [steps]:	7

Injection volume:	10 µL
Tray temperature:	10 °C
Column oven:	40 °C

13. Mass Spectrometric Conditions

MS analysis is carried out using the Exactive Orbitrap high-resolution benchtop mass spectrometer controlled by Aria™ MX software version 1.1. Data acquisition and processing is performed using Thermo Scientific Xcalibur 2.1 software. The Exactive MS was calibrated in positive and negative mode every 48 hours.

The MS Conditions are as follows:

Ionization:	Heated electrospray (HESI)
Polarity:	Positive/negative switching mode
Sheath gas flow rate [arb]:	60
Aux gas flow rate [arb]:	20
Spray voltage [kV]:	3.60
Capillary temperature [°C]:	260
Capillary voltage [V]:	60
Tube lens voltage [V]:	120
Skimmer voltage [V]:	25
Heater temperature [°C]:	250
Scan mode:	Full scan
Scan range [m/z]:	100–900
Microscans:	1
Resolution:	100,000
AGC target:	Balanced

14. Calculation of Results

14.1 Identification

Identification of mycotoxins was indicated by the presence of accurate mass ions obtained at a resolving power of 100,000 FWHM at *m/z* 200 and a mass accuracy window below 5 ppm, corresponding to the retention times of appropriate standards. Additional mass confirmation was given by the simultaneous detection of HCD fragments. Theoretical masses and detected masses in standards in methanol are listed in Table 2. Detected masses of target standards in maize, wheat and animal feed are listed in Table 3. Accurate mass deviation was determined to be below -1.8 ppm.

Table 2. Theoretical and found accurate masses in standards in methanol and fragment ions detected by HCD fragmentation

Mycotoxin	Molecular Formula	Exact Molecular Mass (*m/z*)	Adduct	Found [in MeOH]	Mass Deviation (ppm)	Fragment Ion Formula	Fragment Ion Exact Mass (*m/z*)	eV HCD Fragmentation
DON	$C_{15}H_{20}O_6Na$	319.1152	$+Na^+$	319.1150	+0.6	$C_{14}H_{17}O_4$	249.1124	20
T-2	$C_{24}H_{34}O_9Na$	489.2095	$+Na^+$	489.2095	0	$C_{17}H_{21}O_5$	305.1397	20
HT-2	$C_{22}H_{32}O_8Na$	447.1989	$+Na^+$	447.1989	0	$C_{17}H_{17}O_4$	285.1095	20
FB-1	$C_{34}H_{60}NO_{15}$	722.3957	$+H^+$	722.3955	+0.3	$C_{22}H_{42}NO_2$	352.3227	20
FB-2	$C_{34}H_{60}NO_{14}$	706.4008	$+H^+$	706.4006	+0.3	$C_{22}H_{42}NO$	336.3276	20
ZON	$C_{18}H_{21}O_5$	317.1395	$-H^+$	317.1397	-0.6	C_9H_7O	131.0492	20

Table 3. TLX-HRMS Exact masses and mass deviation in maize, wheat and animal feed

Mycotoxin	Adduct	Found Maize (*m/z*)	Mass Deviation (ppm)	Found Wheat (*m/z*)	Mass Deviation (ppm)	Found Animal Feed (*m/z*)	Mass Deviation (ppm)
DON	$+Na^+$	319.1151	+0.3	319.1151	-0.3	319.1150	+0.6
T-2	$+Na^+$	489.2098	-0.6	489.2098	-0.6	489.2101	-1.2
HT-2	$+Na^+$	447.1993	-0.9	447.1994	-1.1	447.1997	-1.8
FB-1	$+H^+$	722.3962	-0.7	722.3965	-1.1	722.3969	-1.7
FB-2	$+H^+$	706.4010	-0.3	706.4008	0	706.4015	-1.0
ZON	$-H^+$	317.1397	-0.6	317.1398	-0.9	317.1396	-0.3

14.2 Quantification

By comparing peak areas of the samples with those of external matrix-matched calibration standards, quantification of the mycotoxins was carried out. Calibration curves were plotted as relative peak areas (analyte) as a function of concentrations. The mycotoxin concentration (cMyco) in the samples was determined from the equation:

$$C_{Myco} = (A-b)/a$$

C_{Myco} – mycotoxin concentration in µg/kg
A – peak area of the mycotoxin
b – y-intercept
a – slope of calibration curve

15. Method Performance

Single laboratory method performance characteristics were established by spiking experiments in three matrices (maize, wheat and animal feed) with mycotoxin standards. Method recovery and precision was assessed at three different spiking levels (50%, 100% and 200% of the legislative limit for mycotoxins). Method accuracy was confirmed by the analysis of representative, certified reference materials. Other validation parameters included linearity range, LOD, LOQ, intermediate precision and matrix effect. A TLX-HRMS chromatogram of target compounds in maize is presented in Figure 1.

15.1 Specificity

The specificity was confirmed based on the presence of accurate parent masses and HCD fragment ion at the correct retention time corresponding to the mycotoxin standards in methanol (Table 4). The retention times were within ±2.5%. A minimum of 12 data points were required for each peak.

Table 4. Retention time comparison in matrices (specificity), ±2.5% deviation allowed

Mycotoxin	Retention Time (min)			
	Methanol	Maize	Wheat	Animal Feed
DON	2.97	2.98	2.98	2.96
T-2	8.01	8.03	8.02	8.01
HT-2	7.63	7.64	7.63	7.62
FB-1	7.58	7.59	7.56	7.53
FB-2	8.19	8.18	8.15	8.13
ZON	8.39	8.40	8.38	8.38

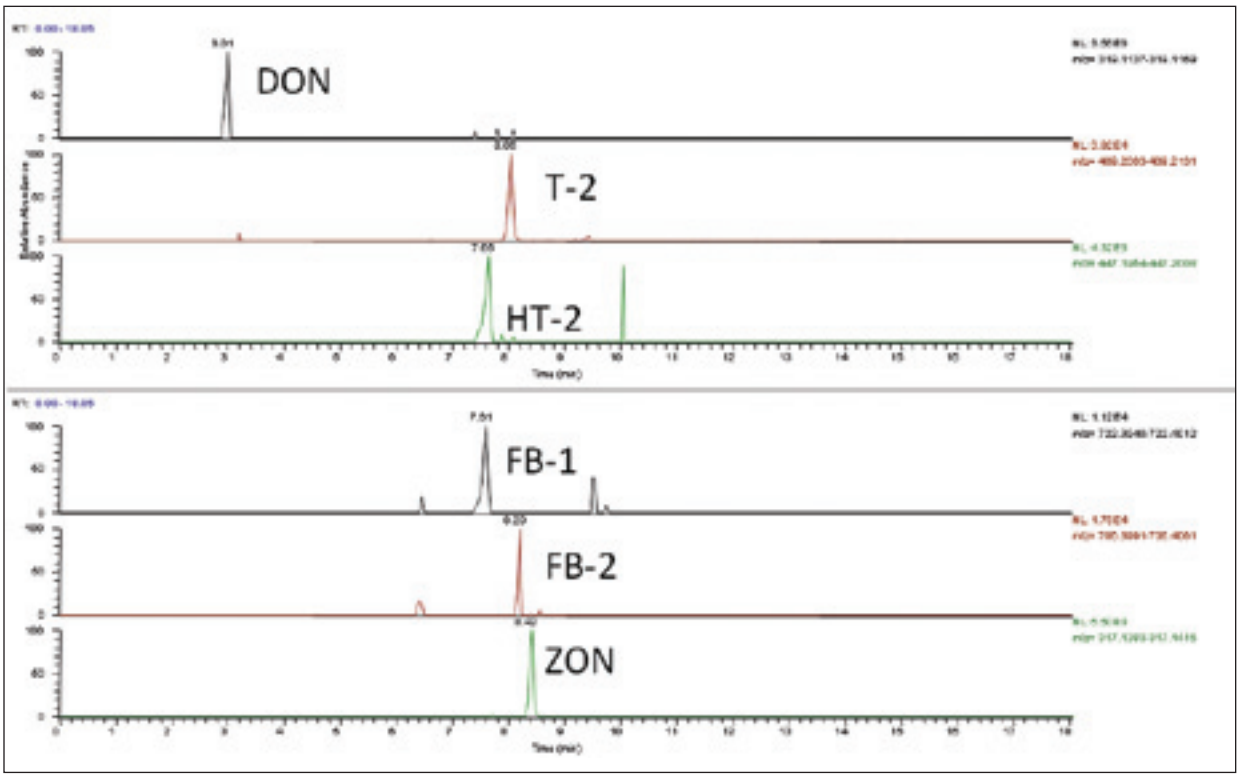

Figure 1. TLX-LC-HRMS chromatogram of DON (200 µg/kg), T-2 (20 µg/kg), HT-2 (20 µg/kg), FB_{-1} (75 µg/kg), FB_{-2} (75 µg/kg) and ZON (10 µg/kg) spiked in maize sample

15.2 Linearity & calibration curve

The linearity of the calibration curves was checked in standard solutions by monitoring the molecular ion of each mycotoxin analyzed (Table 5). In all cases, the correlation coefficients of linear functions were >0.985. The calibration curves were created from eight calibration standards, which were injected in duplicate in each batch starting from zero up to the highest calibration concentration.

Table 5. Linearity ranges of mycotoxin standards in solvent, maize, wheat and animal feed

Mycotoxin	Linearity Range (µg/kg)				
	Methanol		Maize	Wheat	Animal Feed
DON	125– 2190	225–2500	125–2190	125–2190	225–2500
T-2	12–400	12–400	12–400	12–400	12–400
HT-2	12–400	12–400	12–400	12–400	12–400
FB-1	50–500	500–5000	50–500	50–1250	500–5000
FB-2	50–500	500–5000	50–500	50–1250	500–5000
ZON	5–250	25–630	5–250	5–250	25–630

15.3 Recovery, precision and intermediate precision

Method recovery, precision and intermediate precision were evaluated by recovery studies in which maize, wheat and animal feed were spiked at three concentration levels (50%, 100% and 200% of the legislative limit of mycotoxin). Six replicates were prepared for each experiment in accordance with EU guidelines.[4] The samples were spiked as listed in Table 6. Determined concentration (µg/kg), recovery, and relative standard deviation (% RSD) were calculated (Tables 7a-c). Intermediate precision was determined by spiking maize, wheat and animal feed at one level (100% legislative limit) repeated on three days with six replicates (Table 8). Recovery and %RSD values were in the range of 71.6% – 120.2% and 1% – 19%, respectively. The intermediate precisions were found to be below 19%, demonstrating method repeatability. These results conformed to the requirements of Regulation EC 401/2006.[5]

Table 6. Spiking levels for maize, wheat and animal feed at 50%, 100% and 200% of legislative limit

Mycotoxin	Maize and Wheat Spiking Levels (µg/kg)			Animal Feed Spiking Levels (µg/kg)		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
DON	250	500	1000	450	900	1800
T-2	25	50	100	25	50	100
HT-2	25	50	100	25	50	100
FB-1	100	200	400	625	1250	2500
FB-2	100	200	400	625	1250	2500
ZON	25	50	100	50	100	200

Table 7a. Average determined concentration, recovery, and relative standard deviation in maize at three different concentration levels (n=6 each level)

Mycotoxin	Maize								
	Level 1 (µg/kg)	Level 2 (µg/kg)	Level 2 (µg/kg)	Level 1 (%)	Level 2 (%)	Level 3 (%)	Level 3 (%RSD)	Level 2 (%RSD)	Level 3 (%RSD)
DON	215.7	512.6	952.8	86.3	102.5	95.3	18	10	10
T-2	24.8	50.7	103.8	99.3	101.5	103.8	8	3	1
HT-2	26.3	48.6	102.2	105.1	97.3	102.2	4	2	3
FB-1	95.4	193.1	407.6	95.4	96.6	101.9	5	5	2
FB-2	94.7	181.5	376.3	94.7	90.7	94.1	10	10	4
ZON	26.2	50.4	102.8	104.7	100.8	102.8	4	5	2

Table 7b. Average determined concentration, recovery and relative standard deviation in wheat at three different concentration levels (n=6 each level)

Mycotoxin	Wheat								
	Level 1 (µg/kg)	Level 2 (µg/kg)	Level 2 (µg/kg)	Level 1 (%)	Level 2 (%)	Level 3 (%)	Level 3 (%RSD)	Level 2 (%RSD)	Level 3 (%RSD)
DON	247.8	513.4	1087.0	99.1	102.7	108.7	19	19	5
T-2	17.8	46.9	111.6	71.2	93.8	111.6	2	19	12
HT-2	26.9	49.9	98.9	107.5	99.8	98.9	4	5	4
FB-1	82.8	167.3	335.0	82.8	83.6	83.7	7	6	3
FB-2	99.4	183.1	386.6	99.4	91.6	96.6	13	10	5
ZON	29.8	52.4	102.4	119.1	104.9	102.4	4	4	2

Table 7c. Average determined concentration, recovery and relative standard deviation in animal feed at three different concentration levels (n=6 each level)

Mycotoxin	Animal Feed								
	Level 1 (µg/kg)	Level 2 (µg/kg)	Level 2 (µg/kg)	Level 1 (%)	Level 2 (%)	Level 3 (%)	Level 3 (%RSD)	Level 2 (%RSD)	Level 3 (%RSD)
DON	518.7	1024.2	2163.8	115.3	113.8	120.2	15	19	15
T-2	19.0	47.2	95.1	76.0	94.3	95.1	13	3	4
HT-2	26.2	49.3	94.3	104.9	98.5	94.3	8	3	5
FB-1	598.0	1227.7	2456.8	95.7	98.2	98.3	7	6	3
FB-2	571.9	1200.4	2320.3	91.5	96.0	92.8	7	10	6
ZON	58.1	103.6	201.5	116.2	103.6	100.8	2	3	4

Table 8. Average intermediate precision as RSD (%) – mid level (n= 6 on each day) – one level repeated on three days

Mycotoxin	Intermediate %RSD		
	Maize	Wheat	Animal Feed
DON	19	14	19
T-2	4	12	9
HT-2	6	7	6
FB-1	5	7	6
FB-2	7	10	13
ZON	4	5	4

15.4 Accuracy

Quality control materials were analyzed for the determination of method accuracy. All results were in the satisfactory range, thus confirming the accuracy of the method (Table 9). Additionally, the successful participation in a proficiency test confirmed the accuracy of the method (Table 10).

Table 9. Results of quality control materials

QC Material	Matrix	Target Analyte Assigned Value µg/kg (Satisfactory Range)	Found µg/kg (%RSD)
FAPAS 2273	Maize	ZON - 43.7 (24.5-63)	24.9 (1)
FAPAS 2275	Maize	FB 1 - 501 (323-679)	622.7 (7)
		FB 2 - 369 (232-506)	437.2 (6)
FAPAS 2278	Wheat	ZON - 27.7 (15.5-40)	26.1 (4)
FAPAS 2268	Wheat	DON - 618 (405-830)	453.9 (4)
FAPAS 2276	Feed	ZON - 129 (73-184)	100.2 (11)
FAPAS 2258	Feed	DON - 991 (674-1309)	794.1 (11)

Table 10. Results of proficiency testing

PT Material	Matrix	Target Analyte	Assigned Value (µg/kg)	Found (µg/kg)	z-Score
FAPAS 2276	Feed	T-2	T-2 331	373.8	0.7
FAPAS 2258	Feed	HT-2	HT-2 431	458.0	0.3

15.5 Limits of detection (LOD) and quantification (LOQ)

With HRMS detection, no constant noise is detectable and the target peak disappears after a certain concentration level. Therefore, it is not possible to determine the LOD and LOQ values by evaluation of the signal-to-noise ratio as can be done with MS/MS detection. Instead, the LOQ level was defined as 2.5 x LOD as the lowest calibrant, which was possible to integrate with a standard deviation below 20%. The values for mycotoxins detected in matrix had to fulfill requirements to test for compliance with regulatory limits. The individual LOD and LOQ values of mycotoxins are listed in Table 11.

Table 11. LOD, LOQ and maximum limits for all matrices

Mycotoxin	Methanol		Maize		
	LOD (µg/kg)	LOQ (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)	Regulated Limit (µg/kg)
DON	30	75	50	125	500
T-2	2	4	3	8	50
HT-2	2	6	4	10	50
FB-1	12	30	18	45	200
FB-2	14	35	20	50	200
ZON	1	3	2	5	50

Mycotoxin	Wheat			Animal Feed		
	LOD (µg/kg)	LOQ (µg/kg)	Regulated Limit (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)	Regulated Limit (µg/kg)
DON	48	120	500	150	375	900
T-2	3	8	50	4	10	50
HT-2	4	10	50	5	12	50
FB-1	16	40	200	32	80	1250
FB-2	20	50	200	28	70	1250
ZON	2	5	50	10	25	100

16. Conclusion

A generic method for the determination of deoxynivalenol, zearalenone, T-2 toxin, HT-2 toxin and fumonisins B_1 and B_2 from complex matrices of maize, wheat and animal feed was developed. Rather than using different extraction solvents for efficient extraction of target compounds with different chemical properties, a mixture of water containing 0.1% FA/ACN (43:57) enabled satisfactory recovery (71.6%–120.1%) of all target compounds. By using automated cleanup, more than 200 samples were analyzed without any maintenance of the Transcend TLX- HRMS system. Sample cleanup with the TurboFlow column followed by HPLC analysis took 18 minutes. In comparison to disposable cleanup cartridges, the TurboFlow column was used for a minimum of 500 injections of extracted samples. Relative standard deviations and intermediate precision below 19% demonstrated good repeatability. Certified reference materials, which have been analyzed as representative samples of maize, wheat and animal feed for target compounds, and successful proficiency testing demonstrated method accuracy. The results confirm that this method can be used for routine analysis with respect to legislative limits of regulated *Fusarium* mycotoxins.

17. References

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Application Notebook

Marine Toxins

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Determination of Lipophilic Marine Biotoxins in Molluscs by LC-MS/MS using Offline Extraction

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Application Note 63552

Key Words
TSQ Quantum Ultra, Food Safety, Marine Biotoxins

Introduction

In recent years many countries have had to deal with the consequences of toxic microalgal blooms in both marine and fresh water, such as the deaths of wild animals and domestic livestock.

Several cases of poisoning in humans have been associated with the direct consumption of shellfish, fish, or water contaminated by algal toxins. People may also come into contact with toxins during recreational activities along sea coasts affected by episodes of algal blooms. Depending on the type of toxin involved, there are forms of mild and usually self-limiting symptoms, characterized by gastrointestinal disorders or allergic-like episodes. Much more severe symptoms of the neurological type can lead to death.

The foods most frequently involved in episodes of human poisoning are represented by bivalve molluscs. These shellfish can accumulate and concentrate any biotoxins present in the plankton they ingest through filtering large quantities of water for trophical reasons. It is not possible to evaluate shellfish edibility by an organoleptic examination alone. While human ingestion of contaminated food with biotoxins can lead to the onset of different clinical symptoms, in shellfish it usually has only marginal effects. An important risk factor lies in the thermostability of such molecules which are not completely inactivated by common physical treatments for fish products (cooking, smoking, salting, freezing, housing), but remain virtually unchanged in the finished product.

There are a series of regulations issued by the European Union (EU) that relate to marine biotoxins. One is Regulation (EC) No 853/2004 which concerns the control of lipophilic toxins, establishing maximum levels for lipophilic toxins in bivalve molluscs destined for human consumption:[1]

- For okadaic acid, dinophysistoxins, and pectenotoxins together – 160 micrograms of okadaic acid equivalents per kilogram
- For yessotoxin – 1 milligram of yessotoxin equivalent per kilogram
- For azaspiracids – 160 micrograms of azaspiracid equivalents per kilogram

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In the past, aside from bioassays on mice, most analytical techniques developed for the determination of marine biotoxins in bivalve molluscs have been based on offline methodologies. These include methods involving solid phase extraction (SPE) or liquid-liquid extraction (LLE) followed by high pressure liquid chromatography (HPLC) with fluorimetric or UV-diode array detection, as well as detection by liquid chromatography coupled with mass spectrometry (LC-MS).

The EU Commission Regulation (EC) No 15/2011, amending Regulation (EC) No 2074/2005 about the testing methods for detecting marine biotoxins in bivalve molluscs, describes an LC-MS/MS procedure as the reference method for the quantification of lipophilic marine biotoxins – namely okadaic acid, pectenotoxin 2, azaspiracid 1, and yessotoxin.[2,3] Moreover, dinophysistoxin 1 (DTX-1) and dinophysistoxin 2 (DTX-2) can be quantified by the calibration curve of okadaic acid, pectenotoxin 1 by calibration of pectenotoxin 2, azaspiracid 2 and 3 by calibration of azaspiracid 1 and 45-OH-, and 45-homo-OH-yessotoxin by the calibration of yessotoxin.

In accordance with current European regulations, we propose a quick, selective, sensitive, and accurate analytical method for the determination of lipophilic marine biotoxins in bivalve molluscs using an LC-MS/MS method.

Goal

Our goal is to validate analytical procedures proposed in "*EU-Harmonised Standard Operating Procedure for determination of Lipophilic marine biotoxins in molluscs by LC-MS/MS – Version 3*" by LC-MS/MS using offline extraction.[4]

Experimental

Sample Preparation

About 1 kg of bivalve molluscs (*Mytilus Galloprovincialis*) were cleaned with water and put in a solution of NaCl (3.5 g/L). After opening, the molluscs were washed with fresh water, their flesh was removed and placed on a stainless steel net, and they were washed again with deionized water. The whole collected raw tissue, not less than 150 g, was chopped and blended by a mixer.

Extraction procedure

9 mL of 100% methanol (gradient quality) were added to 2.00 ± 0.05 g of blended tissue, put into a centrifuge tube, and mixed by vortex for 3 minutes at maximum speed. After centrifugation at 4000 rpm for 10 minutes, the supernatant solution was transferred into a vial.

A second aliquot of 9 mL of 100% methanol was further added to the residual tissue pellet and homogenized for 1 minute by Ultra-turrax® (*IKA®, USA*) at 12,000 rpm and the mixture was centrifuged at 4000 rpm for 10 minutes. Then the supernatant solution was transferred and combined with the first extract and made up to 20 mL with 100% methanol. When not immediately analyzed, the solution was stored at -20 °C.

Spikes of toxin standard solutions can be added to the blended tissue before the extraction procedure.

Purification Procedure

The organic extract was purified by being passed through a C18 SPE cartridge preliminarily conditioned with 1 mL of 100% methanol. A 0.45 µm syringe filter was placed at the end of the cartridge to improve purification.

LC Conditions for the Thermo Scientific Hypersil GOLD Column

System	Thermo Scientific Accela UHPLC
Solvent A	100% water with 2 mM ammonium formate and 50 mM formic acid
Solvent B	95% acetonitrile + 5% water with 2 mM ammonium formate and 50 mM formic acid
Flow Rate	200 µL/min
Gradient	The mixture started at 30% solvent B (8.0 min) followed by a linear gradient up to 90% solvent B in 3.0 min. It went up to 30% of solvent B in 0.5 min. This composition was maintained for 5.5 min.
Analytical Column	Hypersil GOLD™; 50 × 2.1 mm, particle size 1.9 µm, part number 25002-052130

H-ESI II Source Conditions

Ion Source Polarity	Positive Ion Mode	Negative Ion Mode
Spray Voltage	3000 V	2700 V
Capillary Temperature	270 °C	270 °C
Vaporizer Temperature	240 °C	240 °C
Sheath Gas Pressure (N_2)	15 units	15 units
Auxiliary Gas Pressure (N_2)	5 units	5 units

MS/MS Setup

MS analysis was carried out on a Thermo Scientific TSQ Quantum Ultra triple quadrupole mass spectrometer equipped with a heated electrospray ionization probe (H-ESI II).

Collision Gas (Ar)	1.5 mTorr
Q1/Q3 Peak Resolution	0.7 u (unit mass resolution)
Scan Time	0.100 s
Scan Width	0.500 *m/z*
Data Acquisition Mode	SRM

Analyte	ESI Mode	Parent Mass	Product Mass	Collision Energy	Tube Lens
AZA-1	ESI+	842.3	806.1	51	207
			824.2	42	207
AZA-2	ESI+	856.3	838.1	42	214
			820.2	49	214
AZA-3	ESI+	828.3	792.2	48	192
			810.0	40	192
PTX-2	ESI+	876.3	841.3	35	205
			823.0	40	205
			805.3	41	205
DTX-1	ESI-	817.0	255.0	69	197
			113.1	67	197
DTX-2	ESI-	803.15	255.3	61	207
			113.1	50	207
YTX	ESI-	1141.5	1061.7	50	240
		570.2	467.3	40	240
OA	ESI-	803.3	254.9	68	216
			113.1	50	216

Table 1: Selected ion transitions (*m/z*) of the studied compounds and optimized collision energy and tube lens value for the TSQ Quantum Ultra triple quadrupole mass spectrometer

The optimization of selective reaction monitoring (SRM) parameters was performed by direct infusion of standards. Collision-induced dissociation (CID) data were recorded for each analyte including optimum collision energies for the selected ion transitions.

Table 1 summarizes all the mass transitions found for each analyte and its relative collision energy (CE) and tube lens values.

Results and Discussion

To ensure thorough validation of the method, neat standard solutions, standard addition on purified extracts, and spiked blank tissue extracts were prepared and compared.

Table 2 lists the correlation coefficients (r^2) indicating the linearity of the calibration curves for the three types of samples analyzed; five concentrations of the sample solution are considered (2, 5, 10, 20, and 50 µg/kg or similar).

To assess the inter-day repeatability of the method, ten replicates of spiked samples were analyzed between days. Solutions were prepared containing all the toxins in the five different concentrations used to perform the calibration curves (2, 5, 10, 20, and 50 µg/kg or similar).

The repeatability of the method expressed as the coefficient of variation percentage (CV %) has been rated less than 20% as shown in Table 3.

Analyte	Neat Solution	Spiked Purified Extract	Spiked Extract
AZA-1	0.9932	0.9965	0.9970
AZA-2	0.9973	0.9964	0.9901
AZA-3	0.9972	0.9958	0.9993
DTX-1	0.9964	0.9995	0.9953
DTX-2	0.9973	0.9966	0.9965
YTX	0.9999	0.9923	0.9988
PTX-2	1.0000	0.9977	0.9927
OA	0.9955	0.9924	0.9927

Table 2: Correlation Coefficient (r^2) of the calibration curves for the three types of samples analyzed in the concentration range of 2–50 µg/kg)

Analyte	Standard Deviation	Repeatability	CV%
AZA-1	0.25	0.79	20
AZA-2	0.29	0.90	18
AZA-3	0.43	1.37	17
DTX-1	0.18	0.55	2
DTX-2	0.22	0.68	9
YTX	0.12	0.40	12
PTX-2	0.39	1.36	20
OA	0.16	0.48	4

Table 3: Values of CV% obtained for the repeatability of the lower concentrated curve point (2 µg/kg)

The calculations, of limit of detection (LOD) and limit of quantification (LOQ) were made in accordance with the *UNICHIM Manual N. 179/0* where the calculation of the limit of detection is made through the calibration curve of the instrument used for analysis.[5]

To estimate the LOD and LOQ of the method (Table 4), ten samples were prepared by adding standard solution to 500 mg of homogenized mussel flesh and repeating the extraction procedure according to the method in "*EU-Harmonised Standard Operating Procedure for determination of Lipophilic marine biotoxins in molluscs by LC-MS/MS – Version 3*". LOD and LOQ are expressed in µg/Kg. Recoveries are shown in Table 5.

	Hypersil GOLD		
Analyte	**LOD (µg/Kg)**	**LOQ (µg/Kg)**	**Outliers (Huber Test)**
AZA-1	0.56 ± 0.18	1.11	NO
AZA-2	0.93 ± 0.31	1.86	NO
AZA-3	1.28 ± 0.43	2.57	NO
DTX-1	5.66 ± 1.02	10.45	NO
DTX-2	0.71 ± 0.23	1.42	NO
YTX	1.67 ± 0.56	3.33	NO
PTX-2	1.40 ± 0.46	2.79	NO
OA	3.95 ± 1.32	7.91	NO

Table 4: LOD and LOQ of the method

Analyte	**Spiked Purified Extract**	**Spiked Extract**
AZA-1	96 ± 11	97 ± 11
AZA-2	101 ± 9	94 ± 14
AZA-3	104 ± 10	99 ± 6
DTX-1	101 ± 6	101 ± 7
DTX-2	101 ± 6	108 ± 42
YTX	99 ± 15	102 ± 17
PTX-2	103 ± 13	102 ± 20
OA	95 ± 7	93 ± 18

Table 5: Recovery values, where
$R\% = \{[(\mu g/Kg)_{CALCULATED}/(\mu g/Kg)_{THEORETICAL}]*100\}$

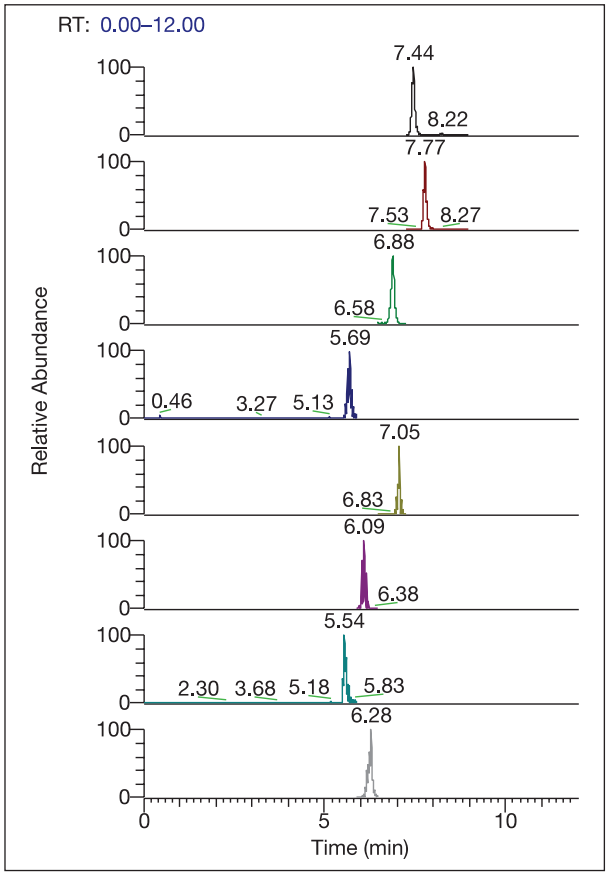

Figure 1: Chromatogram of sample containing 40 ppb of toxins (Retention Time: 7.44 min – AZA-1; 7.77 min – AZA-2; 6.88 min – AZA-3; 5.69 min – OA; 7.05 min – DTX-1; 6.06 min – DTX-2; 5.54 min – YTX; 6.28 min – PTX-2

Conclusion

This method proved to be selective, sensitive, accurate, and reproducible. It can be successfully applied for the quantitative determination of several classes of lipophilic marine biotoxins in bivalve mollusc samples.

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Identification and Quantitation of Microcystins by Targeted Full-Scan LC-MS/MS

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Application Note 569

Key Words
Velos Pro, UltiMate, Water Analysis, Cyanobacteria, Microcystin

Goal

Develop a simple and sensitive LC-MS method for definitive identification and quantitation of microcystins in water.

Introduction

Cyanobacteria, commonly referred to as blue-green algae, are photosynthetic prokaryotes that occur naturally in surface waters. They contribute significantly to primary production and nutrient cycling. Eutrophic, warm and low turbulent conditions in freshwater bodies typically promote the dominance of cyanobacteria within phytoplankton communities. Excessive proliferation of cyanobacteria leads to blooms that disrupt ecosystems, adversely affect the taste and odor of water, and increase water treatment costs. Blooms of toxic cyanobacteria species in surface drinking water sources and recreational waters threaten human health. Gastrointestinal illness, skin irritation, and death following renal dialysis have been attributed to acute cyanotoxin exposure. Chronic exposure can cause liver damage and may be associated with primary liver cancer.[1] The incidence and severity of cyanobacterial blooms are increasing globally, underscoring the importance of cyanotoxin monitoring.

The most commonly encountered cyanotoxins are the microcystins, a group of hepatotoxic cyclic heptapeptides produced by various genera of cyanobacteria, including Microcystis, Planktothrix, and Anabaena. The chemical structure of a microcystin, depicted in Figure 1, is characterized by the presence of the amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid (Adda), which modulates the biological activity of these toxins, and N-methyldehydroalanine (Mdha). Microcystin nomenclature is based on the L-amino acids present at two positions (X and Y in Figure 1) in the molecule. Over 80 structural variants are known, differentiated by the two variable L-amino acids as well as by chain modifications. The inhibition of serine/threonine protein phosphatases type 1 and 2A is considered the major mechanism of microcystin toxicity. Microcystin-LR, one of the most prevalent and potent microcystins, is designated as possibly carcinogenic to humans by the International Agency for Research on Cancer (IARC).[2] The potential risk of chronic exposure to microcystins in drinking water supplies prompted the World Health Organization (WHO) to issue a provisional guideline of 1 µg/L as the maximum concentration of total microcystin-LR (free plus cell-bound) in drinking water.[3] Many national and regional governments have since adopted this guideline value directly or have established slightly modified variants.

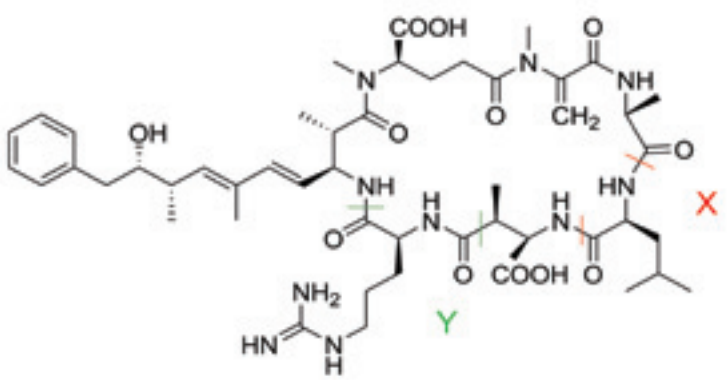

Figure 1. The chemical structure of MC-LR contains leucine (L) and arginine (R) at positions X and Y, respectively. Microcystin nomenclature is based on the L-amino acids present at these two positions.

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A toxic cyanobacterial bloom usually consists of multiple microcystin congeners in varying concentrations. Several techniques for the analysis of microcystins have been developed. Mouse bioassays, protein phosphatase inhibition assays, and enzyme-linked immunosorbent assays (ELISA) are effective for rapid screening but lack specificity. Reversed-phase high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection is the most common approach used for the separation, detection and quantitation of microcystins. An ISO method for microcystin analysis by HPLC-UV has been validated for MC-RR, MC-YR and MC-LR.[4] However, UV detection is susceptible to interferences from water matrices and requires sample cleanup and concentration to achieve desirable detection limits. Furthermore, UV-based methods do not provide unequivocal identification of known microcystins nor enable identification of unexpected variants. Liquid chromatography in combination with multi-stage mass spectrometry (LC-MS^n) enables structural characterization and unambiguous identification of trace levels of microcystins. LC-MS/MS in multiple reaction monitoring (MRM) acquisition mode allows highly selective and sensitive quantitation and confirmation of target microcystins, but this approach requires extensive compound-dependent parameter optimization and cannot be used to detect unexpected toxins. Full-scan MS/MS approaches obviate the need for compound optimization and enable determination of all microcystins present in a sample.

The Thermo Scientific Velos Pro dual-pressure linear ion trap mass spectrometer delivers sensitivity and speed for qualitative and quantitative applications. High-quality full-scan MS^n spectra enable confident structural elucidation and identification. Rapid scanning and fast cycle times generate more scans across chromatographic peaks for robust quantitation and allow the acquisition of more MS^n spectra in shorter chromatographic runs. A wide dynamic range of up to six orders of magnitude facilitates identification and quantitation of low-abundance compounds in complex matrices. Complementary fragmentation techniques may be performed in parallel to enable more MS^n information to be obtained from a single sample. In this application note, we describe a simple and sensitive targeted full-scan LC-MS/MS method for the identification and quantitation of the microcystins MC-RR, MC-YR, and MC-LR using the Velos Pro™ ion trap mass spectrometer coupled to a Thermo Scientific Dionex UltiMate 3000 x2 Dual RSLC system.

Experimental

Sample Preparation

MC-RR, MC-YR and MC-LR standards were purchased from Sigma-Aldrich®. A stock solution of a mixture of these three microcystins was prepared at a concentration of 5 µg/mL. Calibration solutions, with concentrations of 0.025 µg/L to 50 µg/L, were prepared by serial dilution of the stock solution.

LC-MS/MS Analysis

A 50 µL sample was injected on a Thermo Scientific Acclaim 120 guard cartridge with 150 L/min, washed for two minutes to waste and then eluted onto a Thermo Scientific PepMap100 analytical column for separation. LC-MS/MS analysis was performed on an UltiMate™ 3000 x2 Dual RSLC system coupled to an Velos Pro mass spectrometer.

LC Parameters	
Guard cartridge:	Acclaim™ 120 C18 (10 x 3.0 mm i.d., 5.0 µm particle size, 120 Å pore size)
Analytical column:	Acclaim PepMap100 C18 (150 x 1.0 mm i.d., 3.0 µm particle size, 100 Å pore size)
Mobile Phase A:	Water containing 0.1% formic acid
Mobile Phase B:	Acetonitrile containing 0.1% formic acid
Column temperature:	40 °C
Sample injection volume:	50 µL
Flow rate:	150 µL/min
Gradient:	Table 1

Table 1: LC Gradient

Time	% A	% B
0.1	98	2
1.5	98	2
2.0	80	20
3.0	60	40
7.4	40	60
7.5	2	98
7.9	2	98

MS Parameters	
Ionization mode:	Positive electrospray ionization (ESI)
Collision energy:	35%
Isolation window:	2
Targeted full-scan MS/MS:	MC-RR $[M+2H]^{2+}$ at m/z 520 [m/z 150-1100] MC-YR $[M+H]^{+}$ at m/z 1045 [m/z 285-1100] MC-LR $[M+H]^{+}$ at m/z 995 [m/z 285-1100]

Structural Identification and Confirmation

Figure 2 shows extracted ion chromatograms and MS/MS spectra obtained from full-scan LC-MS/MS analysis of a mixture containing MC-RR, MC-YR and MC-LR at concentrations of 0.5 µg/L. MC-RR, MC-YR and MC-LR eluted at 5.62, 6.85, and 6.93 minutes, respectively. The MS/MS spectrum of MC-RR was generated by collision-induced dissociation (CID) of the $[M+2H]^{2+}$ ion and is characterized by major fragment ions at *m/z* 505, 452 and 887, which correspond to $[M+2H\text{-}CO]^{2+}$, $[M+2H\text{-}C_9H_{10}O]^{2+}$ and $[M+H\text{-}C_9H_{10}O\text{-}NH_3]^{+}$, respectively ($C_9H_{10}O$ is a fragment of the Adda residue). The closely eluting compounds MC-YR and MC-LR are easily distinguished by their MS/MS spectra. The MS/MS spectrum of MC-YR, generated by CID of the $[M+H]^{+}$ ion, contains major fragment ions at *m/z* 1017, 599, and 916, which correspond to $[M+H\text{-}CO]^{+}$, [Arg+Adda+Glu + H]$^{+}$, and [Arg+Adda+Glu+Mdha+Ala+Tyr + H]$^{+}$, respectively. The CID MS/MS spectrum of the $[M+H]^{+}$ ion of MC-LR is characterized by major fragment ions at *m/z* 967, corresponding to $[M+H\text{-}CO]^{+}$; *m/z* 599, corresponding to [Arg+Adda+(Glu or MeAsp) + H]$^{+}$; *m/z* 866, corresponding to [Ala+Adda+Arg+(Glu or MeAsp) +Leu+Mdha + H]$^{+}$; and *m/z* 553, corresponding to [Ala+Arg+(Glu or MeAsp) +Leu+Mdha+ H]$^{+}$.

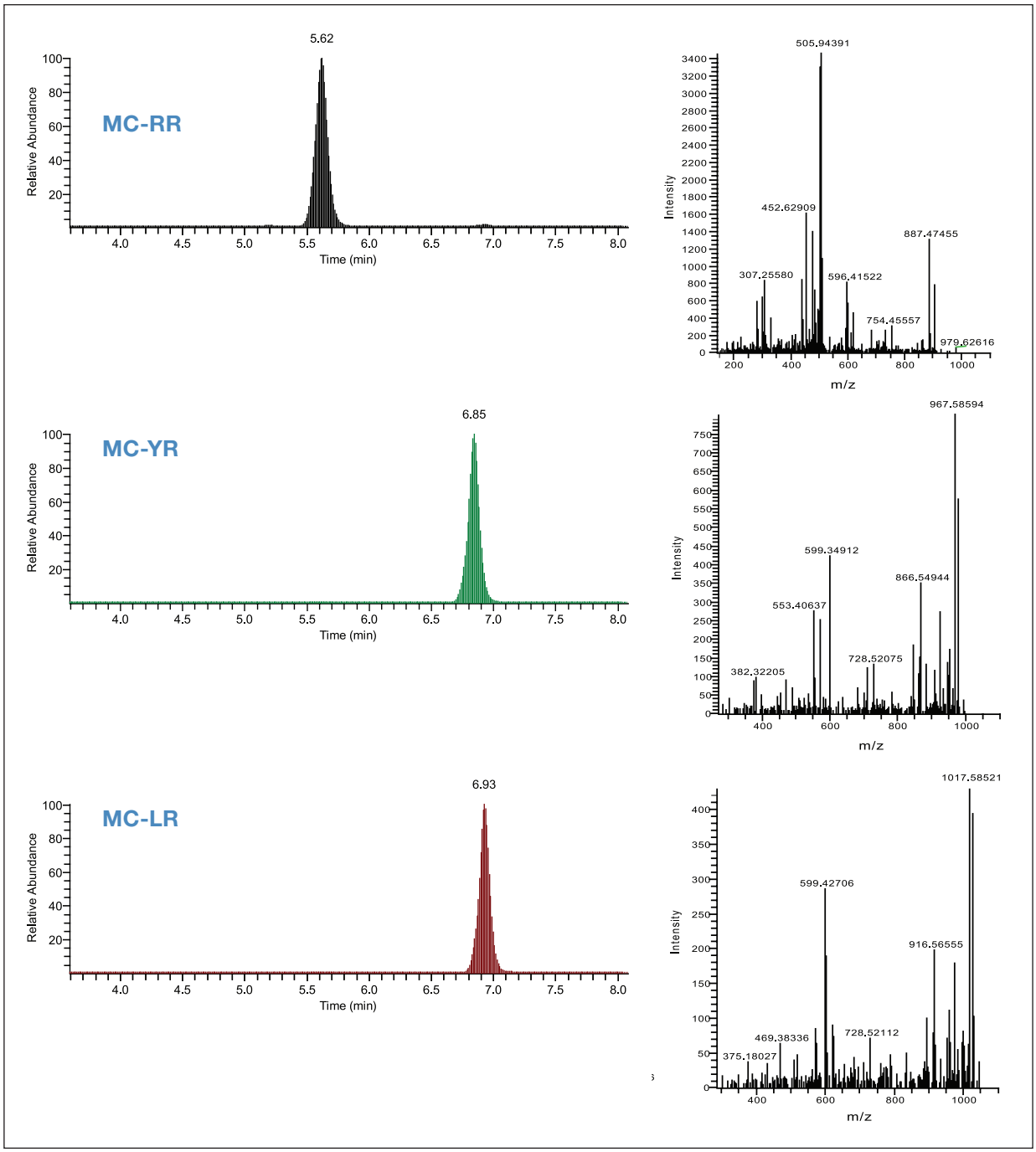

Figure 2. Extracted ion chromatograms and MS/MS spectra for MC-RR, MC-YR and MC-LR at concentrations of 0.5 µg/L

Quantitative Analysis

The high scan speeds and fast analytical cycle time of the Velos Pro mass spectrometer enabled higher numbers of analytical scans across chromatographic peaks for optimal quantitative reliability (Figure 3). Excellent linearity in detector response was observed over the range of 0.05-50 µg/L for all three microcystins. Calibration curves for MC-RR, MC-YR and MC-LR are shown in Figure 4, with coefficients of determination of 0.9986, 0.9994, and 0.9994, respectively. The lowest detectable amount (LOD) of 0.025 µg/L and quantifiable amount (LOQ) of 0.05 µg/L were achieved for each microcystin. Both QC samples, at levels of 0.5 and 5 µg/L, achieved quantitation accuracy better than 94% for all three microcystins. Signal-to-noise ratios of >25 with automatic ICIS algorithm integration in Thermo Scientific Xcalibur software were obtained for MC-LR at the LOQ (Figure 5), demonstrating that this LC-MS/MS method can be used to determine MC-LR at concentrations well below the WHO's recommended guideline level of 1 µg/L.

Method reproducibility was investigated by analyzing five replicate injections of each analyte. Peak area RSDs for MC-LR and MC-YR were less than 7% and 11%, respectively, over the entire linear dynamic range (Table 2). For MC-RR, peak area RSDs over the range 0.10-50 µg/L were under 6%; at the LOQ, the peak area RSD was 16% (Table 2). Retention time precisions were 0.3% RSD or less over the entire dynamic range (Figure 6) for all three microcystins. Tap water, filtered water and surface pond water were analyzed using this method. No microcystins were in any of the three water sources.

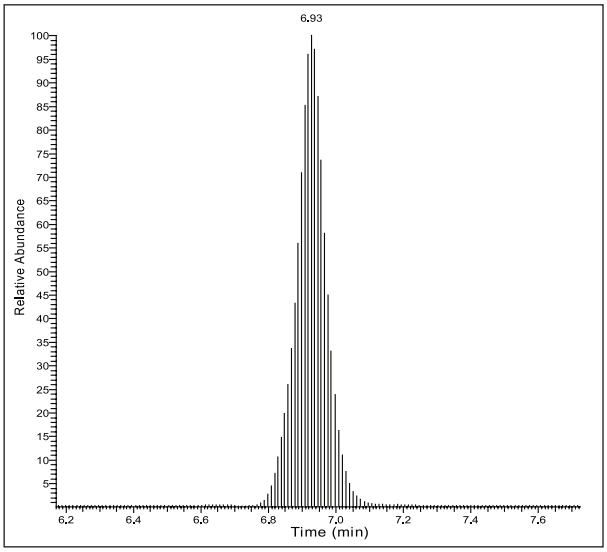

Figure 3. High scan speeds and fast cycle times enable more than 20 data points to be acquired across the MC-LR chromatographic peak.

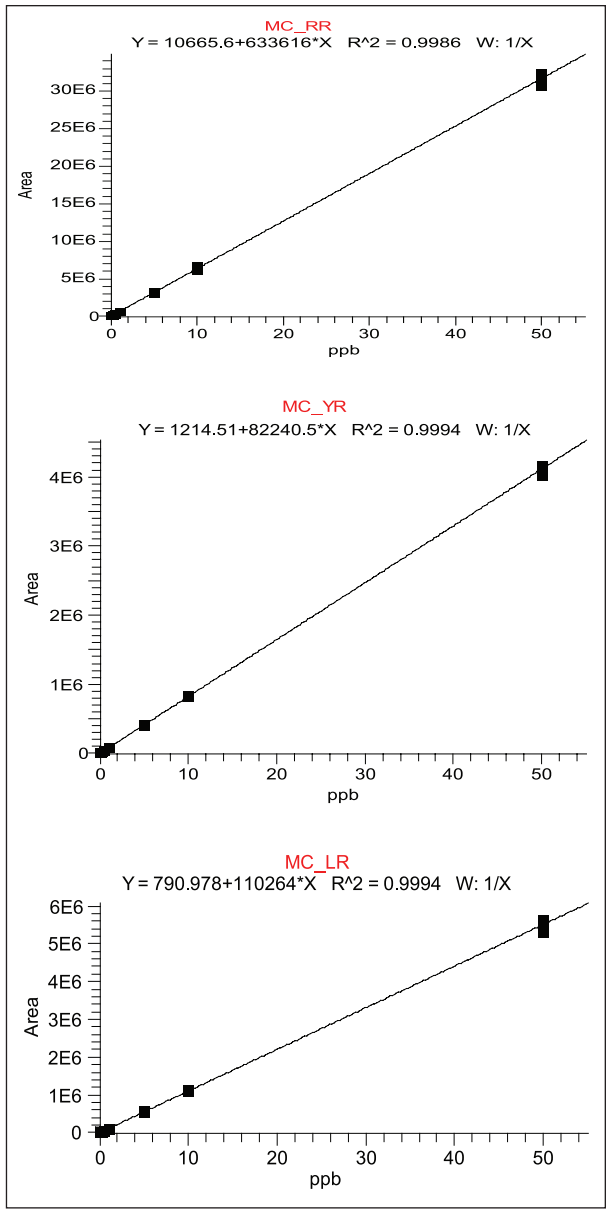

Figure 4. Calibration curves for quantitation of MC-RR, MC-YR and MC-LR

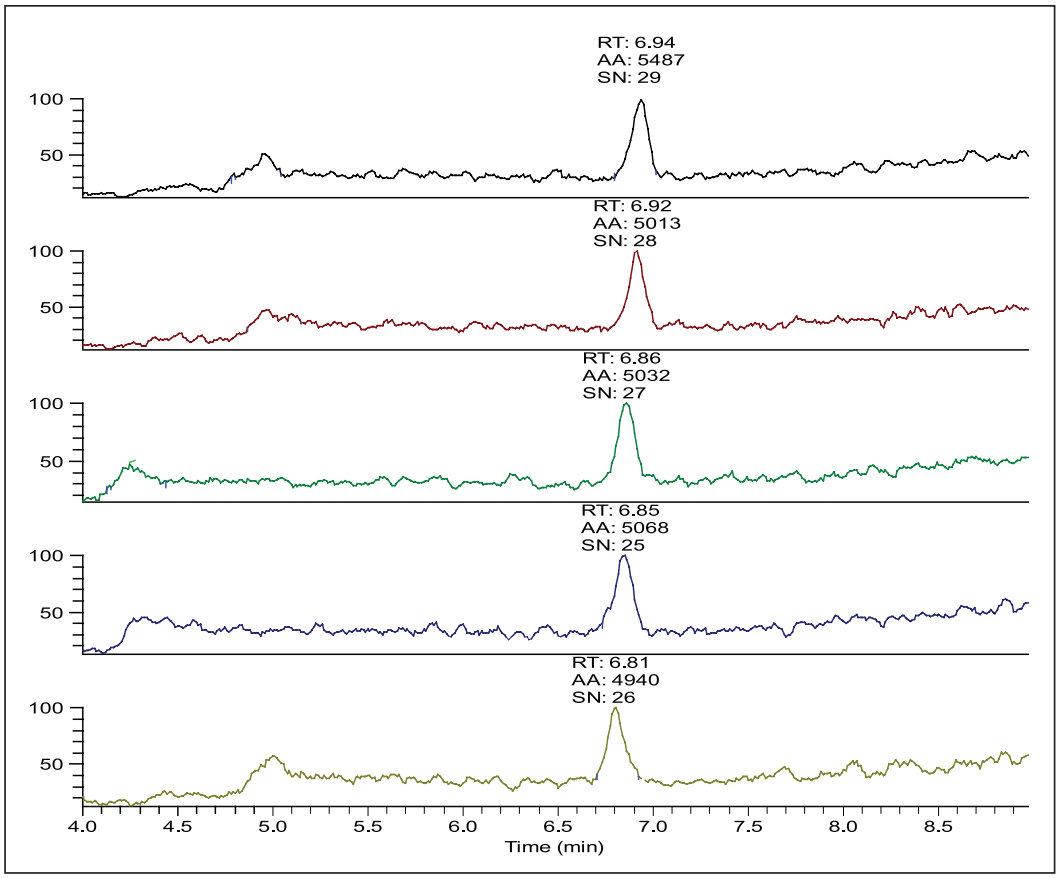

Figure 5. For MC-LR at the LOQ (0.05 µg/L), S/N › 25 and peak area RSD = 6.91%

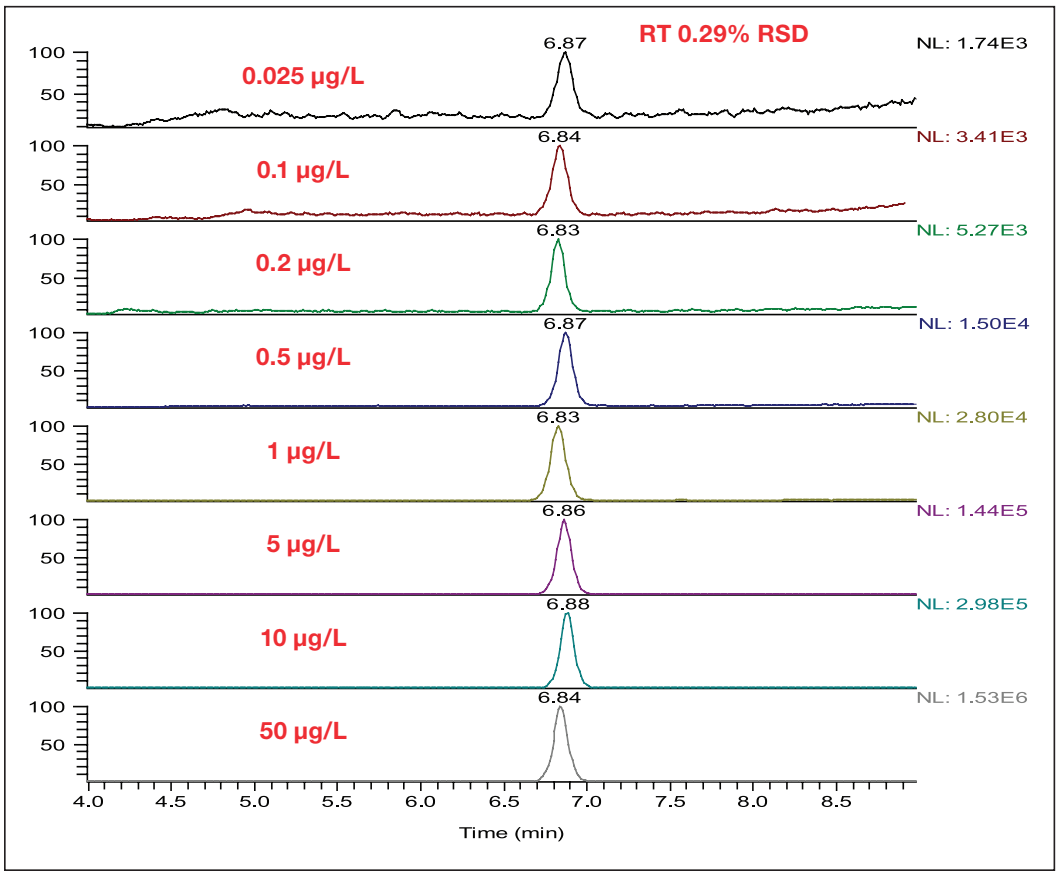

Figure 6. High retention-time precision (‹ 0.3% RSD) over a wide linear dynamic range

Table 2. Peak area precision (from five replicate injections) for LC-MS/MS assay of MC-RR, MC-YR and MC-LR

Levels µg/L	MC-RR	MC-YR	MC-LR
0.05	16.01	10.5	6.91
0.10	2.82	5.88	3.97
0.20	3.54	5.25	4.89
0.50	4.86	8.54	3.03
1.00	5.84	1.76	4.25
5.00	2.28	2.13	2.47
10.00	4.54	1.30	1.31
50.00	2.40	1.76	2.66

Conclusion

A simple, sensitive and robust LC-MS method for quantitative determination of microcystins was developed. Targeted full-scan MS/MS analysis using the LTQ Velos Pro linear ion trap mass spectrometer provided excellent selectivity and sensitivity for the identification and quantitation of MC-RR, MC-YR and MC-LR across a wide linear dynamic range. The LOD and LOQ were 0.025 µg/L and 0.05 µg/L, respectively. The LOQ was significantly lower than the provisional guideline value established by the WHO for MC-LR concentrations in drinking water. Assays performed in full-scan MS/MS mode enable compound confirmation and quantitation without the need for compound-dependent parameter optimization. The method was used to analyze tap, filtered and surface pond water. No microcystins were detected from these three water sources.

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Application Note: 52154

Non-Targeted Screening of Lipophilic Marine Biotoxins by Liquid Chromatography – High-Resolution Mass Spectrometry

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Key Words

- Exactive
- Hypersil GOLD
- Liquid Chromatography
- Marine Biotoxin
- Non-Targeted Screening
- Orbitrap Technology

Introduction

Marine biotoxins are produced by naturally occurring microalgae, whose populations can increase significantly under certain environmental conditions to form a harmful algal bloom (HAB). During the incidence of a bloom, marine biotoxins pose a significant food safety risk when bioaccumulated in shellfish that are ingested by humans. Therefore, adequate testing for biotoxins in shellfish is required to ensure public safety and long-term viability of commercial shellfish markets.

The lipophilic marine toxins class includes the dinophysistoxins, azaspiracids, pectenotoxins, and yessotoxins. The compounds are structurally diverse, as shown in Figure 1, and thus do not contain a common UV chromophore or reactive functional group for fluorescence derivatization. Therefore, LC-MS is the method of choice for their analyses and several MRM-based methods have been reported.[1-3]

In response to the need for non-targeted methods that can potentially detect unknowns, high-resolution LC-MS has been successfully implemented for screening and quantification in food safety applications.[4-6] The lower-cost, higher-mass accuracy, and ease-of-use of modern quadrupole time-of-flight (QTOF) and Thermo Scientific Orbitrap based mass spectrometers have made high-resolution systems viable alternatives to triple-quadrupole systems for routine analysis. After full-spectrum data acquisition, specificity is typically achieved by extracting narrow mass windows (ie. 2–5 ppm) centered around a list of target analytes. Using this approach, it has been demonstrated that a resolving power of 50,000 or greater is required for correct mass assignments in complex matrices.[6] This report describes the use of the Thermo Scientific Exactive benchtop LC/MS system powered by Orbitrap™ technology for screening lipophilic marine biotoxins commonly found in shellfish.[7] The method was optimized using a standard mixture of marine biotoxins, and then applied to a mussel tissue extract.

Experimental

Chemicals and Materials

Certified calibration solutions and mussel tissue reference materials were purchased from the NRC Certified Reference Materials Program (Halifax, Nova Scotia, Canada). Certified calibration solutions were used for the following biotoxins: okadaic acid (OA), dinophysistoxin-1 (DTX1), dinophysistoxin-2 (DTX2), pectenotoxin-2 (PTX2), azaspiracid-1 (AZA1), azaspiracid-2 (AZA2), azaspiracid-3 (AZA3), and yessotoxin (YTX). As a test sample, a mussel tissue containing certified levels of OA and DTX1 was used (CRM-DSP-Mus-b).

HPLC grade acetonitrile and formic acid (98%) were purchased from EMD chemicals (Gibbstown, NJ, USA). Distilled-in-glass grade methanol was acquired from Caledon Laboratories (Georgetown, ON, Canada), and ammonium formate (≥ 99.0%) was from Fluka (St. Louis, MO, USA).

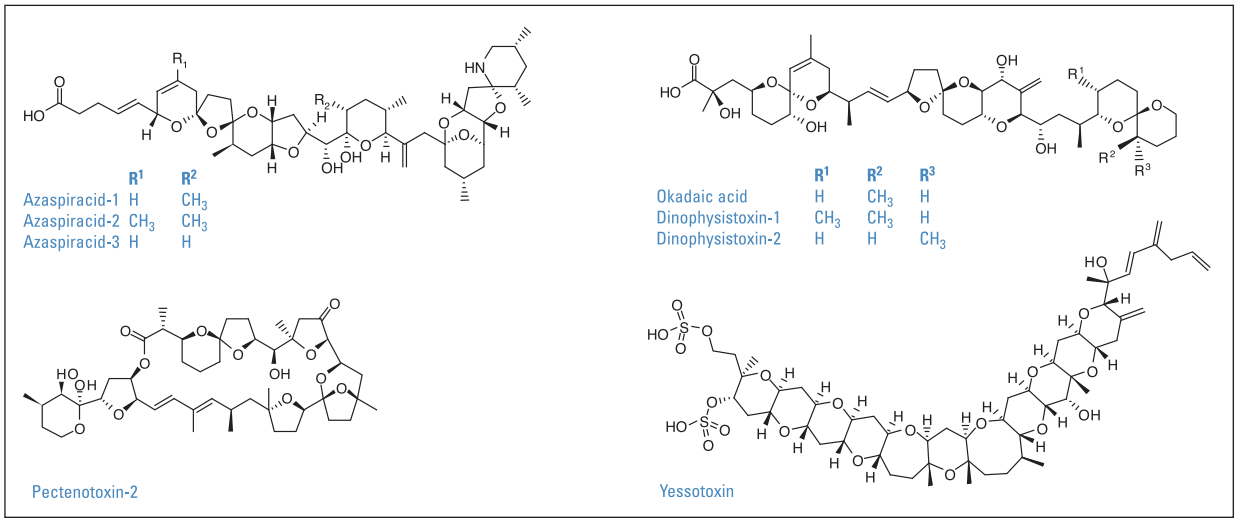

Figure 1: Chemical structures of the primary analogs of the regulated lipophilic marine biotoxins

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Extraction of Lipophilic Toxins From Mussel Tissue

Approximately 4 g of tissue was homogenized with 4 mL of 80% methanol solution using a Polytron PT3000 mixer (Brinkmann, USA) at 10,000 rpm with ice cooling. The sample was then centrifuged at 7,000 rpm for 15 minutes and the supernatant was decanted into a flask. Another 8 mL of 80% was used to clean the mixer by running the homogenizer briefly. The rinsate was centrifuged as before and this supernatant was combined with the first supernatant. 6 mL of 80% methanol was then added to the original pellet, which was homogenized again. After centrifugation, the final supernatant was combined with the previous two. The final volume was made up to 25 mL with 80% methanol solution. Approximately 0.5 mL of this solution was filtered through a 0.45 µm spin-filter (Millipore, Billerica, MA, USA) prior to analysis.

LC-MS Instrumentation and Method

LC-MS analysis was carried out on a Thermo Scientific Accela High-Speed LC coupled to an Exactive™ mass spectrometer, equipped with an Orbitrap mass analyzer and a HESI-II probe for electrospray ionization. The instrument was mass-calibrated daily for positive and negative modes, and the capillary and tube lens voltages were optimized daily, using the automated script within the Exactive acquisition software in both cases. For positive mode, mass calibration was performed with a mixture consisting of caffeine, MRFA tetrapeptide, and Ultramark 1621, while the negative mode calibration was performed with sodium dodecyl sulfate, sodium taurocholate, and Ultramark 1621. All analyses were performed using the 'balanced' automatic gain control (AGC) setting with a 50 ms maximum inject time. Data acquisition was carried out using Thermo Scientific Xcalibur 2.1. Optimal ion source and interface conditions consisted of a spray voltage of 3 kV, sheath gas flow of 50, capillary temperature of 360 °C, and a heater temperature of 250 °C. Alternating positive and negative polarity scans were acquired at a scan rate 2 Hz (50,000 resolution) for an overall cycle time of 1.25 seconds.

Lipophilic toxins were separated on a Thermo Scientific Hypersil GOLD C18 column (2.1 × 100 mm, 1.9 µm particle size), at a flow rate 400 µL/min and using 3 µL injections. Mobile phases were prepared from a stock solution of 1% formic acid solution in water with the pH adjusted to 3.0 using concentrated ammonium hydroxide. This stock solution was then diluted 10-fold with water (A) or acetonitrile (B), resulting in 0.1% formic acid in water for mobile phase A and 0.1% formic acid in 90% acetonitrile for B. Analytes were eluted with a linear gradient from 10 to 90% B from 0 to 2 min, held for 1 min, before returning to the initial conditions of 10% B.

Results

Lipophilic toxins were separated by reversed phase chromatography coupled to the Exactive mass spectrometer. As shown in Figure 2, eight lipophilic toxin standards were baseline separated in just under 6 min and the data shown represents 5 ppm extracted mass chromatograms centered around the masses of the target analytes. As OA, DTX1, DTX2, and YTX ionize significantly better in negative mode, alternative positive and negative polarity scans were acquired to achieve maximum signal for all analytes. To maintain a sufficient number of data points across chromatographic peaks, data was collected at a scan rate of 2 Hz. The scan rate of 2 Hz generates resolution of roughly 50,000, much lower than the maximum resolution possible with the mass spectrometer, but was selected as a reasonable compromise between selectivity and quantitative performance. In addition, it has been demonstrated that a

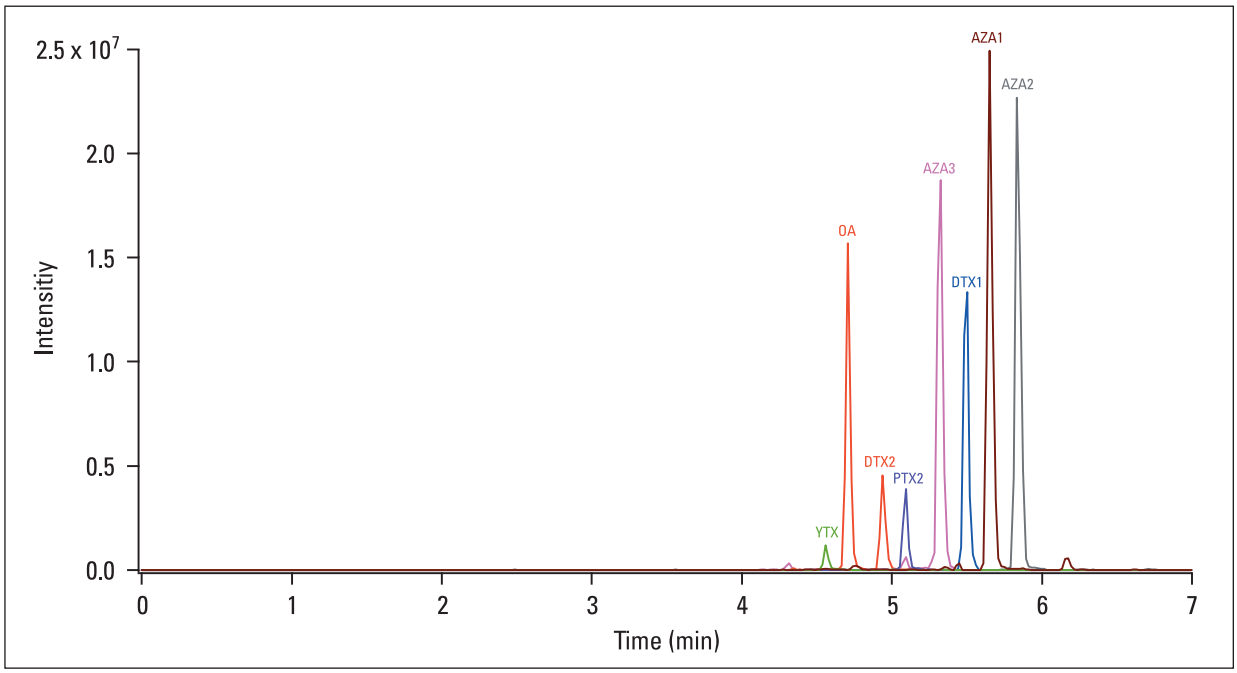

Figure 2: LC-MS chromatograms of eight lipophilic biotoxin standards acquired with alternating positive (PTX2, AZA1,-2,-3) and negative (YTX, OA, DTX1,-2) scans at 2 Hz. Data shown represents 5 ppm mass windows centered around the analyte mass.

resolving power of 50,000 provides sufficient specificity in complex matrices.[6] The ability to rapidly scan both positive and negative polarities allows data collection in a true non-targeted fashion and permits independent optimization of the LC method without consideration of the retention time of positive and negative analytes.

Listed in Table 1 are accurate masses and limits of detection for the lipophilic toxins using external calibration exclusively, without any mass correction on an internal standard or a background ion. In general, accurate masses are below 1 ppm error for analytes detected in positive mode, while those detected in negative mode range between 1–3 ppm error. Similarly, limits of detection ranged from 0.052–0.10 µg/L (ppb) for the positive ions, while those detected in negative mode were distinctly higher at 1.6–5.1 µg/L.

The utility of the screening method for lipophilic toxins was evaluated by analyzing a mussel tissue reference material containing certified levels of okadaic acid and DTX1, as shown in Figure 3. The top trace of Figure 3 represents the total ion chromatogram (TIC), revealing the complex matrix of the mussel tissue. Excellent specificity was demonstrated by the minimal background peaks detected in the 5 ppm mass windows associated with OA and DTX1 (lower trace), and OA and DTX1 are clearly discriminated from the complex matrix. Quantification against calibration with toxin standards in methanol yielded levels of OA and DTX1 of 4.1 µg/g and 0.58 µg/g, respectively, with precision of roughly 10% RSD for both analytes. These concentrations represent roughly half of the certified values for OA and DTX1, with ion suppression by the matrix being the likely cause for these discrepancies. Ion suppression effects are generally observed for all types of mass spectrometers employing electrospray ionization, and can be mitigated with the use of matrix-matched standards if accurate quantification is desired.[8]

Toxin	Tret (min)	Chemical Formula	Ion Detected	Calculated (*m/z*)	Observed (*m/z*)	Error (ppm)	LOD (µg/L)
YTX	4.63	$C_{55}H_{82}O_{21}S_2$	$[M-H]^-$	1141.47172	1141.47433	2.3	5.1
OA	4.81	$C_{44}H_{68}O_{13}$	$[M-H]^-$	803.45872	803.45963	1.1	2.8
DTX2	5.04	$C_{44}H_{68}O_{13}$	$[M-H]^-$	803.45872	803.46002	1.6	1.6
PTX2	5.19	$C_{47}H_{70}O_{14}$	$[M+NH4]^+$	876.51038	876.51067	0.33	0.10
AZA3	5.45	$C_{46}H_{69}NO_{12}$	$[M+H]^+$	828.48925	828.48973	0.58	0.062
DTX1	5.59	$C_{45}H_{70}O_{13}$	$[M-H]^-$	817.47427	817.47639	2.6	2.0
AZA1	5.78	$C_{47}H_{71}NO_{12}$	$[M+H]^+$	842.50490	842.50477	0.15	0.052
AZA2	5.96	$C_{48}H_{73}NO_{12}$	$[M+H]^+$	856.52055	856.52062	0.080	0.064

Table 1: Accurate masses and LODs for the lipophilic marine biotoxins

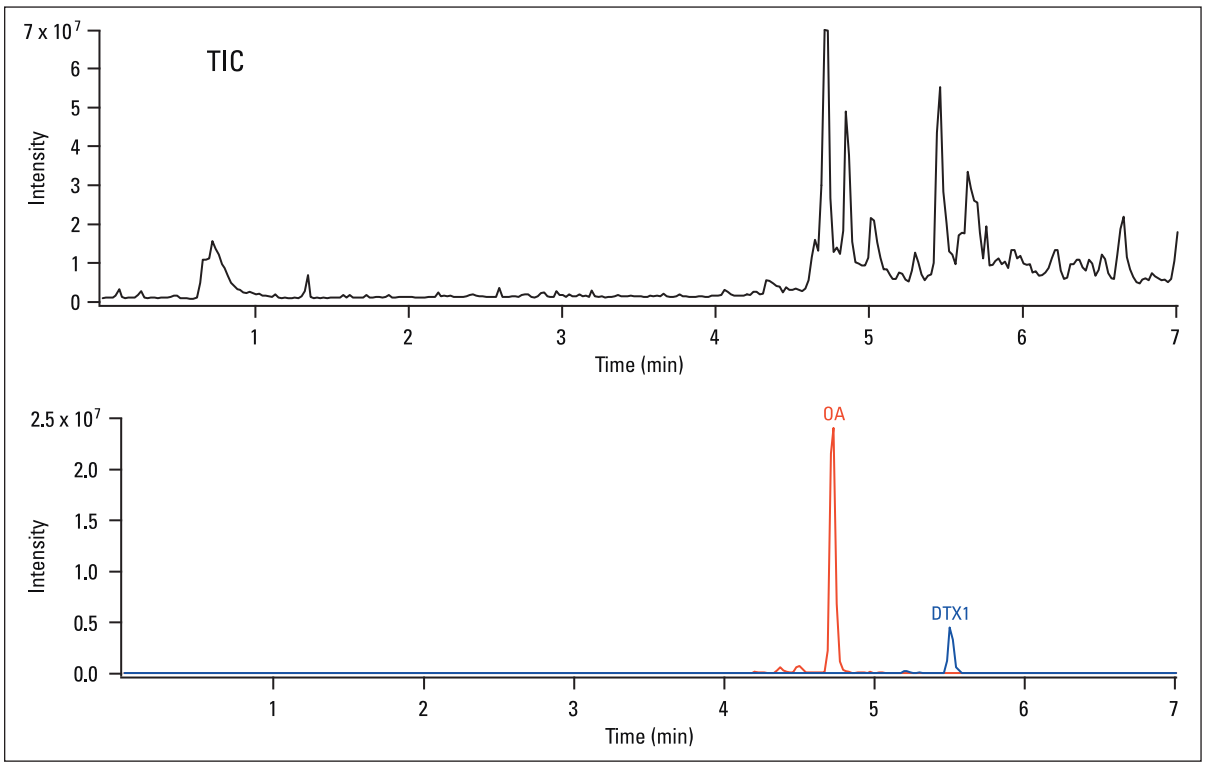

Figure 3: Exactive analysis of a mussel tissue extract showing the total ion chromatogram (TIC; top trace) and 5 ppm mass chromatograms for okadaic acid and DTX1 (lower trace)

Conclusions

The Exactive benchtop LC-MS system was successfully applied to the screening of lipophilic marine biotoxins commonly found in shellfish. This non-targeted approach provides high-resolution data over the entire chromatographic separation, allowing detection of new or unknown compounds in addition to those of interest. Furthermore, the approach requires little method development, as settings are not tuned for individual analytes. Although the results described above were limited to a relatively small subset of biotoxins for which calibration standards are available, extending the approach to other toxins or toxin analogues can be simply accomplished by expanding on the target list of analyte masses during data processing.

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Application Note: 379

Analysis of Microcystins from Blue-green Algae Using the TSQ Quantum Ultra LC-MS/MS System

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Key Words

- TSQ Quantum Ultra™
- Blue-green algae
- LC-MS/MS
- Microcystin
- SRM

Introduction

Overgrowth of algae is a common problem in many wetlands with advanced stages of eutrophication (the enrichment of chemical nutrients containing nitrogen or phosphorus in an ecosystem). This often results in a thick, colored layer on the water's surface, known as an algal bloom. Some of the algae that grow in these bodies of water, known as Cyanobacteria or blue-green algae, produce toxic compounds known as microcystins.

Microcystins have a ring peptide structure consisting of seven amino acids, and more than 80 homologs are known. One of the most widely studied of the microcystins is known as Microcystin-LR, and is shown in Figure 1. Many of the microcystins are particularly toxic to the liver. (*See References.*) Among them are Microcystin-LR, YR and RR, which have been detected in wetlands in Japan. This application note reports on the analysis of these microcystins by using LC-MS/MS.

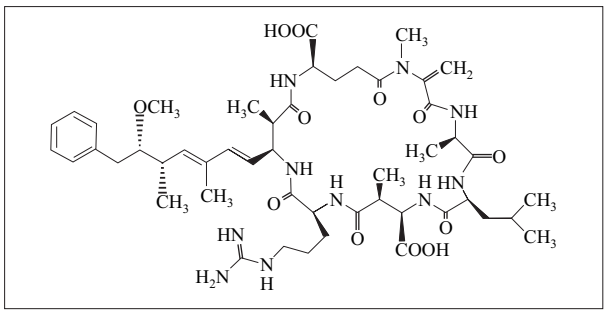

Figure 1: Microcystin-LR

Method

HPLC: HTC PAL Autosampler and Surveyor™ MS pump
Column: HyPURITY™ C18 2.1×50 mm, 5 µ (Thermo Scientific)
Mobile Phase A: Water with 0.1% Formic Acid
Mobile Phase B: Acetonitrile
Gradient: 30%B (0.5 min) → 80%B (in 3 min) → 80%B (2 min hold) → 30%B (7 min hold)
Injection Volume: 20 µL
Flow: 0.2 mL/min
Column temperature: Room temperature
MS: TSQ Quantum Ultra
Ionization: Positive ESI
Spray voltage: 5000 V
Sheath gas: 45 arbitrary units
Auxiliary gas: 15 arbitrary units
Sweep gas: 2 arbitrary units
Capillary T: 350°C
Source CID: Off
Collision gas: Ar, 1.2 mTorr
Scan Time: 0.15 sec
SRM setting: 519.9 → 135.0 @ 32 V (RR)
995.7 → 135.0 @ 65 V (LR)
1045.8 → 135.0 @ 70 V (YR)

SRM Chromatogram (STD 1.0 ppb)

The SRM chromatograms for 1.0 ppb standards are shown in Figure 2. The linear calibration curves of the standards (0.1 ppb–1.0 ppm) are shown in Figure 3.

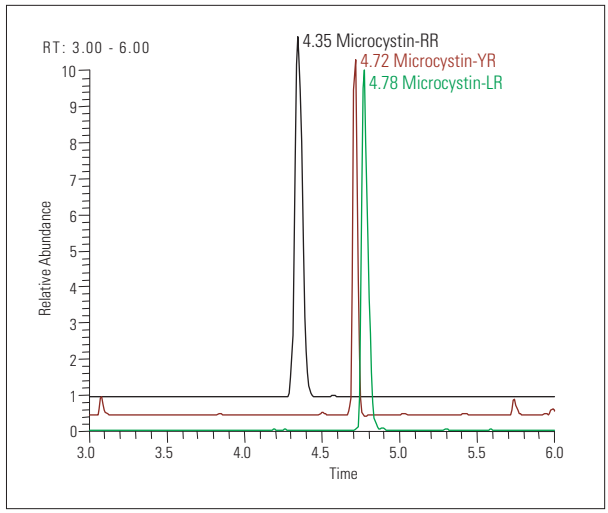

Figure 2: SRM Chromatogram (RT 4.35: Microcystin-RR, RT 4.72: Microcystin-YR, RT 4.78: Microcystin-LR)

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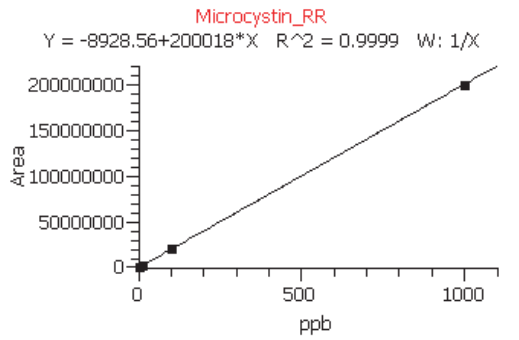

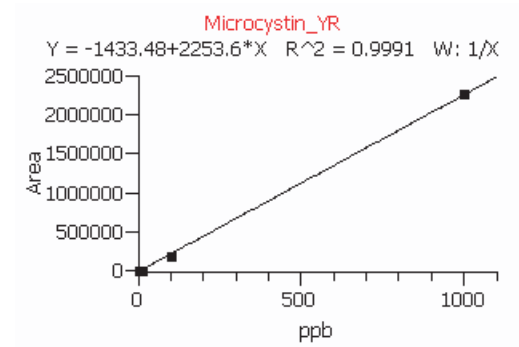

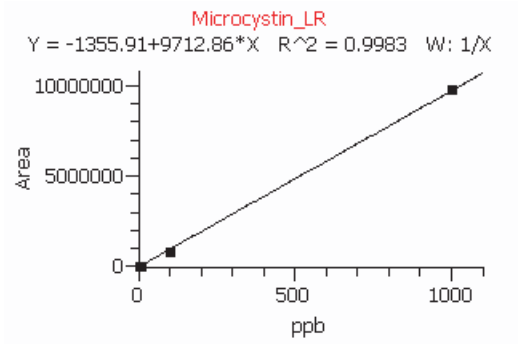

Figure 3: Calibration Curves 0.1 ppb – ~1.0 ppm

Conclusion

Microcystin-LR, YR and RR can be quantitatively analyzed over four orders of dynamic range (0.1 ppb–1.0 ppm) by using the TSQ Quantum Ultra triple quadrupole LC-MS/MS system from Thermo Fisher Scientific.

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Application Notebook

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Adulteration

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Characterization of Triacylglycerides (TAGs) in Vegetable Oils using MALDI LTQ Orbitrap XL Instrumentation

Chris Weise, Tabiwang Arrey, Kerstin Strupat
Thermo Fisher Scientific, Bremen, Germany

Key Words

MALDI LTQ Orbitrap XL, MS^n, triacylglycerides (TAGs), saturated and unsaturated TAGs, Matrix-Assited Laser Desorption/Ionization (MALDI), Orbitrap technology, food safety, structural confirmation, structural elucidation, accurate mass, high resolution

Goal

Uncover structures of specific TAGs and TAG distribution patterns among various vegetable oils exploiting full MS and CID-based MS^n experiments using the Thermo Scientific™ MALDI LTQ Orbitrap™ XL.

Overview

The Power of MS^n Combined with the Simplicity of MALDI

Purpose: Characterization of triacylglycerides of four different vegetable oils

Methods: Regular MALDI sample preparation followed by full MS and MS^n approaches using Orbitrap-based instrumentation

Results: Discriminability of vegetable oils based on their triacylglyceride (TAG) distributions

Introduction

Recognition of authentic oils

Upon the growth of the world's population, consumption of vegetable oils had increased by 4.5% in 2011. By 2020, the annual production of vegetable oils is expected to expand by 23% to 507 million tons, 30 tons will be produced within EU. Therefore, import rates for vegetable oils into the EU are predicted to rise up to 42%, which will be 18% of the entire production worldwide.[1]

These facts, as well as excessive fluctuation of the price, give raise to questions about product safety. Due to the current market situation, cases of economic adulteration of vegetable oils with non-authentic and less expensive oil adjuncts occur frequently.[2]

Economic adulteration potentially provokes economic and health care issues. This resulted in costs of US$ 12 billion (economy) and US$ 290 billion (health care).[2] A fast, comprehensive and reliable mass spectral lipid analysis contributes to quality control and consumer's health.

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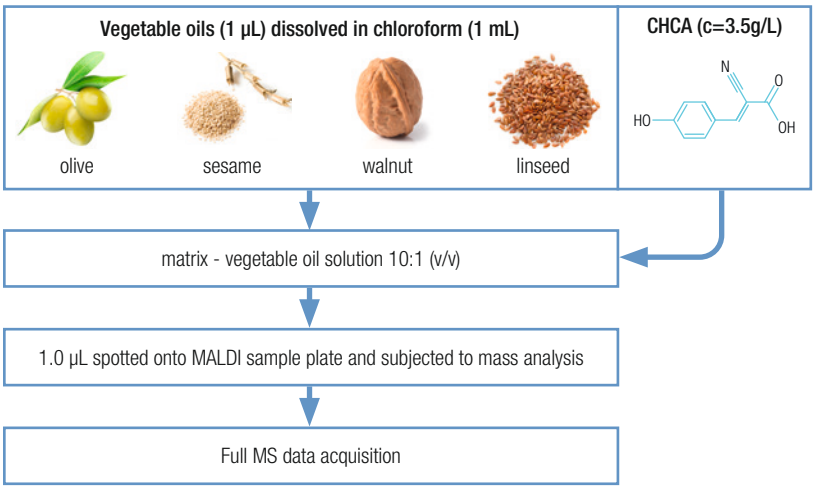

Figure 1: Workflow applied to characterize triacylglycerides (TAGs) in various vegetable oils.

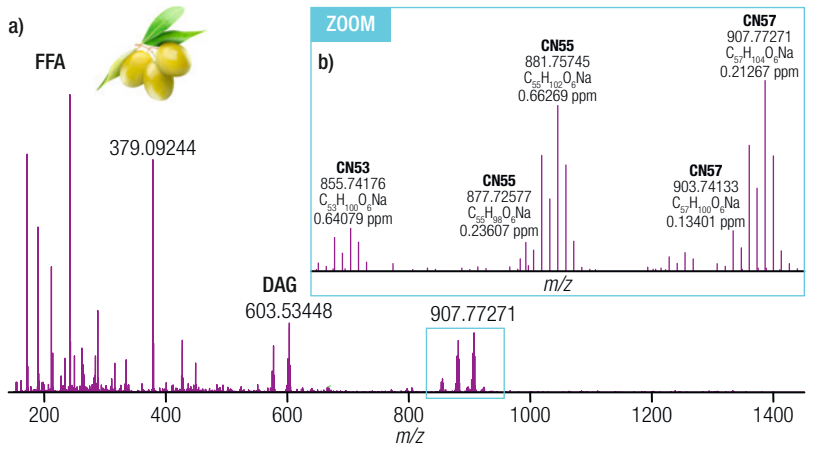

FIGURE 2. : (a) MALDI FTMS full scan (FTMS + p MALDI full MS [150-2000]) of olive oil; an average of 25 scans detected with resolving power 60,000 at *m/z* 400, mass range *m/z* 150-2000, (b) Inset into MALDI FTMS full scan of olive oil; abundance of TAGs containing CN 53, CN55 and CN57 is displayed.

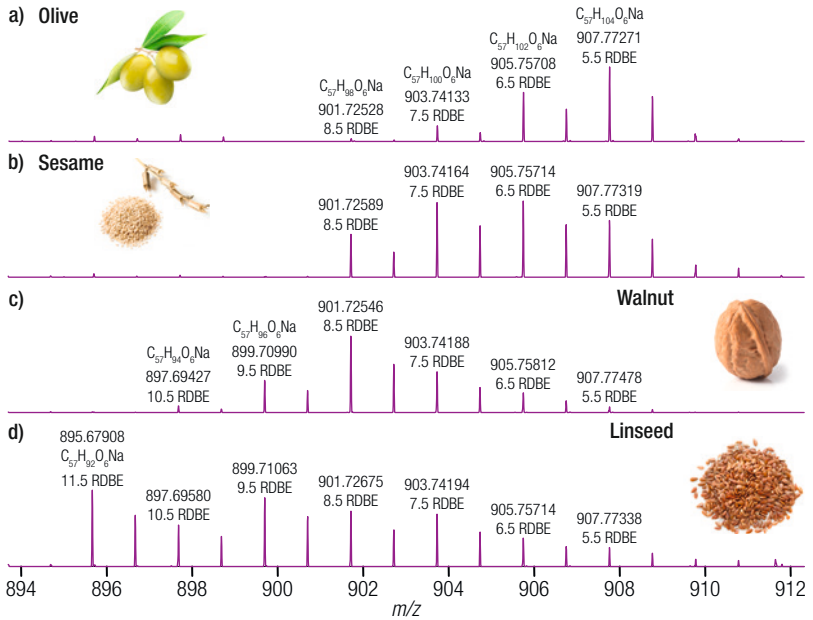

Figure 3: Insets into MALDI FTMS full scan information of various vegetable oils (inset into full scan MS data around *m/z* 900), average of 25 scans each.
a) olive oil, b) sesame oil, c) walnut oil, d) linseed oil.
Detected with resolving power 60,000 at *m/z* 400 in the Orbitrap detector.

Methods

Sample Preparation

α-cyano-4-hydroxycinnamic acid (CHCA) matrix (c=3.5 g/L, dissolved in 84% Acetonitrile, 13% Ethanol, 0.003% TFA) and vegetable oil (1µL dissolved in 1mL 100% chloroform) are used. Finally 1µL of 10:1 premixed matrix/analyte molecule solution is spotted and allowed to dry onto the MALDI plate.

Mass Spectrometry

The MALDI LTQ Orbitrap XL was used for the analysis of triacylglycerides (TAGs) from vegetable oils (linseed oil, olive oil, sesame oil, walnut oil).[3] FTMS full scan information (60,000 resolution @ *m/z* 400) are obtained in positive ion mode. Additionally, tandem MS data (MS^n using FTMS (Orbitrap detector) and ITMS (Ion trap device) of selected triacylglycerides using Collision Induced Dissociation (CID) experiments are collected to reveal the structural compositions of TAGs. The workflow is outlined in **Figure 1**.

Data Analysis

MS and MS^n data are post-processed using QualBrowser, part of Thermo Scientific Xcalibur Software Version 2.2.

Results

Different vegetable oils reveal species-specific triacylglyceride patterns

In FTMS full scan data several compounds are identified upon MALDI. triacylglyceride (TAG) ions are almost exclusively detected as $[M+Na]^+$ adduct ions with negligible abundance of corresponding $[M+K]^+$ adduct ions. Furthermore, presence of diacylglycerides (DAGs) and free fatty acids (FFA), both as protonated ion species, are observed in FTMS full scan data (see **Figure 2**) displayed for olive oil. Sodiated TAGs consisting of 57 carbon atoms (=CN57) display the most intense signal, followed by decreasing signal intensities for different TAG clusters providing carbon numbers CN55 and CN53, both labeled as sodiated ion species (inset in **Figure 2**). Based on the FTMS full scan data structural properties, such as number of carbon atoms (CN), elemental composition and degree of saturation - illustrated through Ring Double Bond equivalents (RDBe) - of a given TAG are determined.

Different vegetable oils exhibit species-specific TAG distributions; i.e. the most abundant TAG molecule in olive oil, *m/z* 907.772, is more saturated in hydrogens (RDBe 5.5) than the most abundant TAG compound in linseed oil, *m/z* 895.679 (RDBe 11.5, see **Figure 3**).

Measurements of these vegetable oils using Electrospray Ionization (ESI) MS reveal mass spectra which are significantly more complex and more difficult to interpret as a result of the Na^+- and NH_4^+ - adduct ions of TAGs formed in ESI mode.[4]

Full MS and MS^n are keys to elucidate structure

Tandem MS experiments of selected monoisotopic TAGs reveal a neutral loss of a fatty acyl residue, i.e. Δm=282.256 is indicative of an oleic acyl residue and equals to a neutral loss of $C_{18}H_{34}O_2$ (CN18:1, see **Figure 4**). MS² spectra are dominated by sodiated and protonated DAG ion signals. This is demonstrated in **Figure 5**, including possible proposals about structural composition.

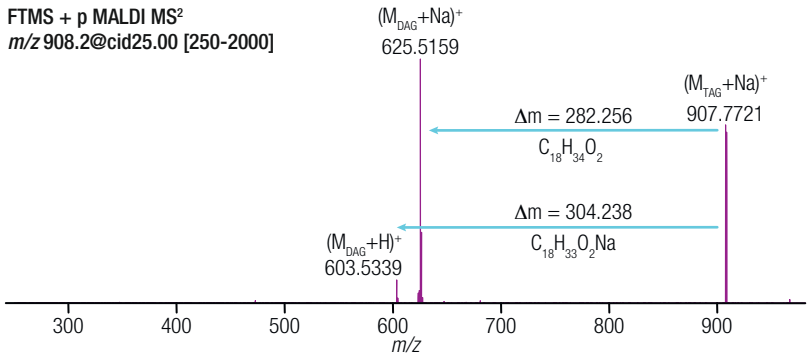

Figure 4: MALDI FTMS MS² Scan of TAG [M+Na]⁺ 57:3 (*m/z* 907.772) from olive oil using 25% Normalized Collision Energy for CID, isolation width 2 u, an average of nine scans, detected with resolving power 60,000 at *m/z* 400.

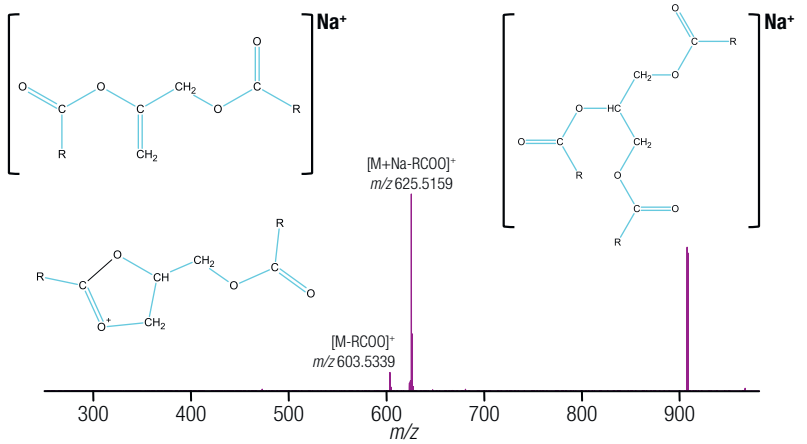

Figure 5: same as Figure 4, MALDI FTMS MS² Scan of TAG 57:3 (*m/z* 907.772) from olive oil using CID, including structural proposals with R = $C_{17}H_{33}$.

Determination of the fatty acyl compositions

In MS³ experiments neutral losses, corresponding to a fatty acyl residue occur; furthermore compound specific fragment ions, are observed. Literature suggests that these fragmentations occur with five or six- membered transition states; these depend on the stereochemical position of the fatty acyl residue on the glycerol backbone.[5]

MS³ spectra of protonated *m/z* 603.339 or sodiated *m/z* 625.5159 DAG show evidence of neutral loss of Δm=56 (C_3H_4O) and Δm=74 ($C_3H_6O_2$). The observed neutral losses in combination with observed fragment ions at *m/z* 529 [M-RCOO-74]⁺, m/z 265 [M-(RCOO)$_2$-56]⁺ and *m/z* 321 [M+Na-(RCOO)$_2$]⁺ are characteristic for MS³ spectra and fragmentation pathway of this compound class (**Figure 6 and Figure 7**). Sodiated fragment of Diacyl- and triacylglycerides ion at *m/z* 625.51 reveals a corresponding fragmentation pathway (not illustrated here). Due to occurrence of two or more fatty acyl residues in tandem MS and MS³ experiments several fatty acyl compositions are determined (see **Table 1**). Alternative fatty acyl compositions are assigned as well.

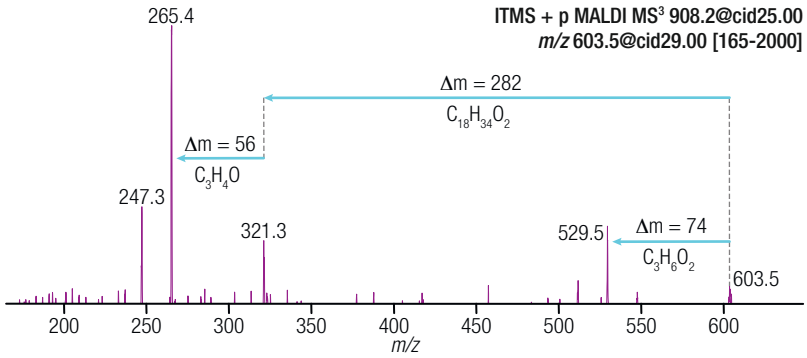

Figure 6: MALDI ITMS MS³ spectrum (ITMS + p MALDI MS³ 908.2@cid25.00 603.5@cid29.00 [165-2000]) of TAG 57:3 (*m/z* 907.772) ⇒ DAG 39:2 (protonated DAG, *m/z* 603.5), average of 21 scans, resolving power 60,000 at *m/z* 400.

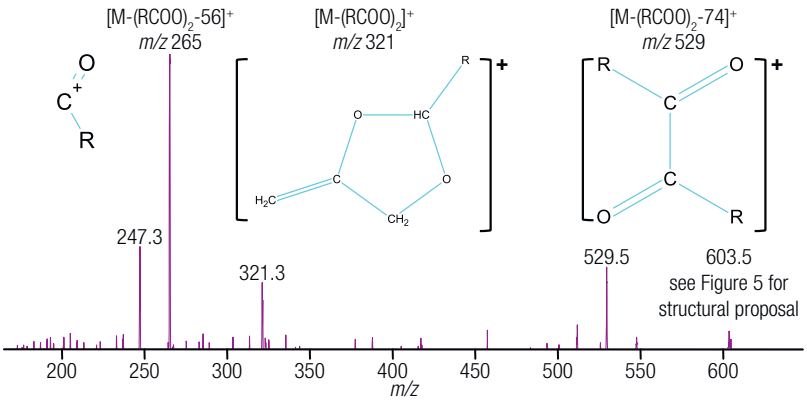

Figure 7: Same as Figure 6, MALDI ITMS MS³ spectrum of TAG 57:3 (*m/z* 907.772) ⇒ DAG 39:2 (protonated DAG, *m/z* 603.5), including structural proposals with R = $C_{17}H_{33}$.

Molecules observed upon FTMS detection

Sodiated TAG molecules observed upon FTMS detection of olive oil, sesame oil, walnut oil and linseed oil (only TAGs with CN57 are illustrated); alternative fatty acyl composition are illustrated in brackets.

type of vegetable oil	*m/z* $[M+Na]^+$	fatty acid composition (non-stereospecific)
olive 	901.726	LLL
	903.741	OLL
	905.757	OOL
	907.773	OOO
sesame 	901.726	LLL
	903.741	OLL
	905.757	OOL
	907.773	OOO (SOL)
	909.788	SOO (SSL)
walnut 	897.694	LLnLn
	899.710	LLLn
	901.726	LLL (SLnLn)
	903.741	OLL (SLLn)
	905.757	OOL (SLL)
	907.773	OOO (SOL, SSLn)
linseed 	895.679	LnLnLn
	897.694	LLnLn
	899.710	LLLn (OLnLn)
	901.726	LLL (OLLn)
	903.741	OLL (OOLn)
	905.757	OOL
	907.773	OOO

Figure 8: Ln: linolenic acid, L: linoleic acid, O: oleic acid, S: stearic acid.

Conclusion

To ensure the natural quality of vegetable oil

- MALDI in combination with high resolution accurate mass detection is a fast, reliable, valuble tool to distinguish vegetable oils qualitatively.
- Full scan MS and MS^n are the key to sum formula and structural composition proposals of endogenous TAGs.
- Despite of the general simplicity of ESI mode, MALDI proves to be more beneficial – as only Na^+-adduct ions are formed. This facilitates mass spectral interpretation of TAGs by MALDI over ESI.
- Results of this study contribute to investigations of economic adulteration of vegetable oils.

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Application Note: 432

Identification of Lysergic Acid Diethylamide (LSD) in Candy by UHPLC/MS

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Key Words

- Accela™ UHPLC
- MSQ Plus™
- Hypersil GOLD™ PFP
- Forensic Analysis
- LSD

Goal

Positively identify trace amounts of lysergic acid diethylamide (LSD) in sugar candy quickly, with minimal sample preparation and no chemical derivatization.

Introduction

Lysergic acid diethylamide (LSD) is a controlled substance in forensic chemistry that is notorious for being difficult to identify. Its myriad evidentiary forms include paper tabs, eye drops, sugar cubes and small sugary candies such as sweet tarts, valentine hearts or mints. Because it is such a potent hallucinogen, typical street doses require only 40 to 120 µg of LSD. The small personal-use amounts seized by state and local law enforcement often lack sufficient drug to allow both forensic analysis by traditional means and archiving of some of the evidence for follow-up testing.

Most forensic laboratories confirm the presence of LSD by using gas chromatography with mass spectrometry (GC/MS). LSD is extracted from the evidence with an organic solvent, derivatized, and determined by GC/MS. GC/MS resolves LSD from other compounds and provides structural information that can be compared to reference spectra in a searchable library.

The disadvantages of GC/MS are its requirements for extensive sample preparation, including chemical derivatization of LSD to a more volatile form, and its impaired performance with analytes that are polar, thermally labile, or nonvolatile. LSD has a high affinity for active sites in liners that can spoil chromatographic resolution. LSD-doped sugar cubes or candy can foul the GC with sugars, increasing the burden of instrument maintenance and hindering throughput.

An alternative method to positively identify LSD in complex food matrices is to use ultra high performance liquid chromatography with mass spectrometric detection (UHPLC/MS). UHPLC/MS offers a threefold benefit compared to GC/MS; simpler sample preparation, no derivatization, and less time wasted baking out or cleaning the instrument. This application note demonstrates how a working forensic laboratory uses UHPLC/MS to analyze sugar candies for LSD. LSD in doped sugar cubes and candy hearts is simply extracted, separated within 5 minutes on a Hypersil GOLD PFP 1.9 µm column, and confirmed by a fast scanning single quadrupole mass spectrometer.

Experimental Conditions

1. Standard and Sample Preparation

A 1000 mg/L solution of LSD in methanol was purchased from Alltech (State College, PA, USA) and diluted to about 5 mg/L with methanol.

The sugar cubes and candy hearts were purchased from a local grocery store. The candy hearts and sugar cubes were treated with 3-5 drops of this LSD solution and allowed to stand for 24 hours prior to use. Ten (10) mg scrapings from the sugar cube or candy heart were added to 2 mL methanol. This mixture was vortexed for 30 sec, allowed to settle for 1 min, and the supernatant was filtered through a cotton-plugged Pasteur pipette. The filtrate was centrifuged for 90 sec, and the supernatant was filtered through a second cotton-plugged Pasteur pipette and transferred to the autosampler vial.

2. Chromatographic Conditions

Chromatographic analyses were performed using the Accela UHPLC system (Thermo Fisher Scientific, San Jose, CA). The chromatographic conditions were as follows:

Column:	Hypersil GOLD PFP (perfluorinated phenyl) 1.9 µm, 100 x 2.1 mm			
Flow Rate:	1 mL/min			
Mobile Phase:	A: Water with 0.06 % acetic acid B: Acetonitrile (ACN) with 0.06% acetic acid C: Methanol with 0.06% acetic acid			
Gradient:	T (min)	A%	B%	C%
	0.00	95.0	0.0	5.0
	1.00	95.0	0.0	5.0
	1.50	90.0	5.0	5.0
	2.70	70.0	10.0	20.0
	3.00	5.0	15.0	80.0
	7.00	5.0	0.0	95.0
	7.10	95.0	0.0	5.0
	8.00	95.0	0.0	5.0
Column Temperature:	45 °C			
Injection:	2 µL partial loop injection, 25 µL loop size Syringe Speed: 8 µL/sec Flush Speed: 100 µL/sec Flush Volume: 400 µL Wash Volume: 100 µL Flush/Wash source: Bottle with methanol			

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3. Mass Spectrometer Conditions

MS analysis was carried out on a MSQ Plus single quadrupole LC/MS detector (Thermo Fisher Scientific, San Jose, CA). The MS conditions were as follows:

Ionization:	Electrospray (ESI)
Polarity:	Positive
Probe Temperature:	500 °C
Cone Voltage:	90 V
Scan Mode:	Full scan with mass range of *m/z* = 125-425 amu
ESI Voltage:	3.5 kV
Scan Time:	0.2 s

Results

LSD elutes at 4.49 min and is detected by using full scans (*m/z* = 125 - 425) of the single quadrupole mass spectrometer. The extracted ion chromatograms from *m/z* 324 ± 0.5 are displayed in Figure 1A. The MS spectrum of the LSD standard shows two molecular ion signals: $[M+H]^+$ at *m/z* 324.1, and $[M+ACN+Na]^+$ at *m/z* 387.1. The MS spectrum of LSD also shows two fragment ion signals at *m/z* 223.3 and 281.3 (Figure 2A).

The methanol extracts from the candy hearts and sugar cubes, doped with LSD, were analyzed using the same UHPLC/MS method as for the standard LSD (Figure 1B, 1C). Positive confirmation of LSD in the samples is assured both by retention time matching and MS spectra matching of the samples (Figure 2B-C) with the LSD standard.

Conclusion

UHPLC/MS can positively identify trace amounts of lysergic acid diethylamide (LSD) in sugar candy in 8 min, after a simple 10 min sample prep involving no chemical derivatization.

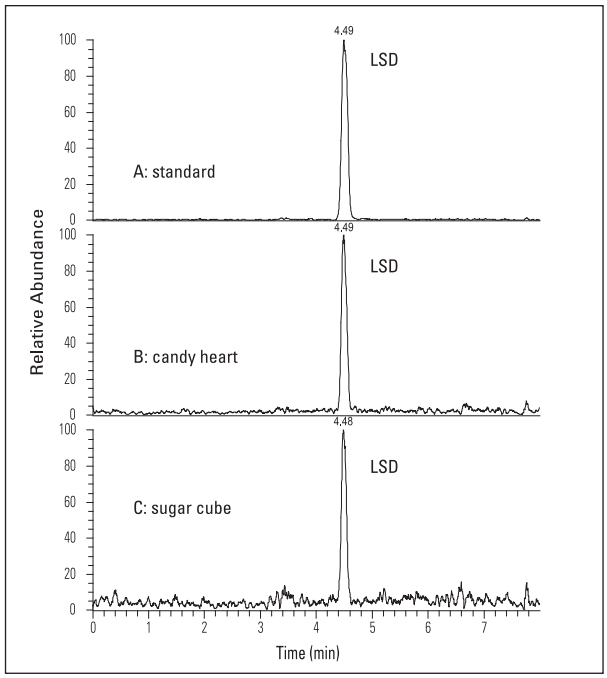

Figure 1: Extracted ion chromatograms at *m/z* = 324 ± 0.5 amu obtained by UHPLC/MS on a Hypersil GOLD PFP column: (A) LSD standard; (B) methanol extract of LSD-doped candy heart; (C) methanol extract of LSD-doped sugar cube. See text for details.

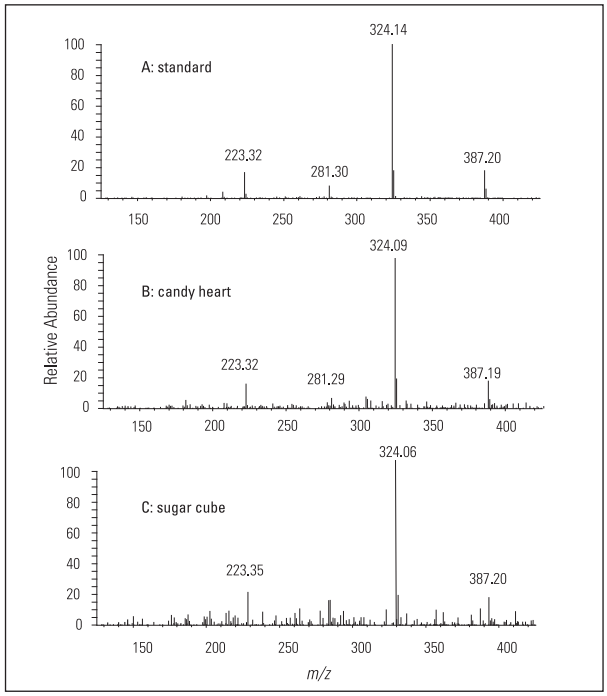

Figure 2: MS spectra of LSD obtained by UHPLC/MS on a Hypersil GOLD PFP column: (A) LSD standard; (B) methanol extract of LSD-doped candy heart; (C) methanol extract of LSD-doped sugar cube. See text for details.

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Application Note: 433

Identification of Cannabinoids in Baked Goods by UHPLC/MS

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Key Words

- Accela™ UHPLC
- Hypersil GOLD™ PFP
- MSQ Plus™
- Δ^9-tetrahydro-cannabinol (THC)
- Forensic Analysis

Goal

Positively identify trace amounts of cannabinoids in a complex food matrix quickly, with minimal sample preparation and no chemical derivatization.

Introduction

Marijuana is the most common illegal drug in the United States, and each year U.S. law enforcement agencies seize more than two million pounds of marijuana in various forms. Seized evidence submitted to forensic laboratories is screened for marijuana by microscopic inspection and simple chemical tests such as the Duquenois-Levine test. Presumptive positive results are confirmed by using gas chromatography-mass spectrometry (GC/MS) to positively identify cannabinoids including Δ^9-tetrahydrocannabinol (THC, the main psychoactive component), cannabinol (the main degradation product of THC) and cannabidiol. This traditional approach works fairly well for leaf marijuana, hashish, hash oil and residue collected from smoking paraphernalia.

GC/MS is less useful for confirming the presence of marijuana in complex food matrices such as baked goods. Simple sample preparation procedures using methanol or methylene chloride coextract many small molecules found in baked goods that can coelute with the target cannabinoids. Cholesterol, fatty acids, and caffeine can contaminate the gas chromatograph, forcing the analyst to clean the instrument and rerun all subsequent samples. More extensive sample preparation methods are time-consuming and often require greater amounts of the controlled substance than are present in the evidence.

An alternative method to positively identify marijuana cannabinoids in complex food matrices is to use ultra high performance liquid chromatography with mass spectrometry detection (UHPLC/MS). UHPLC/MS offers a threefold benefit compared to GC/MS; simpler sample preparation, no derivatization, and less instrument clean up time. This application note demonstrates how a working forensic laboratory uses UHPLC/MS to analyze baked goods for three cannabinoids of forensic importance. The cannabinoids are extracted, separated within 8 minutes on a Hypersil GOLD PFP 1.9 µm, 100 x 2.1 mm column and detected by a fast scanning single quadrupole mass spectrometer.

Experimental Conditions

1. Standard and Sample Preparation

The standard compounds THC, cannabidiol and cannabinol were purchased from Alltech (State College, PA, USA) and used as received. These compounds were mixed and diluted to about 10 ppm with methanol to prepare a stock standard solution.

Brownie and cookie samples were obtained from evidence archived after adjudication. Two (2) mL methanol was added to 25 mg of baked-good material. This mixture was vortexed for 30 seconds, allowed to settle for 2 min, and the supernatant was filtered through a cotton-plugged Pasteur pipette. The filtrate was centrifuged at 12,000 rpm for 90 seconds, and filtered again. The second filtrate was diluted 50 fold with methanol prior to analysis.

2. Chromatographic Conditions

Chromatographic analyses were performed using the Accela UHPLC system (Thermo Fisher Scientific, San Jose, CA). The chromatographic conditions were as follows:

Column:	Hypersil GOLD PFP (perfluorinated phenyl) 1.9 µm, 100 x 2.1 mm			
Flow Rate:	1 mL/min			
Mobile Phase:	A: Water with 0.06 % acetic acid B: Acetonitrile (ACN) with 0.06% acetic acid C: Methanol with 0.06% acetic acid			
Gradient:	T (min)	A%	B%	C%
	0.00	95.0	0.0	5.0
	1.00	60.0	32.5	7.5
	2.00	50.0	40.0	10.0
	5.00	45.0	45.0	10.0
	6.00	25.0	60.0	15.0
	6.50	5.0	0.0	95.0
	7.50	5.0	0.0	95.0
	7.51	95.0	0.0	5.0
	8.00	95.0	0.0	5.0
Column Temperature:	45 °C			
Injection:	2 µL partial loop injection, 25 µL loop size Syringe Speed: 8 µL/sec Flush Speed: 100 µL/sec Flush Volume: 400 µL Wash Volume: 100 µL Flush/Wash Source: Bottle with methanol			

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3. Mass Spectrometer Conditions

MS analysis was carried out on a MSQ Plus single quadrupole LC/MS detector with Xcalibur 2.05 (Thermo Fisher Scientific, San Jose, CA). The MS conditions were as follows:

Ionization:	Electrospray (ESI)
Polarity:	Positive
Probe Temperature:	500 °C
Cone Voltage:	90 V
Scan Mode:	Full scan with mass range of 50-500 *m/z*
ESI Voltage:	3.5 kV
Scan Time:	0.2 s

Results

The cannabinoid standards elute with good resolution at 4.1 min (cannabidiol), 5.1 min (THC) and 5.4 min (cannabinol). The cannabinoids were detected by using full scans (50-500 *m/z*) of the single quadrupole mass spectrometer, and the extracted ion chromatograms from *m/z* 310.5-311.5 + 314.5-315.5 are displayed in Figure 1A. Molecular ions of each compound (*m/z* 315 for cannabidiol and THC and *m/z* 311 for cannabinol) are observed (Figure 2A-C).

The brownie sample, which was taken from an adjudicated case and was known to contain THC, tested positive for THC (Figure 1B, 2D), demonstrating that the sample preparation required for this LC/MS method is simpler, faster and requires less sample than the GC/MS method employed for the original casework.

After ten years in the forensic laboratory's training vault, cannabinoids in the cookie sample had degraded significantly, but by increasing the sample injection from 2 µL to 10 µL, THC was detected with good signal-to-noise (Figure 1C, 2E).

Solvent blanks were analyzed after each sample run, with no apparent carryover from one run to the next (results not shown).

Conclusion

Cannabinoids in baked goods can be identified using UHPLC/MS with minimal sample preparation. The preparation time (10 min) and run time (8 min) make this a very efficient analytical method.

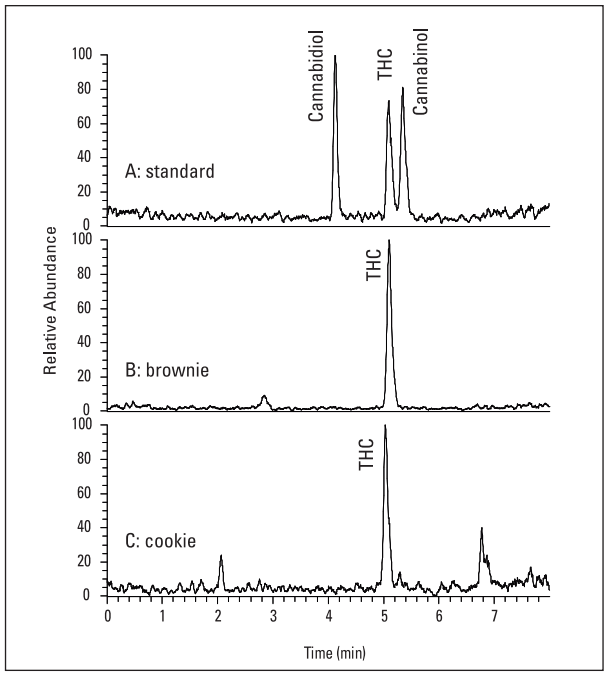

Figure 1: Extracted ion chromatograms (*m/z*, 310.5-311.5, 314.5-315.5) of cannabinoid standards (A) and extracts from brownie (B) and cookie (C) by UHPLC/MS

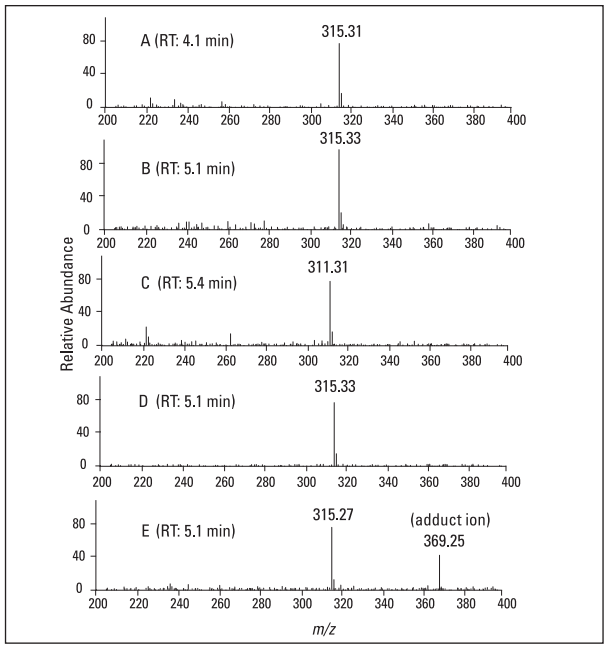

Figure 2: MS spectra of cannabinoid standards, cannabidiol (A), THC (B), cannabinol (C), eluted at 4.1 min, 5.1 min and 5.4 min respectively, and extracts from brownie (D) and cookie (E), eluted at 5.1 min

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Application Note: 502

Simple and Rapid Screening of Melamine in Milk Products with High Resolution Accurate Mass Benchtop Orbitrap LC-MS

Kefei Wang, Chunang (Christine) Gu
Thermo Fisher Scientific, San Jose, CA, USA

Key Words

- Exactive
- HRAM High Resolution Accurate Mass
- High throughput screening
- Milk products

Introduction

Generally used for industrial manufacturing, melamine, a nitrogen-rich white crystal, has been found as an adulterant used to falsify the protein levels in many milk products. In the 2008 Chinese milk scandal, thousands of young children who consumed melamine-contaminated milk products were reported to have developed sickness related to kidney stones and renal failure. More recently in January 2010, China reported another recall of melamine-tainted condensed and powdered milk products. Contaminated milk products were also found during the 2008 scandal in many other countries and regions, causing widespread concern and demand for monitoring melamine in various milk products.

Different countries vary in setting the Maximum Residue Limit (MRL) for melamine, but generally follow the United States Food and Drug Administration (US FDA) MRL of 1 ppm for infant formula and 2.5 ppm for other milk products.[1] Most advanced food testing labs employ mass spectrometry-based methods, particularly liquid chromatography tandem mass spectrometry (LC-MS/MS), for detecting sub-ppm to low ppb levels of melamine.

Milk is a complex matrix containing soluble proteins, sugars and lipids, with additional enriched nutrients such as vitamins and minerals added to infant formula. Sample cleanup is critical and two approaches are generally used. First is the dilute-and-shoot approach in which the milk products are dissolved in diluted acid, followed by protein precipitation with acetonitrile. US FDA uses such a method for reporting a limit of quantitation (LOQ) of 250 ppb on LC-MS/MS.[1] In the second approach, more time-consuming and labor-intensive solid phase extraction (SPE) is used to remove the interferences and enrich the sample melamine for more sensitive quantitation at low ppb level by LC-MS/MS.

Another complication in analyzing melamine with LC/MS is that melamine, being a strong polar small molecule, cannot be retained in conventional reverse-phase HPLC. Ion paring or HILIC (Hydrophilic Interaction Chromatography) mode is used.

In this study, we evaluate a simple and rapid LC/MS method to screen trace levels of melamine in milk products by utilizing a benchtop high resolution, accurate mass Orbitrap mass spectrometer. The sample preparation uses dilute-and-shoot. Analysis is fast and requires only a 1-minute LC separation.

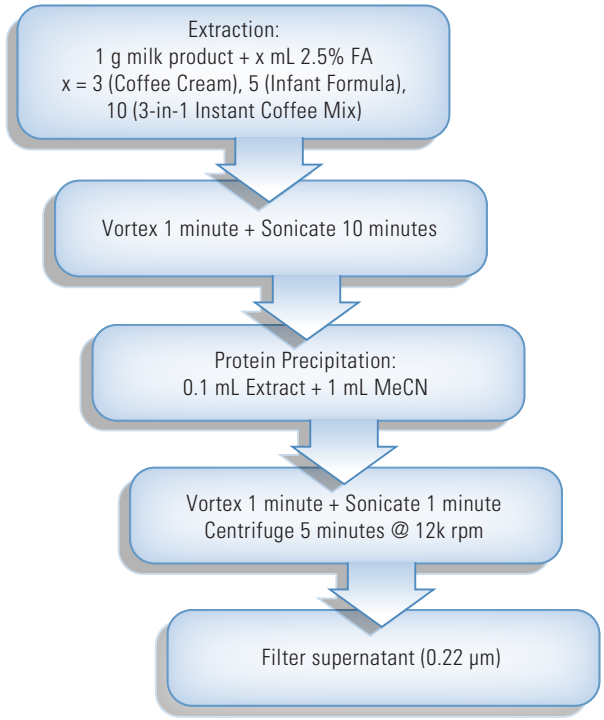

Figure 1: Sample preparation flowchart

Methods

Samples: Concentrated infant formula, instant coffee mix (3-in-1 with coffee, creamer and sugar) and liquid coffee creamer (unflavored) were purchased from local supermarkets.

Sample Preparation: Milk samples were extracted with 2.5% formic acid followed by protein removal with acetonitrile following the US FDA published procedures with modification as shown in Figure 1. The total dilution factors as a result of sample preparation are given in Table 1.

Sample	Total Dilution Factor
Infant Formula	65
Coffee Cream	44
3-in-1 Coffee Mix	110

Table 1. Total dilution factor

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Figure 2: Exactive mass spectrometer and Accela liquid chromatography system

LC Conditions:

LC:	Thermo Scientific Accela liquid chromatography system
Column:	Thermo Scientific BioBasic AX 50 mm x 3 mm, 5 µm
Eluent:	95:5 MeCN (0.1% FA) / Water (0.1% FA), isocratic at 500 µL/min
Injection:	5 µL (with loop)
Run time:	1 minute

Mass Spectrometer Conditions:

Mass spectrometer:	Thermo Scientific Exactive benchtop high resolution, accurate mass system (Figure 2)
MS parameter settings:	See Figure 3
Resolution:	High (R=50,000 FWHM at *m/z* 200)
Lock mass:	*m/z* 195.0877 (Caffeine)
Ion source:	HESI-II, +3.5 KV
Vaporizer temp:	300 °C
Tube lens:	94 V
Sheath/Aux gas:	30/10 units with N_2
Capillary temp:	270 °C

Results and Discussion

The goal of this study was to explore high resolution benchtop mass spectrometry for a simple and rapid method to test melamine in milk products with a detection limit lower than 250 ppb, the reporting LOQ set by the US FDA method for infant formula on a triple quadrupole mass spectrometer. The sample preparation followed the dilute-and-shoot approach without the use of a laborious and time-consuming SPE procedure.

Other than the conventional ion source tuning, the Exactive™ mass spectrometer setup required only one parameter to be changed: resolution was set to High (R=50,000 FWHM at *m/z* 200). The *m/z* 195.0877 of caffeine was used as a lock mass because caffeine was conveniently present in the tuning solution; after each tuning, the residue caffeine peak can be used as a lock mass in subsequent sample analysis. The additional caffeine peak can be found in coffee samples. Under these conditions, melamine (*m/z* 127.0727) can be unambiguously identified with mass accuracy better than 2 ppm.

The Exactive mass spectrometer sensitivity and linear response range were evaluated with the melamine standards. Figure 4 shows the chromatogram and accurate mass spectra of a representative 0.1 ppb solution, and Figure 5 displays a representative calibration curve demonstrating a linear response from 0.1 to 100 ppb.

Milk samples were found to have a strong matrix effect that resulted in severe ion suppression. Preliminary experiments with loop injection without any LC separation failed to detect 1 ppb melamine spiked in any of the three matrices even with a further 5x dilution. Thus it was decided that a simple LC separation is still required.

The LC separation employed a 1-minute run on a BioBasic™ AX weak anion exchange column with a strong organic mobile phase (95% v/v MeCN), creating a HILIC condition[2] that separated the melamine (R.T. ~0.54-0.6 min) from the major interference species eluting either in the void volume (0.35-0.4 min) or after the melamine. An isocratic run was chosen to eliminate the column equilibration time between each injection, thus increasing throughput.

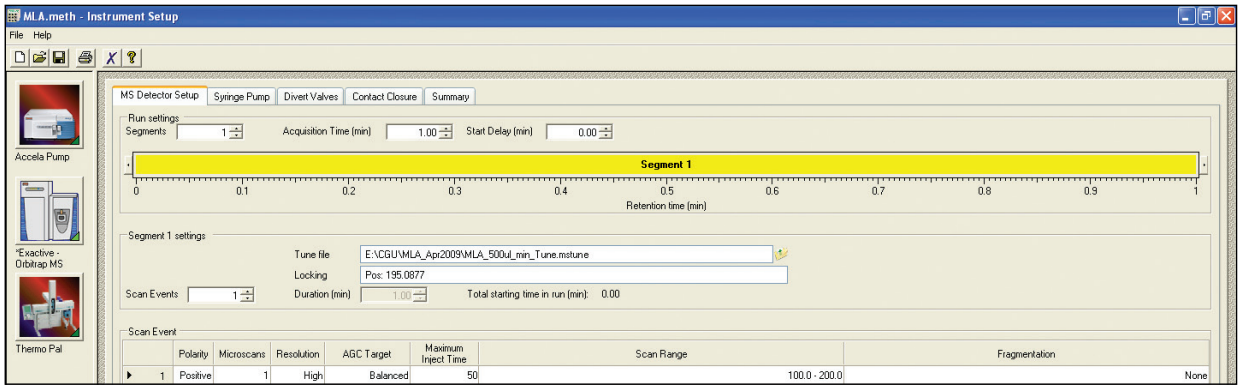

Figure 3. MS parameter settings.

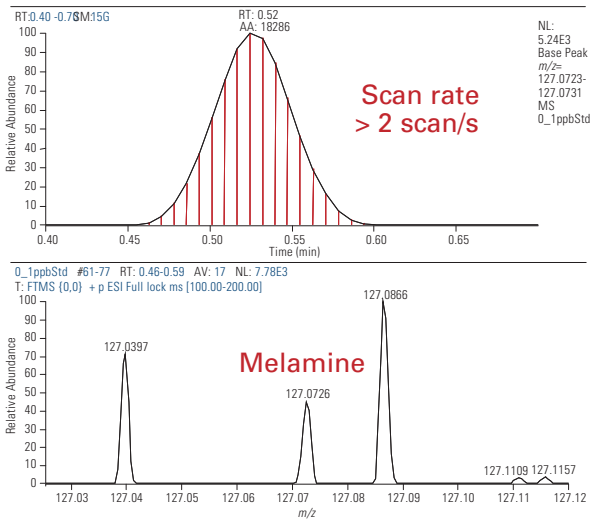

Figure 4: Chromatogram and spectra of 0.1 ppb (0.5 pg on column) melamine standard

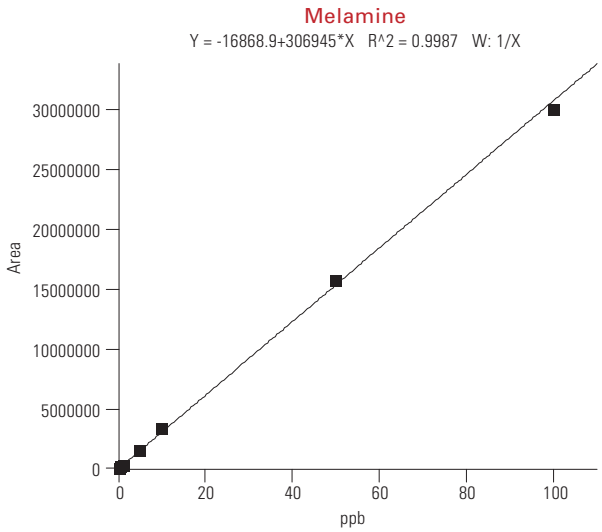

Figure 5: Calibration curve of melamine standard solution (0.1 to 100 ppb). Four orders of linear dynamic range were observed.

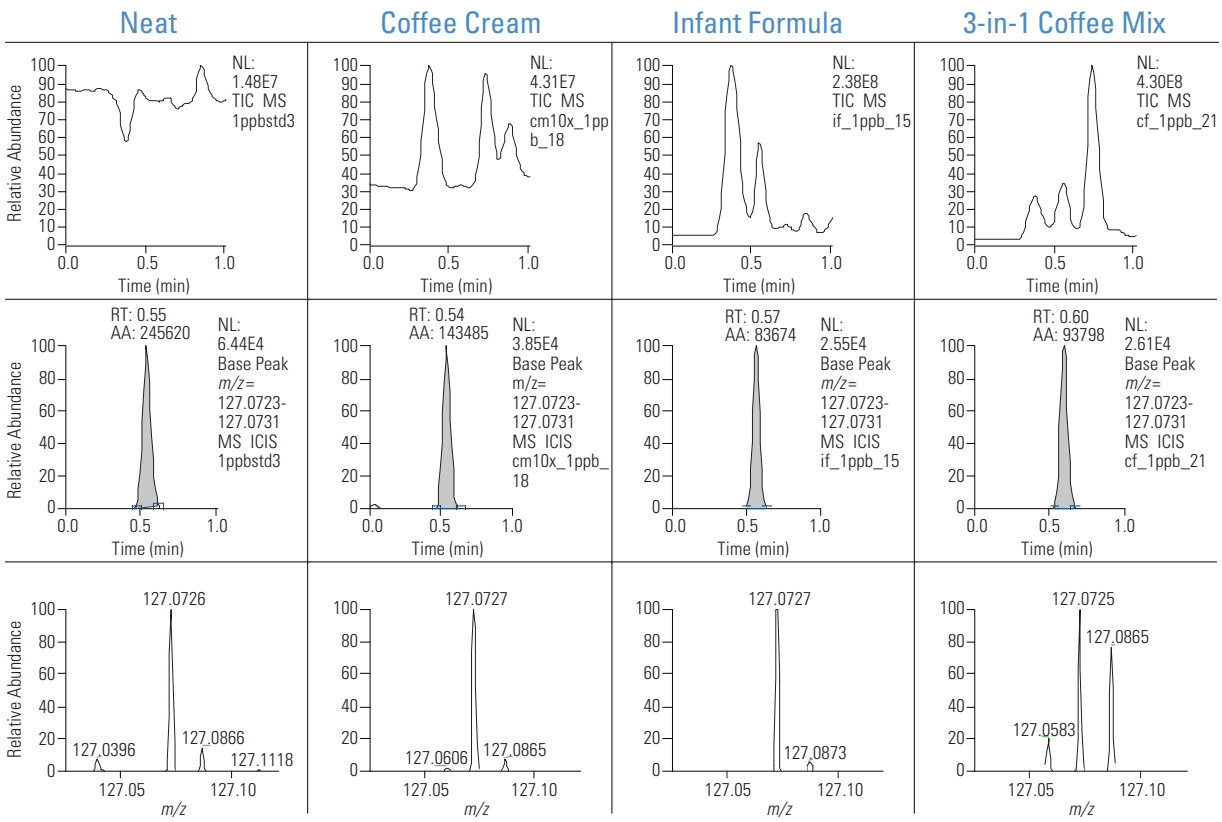

Figure 6: Comparison of 1 ppb melamine in standard (neat) and spiked in extract sample matrix (top: TIC; middle: chromatographic peak, bottom: mass spectra).

Figure 6 shows the comparison of 1 ppb melamine spiked in a mobile phase (neat) and in three extracted sample matrices. As shown, 1 ppb spikes can be detected. Based on the dilution factor from sample preparation in Table 1, the detection of 1 ppb spike corresponds to 65, 44, and 110 ppb in infant formula, coffee cream and 3-in-1 instant coffee mix, respectively.

The responses of 1 ppb melamine in matrices are only 30%-50% of that in the neat solution, but responses were found to be consistent in each sample extract in the spiked 1-10 ppb range evaluated. The average response factor (RF) values from spiking 1, 5, and 10 ppb in each of three sample matrices are given in Table 2. A constant response factor in each extract matrix makes it possible to use the standard addition method for melamine quantitation.

The overall recovery was also evaluated by spiking a 300 ppb level of melamine in three milk products prior to the extraction. The recovery values were found to be 75%-91%.

Sample	RF	RSD%
Coffee Cream	0.50	8.0%
Infant Formula	0.31	7.2%
3-in-1 Coffee Mix	0.34	3.4%

Table 2. Melamine response factor (RF) in sample matrix compared to neat standard (RF=1) and RSD% (n=3)

Conclusions

The high resolution, accurate mass Exactive mass spectrometer was shown to be sensitive in detecting <0.1 ppb melamine (0.5 pg on column) in neat standard, and response is linear from 0.1 to 100 ppb. The error for mass accuracy is <2 ppm with lock mass.

Milk samples prepared by dilute-and-shoot showed severe ion suppression that was reduced with a simple 1-minute isocratic HILIC LC separation, after which a consistent response factor of 0.3-0.5 for each sample matrix was obtained for quantitation.

Quantitation limits were less than 44, 65, and 110 ppb for coffee cream, infant formula and 3-in-1 instant coffee mix, respectively, exceeding the requirement of 250 ppb LOQ as stated by US FDA for infant formula.

References

(1) http://www.cfsan.fda.gov/~frf/lib4421.html, Determination of Melamine and Cyanuric Acid Residues in Infant Formula using LC-MS/MS, Laboratory Information Bulletin No. 4421, October 2008, US FDA.

(2) Varelis, P., Beck, J., Wang, K., and Ghosh, D., Analysis of Melamine and Cyanuric Acid in Food Matrices by LC-MS/MS, Thermo Fisher Scientific App. Note 424.

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Application Note: 424

Analysis of Melamine and Cyanuric Acid in Food Matrices by LC-MS/MS

Peter Varelis, National Center for Food Safety and Technology, Illinois Institute of Technology.
Jonathan Beck, Kefei Wang, and Dipankar Ghosh; Thermo Fisher Scientific, San Jose, CA

Key Words

- TSQ Quantum Ultra™
- Cyanuric Acid
- Food Safety
- LC-MS/MS

Introduction

In March 2007, several North American manufacturers of pet food voluntarily issued nationwide recall notices for some of their products that were reportedly associated with renal failure in pets[1]. The raw material wheat gluten, used to manufacture the pet food, was imported from China and was identified as the source of contamination[2].

Although initial reports suggested that contamination was confined to pet food, further investigations revealed that melamine-tainted fodder may have been used to feed animals intended for human consumption.[3,4,5] In particular, it was discovered that melamine-contaminated ingredients had been used to prepare feed for chickens, swine, and catfish.[3,4] Consequently, the U.S. Food and Drug Administration (FDA)[3] and the U.S. Department of Agriculture (USDA)[4] have developed methods for the analysis of melamine residues in animal tissue. Both methods use tandem mass spectrometric detection and employ disposable strong cation exchange solid phase extraction (SPE) cartridges to prepare samples for liquid chromatographic analysis.

Experimental

Chemicals and reagents

Unless stated otherwise, all organic solvents used in this work were HPLC grade quality and were purchased from Thermo Fisher Scientific (Fair Lawn, NJ, USA). Melamine, cyanuric acid, and 30% (w/w) aqueous ammonia were purchased from Sigma (St. Louis, MO, USA). The internal standards $^{13}C_3$-melamine and -cyanuric acid were prepared using $^{13}C_3$-cyanuric chloride, which was also obtained from Sigma. 18 MΩ water was obtained from a Milli-Q™ (Millipore Corporation, Billerica, MA, US) purification system.

Calibration Standards

Individual solutions (1000 µg/mL) of cyanuric acid and melamine were prepared by dissolving the crystalline compounds in 50% (v/v) aqueous methanol. Aliquots (1 mL) of these solutions were combined and then diluted with 1:3 water-acetonitrile, respectively, to obtain a 10 µg/mL stock solution, from which eight working standards in the range of 1-1000 ng/mL were prepared by serial dilutions with acetonitrile. Calibration standards were prepared by adding 50 µL of the stock solution of the internal standards to 1 mL of each of the eight working standards.

Sample Preparation

Solid samples were homogenized using an Ultra-Turrax® (IKA®-Werke GmbH & Co. KG, Staufen, Germany) homogenizer. After extraction into aqueous 1:1 Water:MeOH, and addition of the internal standards, the samples were prepared by offline ion exchange chromatography using SPE cartridges.

Liquid Chromatography

Aliquots (10-25 µL) of the above extracts were chromatographed on a BioBasic™AX (Thermo Fisher Scientific, Bellefonte, PA) analytical column (2.1×150 mm, 5 µm), which was kept at 30 °C in an oven. The initial mobile phase was composed of acetonitrile-isopropanol-50 mM aqueous ammonium acetate in the ratio of 85:10:5, respectively, and was pumped through the column at a flow of 400 µL per minute.

After 5 min, the mobile phase composition and flow were immediately changed to 9:1 water-acetonitrile, and 500 µL per minute, respectively. These conditions were maintained for 5 min before returning the mobile phase to the initial composition. After 5 min of equilibration, the flow through the column was returned to 400 µL per minute. The column effluent was diverted to waste for the first 1.5 minutes and then switched to the detector for the remaining run time.

MS Conditions – Melamine

MS: Thermo Scientific TSQ Quantum Ultra
Source: Heated Electrospray (H-ESI)
Ionization: Positive ESI
Sheath Gas: 65 units
Auxiliary Gas: 35 units at 250 °C
Ion Transfer Tube Temp: 350 °C
Scan Time: 200 ms/transition
Q1/Q3 Resolution: 0.7 FWHM

SRM Transitions:

Melamine:	*Melamine $^{13}C_3$ (Internal Standard):*
m/z 127→68 @ 32 eV	*m/z* 130→70 @ 32 eV
m/z 127→85 @ 18 eV	*m/z* 130→87 @ 18 eV

QED-MS/MS Conditions:
Collision Energy: 30 eV
Reverse Energy Ramp (RER): 50 eV

MS Conditions – Cyanuric Acid

MS: TSQ Quantum Ultra
Source: Heated Electrospray (H-ESI)
Ionization: Negative ESI
Sheath Gas: 75 units
Auxiliary Gas: 10 units at 250 °C
Ion Transfer Tube Temp: 350 °C
Scan Time: 200 ms/transition
Q1/Q3 Resolution: 0.7 FWHM

SRM Transitions:

Cyanuric Acid:	*Cyanuric Acid $^{13}C_3$ (Internal Standard):*
m/z 128→42 @ 17 eV	*m/z* 131→43 @ 17 eV
m/z 128→85 @ 11 eV	*m/z* 131→87 @ 11 eV

Results

A chromatogram showing a standard mixture of both melamine and cyanuric acid, along with their associated internal standards, is shown in Figure 1. Calibration curves ranging from 1-1000 ppb are shown in Figure 2 and Figure 3 for melamine and cyanuric acid, respectively. The calibrations are linear over the entire range, and a close-up of the lower portion of the calibration curve (1-100 ppb) is shown in the same figure.

Melamine and cyanuric acid were spiked into a matrix of catfish and processed as described in the method section above. A chromatogram of this sample, spiked at 10 ppb for melamine and 50 ppb for cyanuric acid, is shown in Figure 4. Very low noise is observed, emphasizing the effectiveness of the cleanup procedure for a complicated matrix.

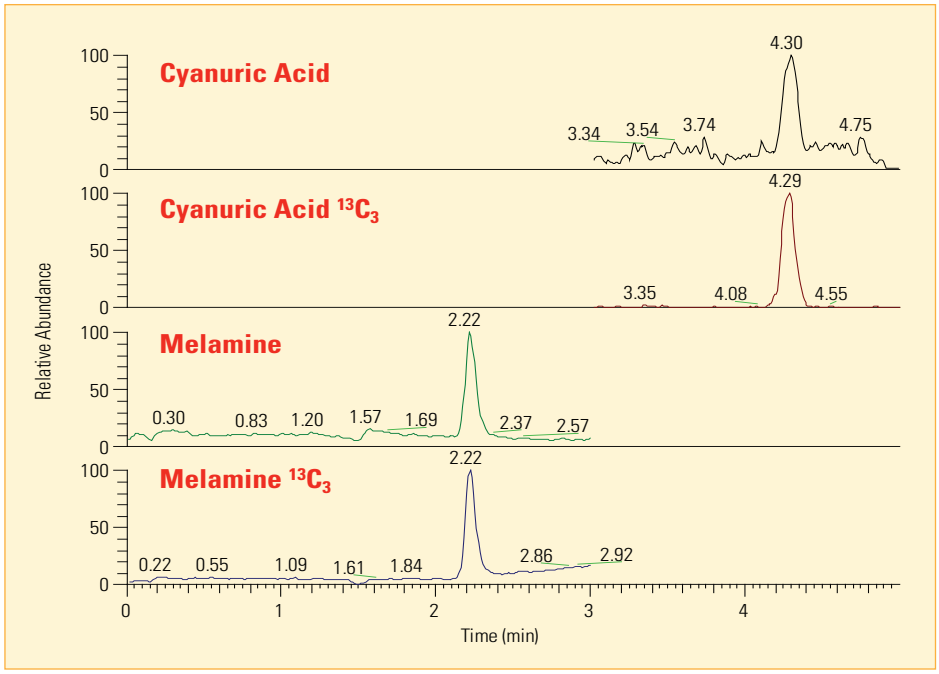

Figure 1. Melamine, cyanuric acid, and their internal standards at a concentration of 1 ppb. From top to bottom, cyanuric acid, cyanuric acid $^{13}C_3$, melamine, and melamine $^{13}C_3$.

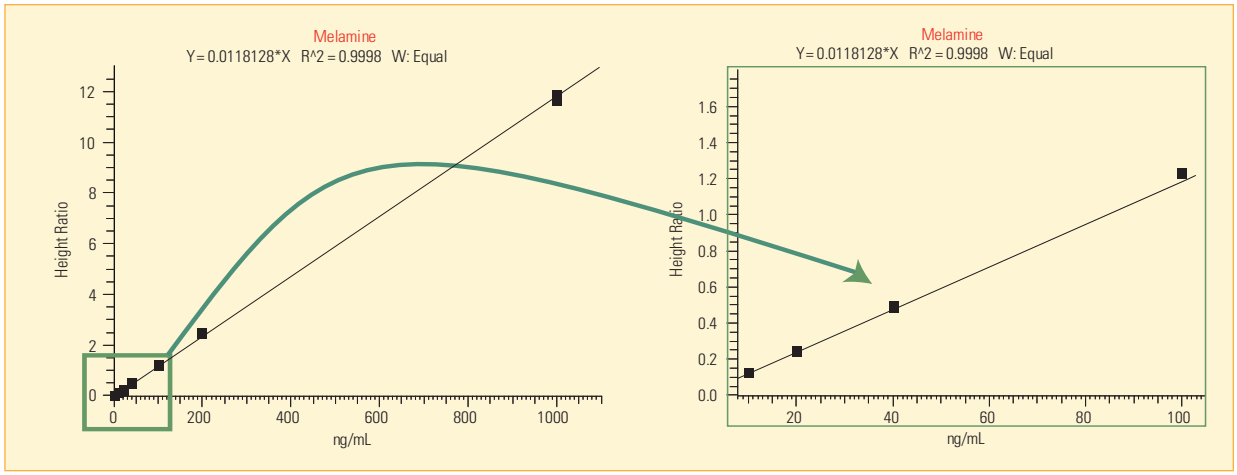

Figure 2. Calibration curve for melamine from 1-1000 ng/mL. The left figure shows the entire calibration range, while the right figure shows the expanded range from 10-100 ng/mL.

Additionally, full spectra data was collected using the standard Quantitation-Enhanced Data-Dependent MS/MS (QED-MS/MS) scan function. QED-MS/MS works by monitoring SRM data, and when the response of a particular SRM reaches a threshold level, the full scan MS/MS is activated. The resulting full scan spectra for melamine at 100 ppb and its internal standard are shown in Figure 5. The full scan data allows for further confirmation of results by eliminating "false positives" and also provides the opportunity to perform a library search. When a full scan QED-MS/MS spectra collected from a catfish sample spiked at 10 ppb was searched against the library, the library search returned melamine as the most likely hit. The results of the library search are shown in Figure 6. The spectrum of the sample and the spectrum that is stored in the library are visible in the lower left quadrant of the figure. The top spectrum is the catfish sample, while the lower spectrum is the reference spectrum. There is good agreement between the two spectra, even though the reference spectrum was generated using standards without matrix.

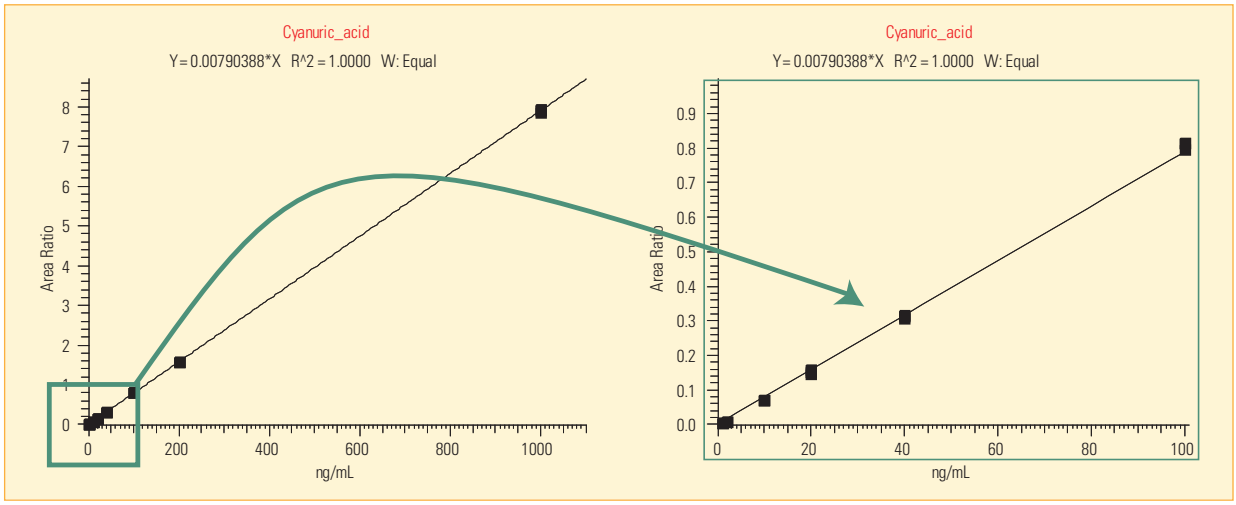

Figure 3: Calibration curve for cyanuric acid from 1-1000 ng/mL. The left figure shows the entire calibration range, while the right figure shows the expanded range from 1-100 ng/mL.

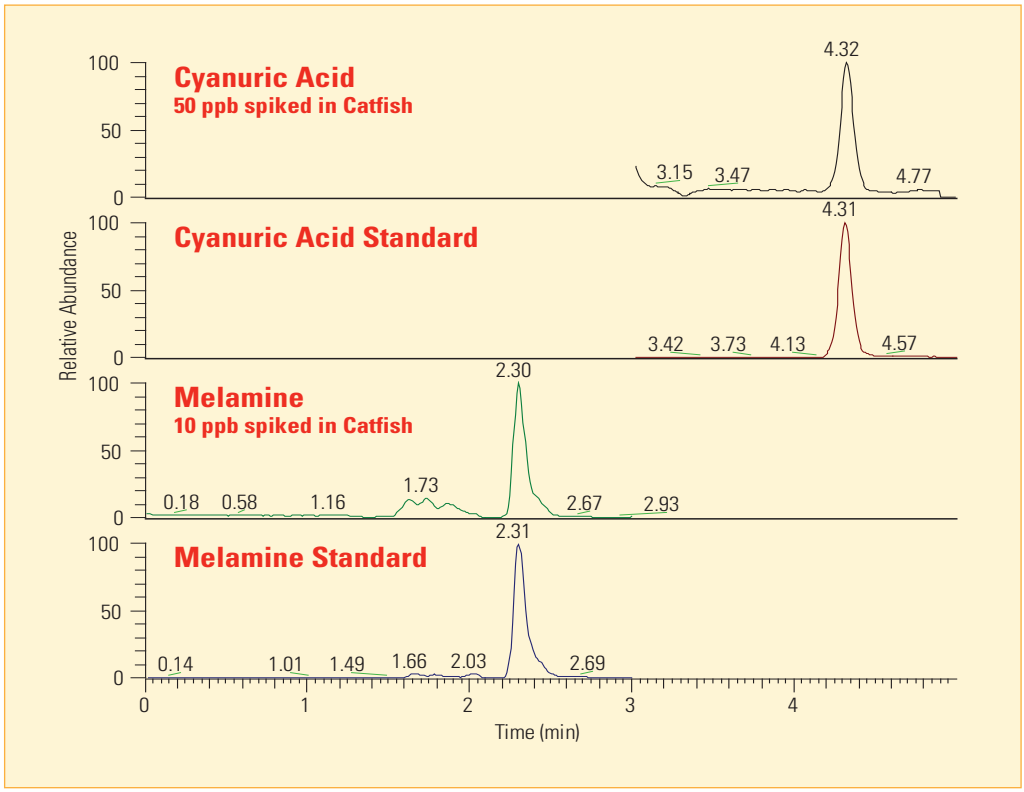

Figure 4: Chromatogram of cyanuric acid and melamine spiked into catfish matrix, at a level of 50 ppb for cyanuric acid, and 10 ppb for melamine

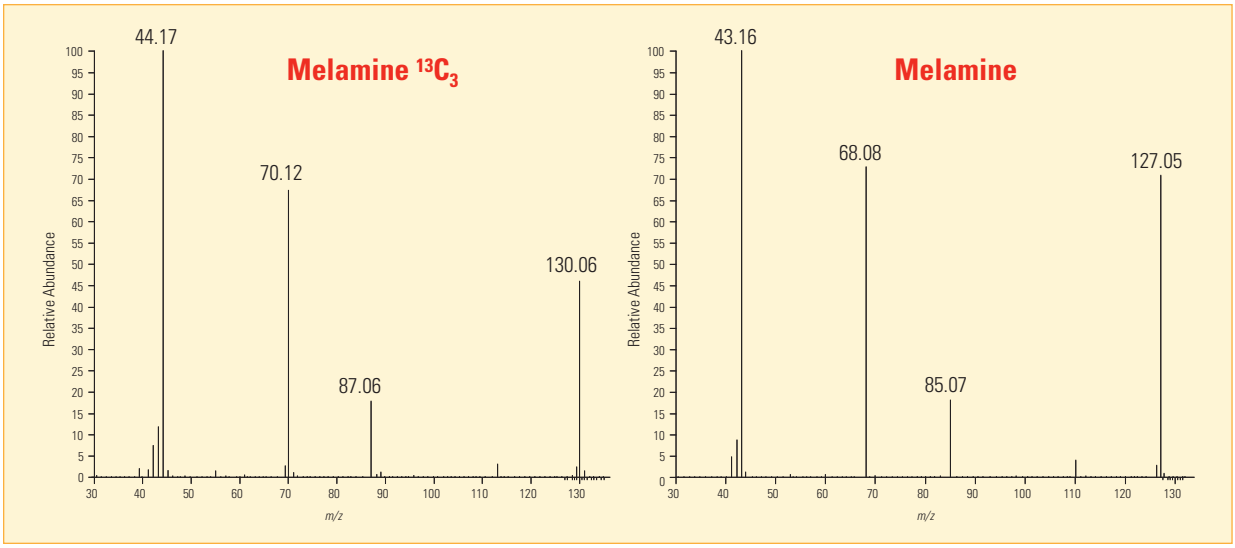

Figure 5: QED-MS/MS spectra for melamine $^{13}C_3$ (left) and melamine (right). Unique, rich, library-searchable spectra are collected in the same chromatographic run, allowing both quantitative and confirmatory full scan data in the same file.

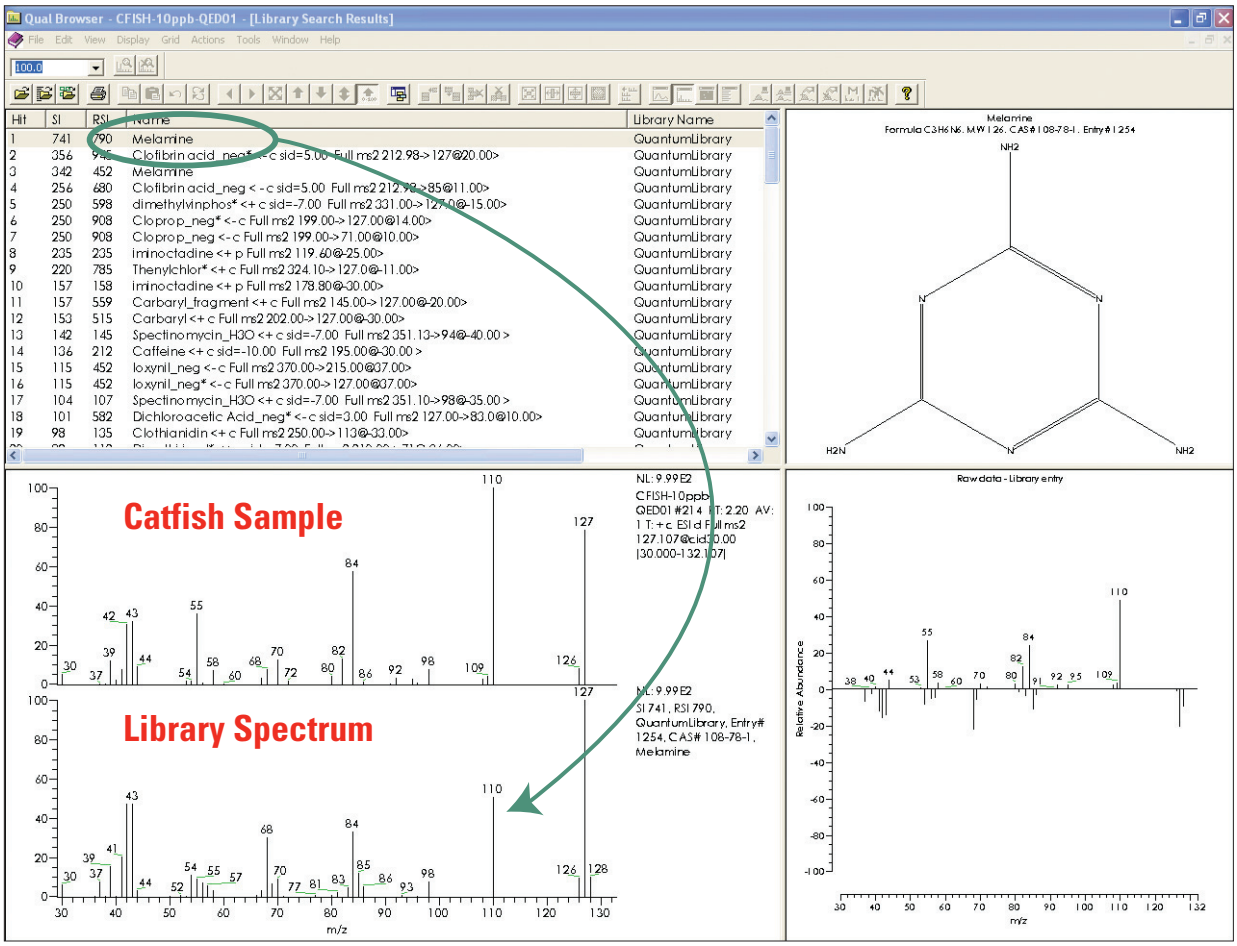

Figure 6: Library search results for melamine spiked at 10 ppb into a catfish matrix. Melamine is the top hit in the search list.

Conclusion

A simple, sensitive, and specific method for the detection and quantitation of melamine and cyanuric acid in food matrices has been demonstrated. The method is robust and allows for the analysis of a large number of samples, without degradation in column performance. Additionally, full scan spectra for Q3 are collected in the same chromatographic run using the QED-MS/MS scan function, permitting a library search of the results to eliminate any false positives.

References:

[1] Weis, E. Nestlé Purina, Hills join pet food recall. *USA Today*. Available at http://www.usatoday.com/news/nation/2007-03-31-pet-food-recall_N.htm. Accessed 10 December 2007.

[2] Aarthi Sivaraman. Melamine in pet food, wheat gluten from China: FDA. Reuters. Available at http://www.reuters.com/article/domesticNews/idUSWEN594320070330. Accessed 10 December 2007.

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Application Notebook

Additional Contaminants

Illegal Dyes

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Analysis of Illegal Dyes in Food Matrices Using Automated Online Sample Preparation with Liquid Chromatography-Mass Spectrometry

Yang Shi, Catherine Lafontaine, and François A. Espourteille

Thermo Fisher Scientific, Franklin, MA, USA

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Overview

Purpose: To develop a rapid and sensitive automated online sample preparation LC-MS/MS method to detect and quantify multiple Sudan dyes in a variety of matrices and also to shorten assay time and increase throughput.

Methods: Automated online sample preparation using Thermo Scientific TurboFlow technology coupled with the Thermo Scientific Exactive benchtop Orbitrap mass spectrometer.

Results: A TurboFlow™ online multi-residue screening method for complicated food matrices utilizing full scan accurate mass detection was developed.

Introduction

Sudan dyes are red dyes used for coloring solvents, oils, waxes, petrol, or as additives in shoe and floor polish. In addition, they have been found in a number of food products such as chili or chili-containing products. Sudan dyes are banned as food additives in the USA[1], the EU[2,3], and many other countries, due to their links to cancer and other negative health effects.

Liquid chromatography-ultraviolet-visible (LC–UV–vis) and liquid chromatography-mass spectrometry (LC–MS) are currently the most popular methods for analysis of Sudan dyes. Traditional sample preparation methods, especially solid phase extraction (SPE), have been widely used in the determination of Sudan dyes. However, these procedures can be labor-intensive, time-consuming and costly, resulting in low sample throughput when performed manually. Lower recoveries have been also noticed associated with SPE cleanup.[4] There is consensus that one of the major scientific challenges in Sudan dyes analysis is to achieve high sensitivity and selectivity while keeping minimal sample clean up.[5]

In this study we describe an easy, comprehensive LC method using a Thermo Scientific Transcend TLX-1 system powered by TurboFlow technology to analyze five illegal dye residues in a variety of sauces.

Methods

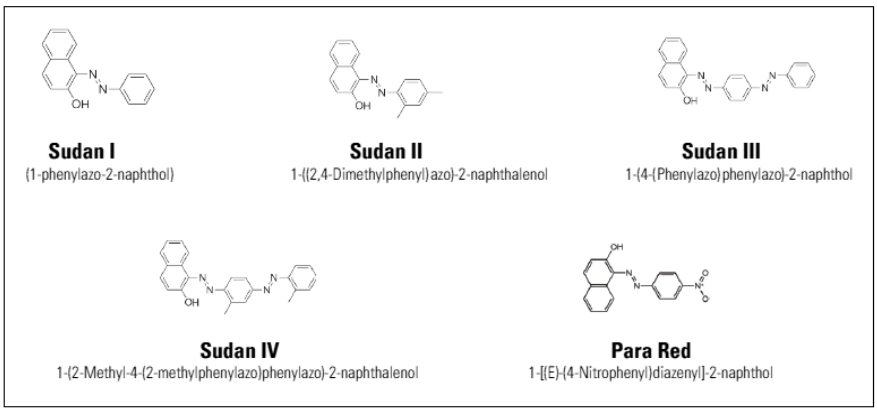
The matrix standard curve

Five analytes, Sudan I, Sudan II, Sudan III, Sudan IV and Para Red (Figure 1) were obtained from Sigma Aldrich (St. Louis, MO). A total of four different food products purchased from local grocery stores were used in this study: Chili Sauce I; Chili Sauce II; Hot Sauce I; Hot Sauce II.

Three grams of each homogenized matrix were weighed into a 50 mL centrifuge tube, followed by the addition of 30 mL acetonitrile (ACN). The tube was vortexed for 10 minutes and then sonicated for another 60 minutes. The resulting solutions were centrifuged at 10,000 RPM for 15 minutes. The supernatant was then filtered through a 0.45 mm syringe filter. No additional clean up of the sample solution was performed. Each milliliter of supernatant corresponds to 0.1 g semi-solid food matrix as the unit of conversion.

A calibrant stock solution was prepared at a final concentration of 1 mg/mL of each analyte in ACN. A range of calibration solutions from 0.5 to 100 ng/mL (equals to 5 ng/g to 1000 ng/g) were made by serial dilutions using individual produced supernatants.

FIGURE 1. Chemical structure of test compounds



LC/MS Methods

Thermo Scientific TurboFlow Method Parameters

Column:	TurboFlow XL C8 column 0.5 x 50 mm
Injection Volume:	25 µL
Solvent A:	0.1% formic acid in Water
Solvent B:	0.1% formic acid in ACN
Solvent C:	1:1
ACN:	isopropanol

HPLC Method Parameters

Analytical Column:	Thermo Scientific Accucore Phenyl Hexyl 3X50 mm 2.6 µm
Solvent A:	0.1% formic acid in water
Solvent B:	0.1% formic acid in ACN

The interference molecules from the matrix were unretained and moved to waste during the loading step of TurboFlow column, while the analyte of interest was retained on the extraction column. This was followed by organic elution to the analytical column and gradient elution to the MS.

Mass Spectrometer Parameters

MS:	Thermo Scientific Exactive benchtop Orbitrap™ MS
MS Ionization Source:	Heated Electrospray Ionization (H-ESI)
Scan Range:	240.0 to 390.0 m/z
Resolution:	50,000
Spray Voltage:	4 KV
Sheath Gas (N_2):	70 arbitrary units
Aux Gas (N_2):	40 arbitrary units
Heater Temperature:	400 °C
Capillary Temperature:	350 °C
Capillary Voltage:	27.5
Tube lens Voltage:	95 V
Skimmer Voltage:	22 V
Polarity:	Positive

Data Analysis

The system was controlled by Thermo Scientific Aria OS software. Data acquisition was performed using Thermo Scientific Xcalibur software. The resulting data were processed with Thermo Scientific LCQUAN quantitative software. The accurate masses of analytes are listed in Table 1.

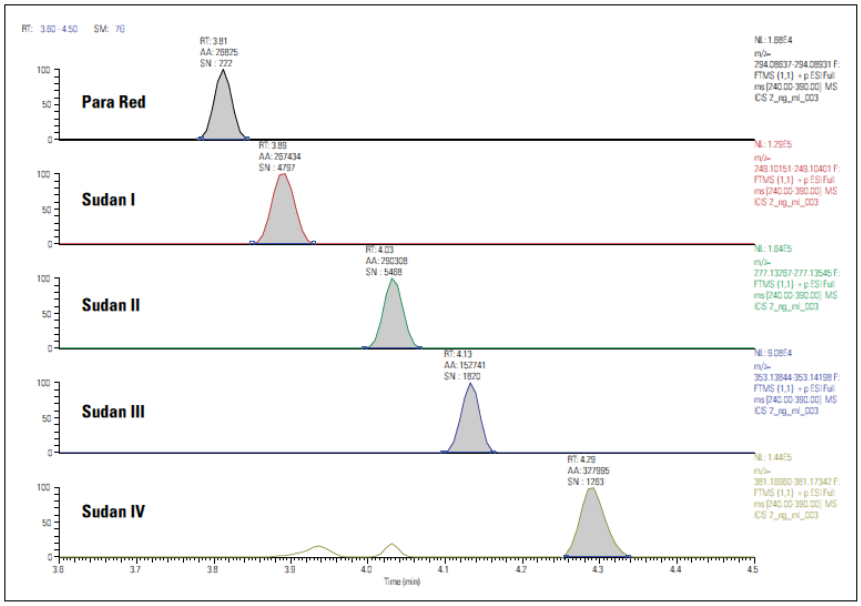
TABLE 1. Molecular formula, theoretical mass of test compounds

Compound	Formula	Exact Mass (m/z)	[M+H]+ (m/z)
Para red	$C_{16}H_{11}N_3O_3$	293.080041	294.087841
Sudan I	$C_{16}H_{12}N_2O$	248.094963	249.102763
Sudan II	$C_{18}H_{16}N_2O$	276.126263	277.134063
Sudan III	$C_{22}H_{16}N_4O$	352.132411	353.140211
Sudan IV	$C_{24}H_{20}N_4O$	380.163711	381.171511

Results and Discussion

Figure 2 shows the representative chromatograms of 5 analytes at 20 ng/g (2 ng/mL) in hot sauce II extract. For the concentration range studied (5-1000 ng/g), limits of quantitation (LOQs) were estimated from triplicate injections (coefficient of variation <15%) of standard solutions at concentration levels. Both the area precision and mean accuracy were below 20% at LOQ. As shown in Table 2, LOQs ranged from 5-20 ng/g for all analytes except para red in 4 studied sauces. A lower LOQ could be achieved by increasing sample injection volume because TurboFlow columns can handle larger injections (up to a few hundred microliters) while regular high performance LC (HPLC) or Ultra HPLC (UHPLC) columns cannot. Good linearity was observed over the entire tested range. The correlation coefficients obtained using weighted (1/x) linear regression analysis of standard curves were greater than 0.99 for all analytes.

FIGURE 2. Representative chromatogram (20 ng/g in Hot Sauce II)



To further assess the reproducibility of the present methodology, a relative standard deviation (%RSD) test was performed on all matrices fortified with analytes at 100 ng/g. Table 2 indicates that the RSDs of six replicate injections were less than 10% for the majority of analytes. These results show the feasibility of the current approach for dyes determination in food matrices.

A recovery study was performed on the four matrices fortified with analytes at 100 ng/g. The recovery was assessed by comparing the detector response of a post-extracted spiked sample with that determined from a spiked neat standard sample at the same concentration. As shown in Figure 3, recoveries were 80-120% for most analytes in all matrices except chili sauce II extract, which indicates no significant matrix effects for the majority of analytes. These matrix-matched calibration curves can be used to overcome matrix effects and calculate concentrations of these illegal dyes in routine lab work.

TABLE 2. Quantitation limit, linearity and relative standard deviation (%RSD) of analytes in four tested matrices

Chili Sauce I

Compound	LOQ (ng/g)	R^2	%RSD (n=6 at 100 ng/g)
Para Red	20	0.9955	9.64
Sudan I	10	0.9960	3.64
Sudan II	10	0.9936	6.24
Sudan III	10	0.9937	7.45
Sudan IV	5	0.9911	4.55

Chili Sauce II

Compound	LOQ (ng/g)	R^2	%RSD (n=6 at 100 ng/g)
Para Red	50	0.9900	4.50
Sudan I	10	0.9906	6.26
Sudan II	10	0.9920	10.82
Sudan III	10	0.9942	10.29
Sudan IV			

The data for Sudan IV was not quantifiable.

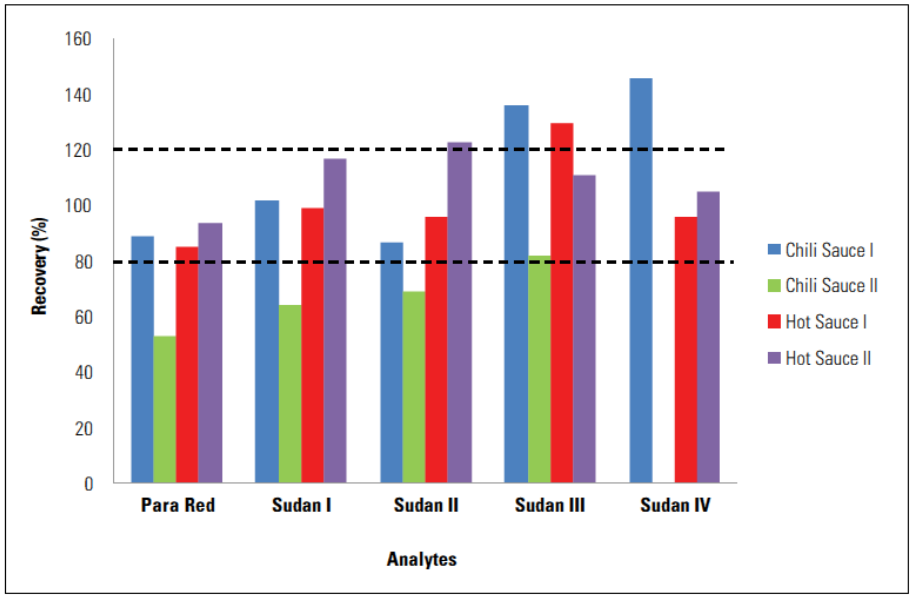
Hot Sauce I

Compound	LOQ (ng/g)	R^2	%RSD (n=6 at 100 ng/g)
Para Red	20	0.9952	4.92
Sudan I	5	0.9980	2.88
Sudan II	10	0.9969	5.11
Sudan III	10	0.9959	2.21
Sudan IV	20	0.9980	4.35

Hot Sauce II

Compound	LOQ (ng/g)	R^2	%RSD (n=6 at 100 ng/g)
Para Red	20	0.9973	7.62
Sudan I	5	0.9981	2.66
Sudan II	5	0.9976	5.36
Sudan III	5	0.9970	6.23
Sudan IV	5	0.9974	2.95

FIGURE 3. Recoveries of 5 analytes fortified in all tested matrices at 100 ng/g



Conclusion

The current method has been tested with four different sauces. Linearity, specificity, recovery and repeatability of the method have been established. Sample preparation time of this strategy was minimal. Not including sonication and centrifugation times, the sample preparation only took 15 minutes. Additionally, since all analytes were eluted within less than one minute of a total six-minute LC run, multiplexing with a Transcend TLX-4 system would further reduce total LC-MS/MS run time four-fold and enable screening of more than 30 samples per hour. Future work could involve screening a larger range of illegal dyes, thus combining a screening method with accurate quantification.

References

1. U.S. Title 21 of the Code of Federal Regulation Part 73, Subpart A
2. Commission Decision of 20 June 2003 on emergency measures regarding hot chili and hot chili products (notified under document number C(2003) 1970) 2003/460/EC
3. Commission Decision of 21 January 2004 on emergency measures regarding chili and chili products (notified under document number C(2004) 68) 2004/92/EC
4. R. Rebane, I. Leito, S. Yurchenko, K. Herodes, A review of analytical techniques for determination of Sudan I–IV dyes in food matrixes, J Chromatogr. A, 1217 (2010) 2747-2757
5. J. Gilbert, Analytical Challenges in Food Safety Advances in instrumental methods for food analysis, Go-Global Seminar, Bangkok, November 27th, 2007

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Application Note: 560

Analysis of Illegal Dyes in Food Matrices using Automated Online Sample Preparation with LC/MS

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Thermo Fisher Scientific, Franklin, MA

Key Words

- Transcend TLX-1
- TurboFlow Technology
- Exactive
- Accucore HPLC Columns

Introduction

Sudan dyes are red dyes used for coloring solvents, oils, waxes, petrol, or as additives in shoe and floor polish. In addition, they have been found in a number of food products such as chili or chili-containing products. Sudan dyes are banned as food additives in the USA[1], the EU[2,3] and many other countries due to links to cancer and other negative health effects.

Liquid chromatography-ultraviolet-visible (LC–UV–vis) and liquid chromatography-mass spectrometry (LC/MS) are currently the most popular methods for analysis of Sudan dyes.[4] Traditional sample preparation methods, especially solid phase extraction (SPE), have also been widely used in the determination of Sudan dyes. However, these procedures can be labor-intensive, time-consuming and costly, resulting in low sample throughput when performed manually. Lower recoveries have also been noticed associated with SPE cleanup.[4] There is consensus that one of the major scientific challenges in the analysis of Sudan dyes is to achieve high sensitivity and selectivity while minimizing sample clean up.[5]

In this study we describe an easy, comprehensive LC method using a Thermo Scientific Transcend TLX-1 system powered by TurboFlow™ technology coupled to a Thermo Scientific Exactive MS to analyze five illegal dye residues in a variety of sauces.

Goal

Develop a rapid and sensitive automated online sample preparation LC-MS/MS method to detect and quantify multiple Sudan dyes in a variety of food matrices and also to shorten assay time and increase throughput.

Experimental

The Matrix Standard Curve

Five analytes, Sudan I, Sudan II, Sudan III, Sudan IV and Para Red (Figure 1) were obtained from Sigma-Aldrich (St. Louis, MO). A total of four different food products purchased from local grocery stores were used in this study: Chili Sauce I; Chili Sauce II; Hot Sauce I; Hot Sauce II.

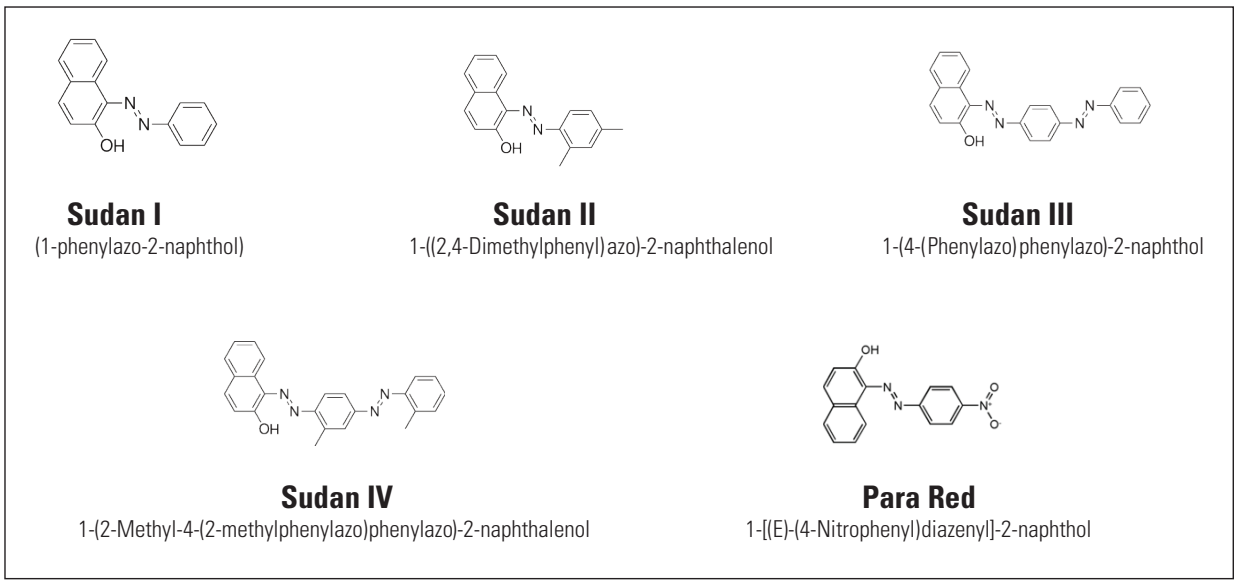

Figure 1. Chemical structure of test compounds

Three grams of each homogenized matrix were weighed into a 50-mL centrifuge tube, followed by the addition of 30 mL of acetonitrile (ACN). The tube was vortexed for 10 minutes and then sonicated for another 60 minutes. The resulting solution was centrifuged at 10,000 RPM for 15 minutes. The supernatant was then filtered through a 0.45-mm syringe filter. No additional clean up of the sample solution was performed. Each milliliter of supernatant corresponds to 0.1 g semi-solid food matrix as the unit of conversion.

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LC/MS Methods

Thermo Scientific TurboFlow Method Parameters

Column:	TurboFlow XL C8 column 0.5 x 50 mm
Injection Volume:	25 µL
Solvent A:	0.1% formic acid in water
Solvent B:	0.1% formic acid in ACN
Solvent C:	1:1 ACN: isopropanol

HPLC Method Parameters

Analytical Column:	Thermo Scientific Accucore Phenyl-Hexyl 50 x 3 mm, 2.6 µm particle size
Solvent A:	0.1% formic acid in water
Solvent B:	0.1% formic acid in ACN

Thermo Scientific Accucore HPLC columns use Core Enhanced Technology™ to facilitate fast and high efficiency separations. The 2.6 µm diameter particles are not totally porous, but rather have a solid core and a porous outer layer. The optimized phase bonding creates a series of high coverage, robust phases. The tightly controlled 2.6 µm diameter of the particles results in much lower backpressures than typically seen with sub-2 µm materials.

Mass Spectrometer Parameters

MS:	Thermo Scientific Exactive high performance benchtop Orbitrap™ MS
MS Ionization Source:	Heated Electrospray Ionization (H-ESI)
Ionization Mode:	Positive
Scan Range:	*m/z* 240.0 to 390.0
Resolution:	50,000
Spray Voltage:	4 KV
Sheath Gas Pressure (N_2):	70 arbitrary units
Auxiliary Gas Pressure (N_2):	40 arbitrary units
Heater Temperature:	400 °C
Capillary Temperature:	350 °C
Capillary Voltage:	27.5 V
Tube Lens Voltage:	95 V
Skimmer Voltage:	22 V

The interference molecules from the matrix were unretained and moved to waste during the loading step of the TurboFlow column, while the analyte of interest was retained on the extraction column. This was followed by organic elution of the analytes to the analytical column and gradient elution to the MS. The system was controlled by Thermo Scientific Aria OS. Data acquisition was performed using Thermo Scientific Xcalibur software. The resulting data were processed with Thermo Scientific LCQUAN quantitative software. The accurate masses of the analytes are listed in Table 1.

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Para Red	$C_{16}H_{11}N_3O_3$	293.080041	294.087841
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Figure 2 shows the representative chromatograms of the 5 analytes at 20 ng/g (2 ng/mL) in Hot Sauce II extract. For the concentration range studied (5-1000 ng/g), all limits of quantitation (LOQs) were estimated from triplicate injections (coefficient of variation < 15%) of standard solutions. The area precision and mean accuracy were below 20% at LOQ. As shown in Table 2, the LOQs ranged from 5-20 ng/g for all analytes except Para Red in four of the sauces studied. A lower LOQ could possibly be achieved by increasing sample injection volume because TurboFlow columns can handle larger injections (up to a few hundred microliters) while regular high performance LC (HPLC) or Ultra HPLC (UHPLC) columns cannot. Good linearity was observed over the entire tested range of each analyte. The correlation coefficients obtained using weighted (1/x) linear regression analysis of standard curves were greater than 0.99 for all analytes.

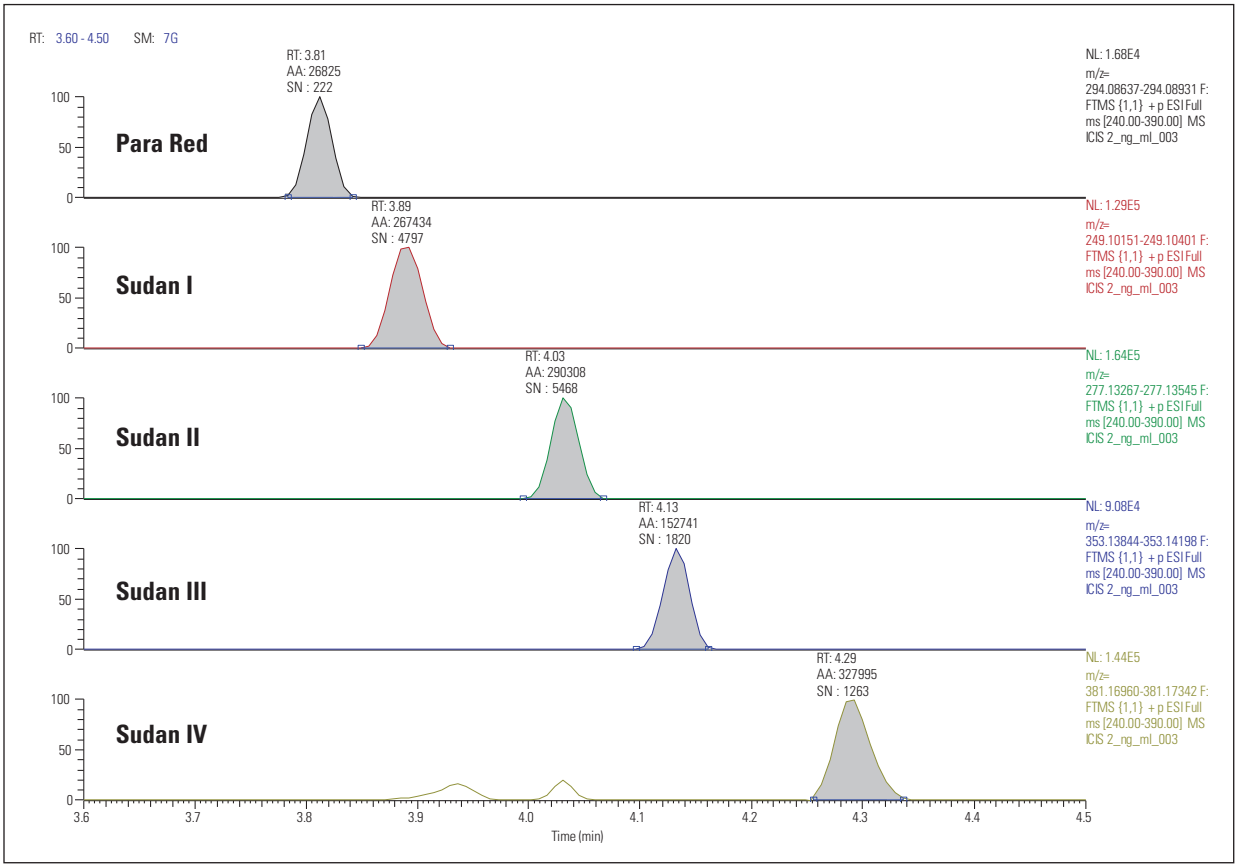

Figure 2. Representative chromatogram (20 ng/g in Hot Sauce II)

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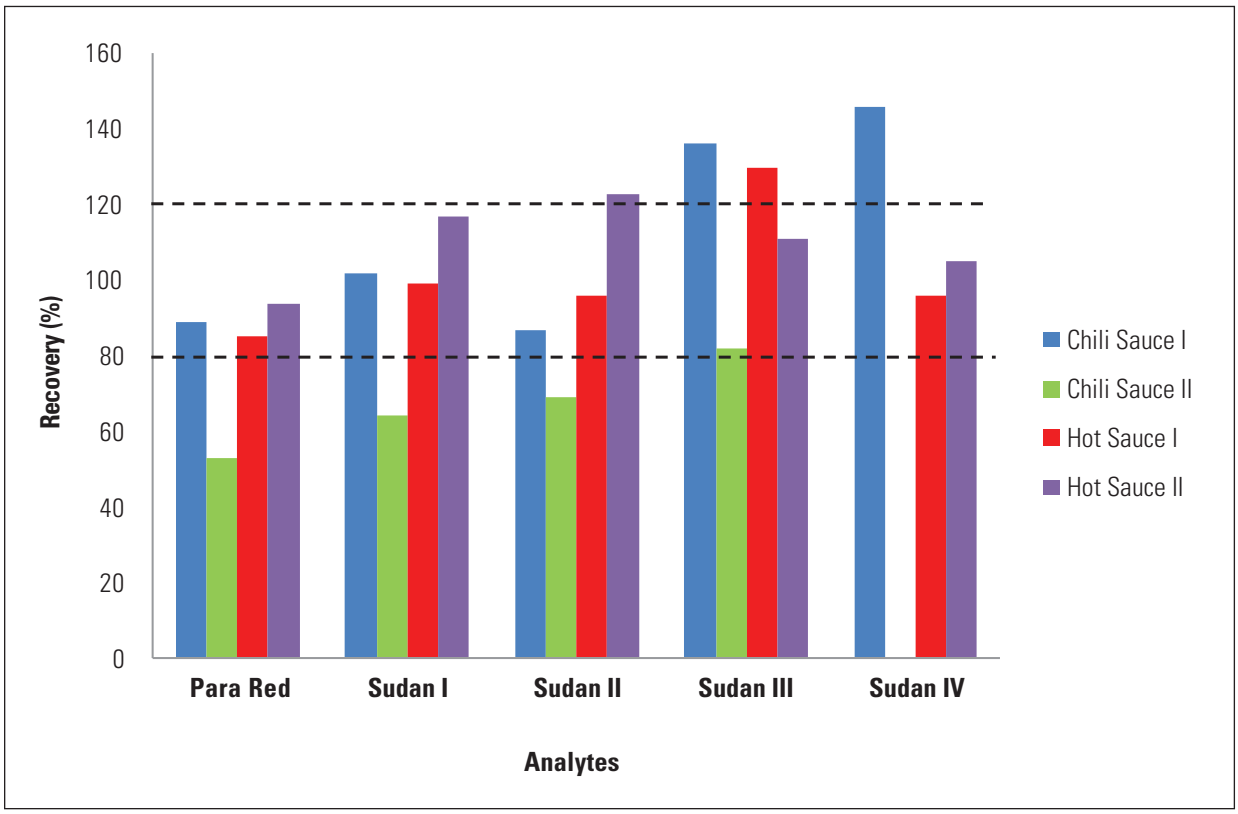

Figure 3. Recoveries of 5 analytes fortified in all tested matrices at 100 ng/g

Application Notebook

Additional Contaminants

Packaging Contaminants

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High-Resolution Accurate-Mass (HRAM) Phthalate Screening using Direct Analysis in Real Time (DART) Ambient Ionization

Catharina Crone,[1] Markus Kellmann,[1] Yue Xuan,[1] Elizabeth Crawford[2]
[1]Thermo Fisher Scientific, Bremen, Germany; [2]IonSense, Inc., Saugus, MA, USA

Thermo SCIENTIFIC

Overview

Purpose:

Demonstrating the analysis of phthalic acid diesters on a high-resolution accurate-mass Thermo Scientific™ Orbitrap™ analyzer-based system coupled to a “Direct Analysis in Real Time” (DART™) ionization source. Due to the presence of a quadrupole mass filter and a collision cell, the system is capable of selective precursor isolation for higher energy collision induced dissociation (HCD) in order to distinguish different isomers and to confirm possible positive hits.

Methods:

A “Direct Analysis in Real Time” (DART) ionization device was coupled to an Orbitrap-based mass spectrometer in order to directly analyze different samples without the need for sample preparation and to avoid phthalate contamination during preparation steps. The background spectra were carefully monitored for ambient phthalate background levels during sample analysis and for instrument inlet contamination. Results of full scan MS analysis and HCD fragmentation of standards were compared with spectra obtained from commercial food packaging and food contact materials.

Results:

Phthalic acid diester standards could be distinguished by their accurate mass in full scan analysis and their specific fingerprint after performing full scan MS2 analysis. Screening of the food packaging materials was done in less than 2 minutes per sample.

Introduction

Phthalic acid diesters (PAEs), also know as phthalates, are widely used in industry as a plasticizer in everyday products like toys, flooring, personal care products and food packages. These compounds can be present up to a high ratio in some materials, for example, an average of 30% w/w in PVC [1]. As substances classified as semi-volatile organic compounds (SVOC), they have been known to evaporate over a long time.

Some of the PAEs have been classified as hazardous, affecting mainly the reproductive system and possibly increasing the risk of cancer [2,3]. The use of these compounds is officially under regulation but still, these phthalates might be present and used during production of goods.

Former studies using high-resolution, accurate-mass (HRAM) mass spectrometry were performed on a benchtop Orbitrap system in full scan mode [4]. In the presented study, advantage was taken of the possibility of using precursor ion selection for MS2. Obtaining a full scan MS2 spectrum, the occurring fragmentation pattern could be used as a fingerprint for the characterization of the different compounds, especially of the different isomers for nominal mass to charge ratios 279 and 391.

Due to their presence in indoor environments, the cross contamination during analysis has to be kept as low as possible. Main sources of contamination are glassware and organic solvents. Therefore, a direct examination without the need of sample extraction and chromatography is of great advantage for these kind of analyses. Still, background coming from carpets etc. has to be critically monitored and subtracted from the sample derived signals.

Methods

Mass Spectrometry

All data was acquired using a Thermo Scientific™ Q Exactive™ mass spectrometer (Figure 1) coupled to a direct analysis in real time (DART) SVPA ion source (IonSense Inc., Saugus, MA, USA). The DART source was operated at 200 ºC using helium as carrier gas.

Source settings for the mass spectrometer were 200 ºC for the capillary transfer tube and a S-Lens level of 50 arbitrary units.

The mass spectrometer was operated in full scan mode (positive ionization mode, mass range *m/z* 100-1000, AGC target 1e6 charges, R=140k), SIM mode (positive ionization mode, isolation width at full width half maximum 2u, AGC target 1e5 charges, R=140k) as well as MS2 mode (positive ionization mode, isolation width at full width half maximum 2u, AGC target 2e5 charges, R=140k).

For HCD fragmentation, the normalized collision energy (NCE) was set to 10 arbitrary units. Targets were chosen for inclusion list with regards to Table 1.

All scan events were scheduled in one method setup resulting in a total method duration of 1.2 minutes (Figure 2). After starting the acquisition, no sample was introduced for up to 0.3 minutes in order to get the background level. Samples were introduced at 0.3 minutes starting with the full scan analysis.

FIGURE 1. Schematics of Q Exactive instrument.

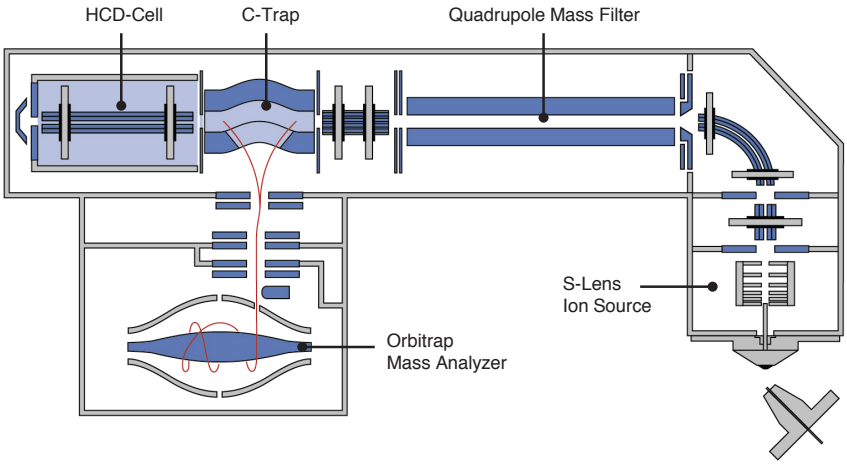


FIGURE 2. Method setup for the phthalate screening method. The method includes full scan analysis as well as targeted SIM and targeted MS2 for the chosen compounds of interest (Table 1). The first 0.3 minutes were scheduled in order to get the background level information without introducing the samples.

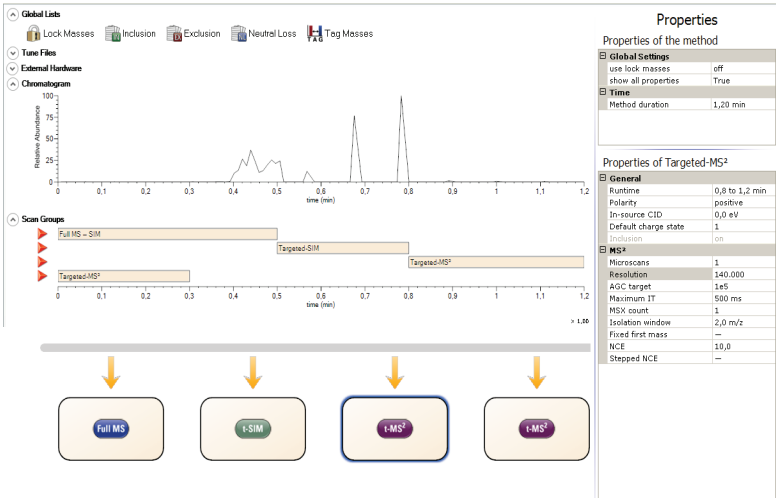


TABLE 1. List of analyzed phthalate standards. Exact masses of [M+H]+ for precursors as well as for a selection of characteristic HCD fragments are displayed.

Compound	Elemental composition	Precursor [M+H]+	Selection of characteristic HCD fragments m/z	Regulation
Di-n-butyl phthalate (DBP)	$C_{16}H_{22}O_4$	279.1591	167.0339; 205.0859; 223.0965	CA Prop 65
Diisobutyl phthalate (DiBP)	$C_{16}H_{22}O_4$	279.1591	167.0339; 205.0859	EU
Benzyl butyl phthalate (BBP)	$C_{19}H_{20}O_4$	313.1434	91.0542; 205.0859	CA Prop 65
Bis(2-ethylhexyl)phthalate (DEHP)	$C_{24}H_{38}O_4$	391.2843	167.0339; 279.1591	CA Prop 65
Di-n-octyl phthalate (DnOP)	$C_{24}H_{38}O_4$	391.2843	167.0339; 261.1485	CA Prop 65
Dioctylitere phthalate (DOTP)	$C_{24}H_{38}O_4$	391.2843	167.0339; 261.1485; 279.1591	-
Diisononyl phthalate (DiNP)	$C_{26}H_{42}O_4$	419.3156	127.1481; 275.1642; 293.1747	CA Prop 65
Diisodecyl phthalate (DiDP)	$C_{28}H_{46}O_4$	447.3469	141.1638; 289.1798; 291.1955; 307.1904	CA Prop 65

Sample preparation

Standards were purchased from Sigma Aldrich (St Louis, MO,USA) and were applied as liquids onto stainless steel mesh (Figure 3). Standards were introduced to the DART source using a motorized sample platform. The commercial packaging samples were introduced to the source by tweezers using an adjustable tweezer base in order to maintain a distinct distance from sample to instrument inlet (Figure 4).

FIGURE 3. DART SVP ionization source attached to a Q Exactive system. The tweezer is showing a lid gasket sample shortly before sampling.

FIGURE 4. Sampling set-up for phthalate standards for DART analysis. Stainless steel mesh screens used as the sampling surface for phthalate standards. Deposited liquid volume was about 3 µL.

Results

Standards and Background Ions

Phthalates are ubiquitously distributed in lab environments. Phthalate contamination during sample preparation might mimic or disturb the actual results. Using DART ionization, these contaminations can be minimized by eliminating sample preparation before sample analysis. Background signal was measured prior to each sample acquisition in order to monitor for ambient phthalates that could be detected with the sample.

The generated HCD fragments (Figure 5-8) were in accordance to the fragments obtained on a linear ion trap (data not shown) and to those described in the literature [5,6]. With regards to product ion ratios, the HCD fragmentation showed higher intensities for lower *m/z* fragments whereas the intensities of higher *m/z* species is lower. *m/z* 149 is MS2 base peak for all standards except DiDP.

FIGURE 5. HCD spectra of DNOP (A), DOTP (B) and DEHP (C) standards. All sample compounds show a precursor *m/z* of 391.2843. Spectra are showing the presence of *m/z* 261 and absence of *m/z* 167 as a specific marker for DNOP. All measured mass-to-charge ratios are within a mass deviation of ≤1.4 ppm compared to the calculated exact masses. Data was acquired with external mass calibration.

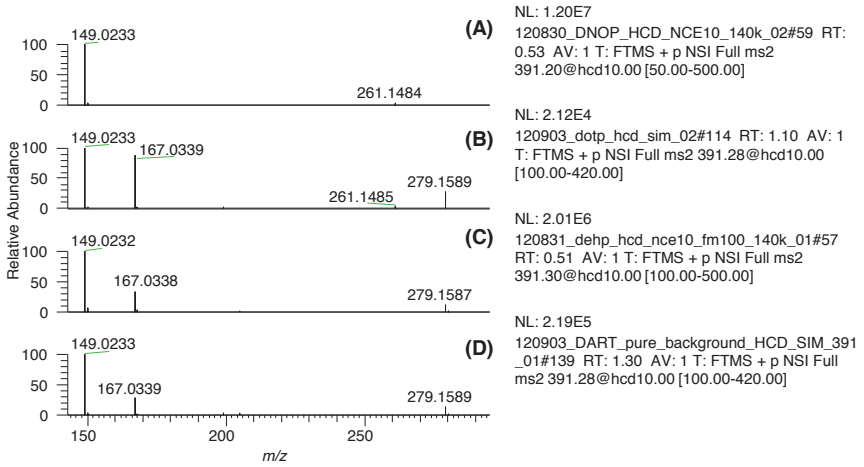


FIGURE 6. HCD spectra of DBP (A) and DIBP (B) standards. Zoom in to mass range 160-250. Proposed HCD fragments are assigned using Thermo Scientific™ Mass Frontier™ software. All measured mass-to-charge ratios are within a mass deviation ≤2.2 ppm compared to the calculated exact masses. Data was acquired with external mass calibration.

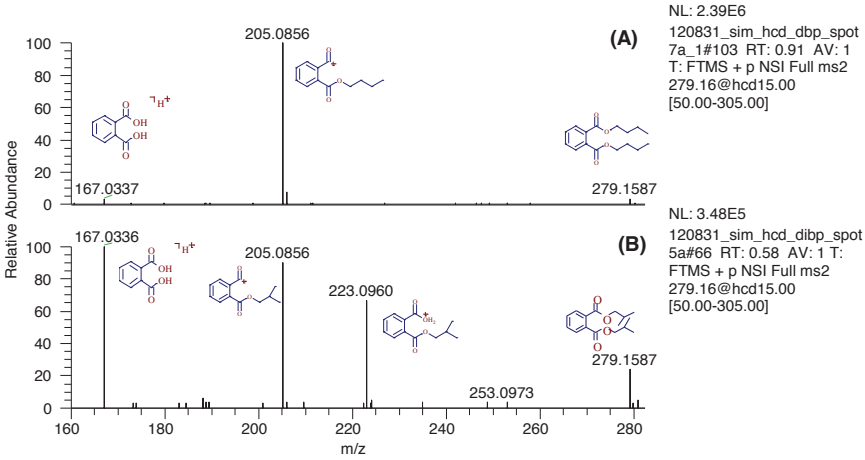


FIGURE 7. HCD spectra of DINP (A) and DIDP (B). Proposed HCD fragments are assigned using Mass Frontier software. All measured mass-to-charge ratios are within a deviation ≤1.9 ppm compared to the calculated exact masses. Data was acquired with external mass calibration.

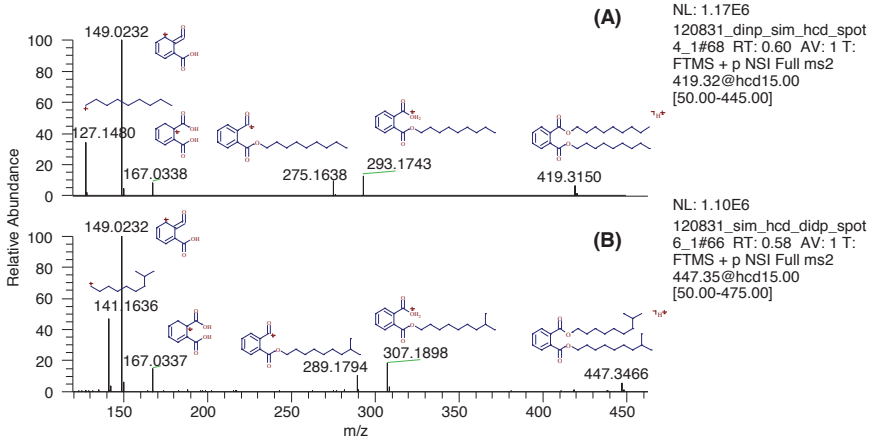
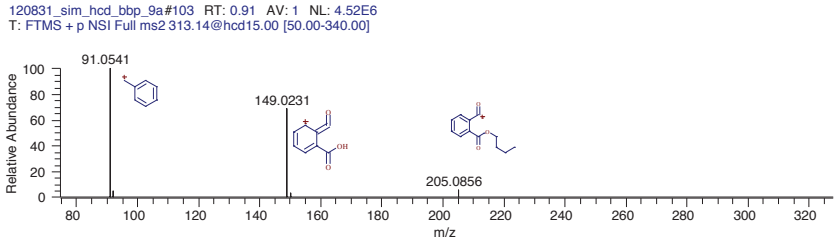


FIGURE 8. HCD spectrum of BBP. Proposed structures are assigned using Mass Frontier software. All mass-to-charge ratios are within a mass deviation ≤1.5 ppm compared to the calculated exact masses. Data was acquired with external mass calibration.



Food packaging material and everyday products

In total, 13 lid gaskets, 9 milk packages, 5 bags and fruit containers were tested. In addition, 7 plastic shoes, 12 wallet samples and 2 sports equipment were tested as well. No positive hit for any of the tested banned standard compounds (DEHP, DBP, DiBP, BBP) could be found in the samples. Full MS data revealed the presence of other compounds used in polymer and plastic industry like acetyl dibutyl citrate (ATBC), diethylhydroxylamine (DEHA), acetylated partial glycerides (AcPG) and erucamide could be identified (data not shown). For future work, reference material should be prepared containing defined ratios of standards for validating the screening method and for developing a quantitative approach.

The presented screening method offers a very fast and convenient setup for getting high-resolution, accurate-mass full scan data as well as getting the whole MS2 fragmentation pattern with the same quality.

Conclusion

- DART combined with Orbitrap-based HRAM LC-MS/MS was shown to be a very fast and convenient way for screening for additives in food packaging and other goods
- Due to ambient phthalate content, the background has to be carefully monitored before starting the analysis of each sample to avoid contamination
- For future work, defined reference material has to be prepared to work on a quantitative approach

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Method: 52251

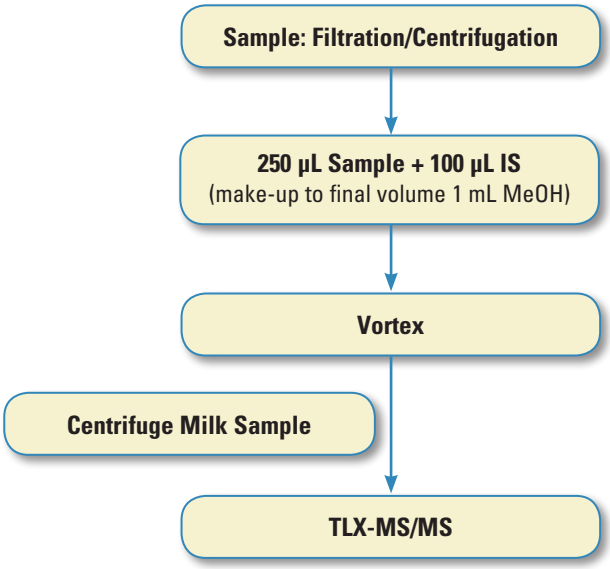
Analysis of Plasticizer Contaminants in Beverages and Milk using an Automated System Based on Turbulent-flow Chromatography Coupled to LC-MS/MS

Ebru Ates, Klaus Mittendorf, Thermo Fisher Scientific Food Safety Response Center, Dreieich, Germany

Key Words

- Transcend TurboFlow (TLX)
- TSQ
- Adipate
- Beverages and Milk
- Phthalates
- Plasticizer

1. Schematic of Method



2. Introduction

Phthalates are endocrine active chemicals used in a variety of consumer products. In some markets, restricted levels of certain phthalates are permitted for use in food contact materials, but they are not permitted as direct food additives.

Phthalates have been used to deliberately adulterate beverages and sports drinks in Taiwan, and phthalates and other plasticizers are widely found as ubiquitous contaminants particularly in fatty foodstuffs. Contamination arises from numerous sources such as the environment and food packaging. Cross-contamination with phthalates can easily arise during trace analysis in the laboratory and there are significant advantages in minimizing sample handling through online automated analysis. A method using online Thermo Scientific TurboFlow chromatography is presented.

3. Scope

This method can be applied to the determination of 10 phthalates and one adipate plasticizer at concentrations ranging from 0.05 to 100 mg/L (DMP, DEP, DPP, BBzP, DiBP, DcHP, DHP, DEHP, DEHA, DiNP, DiDP). The method has been validated for 11 plasticizers in beverages and milk.

4. Principle

This method employs an automated sample preparation technique involving sample concentration, cleanup and analytical separation in a single run using online sample prep with a Thermo Scientific Transcend TLX system powered by TurboFlow™ technology. TurboFlow technology serves as a novel sample preparation technique due to its special flow profile and both size exclusion and reversed phase column chemistry. TurboFlow technology enables very effective separation of matrix and target compounds resulting in relatively clean sample extracts. Identification of plasticizers is based on ion-ratios using selected reaction monitoring (SRM) of characteristic transition ions, and quantification of one of the selected SRM ions using labeled internal standards and external calibration.

5. Reagent List

		Part Number
5.1	Acetone, HPLC grade	A9494
5.2	Acetonitrile Optima, for LC-MS	A9554
5.3	Water Optima, for LC-MS	W64
5.4	Methanol Optima, for LC-MS	A4564
5.5	Formic acid	A11750
5.6	Isopropanol, HPLC grade	A4514

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6. Calibration Standards

6.1 Standards

6.1.1	Dimethyl phthalate (DMP)	Sigma Aldrich
6.1.2	Diethyl phthalate (DEP)	Sigma Aldrich
6.1.3	Diisobutyl phthalate (DIBP)	Sigma Aldrich
6.1.4	Dioctyl phthalate (DOP)	Sigma Aldrich
6.1.5	Butyl benzyl phthalate (BBzP)	Sigma Aldrich
6.1.6	Dihexyl phthalate (DHP)	Dr. Ehrenstorfer
6.1.7	Di-2-ethylhexyl adipate (DEHA)	Sigma Aldrich
6.1.8	Di(2-ethylhexyl)phthalate (DEHP)	Sigma Aldrich
6.1.9	Dicyclohexyl phthalate (DCHP)	Sigma Aldrich
6.1.10	Diisononyl phthalate (DiNP)	Sigma Aldrich
6.1.11	Diisodecylphthalate (DiDP)	Sigma Aldrich

6.2 Internal Standards

6.2.1	Bis(2-ethylhexyl) phthalate-3,4,5,6-d_4 (d_4-DEHP)	Dr. Ehrenstorfer
6.2.2	Diisobutyl phthalate-3,4,5,6-d_4 (d_4-DIBP)	Dr. Ehrenstorfer
6.2.3	Dihexyl phthalate 3,4,5,6-d_4 (d_4-DHP)	Sigma Aldrich

7. Standards Preparation

Rinse all glassware with methanol before pipetting plasticizer standards. Prepare fresh working standard mixtures weekly.

7.1 Stock Solution

Prepare individual standard stock solution of plasticizer standards in methanol at a concentration of 1 mg/mL in amber flasks. Store the standard stock solutions at 4 °C.

7.2 Working Mixture

Prepare two working mixtures. Prepare plasticizer mixture 1 (including DMP, DEP, DPP, BBzP, DiBP, DcHP and DHP) at 40 µg/mL in methanol. Prepare plasticizer mixture 2 (including DEHP, DEHA, DiNP, DiDP) at 200 µg/mL. Store the standard mixtures at 4 °C.

7.3 Stock Standard Solution of Internal Standard

Prepare individual internal standard stock solutions in methanol at a concentration of 1 mg/mL. Store the standard stock solutions at 4 °C.

7.4 Working Standard Solution of Internal Standard

Prepare a standard working mixture of internal standards in methanol at a final concentration of 5 µg/mL. Store the standard stock solutions at 4 °C.

8. Apparatus

		Part Number
8.1	Transcend™ TLX-1 system	
8.2	Thermo Scientific TSQ Quantum Access Max triple stage quadrupole mass spectrometer	
8.3	Precision balance	02225102
8.4	Sartorius analytical balance	14557812
8.5	Thermo Scientific Barnstead EASYpure II water	0905043
8.6	Ultrasonic bath Elmasonic S40H	15335101
8.7	Vortex shaker	14505141
8.8	Vortex standard cap	3205026
8.9	Centrifuge, Thermo Scientific Hereaus, Thermo Scientific Multifuge X3	503260260

9. Consumables

		Part Number
9.1	Thermo Scientific Hypersil GOLD, 150 × 2.1 mm 3 µm	25003152130
9.2	C18 XL 0.5 × 50 mm	CH953280
9.3	LC vials	3205111
9.4	LC vial caps	3151266
9.5	Pipette Finnpipette 10–100 µL	14386318
9.6	Pipette Finnpipette 100–1000 µL	14386320
9.7	Pipette Finnpipette 500–5000 µL	14386321
9.8	Pipette holder	14245160
9.9	Pipette Pasteur soda lime glass 150 mm	136786A
9.10	Pipette suction device	3120891
9.11	Pipette tips 0.5–250 µL, 500/box	21377144
9.12	Pipette tips 1–5 mL, 75/box	2137750
9.13	Pipette tips 100–1000 µL, 200/box	2137746
9.14	Glass tube	14957E

10. Glassware

		Part Number
10.1	Fisherbrand, Beaker, 25 mL	FB100250
10.2	Fisherbrand, Pasteur pipette	1367830
10.3	Fisherbrand, Volumetric flask, 10 mL	FB40010
10.4	Fisherbrand, Volumetric flask, 100 mL	10205C
10.5	Fisherbrand, Volumetric flask, 1000 mL	10205F

11. Procedure

11.1 Sample Preparation and Spiking

Samples of orange juice, bitter lemon and milk were spiked as described below.

Orange Juice

11.1.1 Squeeze one orange into a beaker (rinsed with methanol). Centrifuge (5 min at 5000 rpm) the juice by using 15 mL glass centrifuge tubes (rinsed with methanol). Spike in an LC vial, 250 µL of juice with 100 µL of IS working mixture and the appropriate amount of plasticizer standard working mixture. Fill up to a final volume of 1 mL with methanol and vortex for 10 sec.

Milk, (1.5% Fat) Pasteurized, Homogenized

11.1.2 Pipette 250 µL of milk directly into an LC vial. Add 100 µL of IS working mixture and the appropriate amount of plasticizer standard mixture. Fill up to final volume of 1 mL with methanol, vortex for 10 sec. Put the vial into a 15 mL glass centrifuge tube and centrifuge (5 min at 5000 rpm) to remove protein. Transfer the supernatant into an LC vial by the help of a glass pasteur pipette.

Bitter Lemon

11.1.3 Pipette 250 µL of bitter lemon (market sample) directly into an LC vial and spike with 100 µL of IS working mixture and the appropriate amount of plasticizer standard working mixture. Fill up to a final volume of 1 mL with methanol and vortex for 10 sec.

12. LC Operating Conditions

LC analysis is performed on a Transcend TLX System. The LC conditions are as follows:

TurboFlow Column: C18 XL, 0.5 × 50 mm
Analytical Column: Hypersil™ GOLD C18, 150 × 2.1 mm, 3 µm
Total Run Time: 22 min
Mobile Phase: A: acetonitrile (0.1% formic acid)
B: methanol (0.1% formic acid)
C: water (0.1% formic acid)

12.1 Injector Settings

Injector: Thermo Scientific Pal injector with 100 µL injection syringe volume
Wash solvents for the Autosampler
Solvent 1: acetonitrile/isopropanol/acetone (40/40/20)
Solvent 2: methanol

- Pre clean with solvent 1 [steps]: 3
- Pre clean with solvent 2 [steps]: 3
- Pre clean with sample [steps]: 0
- Filling speed [µL/s]: 50
- Filling strokes [steps]: 1
- Injection port: LC Vlv1 (TX channel)
- Pre inject delay [ms]: 500
- Post inject delay [ms]: 500
- Post clean with solvent 1[steps]: 5
- Post clean with solvent 2 [steps]: 5
- Valve clean with solvent 1[steps]: 5
- Valve clean with solvent 2 [steps]: 5

Injection Volume: 10 µL
Tray Temperature: 10 °C

13. Mass Spectrometric Conditions

MS analysis is carried out using a TSQ Access MAX™ triple quadrupole mass spectrometer controlled by Thermo Scientific Aria software. Data acquisition and processing is performed using Thermo Scientific Xcalibur 2.1 software.

13.1 Mass Spectrometer Conditions

Ionization: Electrospray (ESI)
Polarity: Positive ion mode
Spray Voltage [V]: 3500
Ion Sweep Gas Pressure [arb]: 0
Vaporizer Temperature [°C]: 350
Sheath Gas Pressure [arb]: 50
Aux Gas Pressure [arb]: 15
Capillary Temperature [°C]: 270
Collision Gas Pressure [mTorr]: 0
Cycle Time [s]: 0.8
Scan Mode: timed selected reaction monitoring (tSRM)

The mass spectrometer is programmed with 28 timed segments which is set up for the 11 target plasticizers and 3 labeled internal standards. For each compound one quantifier and one qualifier ion is monitored, including the respective labeled internal standard. The program of segments for tSRM events is shown in Table 2.

	Loading Pump									Eluting Pump				
Step	Step [min]	sec	Flow [mL/min]	Grad	A	B	C	Tee	Loop	Flow [mL/min]	Grad	A	B	C
1	0	30	2.0	Step	–	–	100	–	Out	0.7	Step	40	30	30
2	0.5	60	0.4	Step	40	30	30	T	In	0.3	Step	40	30	30
3	1.5	330	1.0	Step	–	100	–	–	In	0.7	Ramp	1	98	1
4	7.0	480	0.7	Step	–	75	25	–	In	0.7	Step	1	98	1
5	15.0	240	0.5	Step	–	–	100	–	Out	0.7	Ramp	40	30	30
6	19.0	180	0.5	Step	–	–	100	–	Out	0.7	Step	40	30	30

Table 1: TLX-LC gradient program

Analyte	Parent	Production 1 (CE)	Production 2 (CE)	Start Time [min]	Stop Time [min]	Tube Lens	Ion Ratio	Retention Time [min]
DMP	194.84	163.09 (11)	77.26 (33)	0.5	2.0	49	0.23	1.40
DEP	223.08	149.08 (18)	121.13 (28)	0.0	2.0	52	0.11	1.56
DPP	251.10	149.07 (16)	121.13 (32)	0.5	2.5	57	0.10	2.37
DiBP	279.13	149.08 (19)	121.10 (31)	1.0	4.0	57	0.07	3.33
d_4-DiBP	283.13	153.09 (20)	69.30 (46)	1.0	4.0	56	0.07	3.30
BBzP	313.09	91.20 (28)	149.05 (12)	1.0	4.0	60	0.72	3.23
DcHP	331.17	149.08 (25)	167.07 (12)	2.5	4.5	59	0.46	4.70
DHP	335.17	149.05 (16)	121.11 (38)	3.0	6.0	60	0.08	5.75
d_4-DHP	339.19	153.05 (17)	125.10 (39)	3.0	6.0	68	0.08	5.73
DEHA	371.25	129.11 (14)	111.13 (22)	4.0	8.0	72	0.35	7.08
DEHP	391.18	149.06 (25)	121.05 (45)	4.5	7.5	72	0.09	7.03
d_4-DEHP	395.26	153.07 (24)	171.07 (13)	4.5	8.0	68	0.32	7.02
DiNP	419.29	149.03 (26)	85.28 (14)	4.5	7.5	76	0.67	7.48
DiDP	447.31	149.01 (30)	85.23 (17)	5.0	8.5	78	0.77	7.90

Table 2: Parameters for tSRM analysis, ion ratios and retention times of plasticizers in methanol (CE = collision energy)

14. Calculation of Results

14.1 Identification

Identification of plasticizers is indicated by the presence of ions measured in selected reaction ion monitoring mode (SRM) corresponding to the retention times of appropriate standards. For peak identification the ion ratios are checked. The ion ratios for sample extracts should fall within the tolerance of the corresponding standard (657/2002 EC[1]).

15. Interpretation of Results

15.1 Quantification

By comparing the peak areas of the samples with those of external calibration the quantification of the standards in the spiked samples is carried out.

It employs internal standardization using peak area ratios for standards in methanol. D_4-DiBP is used as the internal standard for DMP, DEP, DPP, DiBP and BBzP; d_4-DHP is used as the internal standard for DHP; and d_4-DEHP is used as the internal standard for DEHP, DEHA, DiNP and DiDP. Plot the calibration curves as the relative peak areas (analyte versus the corresponding internal standard) as a function of concentrations. The plasticizer concentration (c_p) in the samples is determined from the equation:

$$C_p = \left(\frac{A_p}{A_{IS}}\right) - b/a$$

C_p – plasticizer concentration in mg/L

A_p – peak area of the plasticizer

A_{IS} – peak area of internal standard

b – the y-intercept

a – the slope of calibration curve

16. Method Performance

Single laboratory method performance characteristics were established by spiking experiments with three samples (homemade orange juice, bitter lemon and milk, see 10.1) with a mixture of plasticizer standards.

Method accuracy was assessed at three different spiking levels of plasticizers (low, mid and high level). Other validation parameters included specificity, linearity range and robustness.

16.1 Specificity

Using tSRM the specificity was confirmed based on the presence of transition ions at the correct retention time corresponding in timed segments to the plasticizer standards in methanol (Table 2). Specificity determination was based on ion ratio confirmation according to allowed variations in 657/2002 EC (Table 2). The deviation for retention time was in the range of ±2.5%.

16.2 Linearity and Calibration Curve

The linearity of calibration curves is assessed over the range from 0.05 to 5 mg/kg (DMP, DEP, DPP, BBzP, DiBP, DcHP and DHP) and 2–100 mg/L (DEHP, DEHA, DiNP, DiDP). In all cases, the correlation coefficients of linear functions are >0.985. The calibration curves are created from eight calibration standards which are injected in each batch in duplicate starting from zero value up to the highest calibration concentration. Between each calibration level methanol is injected as blank.

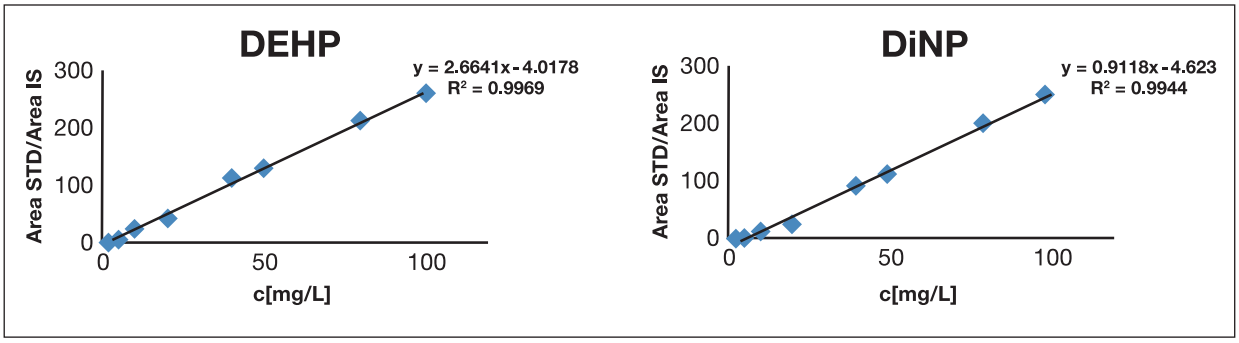

Figure 1: Calibration curve of DEHP and DiNP with d_4-DEHP as internal standard

16.3 Accuracy

Method Accuracy and Precision (Table 3) is evaluated by recovery studies spiking bitter lemon, orange juice and milk at three concentration levels (at low, mid and high levels) of plasticizers. Six replicates are prepared for each experiment, in accordance with EU guidelines. The samples are spiked at 0.2, 2 and 4 mg/L (DMP, DEP, DPP, BBzP, DiBP, DcHP and DHP) and 8, 40 and 80 mg/L (DEHP, DEHA, DiNP, DiDP) levels.

Found concentrations (mg/L), recovery, standard deviation (STDV) and relative standard deviation (%RSD) are calculated.

Analyte	Orange Juice Rec % (%RSD)	Bitter Lemon Rec % (%RSD)	Milk Rec % (%RSD)
DMP	94.5 (5)	102.4 (6)	111.0 (4)
DEP	97.6 (4)	111.0 (5)	108.7 (3)
DPP	106.7 (4)	112.4 (4)	118.0 (3)
BBzP	103.3 (3)	90.0 (4)	98.9 (2)
DiBP	115.8 (4)	90.0 (8)	92.2 (3)
DcHP	77.8 (4)	81.0 (5)	69.0 (3)
DHP	96.3 (4)	96.1 (5)	93.8 (3)
DEHP	97.2 (15)	85.7 (8)	78.2 (9)
DEHA	64.8 (14)	70.4 (7)	76.1 (7)
DiNP	84.5 (12)	75.9 (8)	71.9 (3)
DiDP	72.6 (10)	71.2 (1)	*

Table 3: Average recovery and RSD of plasticizer standards in orange juice, bitter lemon and milk

** DiDP is not applicable for milk matrix*

17. Conclusion

A method for the determination of plasticizers in beverages and milk has been developed to enable fast and cost-effective automated determination of selected plasticizers. Online TurboFlow sample preparation coupled to the analytical HPLC separation equipped with a triple quadrupole detector enables very selective and effective determination of plasticizers. Elimination of time-consuming steps like liquid liquid extraction (LLE) or solid phase (SPE), followed by evaporation and reconstitution enables high sample throughput. Reducing sample treatment steps reduces the probability of cross contamination as well.

18. Reference

1. Commission Decision 657/2002 of August 2002 on implementing Council Directive 96/23/EC concerning performance of analytical methods and the interpretation of results. Official journal of the European communities, L221, 8-36, 2002.

19. Annex

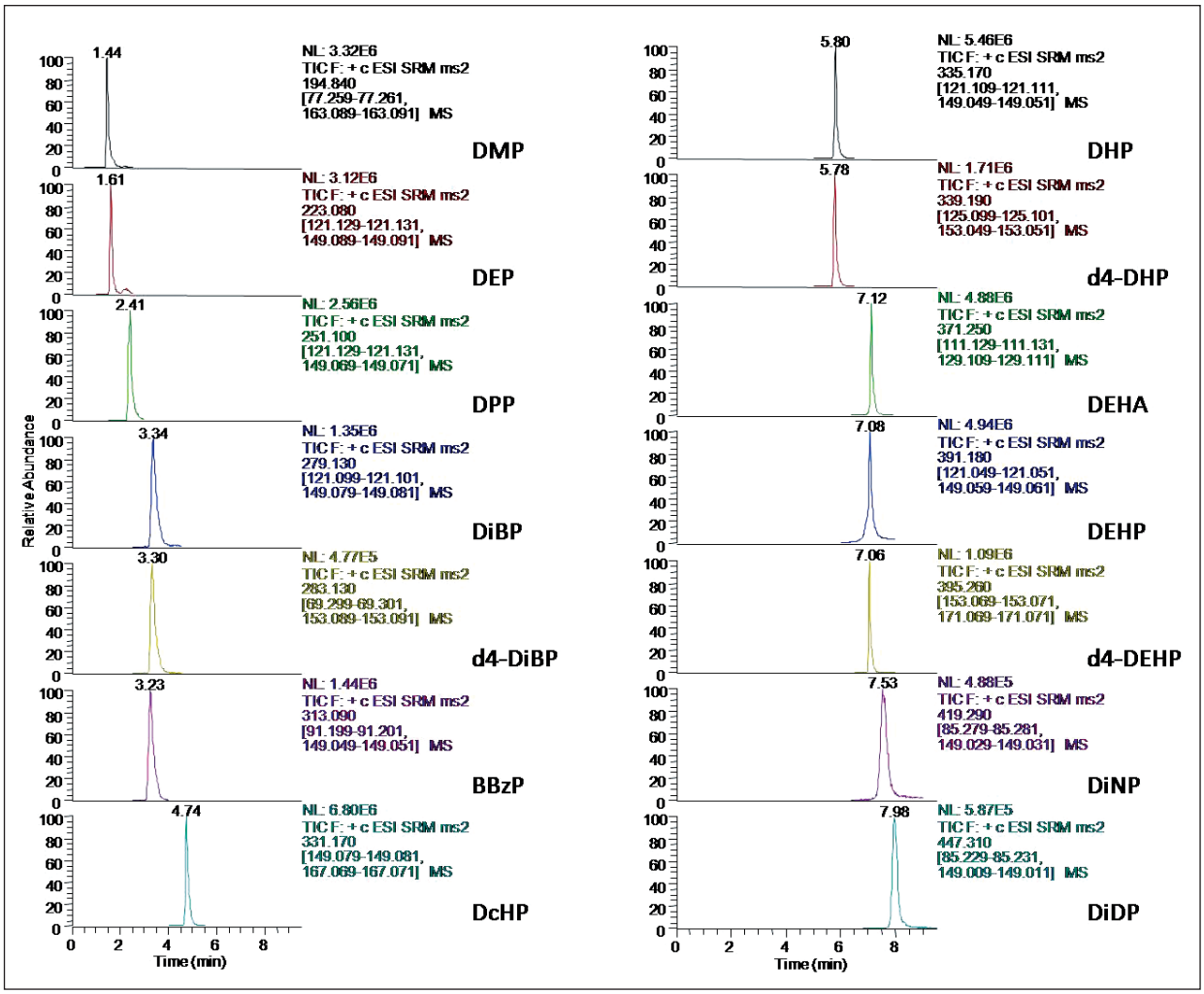

Figure 2: Chromatogram: Separation of plasticizer standards in methanol (c = 1.5 mg/L) at TLX-LC gradient program (Table 1)

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Application Note: 474

Determination of Bisphenol A in Infant Formula by Automated Sample Preparation and Liquid Chromatography-Mass Spectrometry

Yang Shi, Catherine Lafontaine, Matthew Berube, Francois Espourteille, Thermo Fisher Scientific, Franklin, MA, USA

Key Words

- Aria TLX-1
- TurboFlow Technology
- TSQ Vantage
- Infant Formula
- Food Safety

Introduction

2,2-bis(4-hydroxyphenyl) propane, commonly known as Bisphenol A (BPA), is one of the primary chemicals used to make plastics. It is also heavily used in the production of various types of food and drink containers. Because BPA has been known to leach from the plastic lining of metal canned food, the potential risks of exposure to BPA have been a great concern over the past few years. Higher bisphenol A levels are significantly associated with heart disease, diabetes, and abnormally high levels of certain liver enzymes. There is a consensus that infants are at the greatest risk of harm due to exposure to extremely low levels of BPA.[1] The maximum acceptable or "reference" dose for BPA is 50 µg/kg body weight/day, as established by the U.S. Environmental Protection Agency.[2]

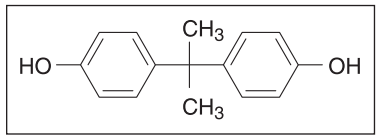

Figure 1: Chemical Structure of Bisphenol A

A liquid chromatography-mass spectrometry (LC-MS) technique has been recently described for the determination of BPA in food.[3] Current strategies for the detection of BPA in canned infant formula employ sample preparations that involve complicated extraction steps such as solid phase extraction, solvent-based extraction, and some micro-extraction techniques. All of these techniques require additional sample concentration and reconstitution in an appropriate solvent. Such sample preparation methods are time-consuming and are more vulnerable to variability due to errors in manual preparation. To offer a high sensitivity (low ppb) BPA detection method and timely, automated analysis of multiple samples, our approach is to use Thermo Scientific TurboFlow technology coupled to the detection capabilities of a high-sensitivity Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer.

Goal

Develop a six-minute LC-MS/MS method using automated sample preparation for the assay of BPA in canned infant formula powder by negative ion atmospheric pressure chemical ionization (APCI) using a deuterated internal standard (BPA-d_{16}).

Experimental

Sample Preparation

Canned infant formula powder, used in this analysis for preparation of blanks, QCs, and standards, was obtained from a local supermarket in Massachusetts. The lid lacquer is low-density polyethylene and the body is polyester. BPA and BPA-d_{16} were obtained from Sigma-Aldrich, US (St. Louis, MO). The diluent (AmAcACN solution) was made using 3% ammonium acetate in acetonitrile-water (70:30, v/v). A BPA working solution was prepared in AmAcACN solution at 10 µg/mL. The infant formula solution was prepared by adding 10 mL of AmAcACN solution to 1 g of infant formula powder and then centrifuging at 10,000 RPM for 30 minutes. BPA standards and QC standards were serially diluted to the target concentrations with the resulting supernatant containing 25 ng/mL BPA-d_{16} as the internal standard. Target standard concentrations ranged from 0.78 ng/mL to 1000 ng/mL. The injection volume was 25 µL.

Method

The extract clean-up was accomplished using a TurboFlow™ method run on a Thermo Scientific Aria TLX-1 LC system using a TurboFlow Cyclone P polymer-based extraction column. Large molecules were not retained and were moved to waste during the loading step while the analyte of interest was retained on the extraction column. This was followed by organic elution to a Thermo Scientific Hypersil GOLD aQ end-capped, silica-based C18 reversed phase analytical column and gradient elution to a TSQ Vantage™ MS with an APCI source. The BPA precursor *m/z* 227 > 133 and 212 high-resolution selective reaction monitoring (H-SRM) transitions were monitored in negative ionization mode. The 133 *m/z* product ion for BPA was used for quantitation, and the 212 *m/z* product ion was used as confirmation. The precursor *m/z* 241 > 223 H-SRM transition was monitored for BPA-d_{16} because BPA-d_{16} is transformed into BPA-d_{14} (MW 242) in water. The total LC-MS/MS method run time was 5.6 minutes.

Aria™ TLX-1 System Parameters

TurboFlow Cyclone P column (0.5 x 50 mm)
Hypersil GOLD™ aQ (4 x 50 mm, 3 µm particle size)

Loading Pump Mobile Phases

Mobile Phase A: 10 mM Ammonium bicarbonate pH 10
Mobile Phase B: 0.1% Formic acid in ACN
Mobile Phase C: 20:40:40 Acetone: Acetonitrile: Isopropanol

Elution Pump

Mobile Phase A: H_2O
Mobile Phase B: Methanol

MS analysis was carried out on a TSQ Vantage triple stage quadrupole mass spectrometer. The MS conditions were as follows:

Mass Spectrometer Parameters

Ion Polarity:	Negative ion mode
Discharge Current:	4.0 V
Vaporizer Temperature:	60 °C
Capillary Temperature:	275 °C
Sheath Gas Pressure (N_2):	30 units
Auxiliary Gas Pressure (N_2):	5 units
Ion Sweep Gas Pressure (N_2):	2 units
Scan Type:	Highly Selective Reaction Monitoring (H-SRM)
Chrom Filter Peak Width:	7.0 s
Collision Gas Pressure:	1.2 mTorr
Declustering Voltage:	0 V
Scan Width:	0.002 *m/z*
Scan Time:	0.05 s
Q1:	0.200 Da
Q3:	0.700 Da
S-Lens (*m/z* 321):	77 V
Collision Energy (*m/z* 227 > 133):	27 V
(*m/z* 227 > 212):	19 V

The entire experiment was controlled by Aria operating software 1.6.2. The data were processed using Thermo Scientific LCQUAN 2.5.6 quantitative software after subtracting background using Thermo Scientific Xcalibur 2.0.7 SP1 data system software.

Results and Discussion

Because BPA exists in air (2 - 208 ng/m^3), dust (0.2 - 199 ng/g), water (5 - 320 ng/L) and in many other sources, it is almost impossible to obtain a real blank of BPA in the laboratory.[3] Therefore, we subtracted the pre-standard double blank peaks from all quantified data using the Xcalibur™ built-in background subtraction tool. Figure 2 shows comparison chromatography of BPA and BPA-d_{16} at the lower limit of quantitation (LLOQ) (0.78 ng/mL) and the upper limit of quantitation (ULOQ) (100 ng/mL). The data were processed using LCQUAN™ 2.5.6 data quantitation software. Matrix-matched calibration standards of BPA showed a linear response at greater than 2 orders of magnitude with r^2 = 0.9921 (Figure 3). All %CVs (n=3) were less than 20% for the LLOQ and less than 10% for all other points of the curve. As shown in Figure 4, the comparison between the pre-blank and post-high blank (before subtraction)

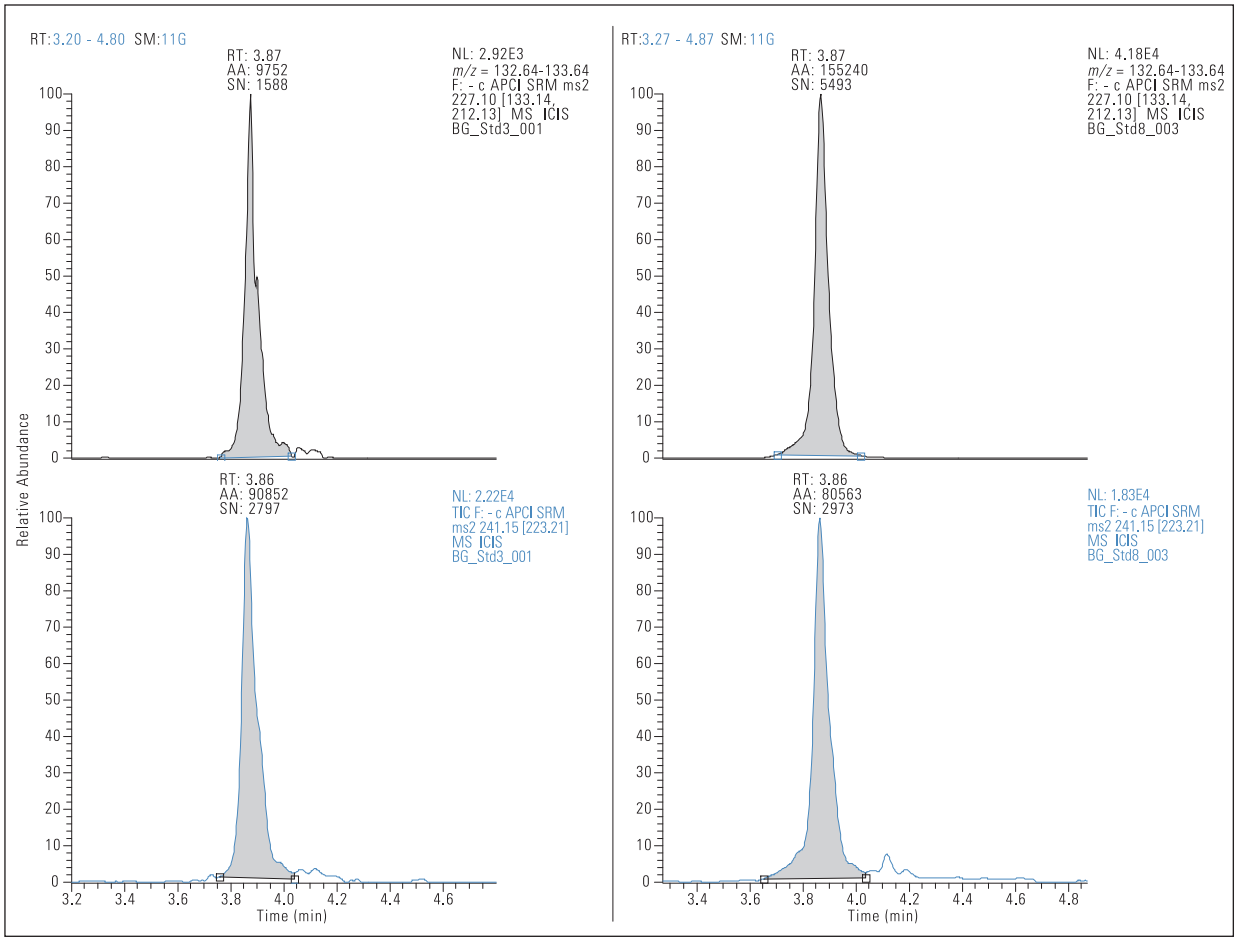

Figure 2: Chromatography comparison of BPA H-SRM *m/z* 133 transition (upper traces) and BPA-d_{16} (lower traces) at LLOQ of 0.78 ng/mL (left panel), and at ULOQ of 100 ng/mL (right panel)

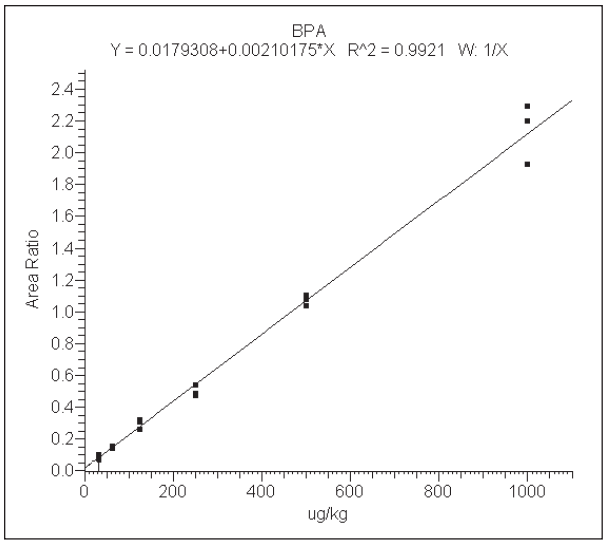

Figure 3: Linear regression curve of BPA standards based on area ratio with internal standard BPA-d_{16}

demonstrated the carryover could be ignored. The matrix interference was evaluated by comparing the chromatogram of the same concentration of BPA spiked in matrix and water. Figure 5 shows such a comparison at 12.5 and 25 ng/mL. As illustrated, the matrix interference was minimal.

We also compared the results of this TurboFlow technology LC-MS/MS study to another popular online solid phase extraction method.[4] Sample preparation times were very close due to few required offline sample treatment steps. The TurboFlow LC-MS/MS method run time, though, was four times faster. Because of differences in food matrices and the number of analytes, it is hard to compare the detection and quantitation limits directly. However, this comparison shows the benefits of using TurboFlow technology in the determination of BPA in food matrices.

Conclusion

A quick, automated sample preparation LC-MS/MS method has been developed that is sensitive enough to detect 7.80 µg/kg (ppb) dry powder (limit of detection) and quantify 31.3 µg/kg (ppb) dry powder (LLOQ) of BPA (background-adjusted) in infant formula powder for screening purposes. Compared to offline liquid/liquid or solid phase extractions, this method eliminates the need for time-consuming sample preparation procedures. The TurboFlow method also shows the advantage of fast separation over other online sample treatment techniques. The LC-MS/MS method run time is only 5.6 minutes, and the sample throughput can be improved by multiplexing on an Aria TLX-2 (or TLX-4) system.

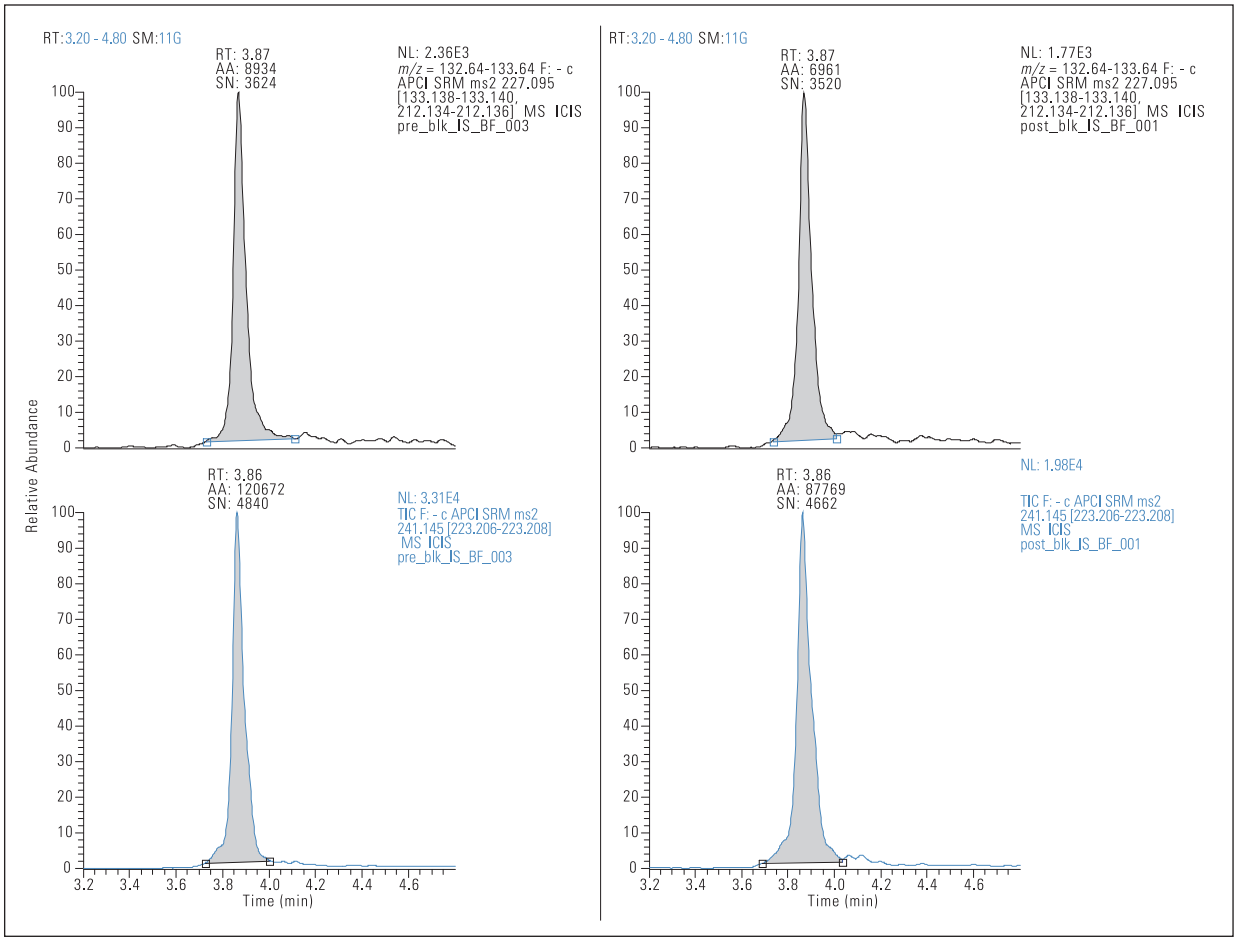

Figure 4: Chromatography comparison of BPA H-SRM *m/z* 133 transition (upper traces) and BPA-d_{16} (lower traces) in pre-blank infant formula matrix (left panel), and in post-high blank (right panel)

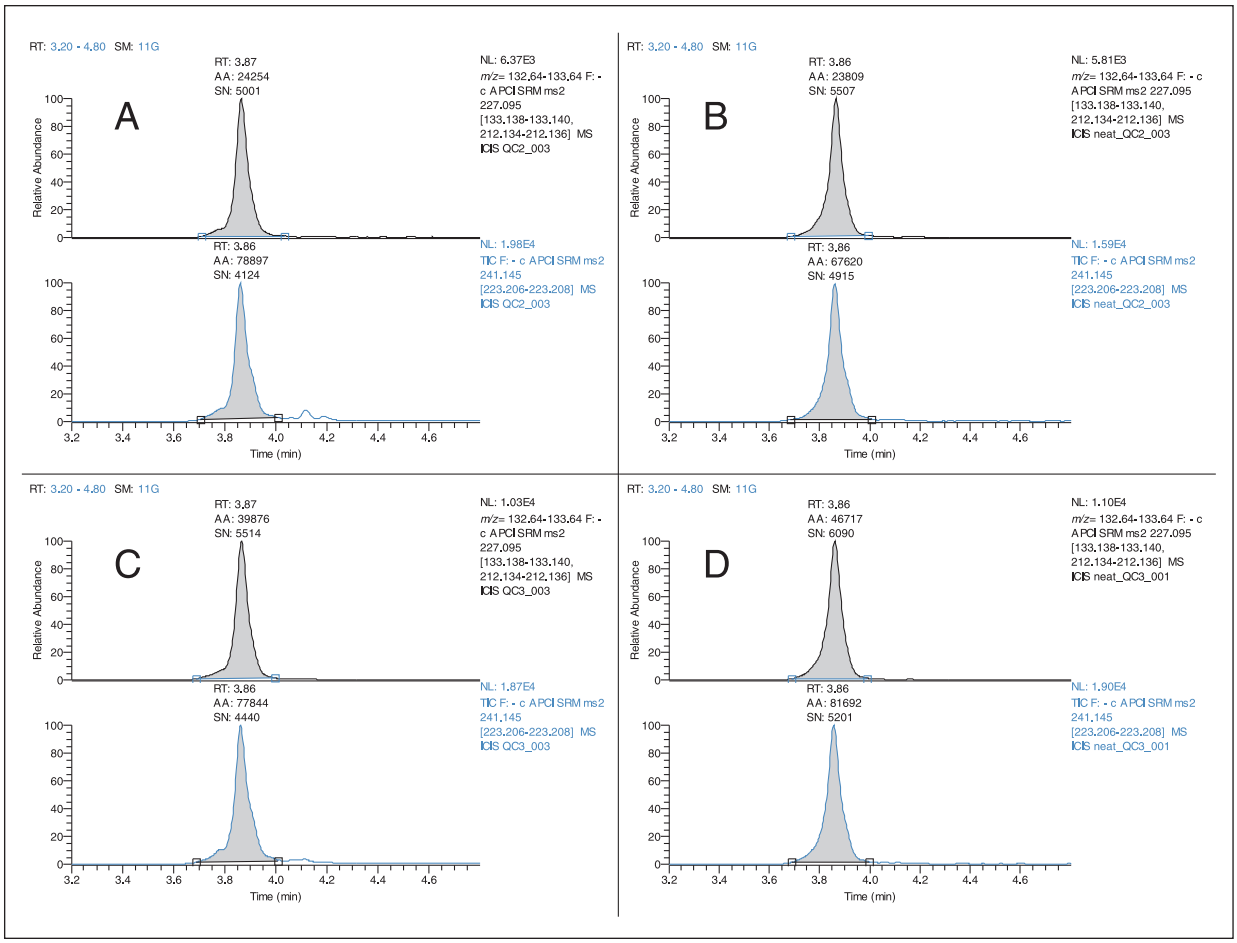

Figure 5: Chromatography comparison of BPA H-SRM *m/z* 133 transition (upper traces) and BPA-d_{16} (lower traces) at 12.5 ng/mL in matrix (panel A), at 12.5 ng/mL in water (panel B), at 25 ng/mL in matrix (panel C), and at 25 ng/mL in water (panel D)

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Application Note: 521

The Analysis of Bisphenol A-diglycidyl Ether (BADGE), Bisphenol F-diglycidyl Ether (BFDGE) and Their Derivatives in Canned Food and Beverages by LC-MS/MS

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Key Words
- BADGE
- BFDGE
- LC-MS/MS
- Food Safety

Introduction

As an attempt to reduce food spoilage and prevent degradation of the container, epoxy-based lacquers or vinylic organosol (PVC) materials are commonly used as coating material in food cans. These lacquers are epoxy phenolic resins based on polymerization products of bisphenol A-diglycidyl ether (BADGE) or bisphenol F-diglycidyl ether (BFDGE). Chlorinated derivatives can be generated during the coating thermal treatment, since BADGE and BFDGE are also used as additives to remove the hydrochloric acid formed in this process. Moreover, hydrolyzed derivatives such as BADGE.2H_2O, BADGE.H_2O, and BFDGE.2H_2O can be produced during storage when the coating comes into contact with aqueous and acidic foodstuffs. The European Union has set specific migration limits (SML) for these compounds: 9 mg/kg for the sum of BADGE and its hydrolyzed derivatives and 1 mg/kg for the sum of BADGE.HCl, BADGE.2HCl and BADGE.HCl.H_2O.[1,2] The presence of this family of compounds in food has received much attention lately due to its suspected mutagenic, genotoxic, and anti-androgenic effects.[3-6]

Goal

To develop a fast and sensitive LC-MS/MS method for the simultaneous quantitative analysis of BADGE, BFDGE, and their derivatives in canned food and beverages.

Experimental

Sample Preparation

Canned Food:

The whole can content was homogenized. A sample of 3 g was mixed with 6 mL of ethyl acetate. The resulting mixture was shaken for 20 minutes and sonicated for 30 minutes in an ultrasonic bath. The mixture was then centrifuged at 4000 rpm for 15 minutes. Five (5) mL of supernatant was transferred to an 8-mL vial and evaporated to dryness under a stream of nitrogen. The extract was reconstituted in 1 mL of MeOH:H_2O (1:1) and filtered before injection (10 µL injection).

Beverages:

A 20-mL sample of beverage was degassed by sonication for 20 min. Then, 3 mL was loaded into a polymeric SPE cartridge that was previously conditioned with 3 mL of MeOH and 3 mL of H_2O. Finally, the analytes were eluted with 4 mL of MeOH. The collected fraction was evaporated to dryness and the extract reconstituted with 1 mL of MeOH:H_2O (1:1) and filtered before injection (10 µL injection).

LC Conditions

Solvent A	Formic acid-ammonium formate (25 mM, pH 3.75, 50 °C)
Solvent B	Methanol
Flow Rate	600 µL/min
Analytical Column	Fused Core™ Ascentis Express C18 150 x 2.1 mm i.d., 2.7 µm (Supelco)

The gradient method was started at 30% solvent B (0.25 min) and linearly increased to 50% solvent B in 0.75 min. The gradient was then increased to 60% of solvent B in 0.5 min, and then to 80% in 4 minutes. This composition was maintained for 0.5 min.

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MS Conditions

MS analysis was carried out on a Thermo Scientific TSQ Quantum Ultra AM mass spectrometer equipped with a heated electrospray ionization probe. The MS conditions were as follows:

Ion Source Polarity	Positive Ion Mode
Spray Voltage	4000 V
Vaporizer Temperature	475 °C
Sheath Gas Pressure (N_2)	60 units
Auxiliary Gas Pressure (N_2)	40 units
Ion Sweep Gas Pressure	2 units
Capillary Temperature	375 °C
Tube Lens	65 V
Collision Gas (Ar)	1.5 mTorr

Results and Discussion

The family of compounds studied tends to form adducts and clusters in positive ionization mode $[M+NH_4]^+$, $[M+Na]^+$, and $[M+K]^+$. The mobile phase used favored the formation of ammonium adducts ions $[M+NH_4]^+$, which dominated the full scan spectra (base peaks). The cleavage of the phenyl-alkyl bond and the α-cleavage of the ether bond were identified as the most intense and characteristic fragmentation of $[M+NH_4]^+$, and therefore selected for quantification and confirmation purposes (Table 1).

Matrix effects were evaluated by analyzing two samples free of BADGEs and BFDGEs – cola soft drink beverage and red pepper. These samples were analyzed by external and matrix-matched calibration. The results showed similar responses for both methods and matched calibration curves, indicating that no matrix effect occurred in the analysis of BADGEs and BFDGEs using the developed LC-MS/MS method.

To evaluate limits of quantification, blank samples were spiked with the studied compounds at low concentration levels (below 2.5 µg/kg) and submitted to the sample pre-treatment detailed above. The results obtained allowed the analysis of this family of compounds in beverages and canned food, given that the LOQs obtained are below (3 to 4 orders of magnitude) the specific migration limits established by the European Union (Table 2).

Good linearity ($r^2 > 0.999$) was observed for calibration curves for standard solutions ranging from 0.5 µg/kg to 5,000 µg/kg.

Run-to-run precision was evaluated by analyzing six replicates of a red pepper sample and a cola sample spiked at two concentration levels. In addition, the ion ratios (quantitative versus confirmatory) were calculated and errors (compared with standards) were always below 10%. Finally, recoveries were calculated by addition of different amounts of the studied compounds (between the LOQ and 250 µg/kg) to blank samples, which were analyzed by external calibration.

Sample Analysis

The LC-MS/MS method developed for the analysis of BADGEs and BFDGEs in canned food and soft-drinks was employed to analyze six aqueous-based canned foods and seven soft-drink samples (Figure 1). In canned soft-drink beverages only BADGE·$2H_2O$ was detected, at concentrations ranging from 2.3 µg/L to 5.1 µg/L, while other BADGEs and BFDGEs were not detected. As an example, Figure 2 shows the LC-MS/MS chromatogram of two canned soft-drinks samples where BADGE·$2H_2O$ was found. In contrast, several BADGEs were found in canned food samples. BADGE·$2H_2O$ was found in all food samples at concentrations between 2.7 µg/kg and 675 µg/kg, with the highest concentration level being

Table 1. Transitions monitored for the analysis of BADGEs and BFDGEs

Compound	Precursor ion *(m/z)*, $[M+NH_4]^+$	Quantitation		Confirmation		Ion Ratio ± SD[b]
		Product Ion *(m/z)*	CE[a] (V)	Product Ion *(m/z)*	CE[a] (V)	
BADGE·$2H_2O$	394.2	209.1	31	135.1	31	1.7 ± 0.1
BADGE·H_2O	376.2	209.1	29	135.1	29	1.9 ± 0.1
BADGE·HCl·H_2O	412.2	227.0	33	135.1	33	1.4 ± 0.1
BADGE	358.2	191.0	30	135.1	30	4.3 ± 02
BADGE·HCl	394.2	227.0	13	135.1	13	2.6 ± 0.3
BADGE·2HCl	430.2	227.0	30	135.1	30	2.0 ± 0.1
BFDGE·$2H_2O$	366.2	133.1	22	181.1	22	1.5 ± 0.1
BFDGE	330.2	163.1	12	189.1	12	1.3 ± 0.1
BFDGE·2HCl	402.1	199.1	20	181.1	20	1.7 ± 0.2

[a]CE: collision energy

[b]SD: Standard deviation (n = 5)

Table 2. MLOQs, run-to-run precision, recoveries, and ion ratio of the LC-MS/MS method

Compound	MLOQ (µg/L)	Soft-drinks and canned food			
		Precision (RSD %)		Recovery (%)	Ion ratio[c]
		Low concentration[a]	Medium concentration[b]		
BADGE·2H$_2$O	0.13 – 1.0	7	3	70 - 95	1.8
BADGE·H$_2$O	0.14 – 1.1	12	3	60 - 83	1.8
BADGE·HCl·H$_2$O	0.14 – 1.1	20	9	69 - 95	1.5
BADGE	0.16 – 1.2	12	10	80 - 86	4.3
BADGE·HCl	0.16 – 1.3	3	11	60 - 70	2.4
BADGE·2HCl	1.6 – 3.4	14	10	80 - 82	2.1
BFDGE·2H$_2$O	1.5	16	8	85 - 90	1.3
BFDGE	0.7 – 4.0	20	10	70 - 89	1.6
BFDGE·2HCl	1.6	13	4	74 - 95	1.9

[a]Low concentration level: Cola sample (0.15 µg/L to 2.0 µg/L) and red pepper (2.0 µg/kg to 15.0 µg/kg).
[b]Medium concentration level: Cola sample (1.5 µg/L to 20 µg/L) and red pepper (20 µg/kg to 150 µg/kg).
[c]Ion ratio calculated at medium concentration level.

in the asparagus sample. Other BADGEs detected in these samples were BADGE·H$_2$O at concentrations ranging from 35 µg/kg to 53 µg/kg, BADGE·HCl·H$_2$O (3.4 – 274 µg/kg) and BADGE·2HCl at concentrations between 0.9 µg/kg and 2.8 µg/kg. In contrast, the original monomer (BADGE) was not found in the samples, probably because it was easily hydrolyzed in these water-based samples. In addition, none of the BFDGEs were found, confirming the decrease in use of BFDGE-based coatings.

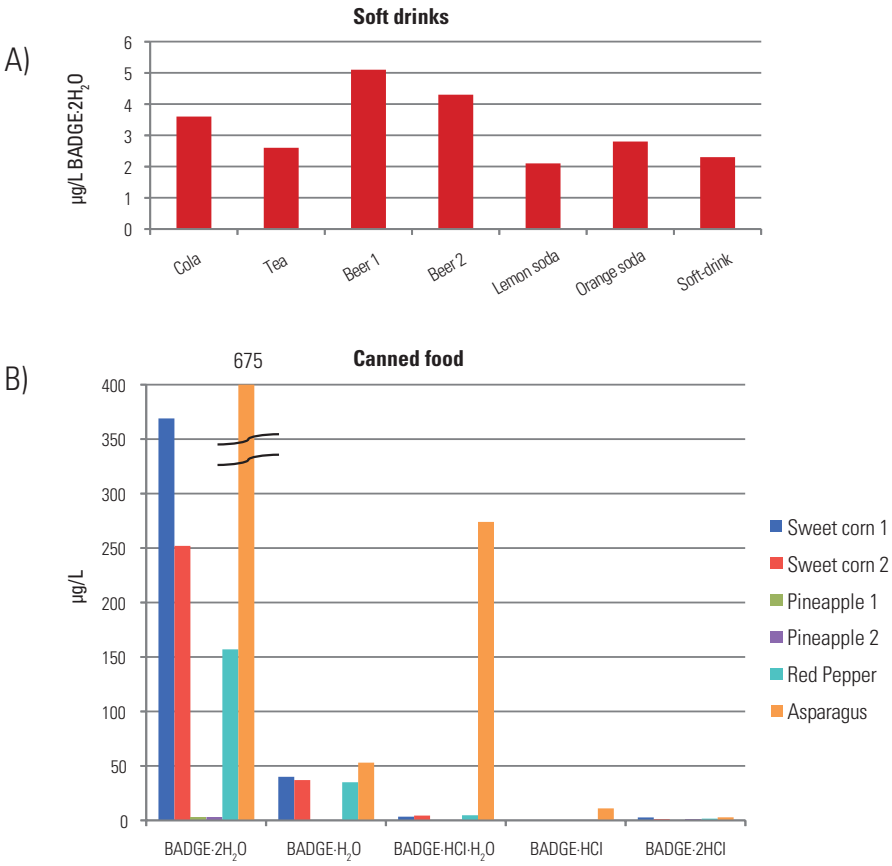

Figure 1. Canned soft-drinks (A) and food samples (B) analyzed using the developed LC-MS/MS method

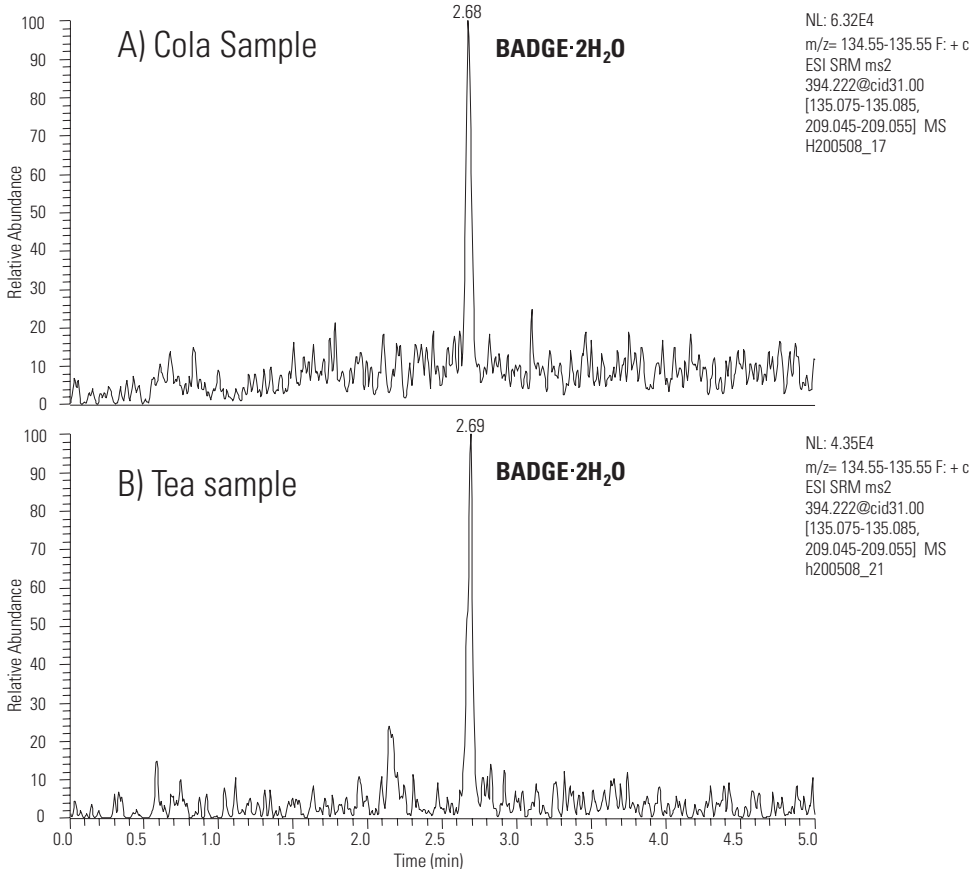

Figure 2. LC-MS/MS chromatograms for cola (A) and tea (B) samples

Conclusions

A fast and sensitive method for the simultaneous analysis of BADGEs and BFDGEs in canned food and beverages is proposed. The limits of quantification of the method vary between 0.13 and 1.6 µg/L for beverages and between 1.0 and 4.0 µg/kg for foodstuff. The method has been applied to real samples. BADGE.2H_2O was detected in all samples at levels between 2.1 and 675 µg/kg. Other derivatives of BADGE were also detected and quantified. No BFDGE or its derivatives were detected.

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More information can be found at Gallart-Ayala, H; Moyano, E.; Galceran, M.T. *J Chrom A*, 2011, 1218, 12.

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Application Note 10315

High Sensitivity Analysis of Nitrosamines Using GC-MS/MS

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Keywords: Nitrosamines, Food Safety, beer, TSQ 8000, GC-MS/MS, quantitation, confirmation, AutoSRM, TraceFinder

Introduction

Nitrosamines is the common term used for compounds of the class of N-nitrosodialkylamines. A large variety of compounds are known and described with different alkyl moieties [1]. The simplest N-nitrosodialkylamine with two methyl groups is the N-nitrosodimethylamine (NDMA). Nitrosamines are in common highly toxic compounds with high cancerogenity for humans and animals, in higher doses leading to severe liver damage with internal bleeding [2,3].

Nitrosamines in food are mainly produced from nitrites. Nitrites are added to food as preservatives in meat and meat products preventing the Botulinus poisoning. Antioxidant food additives like vitamin C can prevent the formation of nitrosamines from nitrites [4]. Another source of nitrosamines is described by the reaction of nitrogen oxides with alkaloids as it is reported from the drying process of the germinated malt in beer production [5]. As nitrosamine levels in malt and beer have been significantly reduced in the brewing process, high analytical performance is required. In addition to the regular control of other food products for daily consumption, malt in beer is also monitored for low levels of nitrosamines.

The “classical” nitrosamine analysis was performed for many years by gas chromatography using a thermal energy analyzer (TEA) as detector. This special TEA detector was used due to its selectivity for nitrosamines with to the specific chemilumniscent reaction of ozone with the detector generated NO from nitrosamines. Today, with increased sensitivity requirements, the detection limits of the TEA, and also its complex operation, no longer comply with the required needs for low detection limits and sample throughput. Mass spectrometric methods have increasingly replaced the TEA.

The EPA method 521 by Munch and Bassett from 2004 provided at that time a suitable GC-MS method based on chemical ionization (CI) using an ion trap mass spectrometer with internal ionization [6, 7], in contrast

to standard quadrupole or ion trap mass spectrometers using a dedicated (external) ion source design. Current developments in GC-MS triple quadrupole technology deliver today very high sensitivity and selectivity also in the small molecule mass range and allow the detection of nitrosamines at very low concentration levels even in complex matrix samples. This is made possible by using a much simpler and standard approach with the regular electron impact ionization (EI) for a very straightforward method for low level nitrosamine analysis.

This application note describes a turn-key GC-MS/MS method for routine detection and quantitation of food borne nitrosamine compounds. The food matrix in this work has been different malt beer products and as a final food product the commercial beer. Special focus in the method development has been made to provide the required high sensitivity for the detection of the nitrosamine compounds for a fast, easy to implement routine method.

The sample preparation is adapted and slightly modified from AOAC Official Method (2000), 982.11 [8]. An SPE column extraction method using a celite column and elution with DCM to isolate the nitrosamines from the beer samples was developed.

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Experimental Conditions
GC-MS/MS Instrument

TRACE 1310 GC

iC Injector Module	Split/Splitless Injector
Injector Temperature	250 °C
Injection mode	splitless
Surge mode	300 KPa
Splitless Time	1.0 min
Analytical Column	TG-WAX MS, 30m×0.25mm×0.5µm
Carrier gas	He （99.999% purity）
Flow rate	1.0 mL/min, constant flow
Oven Program	45 °C for 3 min,
	25 °C/min to 130 °C,
	12 °C/min to 230°C, 1min hold
Transfer line Temperature	250°C
Total analysis time	14.7 min
Total cycle time	18.4 min

TriPlus RSH Autosampler

Injection Volume	1 µL
Solvent	dichloromethane
Standard runs	3 replicate of injections each
Dilution of standard mix	1ppb, 5ppb, 10ppb, 25ppb, 100ppb, 250ppb, 500ppb
Internal standard	NDPA added to each calibration level at 50ppb

TSQ 8000 Triple Quadrupole GC-MS/MS system

Ionization mode	EI
Mass resolution setting	normal
Source temperature	220 °C
Scan mode	MRM, retention time-based SRM mode

MRM Method Setup

The triple quadrupole MS method setup was performed by using the AutoSRM software which is part of the Thermo Scientific TSQ™ 8000 GC-MS/MS software suite. The method generated by AutoSRM was used without any additional manually modification. One autosampler vial containing a standard solution of the nitrosamine compounds to be analyzed has been used only for the AutoSRM process.

The AutoSRM procedure automatically runs the following three steps:

1. First a full scan analysis of the standard solution (Figure 1). Get the most intense ions of the full scan spectra to be used as the precursor ions.
2. Run a next analysis acquiring the product ion spectra from the selected precursor ions (the number of precursor ions to be used can be configured to the analytical needs). Get the most intense product ions from each precursor ion (optionally the desired precursor ions can be selected manually for further optimization).

Table 1. MRM method setup using AutoSRM

	Precursor	Product	Collision Energy (eV)	Retention Time (min)	Time Window (min)
NDMA	74	42.1	15	7.89	1
	74	43.8	5	7.89	1
NDEA	102	44.1	10	8.56	1
	102	85.1	5	8.56	1
NDPA (ISTD)	130	42.9	10	9.76	1
	130	113.1	5	9.76	1
NDBA	158	99.1	5	11.35	1
	158	141.1	5	11.35	1
NPIP	114	41.5	15	11.80	1
	114	83.9	5	11.80	1
NPYR	100	43	10	12.06	1
	100	55.1	5	12.06	1
NMOR	116	56.1	10	12.47	1
	116	86.1	5	12.47	1

** The transitions marked as grey color are quantitation ions*

3. Optimize for all compounds the collision energy of the selected precursor/product ion transitions for maximized compound response and best method sensitivity (Figure 2).

Initiated by the AutoSRM procedure as many as necessary autosampler injections from the one standard vial are scheduled.

As a result of the AutoSRM program, the generated SRM transition table shown in Table 1 has been automatically built. The table represents at the same time the TSQ 8000 GC-MS/MS system MRM acquisition method using the timed-SRM mode with a short acquisition window of 60 s around the compound retention time. No other setting of scan segments is necessary, or will be necessary in case additional compounds need to be added to the acquisition, other than the compound retention time.

Sample Measurements

From the large variety of potential nitrosamines the compounds that had been included in this method are those that are reported to be of relevance in the germinated malt drying process. Samples analyzed included malt beer as unspiked samples and 4% ethanol as sample blanks. In case of the analysis of other food matrices, additional compounds can be added to this method easily at any time as described in the method setup by AutoSRM [9, 10].

Results

The chromatograms of the nitrosamines included in this method show a quick elution of the compounds from 7.87 min NDMA to 12:47 min allowing a short cycle time for increased sample throughput. The peak intensities are retained in Figure 3 at the lowest calibration level of 1 ppb. NDMA can be detected with good S/N values.

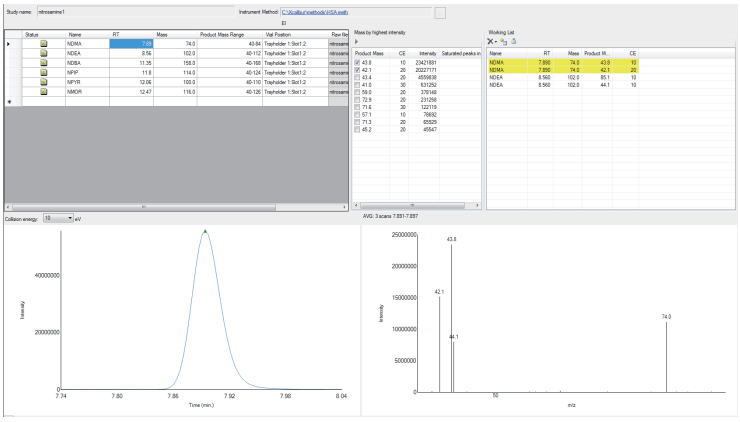

Figure 1. AutoSRM Precursor Ion Selection for NDMA from EI full scan spectra

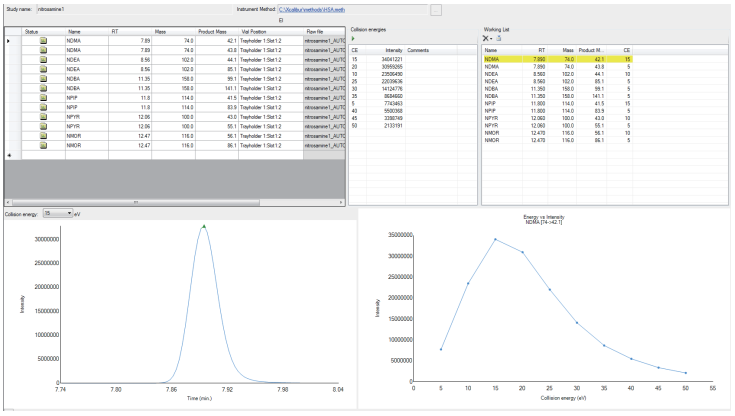

Figure 2. AutoSRM collision energy optimization for all nitrosmaine precursor ions

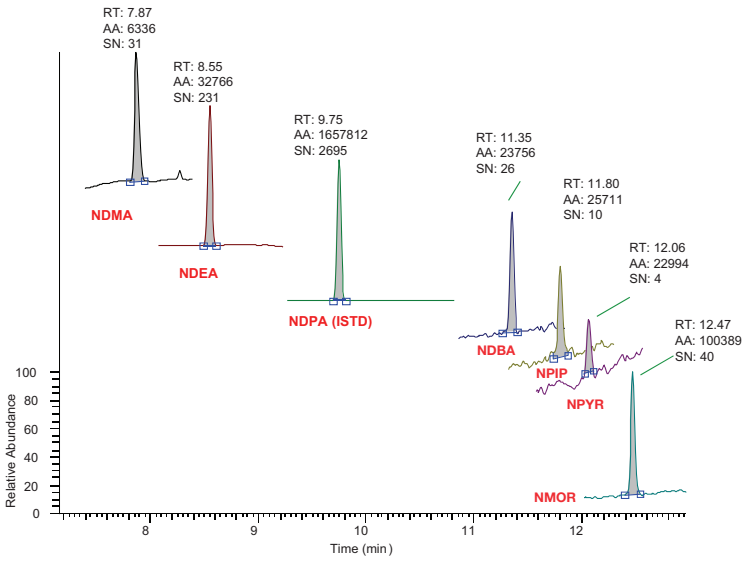

Figure 3. Chromatogram of the standard mix at 1 ppb

The quantitative calibration has been performed in a wide concentration range from 1 ppb to 500 ppb. Figure 4 shows the chromatogram peaks of NDMA from all the calibration runs. In all cases the NDMA peak shape is perfectly symmetrical, no tailing occurs and the peak area integration provides very reliable values without the need for any further manual corrections. The linear calibration of NDMA used to quantify the samples is shown in Figure 5 with very good correlation of R^2 better than 0.99. The same good calibration precision is achieved for all nitrosamines in this TSQ 8000 GC-MS/MS method.

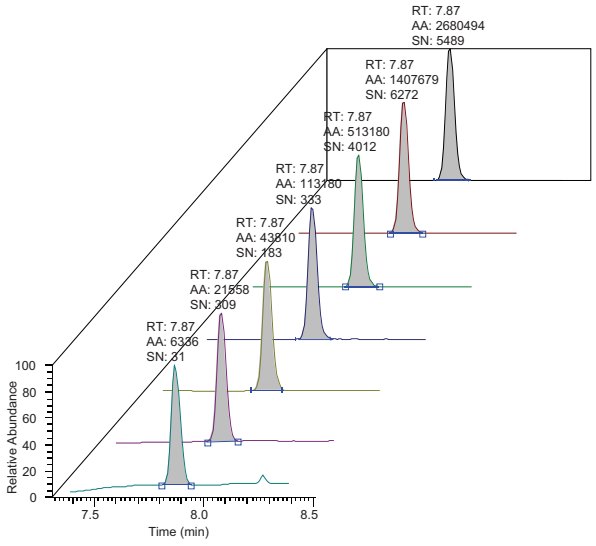

Figure 4. NDMA calibration runs from 1 ppb (bottom) to 500 ppb (top)

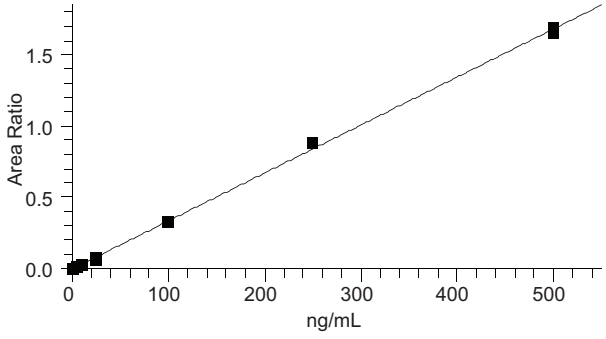

Figure 5. Linear calibration function for NMDA from 1 ppb to 500 ppb

LOQ Determination

The calculation of the LOQ and LOD was based on the S/N achieved for a chromatographic peak.The LOQ calculation is based on the level of S/N 10, and LOD values are calculated based on a S/N of 3.

Table 2. Calculation of the method LOQ and LOD

Compound	S/N @ 1ppb	Calculated LOQ (ppb)	Calculated LOD (ppb)
NDMA	13	1.0	0.25
NDEA	231	0.05	0.02
NDBA	23	0.5	0.20
NPIP	10	1.0	0.50
NMOR	40	0.3	0.10
NPYR	24	3	1.0

Confirmation

For compound confirmation the ion ratio check provided by the Thermo Scientific TraceFinder™ quantitation software was used by comparing the ion intensity of the second acquired SRM transition with the first SRM used for quantitation. The precision for the ion ratio was calculated using the three replicate standard runs over the complete concentration range from 1 ppb to 500 ppb and is shown in Table 3. Although the detected ions all are in the low mass range and potentially subject to many interferences the precision of the product ion ratio is very good in the range of 1-4%.

For quality control purposes in sample analyses the confirmation of a positive result is done by the ion ratio check during the quantitation data processing in TraceFinder software. The ion ratio of the two acquired product ions is required to stay within +/- 5% (10%) for all compounds, compared to the calibrated value from the standard runs. This provides a solid safety margin for routine sample measurements. Table 3 indicates the used average value (AVG) of the ion ratio for all nitrosamines investigated.

Sample Measurements

A number of samples have been measured, including blanks and spiked beer samples. The results of a blank sample are shown in Table 4. The found low NDMA concentration in this sample has been calculated below the calibration, and also below LOQ. The blank sample could be confirmed to be free from nitrosamine compounds at the given LOQ.

Another sample was prepared from beer that has been spiked with different amounts of nitrosamines. All nitrosamine compounds have been detected and quantified in a low concentration range of 9 – 13 ppb, see Table 5. Each quantified peak passed the ion ratio quality control and could be positively confirmed at this low level by calculating the product ion ratios for each of the compounds.

Conclusions

With the described GC-MS/MS method on the TSQ 8000 system all nitrosamine compounds under investigation could be safely detected and precisely quantified at the required low levels for a safe food control.

The LODs of all compounds have been determined to be below 1 ppb, using 1 ppb as the lowest concentration for the quantitative calibration.

The TSQ 8000 GC-MS/MS shows a wide linearity in the range of 1-500 ppb with very good precision. All calibration curves have been shown to be strictly linear with R^2 better than 0.99.

The TSQ 8000 GC-MS/MS shows great ion ratio stability for the confirmation of positive samples. The RSD% of the ion ratio of all compounds is lower than 4% even at LOQ level.

The use, setup and maintenance of a GC-MS/MS method for nitrosamines is easy. The unique AutoSRM software finds and optimizes the SRM transitions and collision energy automatically, even facing new and yet unknown components.

Based on the demonstrated GC-MS/MS method, the TSQ 8000 GC-MS/MS can successfully quantify the concentration of nitrosamine components in real samples without any uncertainty.

The described GC-MS/MS method for food nitrosamines on the TSQ 8000 GC-MS/MS can serve as a turnkey method for routine use in food safety control. It is using standard GC-MS/MS triple quadrupole instrumentation which is also common for many other areas of regular food safety control, e.g. pesticides, POPs or polyaromatic hydrocarbons. The presented method is fast, allows high sample throughput, and provides results with very high sensitivity and precision. With this standard EI ionization method setup this presented method for low level nitrosamine quantitation is recommended to be employed as a productive alternative to the earlier described chemical ionization ion trap procedure using liquid CI reagents.

Table 3. Precision of the confirming ion ratios from 1 ppb – 500 ppb

Concentration (ppb)	1	5	10	25	100	250	500	AVG	RSD (%)
NDMA	70.7	67.9	68.0	69.8	69.1	71.9	69.6	69.6	2.01
NDEA	20.8	22.1	22.5	22.4	22.5	22.5	22.5	22.2	2.84
NDBA	102.4	102.4	98.2	98.6	96.1	93.4	99.2	98.6	3.28
NPIP	6.1	5.5	6.2	5.9	6.0	6.1	6.2	6.0	3.88
NPYR	-	64.6	62.4	66.2	66.9	68.1	66.7	65.8	3.06

Table 4. Results of a blank sample

Compound	Area	ISTD Area	Area Ratio	Ion Ratio Confirmation	Calculated Amount (ppb)
NDMA	2591.368	2028129.842	0.001	Pass (65.1%)	0.74*
NDEA	1875.386	2028129.842	0.001	Fail (0%)	N/A
NDBA	6806.996	2028129.842	0.003	Fail (81.1%)	N/A
NPIP	N/A	2028129.842	N/A	N/A	N/A
NPYR	N/A	2028129.842	N/A	N/A	N/A
NMOR	4415.782	2028129.842	0.002	Fail (0%)	N/A

**Below LOQ*

Table 5. Results from a spiked beer sample

Compound	Area	ISTD Area	Area Ratio	Ion Rato	Calculated Amount (ppb)
NDMA	91318.135	2282168.009	0.040	Pass (68.3%)	12.0
NDEA	480955.478	2282168.009	0.211	Pass (22.0%	9.4
NDBA	402754.561	2282168.009	0.176	Pass (96.8%)	13.2
NPIP	280162.125	2282168.009	0.123	Pass (5.9%)	10.1
NPYR	318081.273	2282168.009	0.139	Pass (68.9%)	13.3
NMOR	1145719.054	2282168.009	0.502	Pass (67.9%)	10.1

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Determination of Acrolein and Other Process Contaminants in Beer, Wine, and Potato Chip Matrices by Liquid Chromatography-Single Quadrupole Mass Spectrometry

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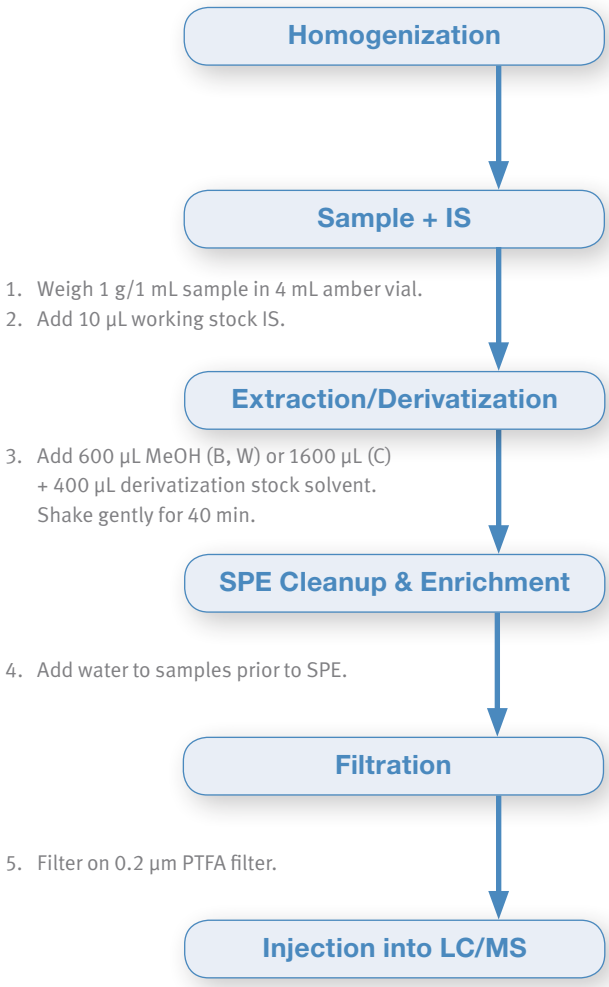
Method 63558

Key Words
Liquid chromatography, single quadrupole LC/MS, DNPH derivatization, reactive carbon compounds

1. Schematic of Method



2. Introduction

Carbonyl compounds are widely found in food products. They can originate from raw materials, alcoholic fermentation, or from a wide range of chemical reactions such as lipid oxidation, Maillard reactions, Strecker degradation, and aldol condensation. Acrolein is the α,β-unsaturated carbonyl compound also called prop-2-enal or acrylic aldehyde. Acrolein has a high volatility and very high reactivity.[1] To evaluate risk assessment, more information on its occurrence needs to be generated. The aim of this study was to develop an in-house validated method for the determination of a wide range of carbonyl compounds to quantify these process contaminants in beer, wine, and potato chips using LC/MS.

3. Scope

This method can be applied to screen for the presence of acrolein and its homologues and other process contaminants (5-hydroxymethylfurfural, acetoin, glyoxal, methylglyoxal, and nonenal) at levels above 0.2 mg/kg in wine, beer, and potato chip products.

4. Principle

The method involves simultaneous extraction and derivatization of the carbonyl compounds from foods to form the corresponding 2,4-dinitrophenylhydrazones. After cleanup on solid phase extraction cartridges, LC/MS analysis was performed for quantification. Non-naturally occurring hydroxyl-acetoin was used as an internal standard.

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5. Reagent List

5.1	Acetonitrile, LC/MS grade
5.2	Methanol, Fisher Chemical™ Optima™ LC/MS grade
5.3	Water, LC/MS grade
5.4	Hydrochloric acid, 37.5%
5.5	Dinitrophenylhydrazine

6. Standard List

6.1	Target compounds: acrolein (ACR), acetoin (ACET), glyoxal (GLX), methyl-glyoxal (MeGLX), 5-hydroxymethylfurfural (HMF), and 9-nonenal (NON) obtained from Sigma-Aldrich®
6.2	Internal standard: hydroxyl-acetoin (ACETOH) from Sigma-Aldrich

7. Standards & Reagent Preparation

7.1 Standard stock solutions (including IS) (1000 µg/mL)
Approximately 30.00 mg of the compound (the amount was re-calculated based on the actual purity of the standard) was weighed into a 40 mL screw cap amber vial and dissolved in 30 mL methanol. The real concentration of solution was calculated gravimetrically. Standard solutions were kept in a refrigerator and in the dark. Long-term exposure (0.5 hour or more) to room temperature or daylight during preparation of working standards was avoided.

7.2 Working standard solution (143 µg/mL)
The same volume of each individual stock standard solution (also from internal standard) was transferred into an amber vial. Working standard solutions were prepared fresh every time before using. All necessary dilutions were performed from this solvent.

7.3 Stock derivatization solvent (DNPH) (4000 mg/L)
600 mg of dinitrophenylhydrazine was weighed in a 250 mL bottle and 75 mL methanol (MeOH) and 37.5 mL water (H_2O) were added. The solution was shaken thoroughly before and while carefully adding 37.5 mL concentrated HCl in the fume hood. A subsequent volume of this solution was filtered on a 0.2 um PTFA filter into a smaller volume bottle and was used for derivatization purposes.

Note: Pellets and precipitates can be observed in this solution after a while. In this case, repeated filtration was applied.

8. Apparatus

8.1	Sartorius® analytical balance *(Sartorius GmbH, Germany)*
8.2	Thermo Scientific™ Barnstead™ Easypure II water
8.3	Horizontal shaker
8.4	Universal top frame for shaker
8.5	BRAND™ accu-jet® pipettor controller *(BRAND GmBh + Co. KG, Germany)*
8.6	pH meter
8.7	SPE vacuum manifold
8.8	Mortar
8.9	Thermo Scientific Heraeus Fresco™ 17 micro centrifuge
8.10	Thermo Scientific Accela™ UHPLC system
8.11	Thermo Scientific MSQ™ mass spectrometer

9. Consumables

9.1	LC vials
9.2	Thermo Scientific Finnpipette™ 100–1000 µL pipette
9.3	Finnpipette 10–100 µL pipette
9.4	Finnpipette 500–5000 µL pipette
9.5	Pipette holder
9.6	Pipette tips 0.5–250 µL, 500/box
9.7	Pipette tips 1–5 mL, 75/box
9.8	Pipette tips 100–1000 µL, 200/box
9.9	Spatula, 18/10 steel
9.10	Spatula, nylon
9.11	Tube holder
9.12	Wash bottle, PTFE
9.13	2 mL vial rack
9.14	15 mL centrifuge plastic tube
9.15	Syringe 1 mL
9.16	Syringe filter 0.2 µm
9.17	Thermo Scientific Accucore™ RP-MS 2.6 µm , 100 x 2.1 mm HPLC column
9.18	Thermo Scientific HyperSep™ C18 SPE cartridges, 3 mL, 200 mg
9.19	Thermo Scientific Uniguard™ holder
9.20	Thermo Scientific Hypersil™ GOLD 10 x 4 mm, 3 µm guard column

10. Glassware

10.1	1 mL glass pipette
10.2	1 L bottle
10.3	500 mL bottle
10.4	30 mL amber screw cap vials
10.5	Caps for vial
10.6	4 mL amber screw cap vials
10.7	Caps for vial

11. Procedure

11.1 Sample Preparation

Liquid samples (beer and wine)

A liquid sample of 1 mL was placed into a 4 mL amber vial and its weight was noted. Then, 600 µL MeOH, 100 µL IS, and 400 µL DNPH solutions were added. After shaking on a horizontal shaker at approximately 250 rpm for 30 min, 570 µL H_2O was added prior to application of solution onto the SPE cartridge.

Solid samples (chips)

Potato chip samples were manually homogenized in a mortar and 1g of the homogenate was placed in a 15 mL centrifuge tube. After recording the exact weight of the sample, 1600 µL MeOH, 100 µL IS, and 400 µL DNPH solutions were added and the tube was shaken on the horizontal shaker at approximately 250 rpm for 30 min. After finishing derivatization, the supernatant was decanted and 3200 µL H_2O was added prior to application of solution onto the SPE cartridge.

11.2 Sample Clean-Up, Enrichment (Solid Phase Extraction)

Solid phase extraction occurred on a Thermo Scientific HyperSep C18 SPE cartridges, 3 mL, 200 mg, as follows:

- Cartridge was conditioned with 2 mL MeOH.
- Cartridge was equilibrated with 2 mL 30% MeOH/H_2O.
- Sample was loaded.
- Cartridge was washed with 1 mL 50% MeOH/H_2O.
- Analytes were eluted with 2 mL MeOH. This fraction was used for further analysis.
- 1 mL eluate was filtered through a 0.2 µL PTFE syringe filter into a standard 2 mL HPLC vial and injected in the LC-MSQ™ instrument.

Note: Precipitation of the derivatization agent still can occur in the LC vial after 24 hrs. For best practice and analysis, check the samples waiting for injection frequently (approximately every 8 hrs). To avoid unwanted precipitation, keep the autosampler temperature at 40 °C or filter unused samples again.

12. Analysis

12.1 LC Conditions

The LC condition were as follows:

LC column:	Accucore RP-MS 2.6 µm, 100 x 2.1 mm
Mobile phase A:	MeOH
Mobile phase B:	H_2O
Column oven temperature:	40 °C
Total measurement time:	7 min
Gradient:	Table 1

Table 1. Gradient program

Time [min]	A%	B%	Flow Rate [µL/min]
0.0	45	55	400
1.0	45	55	400
3.6	86	14	400
4.0	100	0	400
5.9	100	0	400
6.0	45	55	400
7.0	45	55	400

12.1.1 Injector settings

The injector settings were as follows:

Injector:	Accela autosampler
Sample holder temperature:	30 °C
Cleaning solvents:	Acetonitrile
Injection loop volume:	25 µL
Pre-clean solvent volume:	100 µL
Pre-clean solvent:	2 steps
Filling speed:	50 µL/s
Post-clean solvent volume:	100µL
Post-clean solvent:	1 steps
Injection volume:	2 µL

12.2 Mass Spectrometric Conditions

Mass spectrometric detection was carried out by the MSQ single quadrupole mass spectrometer in selected ion monitoring (SIM) mode with atmospheric pressure chemical ionization (APCI). All compounds were individually tuned for optimal cone voltage.

The MS conditions were as follows:

Ionization method:	APCI
Polarity:	Negative
Scan type:	Full scan 150–350 *m/z*
Scan time:	0.2 s
Probe temperature:	350 °C
Needle voltage:	3.2 kV
Time range:	1–7 min
Cone voltage:	15 V

13. Calculation of Results

Calibration by the internal standardization is applied for the determination of process contaminants. This quantification method requires determination of response factors R_f defined by the equation below. The calculation of the final result is performed by using the following equations.

Calculation of the response factor:

$$R_f = \frac{A_{St} \times C_{[IS]}}{A_{[IS]} \times C_{St}}$$

R_f – response factor

A_{St} – area of the target compound peak in the calibration standard

$A_{[IS]}$ – area of the internal standard peak of the calibration standard

c_{St} – target compound concentration of the calibration standard solution

$c_{[IS]}$ – internal standard concentration of the calibration standard solution

Calculations for each sample the absolute amount of analyte that was extracted from the sample:

$$X_{analyte} = \frac{A_{analyte} \times X_{IS}}{A_{IS} \times R_f}$$

$X_{analyte}$ – absolute amount of analyte that was extracted from the sample

$A_{analyte}$ – area of analyte peak in the sample

$A_{[IS]}$ – area of the internal standard peak in the sample

$X_{[IS]}$ – absolute amount of internal standard added to the sample

The concentration of analyte in the sample [µg/g]:

$$C = \frac{X_{analyte}}{m}$$

m – weight of sample [g]

$X_{analyte}$ – absolute analyte amount [µg]

14. Method Performance Characteristics

The method was in-house validated according to the criteria specified in the IUPAC/AOAC guideline for single laboratory validation.[2,3] Representative chromatograms are shown in Figures 1 and 2 for standard derivated carbon compounds in solvent and a spiked beer sample, respectively. Determined validation parameters were specificity, linear range, repeatability, accuracy, limit of detection (LOD) and limit of quantification (LOQ), and method robustness as listed below. Matrix samples purchased in local stores were used for establishing validation parameters after being checked for the presence of target compounds prior to the validation study according to 11.1. After it was concluded that a matrix sample was free of target compounds, it was able to be used as a blank matrix for spiked experiments and the determination of target compounds during method validation. Derivatization of methyl-substituted compounds (HMF and MeGLX) resulted in *cis* and *trans* isomers. Consequently, quantification of these compounds was based on the sum of the isomers.

14.1 Selectivity

Using multiple single ion monitoring (SIM) the specificity was confirmed based on the presence of fragment ions at the correct retention time corresponding to the process contaminant standards in the solvent (Table 2). Acceptance criteria for retention time (less than 2.5% RSD) was set according to Bauer *et al.*[1]

Table 2. LC/MS parameters for selected reaction monitoring of analytes

Analyte	Rt [min]	M [g/mol]	Quantifier mass *m/z*	Ion 2 mass *m/z*	Dwell time [s]	APCI Polarity
ACR	3.79	56	236	237	0.2	negative
ACET	3.38	88	268	269	0.2	negative
ACETOH (IS)	2.56	74	254	255	0.2	negative
GLX	2.30	58	238	239	0.2	negative
MeGLX	2.65 & 3.14	72	252	253	0.2	negative
HMF	3.04 & 3.61	126	306	307	0.2	negative
NON	5.32	140	320	321	0.2	negative

14.2 Linearity and Calibration Curve

The linearity of the calibration curves was assessed by internal standardization over the range 0–100 mg/kg. The calibration curves were created at five levels (matrix-matched) by spiking cleaned-up extracts prior to LC injection. All levels were prepared and injected in duplicate. Calibration levels were 0, 5, 25, 50, 75, and 100 mg/kg. In all cases, the correlation coefficients of linear functions were better than 0.985. Rf values for internal standardization were determined from the calibration curves for each matrix and internal standards by calculating cumulative average response factor over the whole calibration range.

14.3 Accuracy

Method accuracy and precision was assessed by a recovery study using blank matrices spiked at three concentration levels and injected in six individually prepared replicates. Samples were spiked at 10, 50, and 100 mg/kg concentration levels prior to processing. All recovery samples were analyzed within 6 hrs after preparation to avoid or minimize further reaction of compounds. Found concentrations and relative standard deviation (% RSD) were calculated and expressed as recovery and precision (Table 3). The expectation of the method was to meet recovery values between 70%–120%, which was met for all compounds. An additional accuracy experiment was carried out by injection of Food Analysis Performance Assessment Scheme (FAPAS®) 2823 external quality control (QC) material (n=6). However, the QC contained only HMF in honey matrix, which was the only available test material with the target compound(s) and similar matrix. The measured average values for the samples were 54 mg/kg (±8.3%), which fell in the middle of the acceptable range (48–61.2 mg/kg) and corresponded well with the assigned values (55 mg/kg).

Table 3. Mean recovery (%RSD) of method

Compound	Recovery % (%RSD)								
	Wine			Beer			Chips		
	10 mg/kg	50 mg/kg	100 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg
ACR	79 (3)	119 (9)	93 (5)	79 (7)	103 (5)	93 (9)	106 (10)	103 (7)	111 (12)
ACET	119 (9)	79 (8)	87 (10)	119 (13)	93 (11)	87 (17)	116 (6)	84 (5)	107 (15)
GLX	<LOQ	114 (7)	101 (6)	<LOQ	117 (4)	101 (15)	<LOQ	111 (8)	100 (15)
MeGLX	95 (1)	107 (5)	106 (6)	95 (4)	110 (3)	106 (9)	<LOQ	87 (6)	97 (11)
HMF	100 (7)	113 (8)	85 (11)	100 (8)	115 (4)	85 (3)	87 (6)	104 (4)	92 (10)
NON	85 (6)	112 (6)	94 (2)	85 (7)	85 (11)	94 (10)	107 (9)	96 (8)	98 (13)

14.4 Precision

Method within-day precision and between-day precision values were determined with individually prepared samples for each matrix at the middle spiking level (50 mg/kg) each in six replicates and expressed as %RSD over three days. Measured values deemed to be acceptable (below 15%) and are shown in Table 4. All repeatability samples were analyzed within 6 hrs after preparation.

Table 4. Precision and intermediate precision at 50 mg/kg concentration level

Compounds	Repeatability			Intermediate Precision		
	Wine	Beer	Chips	Wine	Beer	Chips
ACR	9	5	7	15	12	9
ACET	8	11	5	9	16	7
GLX	7	4	8	12	10	10
MeGLX	5	3	6	6	4	8
HMF	8	4	4	11	5	10
NON	6	11	8	14	15	11

14.5 Limit of Detection, Limit of Quantification

Limits of detection and quantification were estimated following the IUPAC approach, which consisted of analyzing the blank sample to establish noise levels and then testing experimentally estimated LODs and LOQs for signal-to-noise ratios, 3 and 10 respectively. Due to the lack of legislation values, the expectation of the method was to achieve limits as low as possible. The resulted LOD and LOQ values are listed in Table 5.

Table 5. Method LOD and LOQ values

Analyte	SOLV		Beer		Wine		Chips	
	LOD (mg/kg)	LOQ (mg/kg)	LOD (mg/kg)	LOQ (mg/kg)	LOD (mg/kg)	LOQ (mg/kg)	LOD (mg/kg)	LOQ (mg/kg)
ACR	0.15	0.5	0.25	0.8	0.25	0.8	0.25	0.8
ACET	0.24	0.8	0.6	2	0.6	2	0.6	2
ACETOH (IS)	7.5	25	7.5	25	7.5	25	7.5	25
GLX	0.5	1.7	6	20	4.5	15	4.5	15
MeGLX	0.9	3	5	17	4.5	15	7.5	25
HMF	0.1	0.3	0.36	1.2	0.3	1	0.3	1
NON	0.1	0.3	0.18	0.6	0.18	0.6	0.18	0.6

14.6 Survey Samples

To prove method applicability for real samples, different beer, wine and chip products were purchased (n=16) in local stores and analyzed with the method. Samples covered different types of beer, red wine, and potato chip samples with different flavors (onion, salted, pepperoni, cheese, ketchup). No traces of the main target compound (ACR) were found in any of samples. However traces of NON between 0.4–0.9 mg/kg and ACET between 0.8–1.3 mg/kg were found in three out of seven beer samples, while all beer samples contained HMF in concentrations between 0.4–6.1 mg/kg. In addition, ACET was measured in concentration between 4.8–5.5 mg/kg in two out of four red wine samples.

14.7 Robustness

The following parameters became evident during the robustness study as critical for a repeatable method:

- stability of working stock standard solutions is very limited at room temperature and during daylight
- concentration and amount of added derivatization solvent (analyte-to-derivatization-agent ratio)
- derivatization reaction time
- pH of derivatization solvent

Therefore to achieve comparable results, the instructions need to be followed very carefully.

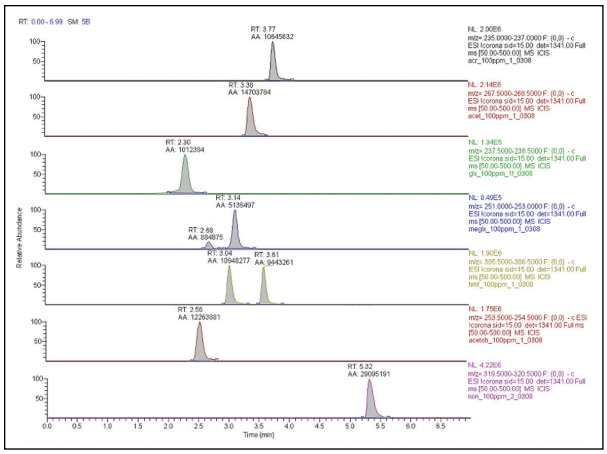

Figure 1. Chromatogram of 100 ppm standard derivated carbon compounds in solvent freshly after the derivatization reaction. (Traces from top: ACR, ACET, GLX, MeGLX, HMF, ACETOH and NON)

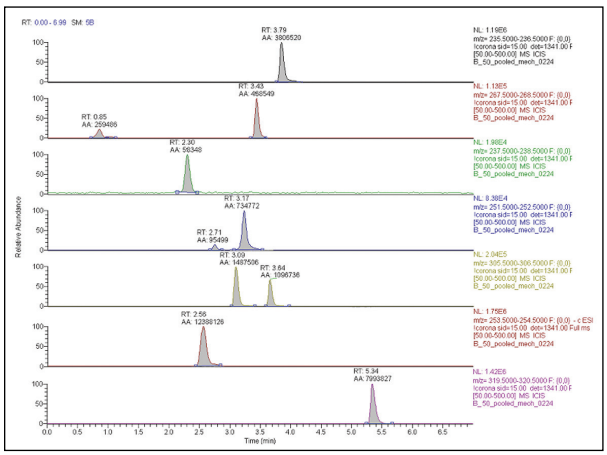

Figure 2. Chromatogram of a beer sample spiked at 50 ppm with carbon compounds and after the derivatization reaction. (Traces from top: ACR, ACET, GLX, MeGLX, HMF, ACETOH and NON)

15. Conclusion

The method presented describes determination of low molecular weight and very reactive food process contaminants (acrolein and other low molecular weight carbon components) in three different matrices by application of *in-situ* derivatization reaction and fast chromatographic determination by LC-MS instrumentation. Due to the very short half life of the target compounds, the derivatization reaction has to be carried out as fast as possible after sampling to be able to recover the maximum amount of analytes. The in-house validation of the method gave detection capability at the sub-ppm level and confirmed the reliability of the method for quantification under the described conditions: selectivity, recovery, and precision values were in accordance with the expectations of the latest method performance guidelines. Consequently, the method is applicable for determination of the target compounds in beer, wine and potato chip matrices by using LC/MS.

16. References

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A Rapid and Specific Method for the Detection of Spiked Toxins Into the Food Supply

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Overview

Purpose: Fast and accurate screening of unknown toxic substances in food supply

Methods: Toxins containing samples were analyzed by UHPLC-HR/AM MS/MS on a Q Exactive benchtop Orbitrap mass spectrometer with full scan at 70,000 resolution and data dependent MS/MS at 17,500 resolution. Chromatograms were analyzed via a data mining program, SIEVE software, using a three-step method including 1) chromatogram alignment, 2) component detection, and 3) identification through online or customized libraries.

Results: All three spiked toxins in acetonitrile sample and one toxin in an apple juice sample, were successfully and fairly easily identified as the spiked toxic unknowns. The second toxin in apple juice sample has in-source fragmentation under the ion source condition. Therefore, the target toxin and its co-eluted fragment products were identified from SIEVE software. In addition, two overlapped toxins in LC chromatograms with short gradient were accurately identified through the workflow.

Introduction

Developing a fast and accurate screening method for detecting a wide range of toxic compounds is an important task for food safety. Recently, there has been a trend toward the use of full scan high-resolution, accurate mass (HR/AM) spectrometry for this purpose. HR/AM spectrometry overcomes the screening limitation via selected reaction monitoring (SRM) on triple stage quadrupoles, because specific compounds need not be selected before analysis. The entire mass range is essentially "selected". HR/AM measurement provides the specificity. Highly confident identification is achieved by accurate mass measurement of both precursor and fragment ions. A novel UHPLC-MS/MS method employing the Thermo Scientific Q Exactive benchtop Orbitrap™ mass spectrometer (Figure 1) is proposed here for the study of possible spiked toxic agents into apple juice.

Methods

Sample Preparation: Two sets of samples (acetonitrile and apple juice matrix) were prepared by spiking 10 ppm level of toxin compounds into 10 ml of the matrix and then adding 10 ml of acetonitrile. Spiked toxins in the acetonitrile sample were Colchicine, Strychnine and Aconitine; Lobeline and Solanine were spiked in the apple juice sample. The mixtures were shaken for 30 minutes and stored at 4 ^{0}C until further analysis. Solvent and matrix blanks were prepared in the same way without spiking any compounds. Samples were analyzed by ultrahigh pressure liquid chromatography-mass spectrometry (UHPLC-MS) with the Thermo Scientific Accela 1250 pump and the Q Exactive™ benchtop mass spectrometer with full scan and data dependent MS/MS with a 6.5-minute gradient. The identity of the spiked compounds was not known by the analyst prior to the analysis. The spiked compounds were thus "unknown" to the analyst.

Liquid Chromatography:

Column:	C18 column (2.1 x 50 mm, 1.8 μm)
Injection Volume:	2 μL
LC:	Accela™ 1250 pump
Solvent A:	Water, 0.1% Formic Acid
Solvent B:	Methanol
Flow Rate:	350 μL/min

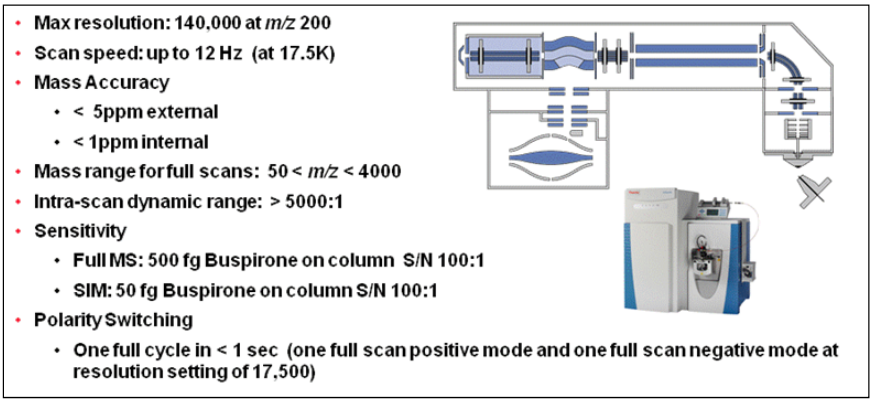
Gradient:

Time	A%	B%
0.0	95	5
3.5	5	95
6.5	5	95
6.6	95	5
9.0	95	5

Mass Spectrometry:

Spray Voltage (+)	3800 kV
Capillary Temperature (+)	320 ºC
Sheath Gas (+)	50
Aux Gas (+)	15
Sweep Gas (+)	0
Heater Temperature (+)	450 ºC
S-lens	50
Positive MS Scan	1 microscan
Full Scan	R = 70,000; AGC = 1e6; Inject = 250 ms; Lock Mass = off
MS/MS	R = 17,500; AGC = 2e5; Inject = 120 ms; HCD = 35±20%

FIGURE 1. Q Exactive Benchtop Orbitrap Mass Spectrometer



Data Analysis: Data acquisition was performed using Thermo Scientific Xcalibur 2.1 software. Differential analysis in Thermo Scientific SIEVE 1.3 software was used to analyze spiked and control samples with principal component analysis(PCA).

Figure 2 shows the overall SIEVE workflow for the unknown screening. Briefly, acquired LC chromatograms are first aligned based on the selected reference raw file to correct the retention time variance from LC runs. Then all peaks found above given intensity threshold will be ordered based on intensity. Frames of 0.30 min x 0.010 amu (see Figure 3) are defined based on the orders from the most intense to second highest peak and so on, without overlapping with previous frames. The capacity of 2,700,000 frames in 9-minute gradient are used to define analytes that may exist in the sample by PCA analysis. The ions found from differential analysis is identified through ChemSpider™ online database search and confirmed with MS/MS spectra.

FIGURE 2. SIEVE Workflow

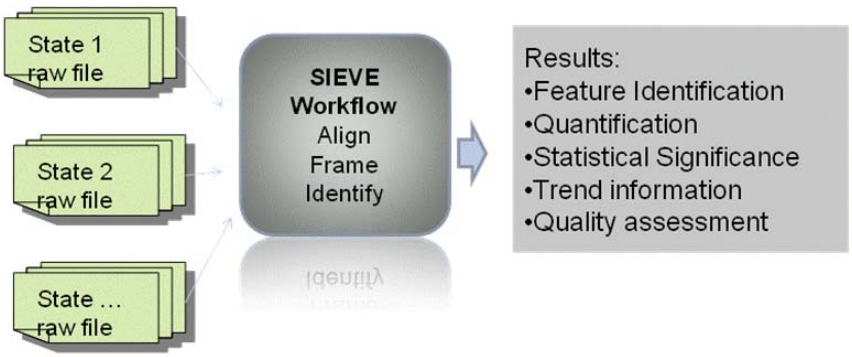
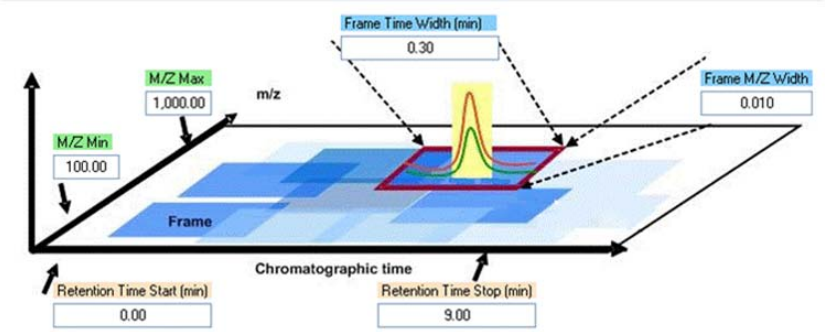


FIGURE 3. Frame Parameters



Results

Solvent Sample Analysis: The results showed a distinct difference between the spiked and controlled blank samples (Figure 4). The ChemSpider search returned with the correct identification of three compounds: Strychnine, Colchicine and Aconitine with the mass tolerance of 2 ppm with external calibration (Figures 5 & 6). Notice that two of the spiked toxins, Colchicine and Aconitine (Figure 6), overlapped with each other in UHPLC chromatograms with a short gradient but were accurately identified by SIEVE software via a differential analysis between samples versus the control blank when searched against KEGG and Sigma-Aldrich online databases in ChemSpider.

FIGURE 4. Chromatographic Alignment, Frame and PCA Analysis of Solvent Sample1 Spiked with 3 Toxins

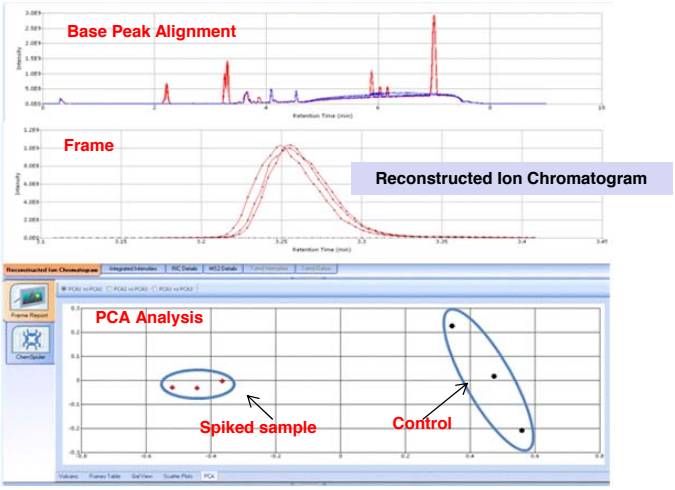


FIGURE 5. Identified Toxin1-Strychnine(*m/z* 335.1751) in Solvent Sample

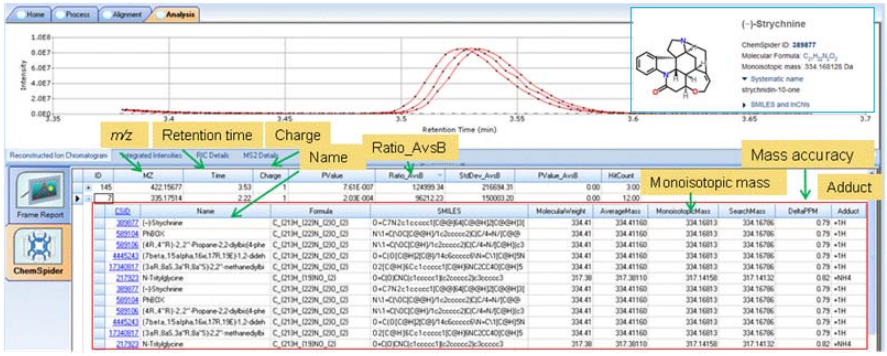
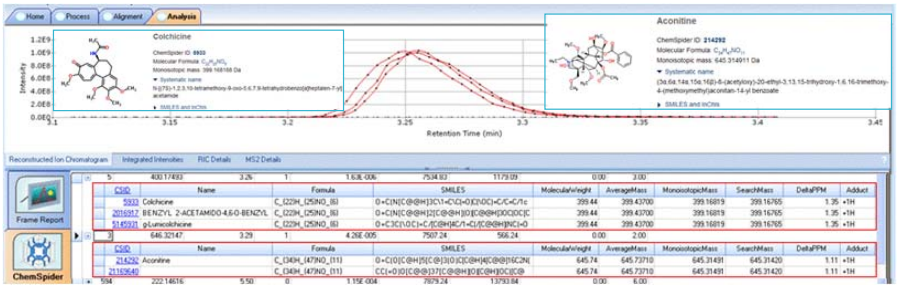


FIGURE 6. Identified Toxin2-Colchicine(*m/z* 400.1749) and Toxin3-Aconitine (*m/z* 646.3215) in Solvent Sample



Apple Juice Sample Analysis: Two toxins were spiked in the apple juice sample. Three replicates were analyzed against a clean apple juice matrix. Results are shown in Figures 7,8 & 10. Lobeline was fairly easily identified as the first spiked toxin (Figure 8). The identity of Lobeline was confirmed by MSMS interpretation as shown in Figure 9.

FIGURE 7. Apple Juice Sample-PCA Analysis

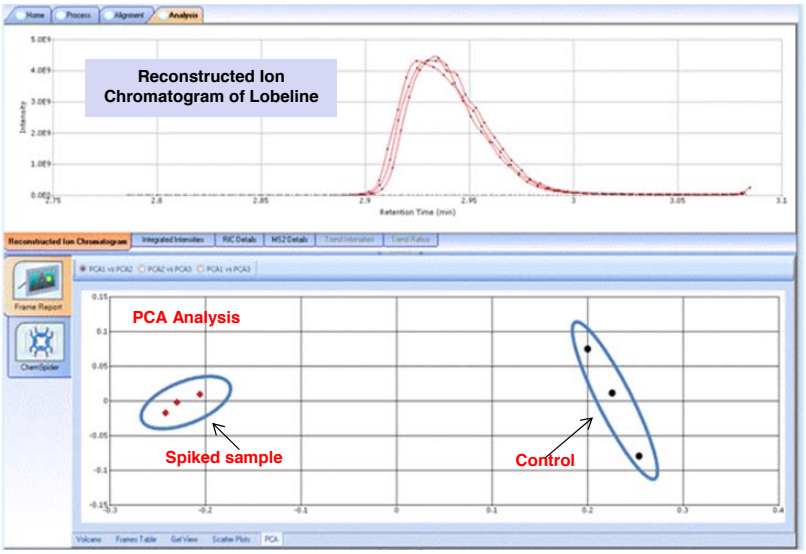


FIGURE 8. Identified Toxin1-Lobeline(*m/z* 338.2113) in Apple Juice Sample

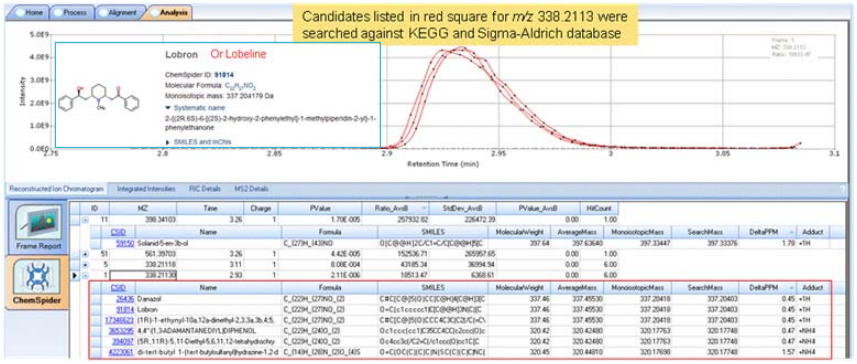
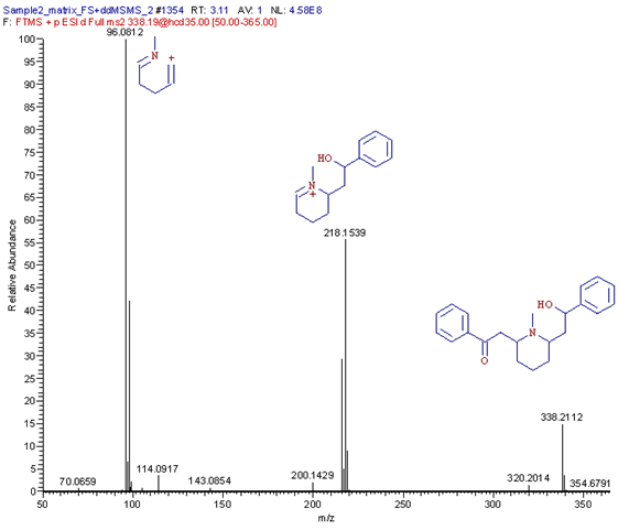


FIGURE 9. Annotated MS/MS spectrum of Lobeline(*m/z* 338.2113)



In apple juice sample, Solanine *m/z* 868.5031 and its in-source fragmentation ions, Solanadine *m/z* 398.3410, 560.3936, 561.3970 (n+1 isotope of 560.3936) co-eluted at 3.26 min and were identified as the 2nd toxic compound (Figures10 & 11).

FIGURE 10. Identification of Toxin2-Solanine(*m/z* 868.5031) and its In-Source Fragment Solanadine(*m/z* 398.3410) in Apple Juice Sample

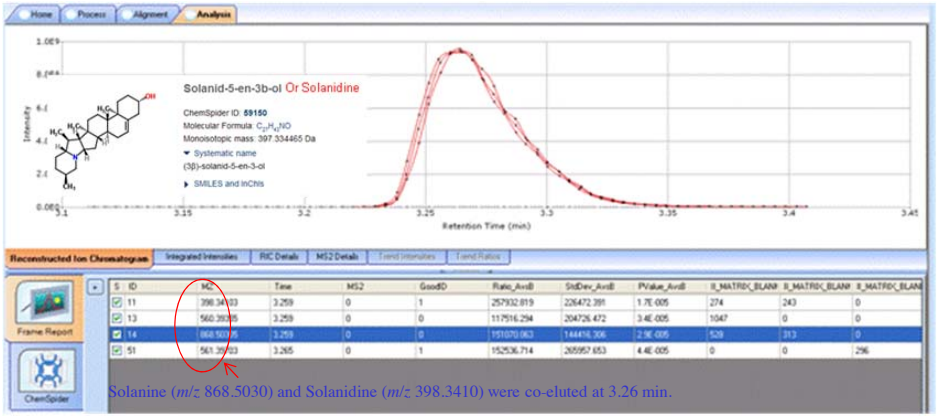
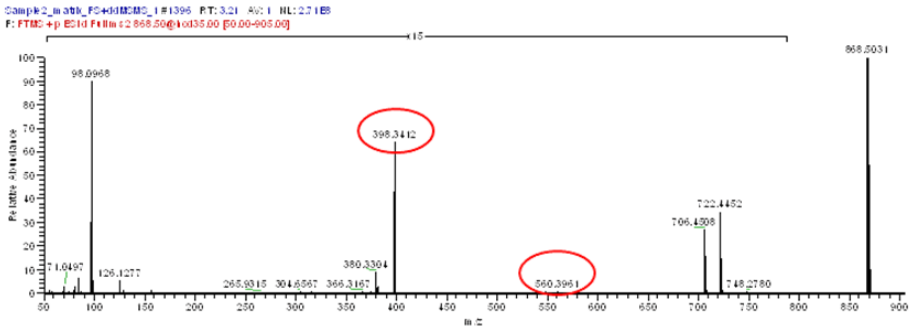


FIGURE 11. MS/MS Spectrum of *m/z* 868.5031



Conclusions

- Acetonitrile solvent sample spiked with 3 toxins, apple juice sample with 2 toxins and matrix blanks were analyzed in triplicate by the Q Exactive MS with Full Scan at 70,000 and Top1 MS/MS at 17500 resolution.
- Mass accuracy of all identified toxins is within 2 ppm with external mass calibration.
- Spiked toxins were screened through SIEVE1.3 software. Four out of five unknowns were easily and successfully targeted. The other toxin was found to co-eluted with its in-source fragmentation products.
- With the function of precursor ion selection for MS/MS and the HR/AM data acquired from the Q Exactive MS, unknown compounds can be screened out at high confidence with the structure elucidation within one injection.

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Advances in Automation of Food-related Analysis and Screening

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Overview

Purpose: To introduce new sample preparation methods aimed to solve formidable analytical challenges in the food-related analysis area.

Methods: Automated online sample preparation using Thermo Scientific TurboFlow technology coupled with Thermo Scientific mass spectrometers.

Results: Through using this technology for food analysis, the need for manual sample preparation is removed, resulting in significantly increased sample throughput .

Introduction

Worldwide food safety concerns have risen dramatically as the number of food contamination incidents and product recalls has increased. Therefore, accurate monitoring of contaminant levels in food and agricultural products is essential to assure the safety of the food supply and to manage human health risks. It is well-known that the basic analytical requirements in food analysis are high resolution, high throughput, high sensitivity detection and quantification of contaminants at or below the maximum residue limit (MRL) or tolerance of the compound in a given food matrix. Liquid chromatography-mass spectrometry (LC/MS) as the central enabling technology has been recognized as an indispensable tool in food safety and quality control fields[1]. LC/MS provides high speed, high resolution and high sensitivity separation of various chemical compounds.

Every food analysis starts with sample preparation, which is widely accepted as one of the most critical steps of LC/MS. Increased demand for higher throughput, accuracy and lower matrix interference from food analysis laboratories has made sample preparation the largest bottleneck. Currently, solvent extraction and solid phase extraction (SPE) are two of the most widely used methods to isolate and/or enrich target analytes from food matrices. When done manually, these offline techniques are often labor-intensive, time-consuming and costly, resulting in low sample throughput. Turbulent flow chromatography technology can eliminate the need for lengthy offline sample preparation steps, thereby eliminating these disadvantages.

Methods

Turbulent flow chromatography

This study will review a number of key applications in food safety using turbulent flow chromatography. All experiments used a Thermo Scientific Transcend TLX system powered by TurboFlow™ technology to separate analytes from various matrices prior to MS/MS analysis. The system injected the sample directly onto a narrow diameter (0.5 or 1.0 mm), TurboFlow chromatography column packed with large particles [Figure 1(A)]. High linear velocities are created inside the column, which force large molecules to quickly flow through to waste while retaining the small molecule analytes. The technology is an improvement over traditional SPE because it utilizes reusable extraction columns in a closed system, reducing the time required for offline sample preparation from hours to minutes. It also allows automatic removal of proteins and larger molecules in complex mixtures by combining turbulence, diffusion and chemistry. Figure 1(B) shows the typical configuration of a single-channel Transcend™ TLX system.

By directly injecting food samples into the LC/MS system, food safety and quality laboratories can achieve significant analytical improvements by eliminating time-consuming, costly sample preparation steps. Turbulent flow chromatography technology also allows the broad selection of stationary phases. These features make the technology a versatile and important tool in the food safety area.

FIGURE 1. Fluid Path in turbulent flow chromatography column and LC/MS system configuration

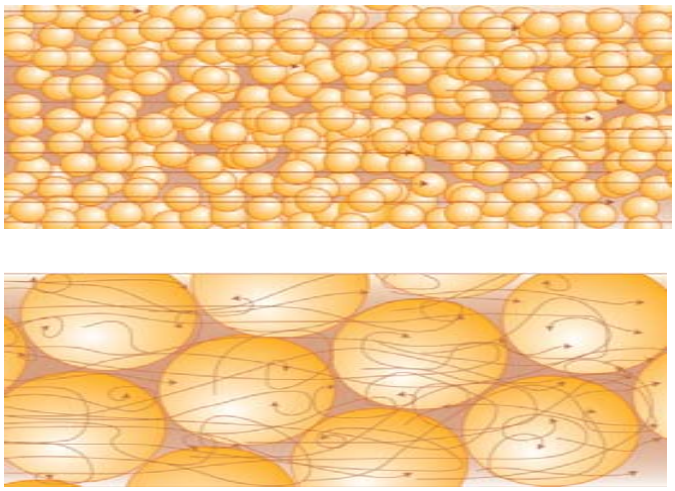


Figure 1(A)

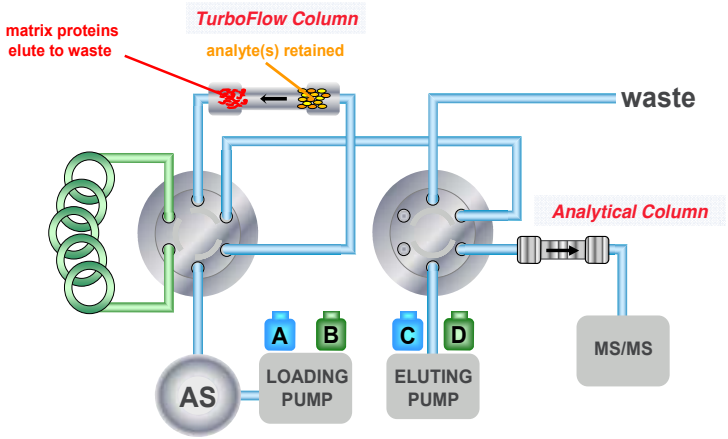


Figure 1(B)

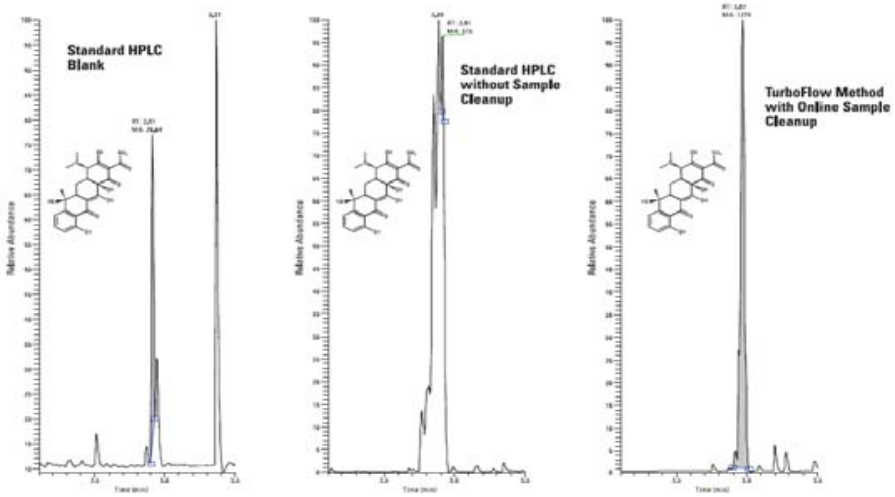
Results and Discussion

1) Veterinary drugs and chemicals

Four common chemical residues, malachite green (MG), leucomalachite green (LMG), ciprofloxacin and tetracycline in fish, shrimp and pig liver were analyzed using a Thermo Scientific TSQ Quantum Access triple stage quadrupole mass spectrometer[2]. The MRLs for these analytes range from 2 μg/kg for the sum of MG and LMG residues in fish muscle to 100 μg/kg for both ciprofloxacin and tetracycline in muscle for all food-producing species.

The total offline sample preparation time was approximately 30 to 40 minutes, including homogenization, centrifugation and calibrator preparation. Figure 2 compares representative standard high pressure liquid chromatography (HPLC) and turbulent flow chromatography method chromatograms of 500 ng/kg (parts per trillion) tetracycline in fish matrix. This indicates the capability of removing endogenous interferences using the LC/MS system, thus reducing ion suppression effects and improving detection limits.

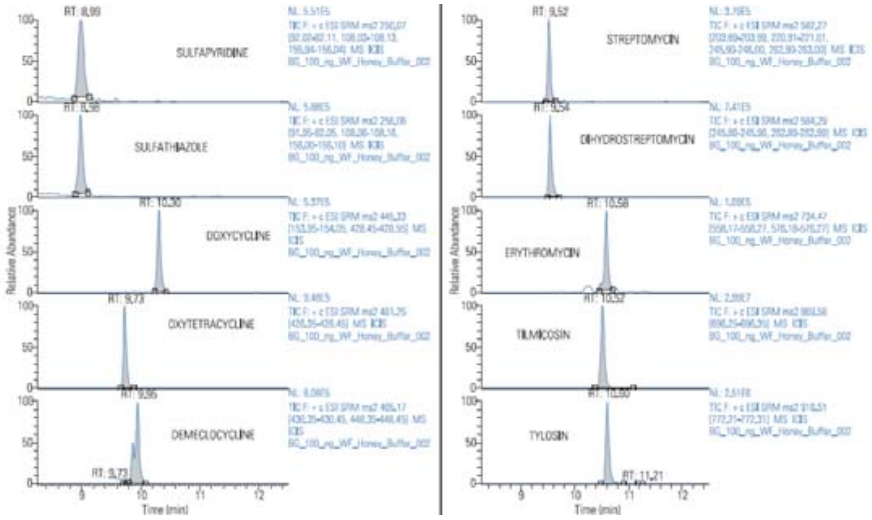
FIGURE 2. Chromatogram comparison of tetracycline at 500 ng/kg in fish (tilapia) matrix in standard HPLC and turbulent flow chromatography method



2) Antibiotics in honey

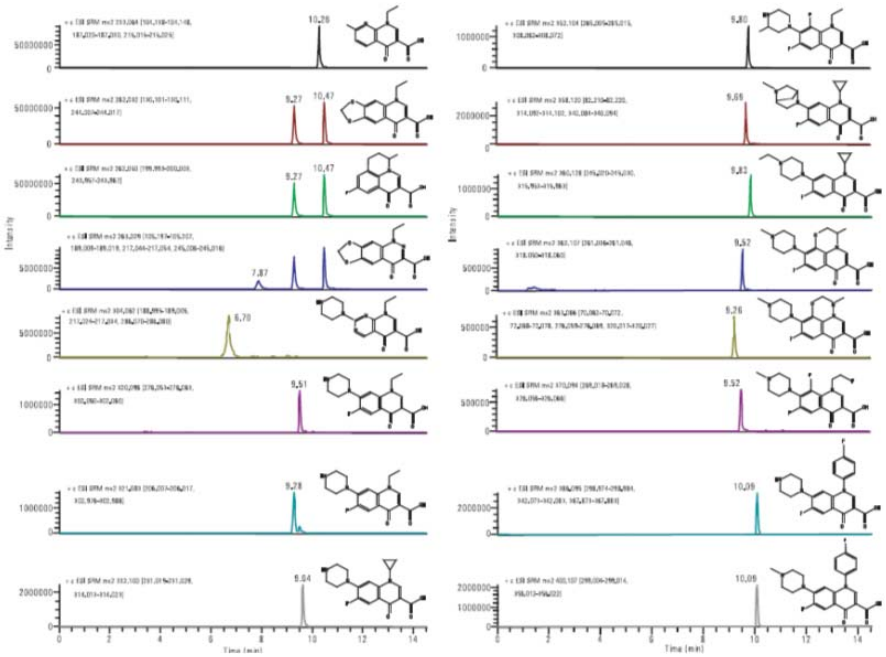
Ten representative antibiotics in honey belonging to four different structural classes were selected: sulfonamides, tetracyclines, aminoglycosides and macrolides[3]. The only offline sample preparation step required was the aqueous buffer dilution of raw honey to reduce the sample viscosity, which took less than 10 minutes. The total LC-MS/MS method run time was less than 18 minutes. A representative chromatogram of the 10 analytes at 100 ng/mL in 1:1 honey/buffer is shown in Figure 3.

Figure 3. Example chromatogram of 100 ng/mL calibration standard in 1:1 honey/buffer



Quinolones, including fluoroquinolones, in honey were also investigated[4]. Instead of using an SPE method, an online extraction method using turbulent flow chromatography was developed. The sample preparation time for the entire batch, including 16 compounds, was reduced from 5 hours to 40 minutes (80% of sample preparation time eliminated). The quantitation limits for the majority of analytes were 1 µg/kg (ppb) with no matrix interference. Figure 4 illustrates representative selected reaction monitoring (SRM) chromatograms at 20 µg/kg, showing the selected ion transitions and retention times for the studied analytes.

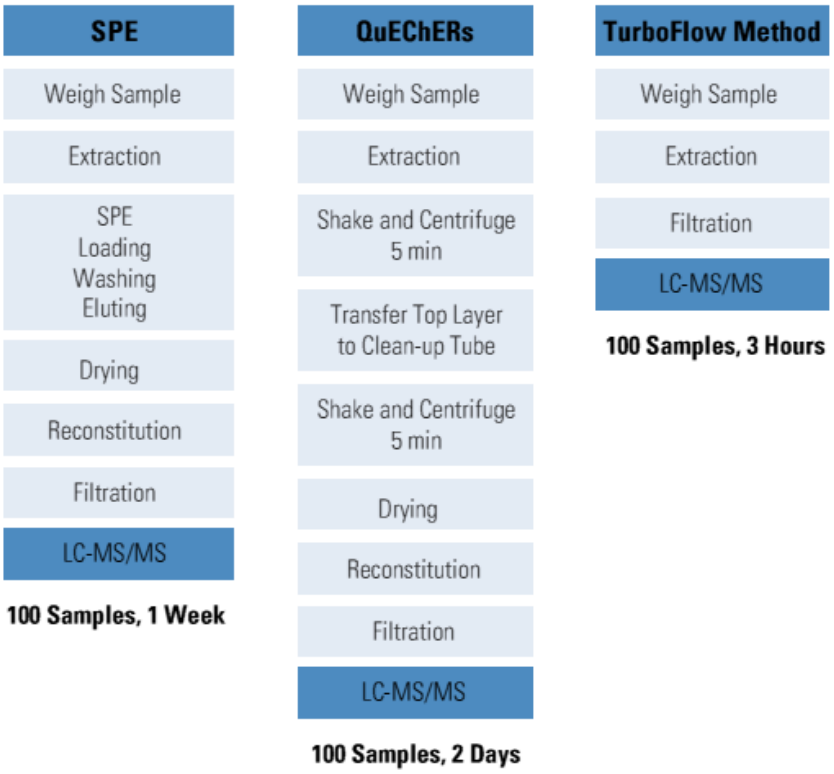
FIGURE 4. Figure 4: Representative SRM chromatograms (20 µg/kg) showing the selected ion transitions and retention times for the studied analytes



3) Pesticides in green tea

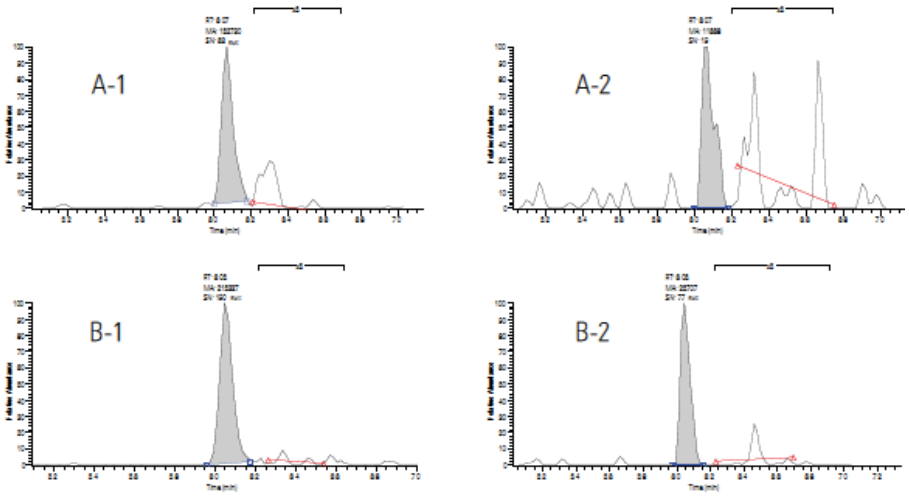
As shown in Figure 5, we compared a TurboFlow method and two currently popular methods for pesticide residue sample preparation, SPE and quick, easy, cheap, effective, rugged, and safe (QuEChERS). A typical SPE method involves equilibrating the cartridge, loading, washing and eluting analytes. It usually takes about 1 week to process 100 samples. Although QuEChERs was designed to simplify sample preparation, it still requires two-step centrifugation and concentration. A few days are typically required to prepare 100 samples with QuEChERs. TurboFlow technology minimizes preparation of 100 samples to less than 3 hours, dramatically improving the efficiency and throughput of this routine lab test[5].

FIGURE 5. Comparison of the TurboFlow method to SPE and QuEChERs



By using the Transcend TLX system with TurboFlow technology, the background noise and interference peaks are reduced significantly. Figure 6 compares chromatograms of Clomazone at 6.25 µg/L in tea extract using standard HPLC (top) and the TurboFlow method (bottom). The left panel (A-1 and B-1) shows the primary transition of m/z 240 > 125. The right panel (A-2 and B-2) shows the secondary transition of m/z 240 > 89. It clearly shows the effectiveness of background reduction using TurboFlow technology while the signal to noise ratio increased by 3 and 4 times for m/z 125 and 89 transitions, respectively. The area responses of both peaks also increase by more than 50% due to the minimization of ion suppression incurred by matrix. We also noticed the mass spectrometry response become more stable across the entire tested concentration range, thus improving the method reliability.

FIGURE 6. Comparison of chromatograms of Clomazone at 6.25 µg/L in tea extract using standard HPLC (top) and the TurboFlow method (bottom).



Conclusion

Online sample extraction utilizing turbulent flow chromatography coupled with LC-MS/MS and complimentary techniques has gained popularity in the food safety arena. The objective of this technology is to provide automated, high resolution, high sensitivity and high specificity separation of target analytes from extremely complex food matrices, removing the need for manual sample preparation and therefore increasing sample throughput. Turbulent flow chromatography also facilitates mass spectrometry detection and quantitative measurement and minimizes ion suppression and matrix effects. In addition, the multiplexing capability of certain LC/MS systems can quadruple the throughput of a turbulent flow chromatography method, providing unmatched productivity and cost savings.

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Analysis of Perchlorate in Infant Formula by Ion Chromatography-Electrospray-Tandem Mass Spectrometry (IC-ESI-MS/MS)

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Key Words

- TSQ Quantum Access
- Ion chromatography
- Food safety
- Sensitivity

Introduction

Ion chromatography-mass spectrometry (IC-MS/MS) can be used to detect, quantify, and confirm a variety of analytes in environmental and food matrices, including haloacetic acids, bromate, and percholorate. Perchlorate, a naturally occurring and man-made contaminant, is widely found in the environment in surface water, groundwater, and soil. Through environmental contamination, perchlorate has entered the food supply chain and has been detected in a wide variety of foods including eggs, milk, vegetables, and fruits. In humans, perchlorate interferes with the ability of the thyroid gland to take up iodine, which is needed to produce hormones that regulate many body functions after they are released into the blood. Because these thyroid hormones are essential for normal growth and development, infants and children could more likely be affected by perchlorate than adults.

A recent study by the Centers for Disease Control and Prevention examined various types of commercially available powdered infant formulas to determine if perchlorate could be found at measurable levels.[1] All of the powdered infant formulas tested contained perchlorate. Significantly higher concentrations of perchlorate were found in cow's milk-based formulas with lactose than in other types.

A simple and ultra-sensitive IC-MS/MS technique for the quantitation of perchlorate in powdered infant formula, liquid infant formula, and milk is described here. Unlike conventional detection methods that require labor intensive solid phase extraction (SPE) sample enrichment, only a simple sample preparation is necessary.

Goal

To develop a simple and ultra-sensitive IC-MS/MS method to separate and quantitate perchlorate without time-consuming sample preparation.

Experimental Conditions

Sample Preparation

Samples of commercially available liquid infant formula, reconstituted powdered infant formula, and milk were prepared for analysis. A 4-mL sample of formula or milk was mixed with 4 mL of ethanol (pre-cooled at 4 °C) and 0.4 mL of 3% acetic acid. The sample was spiked with 40 µL (100 ng/mL) isotope-labeled internal standard. The mixture was vortexed and then centrifuged at 5000 rpm for 30 minutes under refrigeration (-5 °C). The supernatant was run through a syringe filter that had been preconditioned with 5 mL ethanol and 15 mL of deionized water. The filtrate was collected in 10-mL plastic autosampler vials and readied for IC-MS/MS analysis.

Ion Chromatography

IC analysis was performed on a Thermo Scientific Dionex ICS-3000 Reagent-Free ion chromatography (RFIC) system. The IC conditions were as follows:

Column set:	Thermo Scientific Dionex AG16 / AS16 hydroxide selective anion exchange columns
Suppressor:	Thermo Scientific Dionex ASRS 300 self-regenerated suppressor (external water mode)
Column temperature:	30 °C
Injection volume:	100 µL
Eluent:	Isocratic 45 mM hydroxide
Eluent Source:	EGC III KOH
Solvent:	150 µL/min acetonitrile delivered by an AXP-MS pump
Flow rate:	300 µL/min
Detection:	First detector: Suppressed conductivity Second detector: Thermo Scientific TSQ Quantum Access mass spectrometer

Mass Spectrometry

MS analysis was carried out on a TSQ Quantum Access™ triple stage quadrupole mass spectrometer with an electrospray ionization (ESI) source. The MS conditions were as follows:

Ion source polarity:	Negative ion mode
Spray voltage:	4000 V
Sheath gas pressure:	40 arbitrary units
Ion sweep gas pressure:	15 arbitrary units
Auxiliary gas pressure:	5 arbitrary units
Capillary temperature:	300 °C
Collision gas pressure:	1.8 mTorr
Scan mode:	Selected reaction monitoring (SRM)

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Selected reaction monitoring allowed the following fragmentation patterns to be observed:

m/z 99 ($^{35}ClO_4^-$) → *m/z* 83 ($^{35}ClO_3^-$)	primary transition for native perchlorate (quantitative)
m/z 101 ($^{37}ClO_4^-$) → *m/z* 85 ($^{37}Cl^{18}O_3^-$)	secondary transition for native perchlorate (confirmative)
m/z 107 ($^{35}Cl^{18}O_4^-$) → *m/z* 89 ($^{35}Cl^{18}O_3^-$)	primary transition for labeled IS (quantitative)

Results and Discussion

Figure 1 shows a representative SRM chromatogram for a perchlorate standard of 20 pg/mL at the low end of the calibration range. Even with such a low concentration, perchlorate shows a well-defined peak that can be accurately quantified.

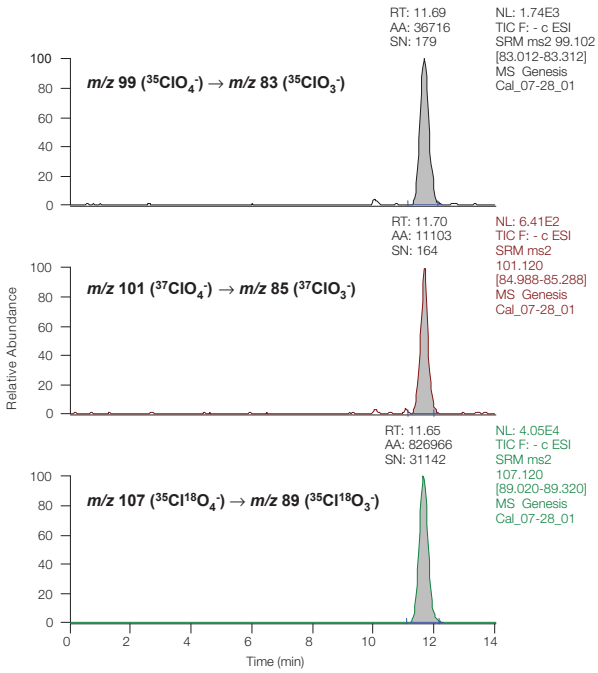

Figure 1: SRM chromatograms of a perchlorate standard at 20 pg/mL

Calibration curves generated on the TSQ Quantum Access mass spectrometer show excellent linearity (Figure 2). For quantitative analysis, the 99 → 83 SRM transition of perchlorate was used (ClO_4^- Quan), and for qualitative analysis, the 101 → 85 SRM transition of perchlorate was used (ClO_4^- Qual). The labeled IS was used with the quantitative and qualitative ions to calculate the squared correlation coefficients (r^2) of the 99 → 83 and 101 → 85 SRM transitions of perchlorate, which were were 0.9996 and 0.9998, respectively. The data was weighted by 1/X to ensure better quantification accuracy for low level samples. The calibration range was 20–10,000 pg/mL.

The instrument lower detection limit was determined to be 5 pg/mL with S/N > 10, which is significantly below the lowest reported value (30 pg/mL)[1]. The upper calibration limit was set at 10 ng/mL, which covered the highest reported value (5.05 ng/mL)[1]. The practical limit of detection (LOD) was determined by $Amt_{Blank} + 3 \times S_0$ = 28.9 pg/mL. The systematic factor Amt_{Blank} was calculated by running deionized water instead of real sample through all sample preparation and instrument analysis procedures.

The IC-MS/MS system provides excellent chromatographic performance and allows separation of perchlorate from complex matrix components. Figure 3 displays the SRM chromatograms for perchlorate in unspiked infant formula and milk samples.

The recovery was evaluated by comparing the differences between unspiked and spiked (5 ng/mL) samples (n=2, n=3). Excellent recoveries were achieved as shown in Table 1.

Table 1. Recovery of perchlorate in infant formula and milk

	Unspiked	Spiked	Recovery
Liquid Infant Formula	1.74 (2.68)	6.91 (4.95)	103%
Milk	2.21 (2.34)	7.93 (2.71)	114%†

†*Reported amounts are in the units of ng/mL with %RSD included in parenthesis.*

The instrument accuracy and precision were evaluated by repeat injections of standards at three levels, as shown in Table 2.

Table 2. Method performance – Accuracy and precision

Specified Amount	Quantified Amount	%RSD	%Deviation	$\%RSD_{R.T.}$
100 pg/mL	98.18 pg/mL	3.05	1.82	0.13
1 ng/mL	1.04 ng/mL	1.62	3.52	0.08
10 ng/mL	10.74 ng/mL	1.45	7.36	0.11

Perchlorate was detected in every sample tested in this study. The method precision, evaluated by repeat assays of each unknown sample, was excellent as shown in Table 3. The powdered infant formula sample was quantified at 2.44 ng/mL, and the milk sample was quantified at 4.64 ng/mL. No interference was detected for any of the samples analyzed.

Table 3. Method performance – Real samples

Sample	Quantified Amount (ng/mL)	%RSD
LIF-1	1.74	2.68 (n=3)
LIF-2	2.21	2.34 (n=3)
LIF-3	1.05	3.63 (n=7)
PIF-1	2.44	1.74 (n=3)
MLK	4.64	1.96 (n=7)

LIF: Liquid infant formula; PIF: Powdered infant formula; MLK: Milk

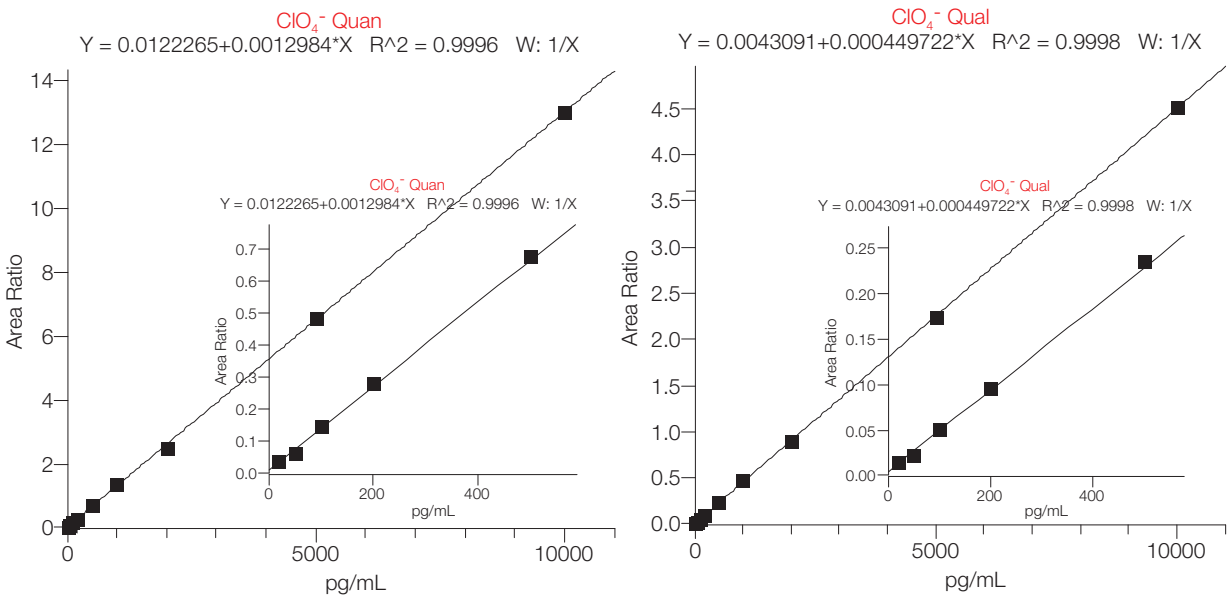

Figure 2. Calibration curves for quantitative and qualitative analysis of perchlorate

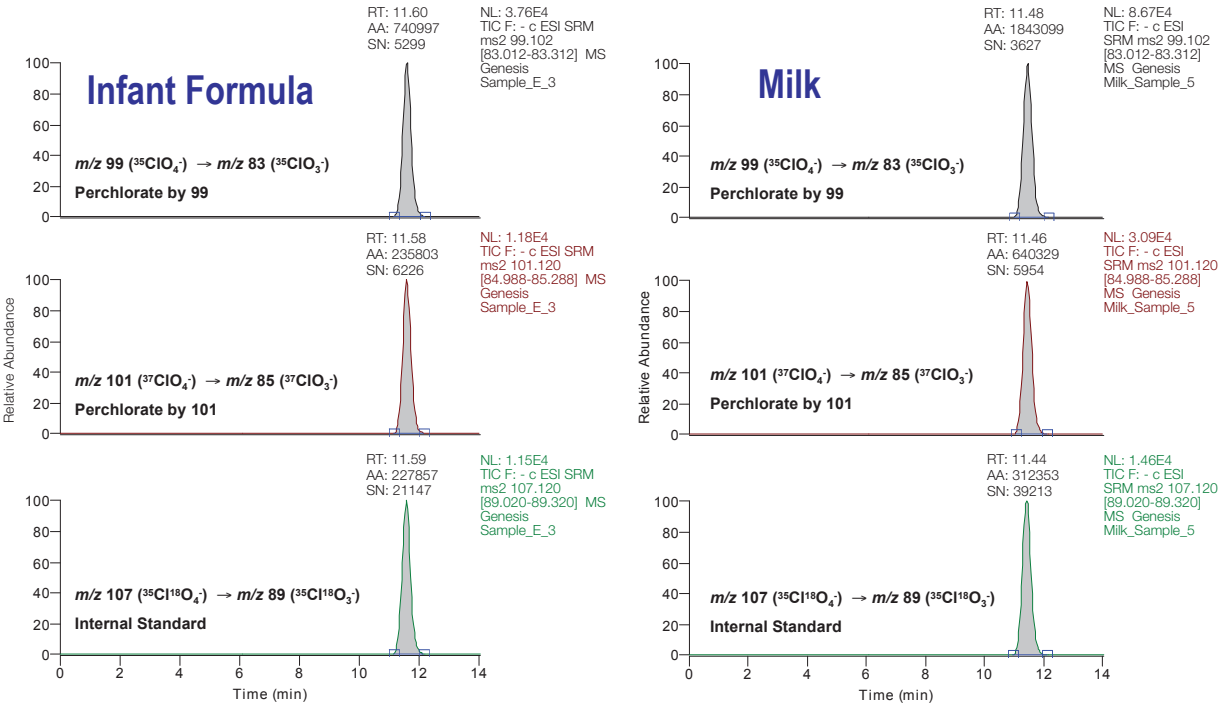

Figure 3. Perchlorate in unspiked infant formula and milk samples. The calculated concentrations are shown in Table 3.

Conclusion

A selective and ultra-sensitive IC-MS/MS method has been successfully applied for the quantification of perchlorate in infant formula and milk. Because the simple sample preparation was deployed and not the long SPE enrichment method, several hours of sample preparation time was saved. The wide linear range covers the reported perchlorate levels in infant formula. Excellent reproducibility (%RSD=3.63, n=7), accuracy, and precision were achieved.

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Application Notebook

Natural Compounds and Food Additives

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Application Note: 52242

Identification and Quantification of Impurities in Wines by GC/MS

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(With special thanks to members of Œnologic Center of Grezillac)

Key Words

- ISQ Single Quadrupole GC-MS
- TRACE GC Ultra
- Food and Beverage
- SPME
- Wine

Introduction

While wine makers have historically used gas chromatography and mass spectrometry (GC/MS) to detect pesticides, they now more commonly use the technique to supplement quality control checks of wine taste. Without GC/MS, wine makers must rely on expert evaluation by oenologists to determine wine quality. By identifying maturation tracers and molecules commonly responsible for taste defects, GC/MS augments expert opinion with objective and quantitative information. When using a SPME extraction method, GC/MS has the additional advantages of requiring very small sample sizes, a minimum of sample preparation, and rapid analysis of target molecules.

Several types of molecules, while not dangerous to humans, affect wine taste and quality, such as volatile phenol compounds derived from *Brettanomyces* yeast metabolism.[1,2] Haloanisoles such as 2,4,6-tricholoranisole that result from cork fungal infections also affect wine taste.[3,4] Methoxypyrazines such as 3-isobutyl-2-methoxypyrazine (IBMP) and 3-isopropyl-2-methoxypyrazine (IPMP) are maturation markers, and detecting their levels can help determine ideal grape harvest time.[5] An automated technique with repeatable results for detecting these compounds is highly desirable, and GC/MS can provide such a method.

Extracted wine samples were analyzed by a sequential full-scan/SIM acquisition on a GC-MS system consisting of a Thermo Scientific ISQ single-quadrupole mass spectrometer and a Thermo Scientific TRACE GC Ultra gas chromatograph. The results were compared to the sensitivity limits of human tasters. This method allows wine makers to obtain precise measurements on the organoleptic parameters that determine wine purity on site rather than having to send samples for expensive, external analysis. In this report, we present the design and results of this study, including the experimental method used to detect impurities and the concentration ranges that compare GC/MS with human detection.

Figure 1: ISQ Single Quadrupole GC-MS system

Methods

For this experiment, several targeted molecule types that affect wine quality were analyzed using an ISQ™ Single Quadrupole GC-MS system (Figure 1). Table 1 contains a brief description of the effects on wine quality of the four target molecule types, and examples of how GC/MS analysis can provide value in quality control.

Molecule Type	Description of Effect on Wine	Benefit of GC/MS Analysis
Volatile Phenols (4-ethylphenol, 4-ethylgaiacol, 4-vinylphenol, 4-vinylgaiacol)	Volatile phenols are produced in various steps of *Brettanomyces* yeast metabolism. The two produced in the final step – 4-ethylphenol and 4-ethylgaiacol – give the wine an "animal" taste and depreciates its quality.	GC/MS can detect 4-ethylphenol and 4-ethylgaiacol in lower concentration than human tasters. GC/MS can also detect the presence of 4-vinylphenol and 4-vinylgaiacol, intermediaries in *Brettanomyces* yeast metabolism and allow wine makers to discard contaminated batches.
Geosmine	This fragrant compound derived from moldy grapes interferes with a wine's taste.	Detecting geosmine in wine alerts makers to the presence of mold in their grapes and allows them to locate and treat a contaminated plot of land.
Haloanisoles (TBA, TCA, TeBA, PCA)	These compounds come from halophenols, compounds used to prevent wood degradation in vines. They give wine a moldy odor.	Assays provide information of an organoleptic default in wine production and help identify contamination sources.
Methoxypyranzines (IBMP, IPMP)	IBMP and IPMP are maturation markers, and their levels decrease as wine matures. IBMP gives wine a "green pepper" taste; IPMP imparts an earthy flavor.	Determining the levels of IBMP and IPMP in wine affects harvesting decisions.

Table 1: Targeted molecules affecting wine purity

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Sample Preparation

To prepare the samples, a 10 mL sample of wine was saturated with NaCl. The sample was placed in a vial and extracted using SPME. A PDMS/DVB 65 µm StableFlex™ SPME Fiber (SUPELCO-57293U) was used, and the fiber was exposed to the sample for agitation for 30 minutes at 70 °C at three-second intervals.

Instrumental Analysis

The ISQ mass spectrometer used for this analysis was set to perform sequential full scan/SIM acquisitions. The TRACE™ GC Ultra was equipped with a standard split/splitless injector. The split/splitless injector temperature was set to 220 °C, and a splitless injection was used. The ISQ GC-MS parameters are summarized in Table 2. The analytical column used was a Thermo Scientific TraceGOLD TG-5MS 15 m × 0.25 mm i.d. × 0.25 µm film (PN 26098-1300). TCA d5 was used as an internal standard; its SIM ions are 215 and 217.

The results were analyzed using Thermo Scientific QuanLab Forms software. QuanLab™ Forms automatically tests the expected retention times (RT), actual ratio versus range of tolerance, and the coelution of ions. QuanLab Forms is also Directorate-General for Health and Consumer Protection (SANCO) compliant and can be used in the European Union.

Results

The spectra of the sequential SIM scan can be seen in Figure 2. The SIM ions monitored using the ISQ are listed in Table 3. Figures 3 through 7 present the calibration curves of several of the target molecules at various linearity ranges. Calibration ranges were established according to the range of human perception – and to the range of interest for oenologists – as opposed to instrument performance.

For all these target molecules, the GC-MS was able to detect lower concentrations than the limits of human perception.

ISQ	
Source Temp (°C)	200
Detector Gain	1×10^5
Start Time (min)	0.2
Acquisition End Time (min)	40
Full Scan Range (u)	35–450
Dwell Time (ms)	20
SIM Ions	See Table 3

TRACE GC Ultra	
Oven Method	
Initial Temp (°C)	40
Initial Time (min)	1.0
Rate #1 (°C/min)	5
Initial Temp #2 (°C)	60
Initial Time #2 (min)	1
Rate #2 (°C/min)	3
Initial Temp #3 (°C)	125
Hold Time #3 (min)	1
Rate #3 (°C/min)	10
Final Temp (°C)	238
SSL Method	Splitless
Temperature (°C)	220
Mode	Splitless
Splitless Time	3 min
Carrier Flow (mL/min)	1.2
Gas Saver	On
Vacuum Compensation	On
Transfer Line (°C)	250

Table 2: Instrument method summary for the full scan/SIM analysis of target molecules on the ISQ and TRACE GC Ultra

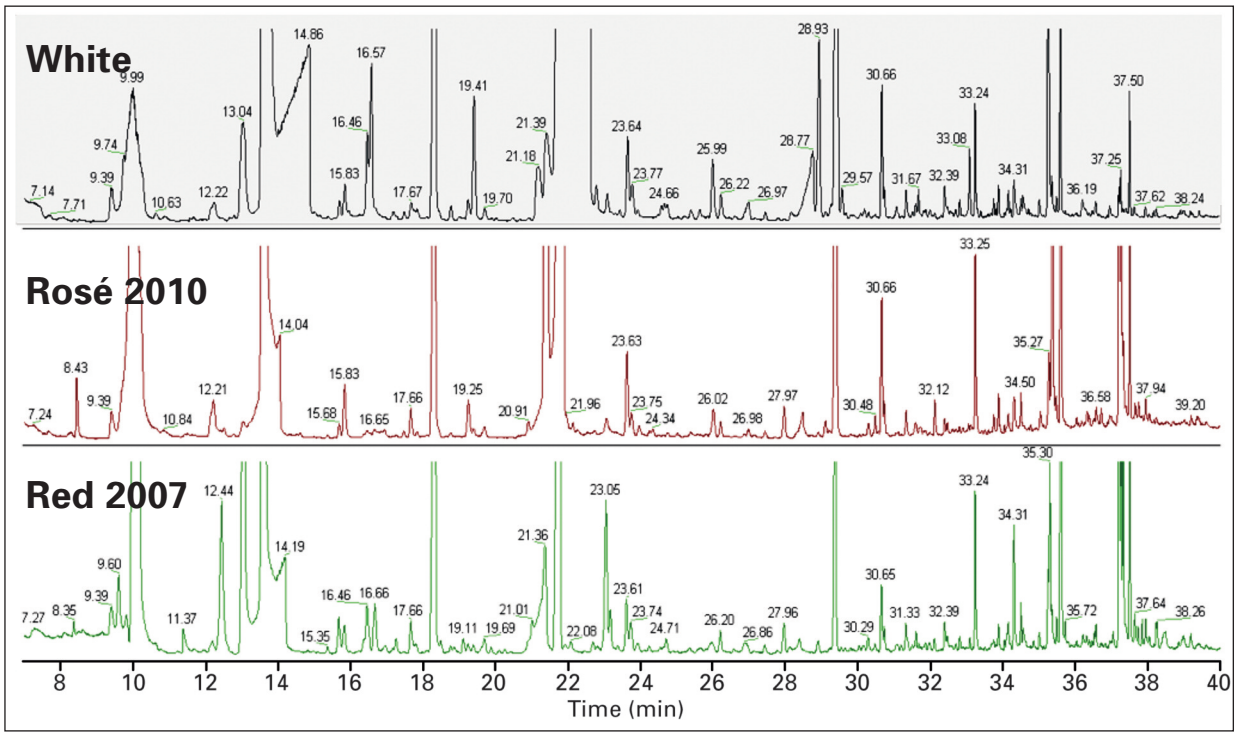

Figure 2: Chromatograms showing full-scan acquisitions for three wine types

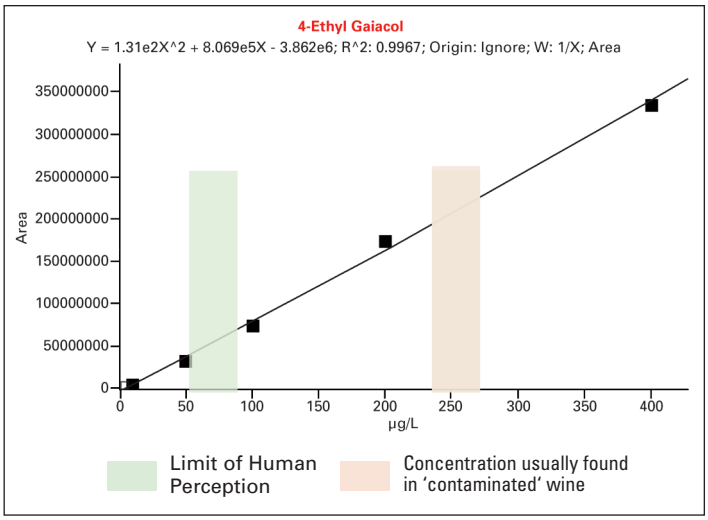

Figure 3: 4-Ethylgaiacol from 50 to 100 µg/L

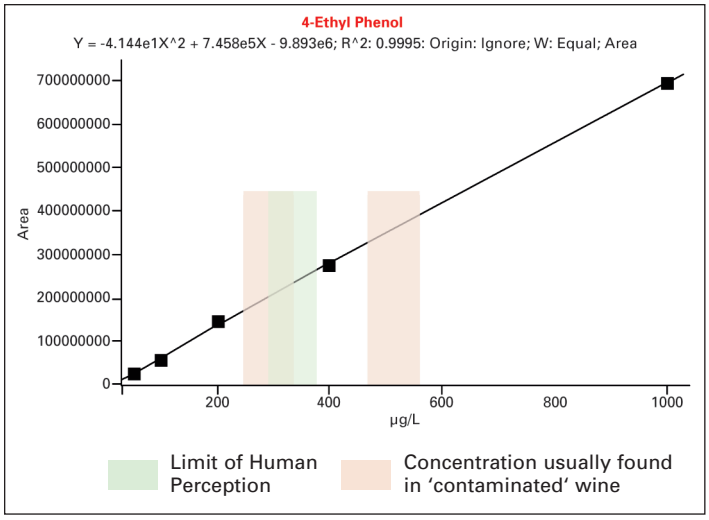

Figure 4: 4-Ethylphenol from 300 to 400 µg/L

Target Molecule	*m/z*
IPMP	124, 137, 152
IBMP	94, 124, 151
4-Ethylphenol	77, 107, 122
4-Ethylgaiacol	122, 137, 153
Trichloroanisole	195, 210, 212
Geosmine	111, 112, 125
Tetrachloroanisole	231, 244, 246
2,4,6-Tribromoanisole	329, 344, 346
Pentachloroanisole	278, 280, 282

Table 3: SIM ions monitored for the target compounds

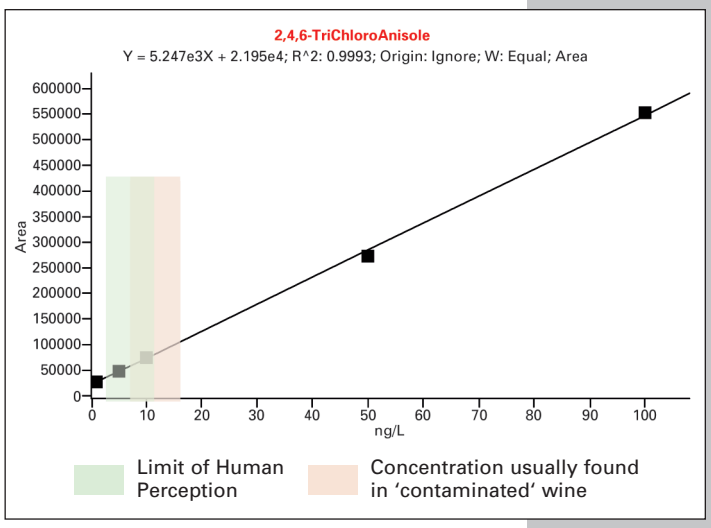

Figure 5: 2,4,6-Trichloroanisole from 2 to 5 ng/L

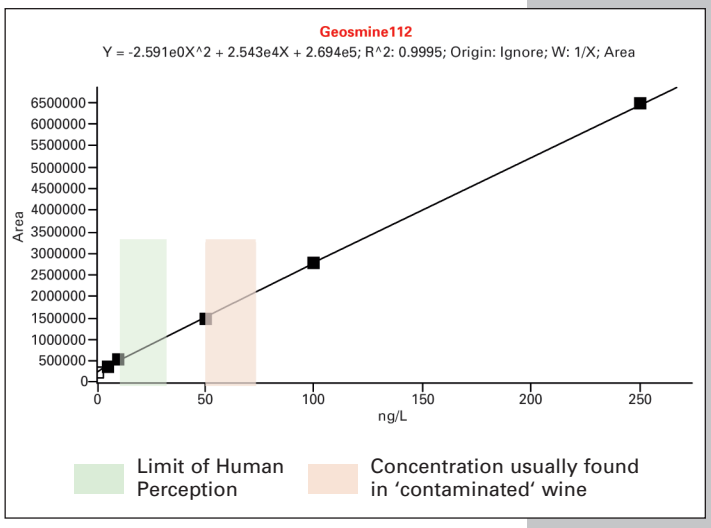

Figure 6: Geosmine from 10 to 50 ng/L

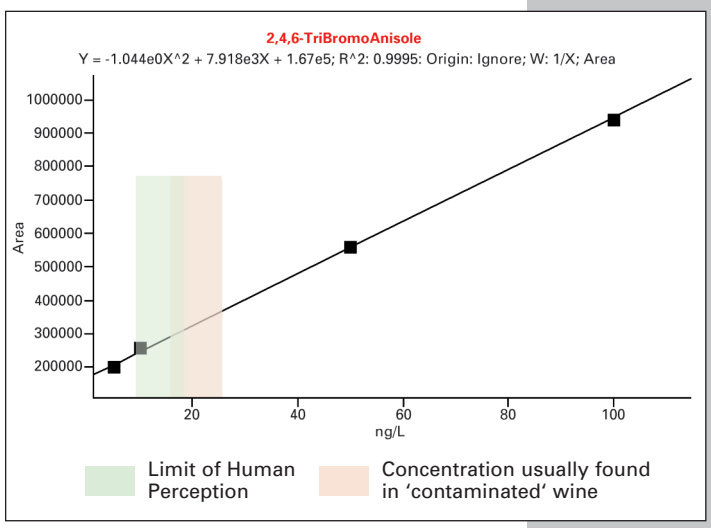

Figure 7: 2,4,6-Tribromoanisole from 10 to 20 ng/L

Conclusion

The ability of the ISQ GC-MS to detect several contaminants in wine at lower concentrations than the limit of human tasters, and its ease of use in combination with a single-step, two-minute sample preparation make it a useful tool for the wine industry. The sequential full-scan/SIM acquisition method for detecting the impurities also does not require extensive training of personnel to provide accurate results. In addition, this general method may be improved or customized to particular wines by incorporating new parameters such as trying other SPME coatings in the extraction phase.

The wine, champagne, and spirit market can be well served by analytical chemistry tools such as GC-MS. There are also other potential uses for this analysis method. For example, wine and other spirit producers risk their recipes being compromised when they outsource their product analysis, and prefer to conduct it on site. In addition, analysis of competitors' products using a GC-MS can help producers quantify what makes one wine superior to another.

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Top Down Milk Protein Identification and Relative Quantification by Q Exactive Mass Spectrometer

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Overview

Purpose: Mixtures of casein and whey proteins were characterized by top-down mass spectrometry in order to determine forms to be quantified in milk samples. These identified forms have been used to calculate the whey protein/casein protein ratio studies.

Methods: Whey and casein proteins were mixed and top-down LC-MS/MS data of these samples were acquired on a benchtop quadrupole-Orbitrap mass spectrometer. ProSightPC 3.0 was used for intact protein identification. SIEVE 2.1 was used for whey protein/casein protein ratio calculations.

Results With top-down approach, we identified α –lactalbumin, beta-lactoglobulin, α–s1-casein, α–s2-casein, k-casein and β–casein in these samples. Different forms of the milk proteins, including intact, truncation products, and with unexpected modifications forms were identified. Using PCA software, whey protein to casein ratios were calculated. Consistent whey to casein ratios were obtained for all different forms of proteins.

Introduction

Whey proteins, most notably alpha-lactalbumin and beta-lactoglobulin, are currently being investigated for their potential positive health benefits. Whey proteins are a substantial percentage of human milk (~60%) while such proteins are less abundant in cow milk (~20%). Currently, the nutritional requirements of infant formulas require comparison of their amino acid composition in comparison to human milk, but the calculation of the ratio of whey protein/casein protein currently is not required in most countries. However, in China, there is an emerging requirement that at least 60% of protein content in infant formula are from whey proteins but there currently is no standard method to calculate this ratio. The work described in this poster describes the identification of the various forms of caseins and whey proteins as a first step and then intact protein-based method for determination of the whey vs. casein protein ratio. A surprising number of casein protein forms were identified, indicating that quantification based on a single target casein or whey mass or using bottom up approach with peptides will underestimate the amount of that protein in a given sample.

Methods

Whey (Hilmar Ingredients) and casein (Sigma-Aldrich, Cat No. C8654-550G) powders were prepared in 5% acetonitrile, 95% water with 0.1% formic acid and were mixed with different whey to casein ratios (1:3, 1:2, 1:1 and 2:1). Each protein solution was analyzed in duplicate by ESI-LC-MS/MS analyses using a Thermo Scientific™ Hypersil™ BioBasic C8 reversed phase 1 mm x 100 mm column packed with 5 um particles in conjunction with a Thermo Scientific™ Q Exactive™ mass spectrometer at 140,000 resolution. The LC/MS/MS runs were subsequently analyzed using a prerelease version of ProSightPC 3.0. ProSightHT was first used to deconvolve the datasets using Xtract as the data reduction algorithm. The resulting .puf files were loaded individually and all spectra were analyzed by appending and searching the three searches described in the next section. Results from each dataset were exported to a ProSightPC repository and the results were reviewed in a single repository report. Proteins with expectation values better than 1e-6 were considered to be valid. The LC/MS elution chromatogram were extracted to get protein's molecular weight. From the extracted MS full chromatogram , the whey to casein protein relative ratios were then calculated using Thermo Scientific™ SIEVE™ 2.1.

Results

Protein Identification Using ProSightPC

The datasets were first searched in ProSightPC against a full bovine protein database with no indexed modifications and a 60 kDa precursor search tolerance. This type of search allows ProSightPC to identify the proteins in the casein and whey samples without fully characterizing them against a large database such as bos taurus (87708 proteoforms). As expected, alpha-S1-casein, alpha-S2-casein, beta-casein, kappa-casein, and beta-lactoglobulin were identified as well as glycosylation-dependent cell adhesion molecule 1 (GLCM1). Alpha-lactalbumin, a major component of whey proteins, was confidently identified after reduced and alkylated.

To identify and characterize as many different forms of the identified proteins above, a flatfile database using the 7 accession numbers above was created using up to 20 concurrent modifications per sequence allowing for all annotated modifications. The resulting sequence database contained 39 basic sequences and 33651 proteoforms. Each spectrum in each dataset was searched 3 times:

1) Absolute mass search with 1.02 Da precursor mass accuracy and 10 ppm fragment accuracy to find forms that exactly match predicted intact proteoforms in the database.

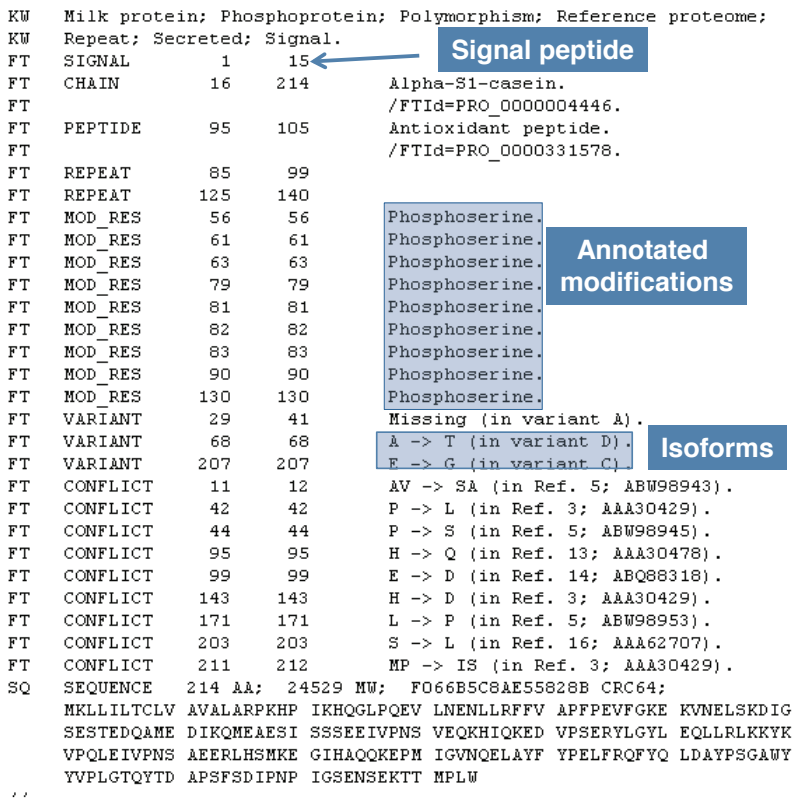
2) Biomarker search with 10 ppm precursor mass accuracy and 10 ppm fragment mass accuracy to find truncation products.

3) Absolute mass search "delta m" with 25000 Da precursor tolerance and 15 ppm fragment tolerance to find forms of the target proteins that were not annotated in the original flatfile and do not match a truncated form.

The identification with the lowest expectation value from the three searches above was chosen as the best candidate for each MS/MS spectrum. The results of the searches produced 14 matched intact forms for the 6 proteins excluding alpha-lactalbumin and in addition 59 truncated and partially characterized forms.

The whey proteins where for the most part detected in only a few primarily intact forms, while the casein proteins were detected in many different forms that included a substantial number of truncation products. One such example of such complexity is alpha-casein. For this protein, there are 9 known phosphorylation sites, a signal peptide that is removed in the mature form, and 3 known sequence variants, as annotated in the Uniprot flatfile (Figure 1).

FIGURE 1. UniProt flatfile entry for alpha-S1-casein, accession number P02662. The entry indicates that up to 9 phosphoserines may be identified on specific sites and there are at least four different known variants. Intact forms of the primary sequence with 6, 7, 8, and 9 phosphorylations were also detected. Also, less abundant proteoforms with the variant C sequence were also detected.



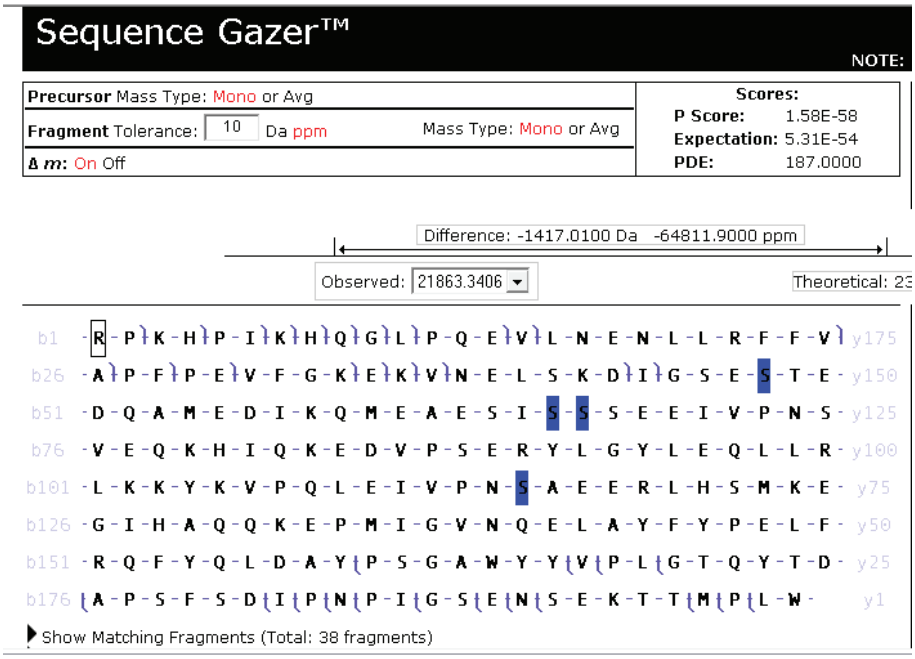
The list of identified forms of alpha-casein can be found in Table 1. For the first search with narrow precursor and product ion tolerances, 6 different intact forms of alpha-casein were detected, all with the signal peptide removed, including two forms of variant C (E->G) with 7 and 8 phosphorylations. The subsequent biomarker search identified 16 different truncated forms of alpha-S1-casein that contained both the N-terminus and the C-terminus. Depending on the site of truncation, many of these forms were also highly phosphorylated. Finally, the error tolerant absolute mass search identified 5 forms that could not be completely characterized. The entries in table 1 denoted as "not fully characterized" were identified by search number 3, the absolute mass search with a large precursor mass tolerance. For these identifications, large stretches of sequence were identified from one or both termini, but the mass difference between the target protein sequence and the measured monoisotopic

Table 1. List of identified forms of alpha-casein. The best E-value column indicates the protein spectral match that produced the most confident result across the 10 raw data files.

Nominal Mass (Da)	RT (min)	Identified form	Best E-value
2617	4.1	Truncation	3.35e-39
7684	5.1	Not fully characterized	5.07e-27
7851	5.6	Not fully characterized	3.27e-29
8018	6.1	Not fully characterized	5.20e-15
8561	7.3	Truncation	2.26e-24
8633	7.3	Truncation	1.05e-59
9519	6.4	Truncation, 7 phosphorylations	4.28e-27
10871	6.7	Truncation, 1 phosphorylation	5.02e-31
11162	6.5	Truncation, 1 phosphorylation	5.64e-31
11290	6.3	Truncation, 1 phosphorylation	1.30e-32
12747	5.3	Truncation, 7 phosphorylations	7.01e-35
13348	6.0	Truncation, 1 phosphorylation	3.12e-22
13734	5.7	Truncation, 5 phosphorylations	9.93e-13
14099	5.9	Truncation, 1 phosphorylation	5.80e-39
14750	5.8	Truncation, 2 phosphorylations	6.07e-26
19338	5.0	Not fully characterized	1.54e-08
19416	5.1	Truncation, 7 phosphorylations	9.34-15
19496	5.4	Truncation, 8 phosphorylations	1.07e-12
21863	7.0	Not fully characterized	5.31e-34
23347	7	Truncation, 8 phosphorylations	3.71e-45
23427	7.1	Truncation, 9 phosphorylations	1.53e-23
23440	6.4	Intact, 6 phosphorylations	8.48e-56
23448	6.5	Intact, 7 phosphorylations, variant C	4.13e-49
23520	6.5	Intact, 7 phosphorylations	5.02e-65
23528	6.7	Intact, 8 phosphorylations, variant C	5.74e-45
23600	6.6	Intact, 8 phosphorylations	4.87e-60
23680	6.9	Intact, 9 phosphorylations	1.39e-52

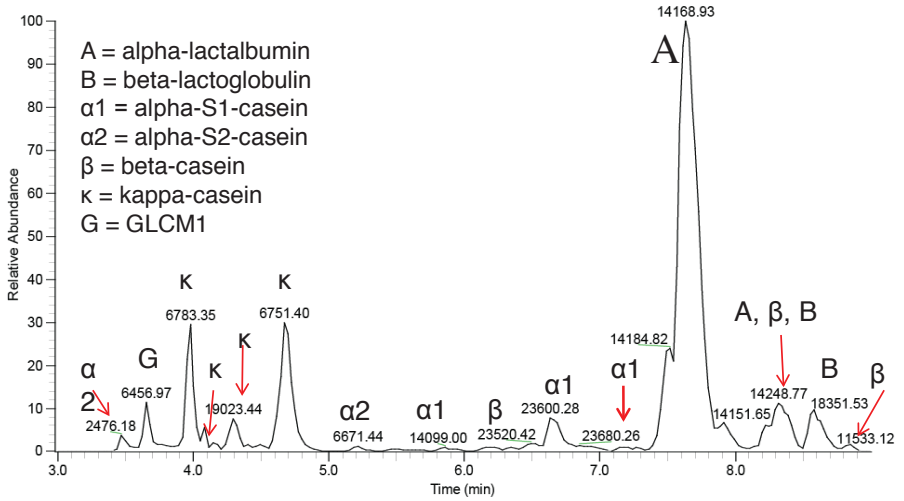
mass could not be exactly localized on the protein sequence. One interesting example of this is the alpha-s1-casein proteoform with nominal monoisotopic mass of 21863 Da. This protein form is relatively abundant and the Sequence Gazer result (Figure 2) shows substantial sequence coverage with a very confident expectation value of 5.31e-54. Given that the N- and C-termini are confirmed by many matching fragments, the mass discrepancy can be localized to a region in the middle of the sequence. This mass discrepancy is actually rather large (>1000 Da) and suggests that this could be due to a removal of a stretch of amino acids.

FIGURE 2. Sequence Gazer results for an unexpected form of alpha casein. There is substantial sequence coverage from both termini and thus the modification to the sequence is somewhere in the middle. Due to the size of the mass discrepancy, it is likely that the modification is a stretch of missing sequence, perhaps due to a splice variant.



Alpha-S2, beta- and kappa-casein were also determined to be highly heterogeneous with 5, 25 and 7 forms detected, respectively. In contrast, only 3 forms were detected for beta-lactoglobulin, 2 of which were intact, as well as 3 forms of GLCM1. Two forms of intact alpha-lactabumin and some truncated forms were identified when the whey proteins were reduced and alkylated. Figure 3 is the annotated extracted chromatogram of one of the whey casein mixture elution profile.

FIGURE 3. Annotated extracted chromatogram for one of the whey:casein protein mixture datasets. There are multiple abundant chromatographic peaks for each of protein, indicating extensive heterogeneity for each of these proteins.



Protein Quantitation Using SIEVE 2.1

For relative protein quantitation, the extracted chromatogram of different whey to casein protein ratio were analyzed by SIEVE 2.1. Figure 4 was the trend intensities of whey protein, α-lactalbumin and casein protein, α-S1-casein. It reproducibly showed clear relation that the α-lactalbumin percentage decreased while the casein concentration increased . Since the proteins are so heterogeneous, using one protein will underestimate the real protein concentration. We sum the peak intensities from whole whey proteins and casein proteins. The whey to casein protein response ratios were calculated using the sum intensities of whey and casein proteins. Surprisingly, it seems yield the linear trend response with both injections (Figure 5). This leads us to the possibility of protein quantitation with intact protein approach. For more accurate protein quantitation, the response factor of each pure protein should be applied.

FIGURE 4. Different ratio whey to casein protein mixture trend intensities analysis by SIEVE 2.1. Whey to casein ratio: Blue (2 to 1), Red (1 to 1), Green (1 to 2) Yellow (1 to 3)

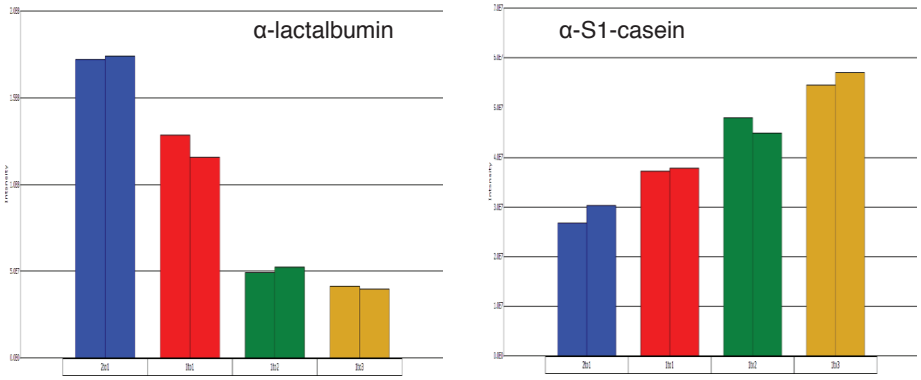
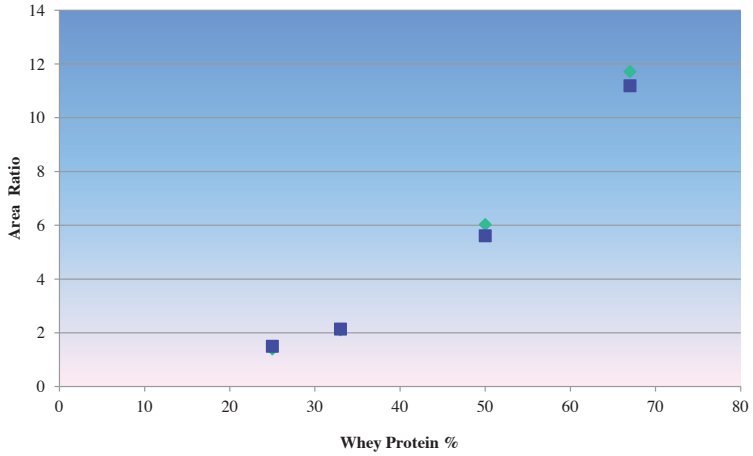


FIGURE 5. Total whey protein to total casein protein response intensity ratios verses percentage of whey protein in protein solutions.



Conclusions

- The casein proteins are surprisingly complex, with numerous truncated forms of the expected proteins
- Alpha-s1-casein is the most complex of the proteins in the mixture, with 27 different identified forms including two different sequence variants.
- The different whey:protein ratios were calculated by using the extracted ion chromatogram.

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Direct Analysis of Red Wine Using Ultra-Fast Chromatography and High Resolution Mass Spectrometry

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Key Words
- LTQ Orbitrap™
- U-HPLC
- Flavonoids
- Red Wine

Overview
Red wine is a very complex mixture and a rich source of beneficial anti-oxidants. Identification and quantitation of these natural products is challenging. Ultra High Pressure Liquid Chromatography (U-HPLC) coupled to the Thermo Scientific LTQ Orbitrap XL mass spectrometer was used for analysis of French red wine, which enabled simultaneous detection and relative quantitation of the wine's anti-oxidant constituents. The phenolic compounds (such as quercetin) responsible for most of the health benefits associated with the consumption of red wine were identified and their variable content across two different harvest years was observed. Direct wine analysis approach was then applied to monitor the progressive changes in red wine after its exposure to air. This work demonstrated the feasibility of analyzing complex mixtures without any prior sample preparation by making use of the high resolving power of both U-HPLC and the Orbitrap™ mass analyzer detector.

Introduction
Free radicals derived from molecular oxygen are considered major causative agents of tissue damage.[1,2] Both recent and historical evidence suggests that regular drinking of wine ***in moderation*** has a positive impact on human health thanks to its high content of anti-oxidants.[3,4] Red wine in particular contains a complex mixture of phenolic compounds which are important contributors to the organoleptic quality of wines as well as essential components in the evolution of wine. Quercetin is of special interest for its commercial use as an anti-oxidant food supplement with a proven record of promoting vascular relaxation, inhibiting human platelet aggregation *in vitro*, and modulating eicosanoid synthesis towards a pattern likely to be protective against coronary heart disease.[5]

Reversed-phase HPLC is well established for the analysis of flavonoids in red wine, including quantitative analysis.[6,7,8] Coupling reversed-phase HPLC to a mass spectrometer adds considerable benefits such as the ability to:

1) analyze complex mixtures without much sample fractionation
2) monitor hundreds of compounds in a single analysis over a wide dynamic range of concentrations
3) provide an unambiguous identification and structural characterization of the compounds based on accurate mass measurement and informative fragmentation spectra.

Recent advances in both HPLC and mass spectrometry techniques are having a significant impact on the analyses of complex mixtures such as those represented by food and agricultural products. First, the use of small particles (< 2 µm) in HPLC columns can provide remarkable increase in speed of analysis while maintaining or even improving the separation efficiency. Second, the new generation of powerful but easy-to-use hybrid mass spectrometers, like the LTQ Orbitrap XL, combines extremely high mass accuracy and resolution with the capability of multiple levels of fragmentation.[9] The combination of these powerful techniques provides a robust and confident means of profiling complex mixtures as well as successful identification and advanced structural characterization of detected compounds. As a result, we are seeing rapidly growing interest in the area of metabolomic analysis being applied in nutrition and health research.[10,11]

We investigated the potential of a direct analysis of red wine using U-HPLC coupled to a linear ion trap – Orbitrap hybrid mass spectrometer. Of particular interest was the ability of the designed workflow to pinpoint statistically significant differences between individual harvest years for wines of a specific origin (area, label). In addition to that, we used the developed methodology to monitor the trend in oxidative changes of red wine after exposure to air.

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Methods

Two bottles of French red wine Les Charmes de Kirwan, Margaux (cuvee, Bordeaux region, France), years 2003 and 2005, were obtained from a specialized wine merchant. The wine was stored at room temperature in the dark until analyzed. Immediately after opening the bottle, a glass vial (20 mL) was filled with the wine to the very top, quickly closed to ensure minimum oxidation, and stored at 4°C in the dark. This sample was collected just in case there was a need for repeated analysis of the profiling experiments or structural elucidation studies. A second 20 mL aliquot of wine was poured from the original bottle into a glass beaker. From this beaker a sample vial was immediately filled to the rim and placed in the chilled (4° C) Thermo Scientific Accela autosampler tray, awaiting analysis. For a wine oxidation trend analysis, further samples were taken from this open beaker 1, 5 and 24 hours after the bottle opening.

Chromatography was performed using an Accela U-HPLC injecting 20 µL sample from a cooled tray (4°C) directly onto a Thermo Scientific Hypersil GOLD column (2.1 mm x 100 mm, 1.9 µm particles, equilibrated in 95% solvent A (0.1% aqueous solution of formic acid), 5% solvent B (acetonitrile containing 0.1% formic acid). The compounds were eluted using flow rate 300 µL/min by linearly increasing solvent B concentration from 5% to final 40% over 15 min, and from 40% to 95% over 1 min. The column was then washed with 95% solvent B (2 min) and re-equilibrated in 95% solvent A, 5% solvent B. The total run time, including column wash and equilibration, was 20 min.

A Thermo Scientific LTQ Orbitrap XL mass spectrometer was operated in positive ion mode at 30,000 resolving power (defined as FWHM @ *m/z* 400) for full scan analysis (mass range 150 – 1500 u) followed by data dependent MS/MS on the most intense ion from the full scan at 7,500 resolving power (~0.3 sec per scan). The measurements were done in triplicate with external calibration. The settings for the higher energy collisional dissociation (HCD) fragmentation mode were 65% normalized collision energy, isolation width 3 u.

Thermo Scientific SIEVE 1.2 software was used for comparative and trend analyses. The software allows for processing a large number of samples, presenting the statistically significant differences between populations and various time points. Data were normalized on total spectral ion current. Results were filtered using pValue < 0.001 and at the same time requiring a minimum 2-fold change in peak height.

The results from SIEVE™ were further subjected to multivariate analysis with SIMCA P+™, version 11 (Umetrics, Umea, Sweden).

Mass Frontier™ (HighChem, Slovakia) software was used to confirm a suggested compound identity and structure based on observed fragmentation patterns.

Results

Due to the large number and the chemical complexity of phenolic compounds in wine matrix, analytical methods in the past involved sometimes difficult and complicated traditional chromatographic techniques. One of the major problems underlying separation of the phenolic compounds is their similarity in chemical characteristics. As many phenolics show similar UV spectra with maxima in a narrow range of 280-320 nm, extensive fractionation steps might be needed prior to HPLC analysis. Rather large initial volumes required and variable losses occuring due to incomplete extraction or oxidation can be an issue. The use of modern chromatographic techniques coupled to mass spectrometric detection can alleviate these problems.

Our approach avoids entirely the sample fractionation step: red wine is injected directly on the reverse phase column. Moreover, the use of small particles (< 2 µm) and relatively high flow rates (300 µL/min) enable swift analysis with excellent chromatographic resolution. The observed peak width for individual compounds was, on average, 7 sec, back pressure not exceeding 350 bar. With 20 min total cycle time per injection, this setup allows for high throughput analysis while the total sample consumption remains negligible (20 µL per injection). U-HPLC coupled to the LTQ Orbitrap XL proved to be very robust, allowing for an uninterrupted analysis of 24 untreated red wine samples which corresponds to an 8-hour continuous analysis without any requirement for a system cleanup or column change.

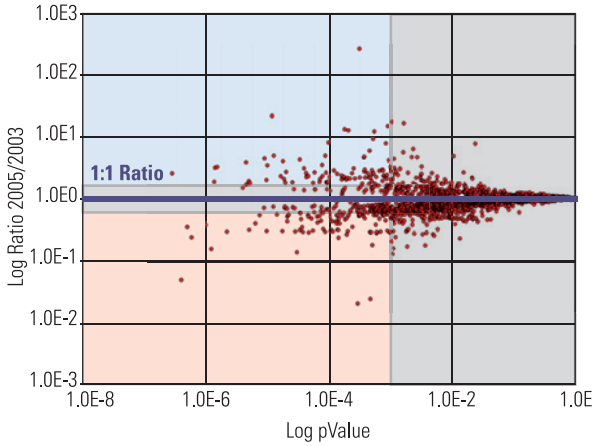

Figure 1: Overview of differences between harvest years 2003 and 2005. The result from differential analysis software (SIEVE 1.2) highlights the compounds having at least two-fold higher concentration in year 2005 compared to year 2003 (blue shaded area) and compounds whose concentration in year 2005 was less than a half of that in year 2003 (red shaded area). The purple horizontal line represents 1:1 ratio between concentrations in the year 2005 and 2003. The grayed area covers the features with less pronounced concentration difference and those with low statistical significance, i.e. pValue > 0.001.

Variables like wine varietals, soil composition, and harvest year will play an important role by providing the basic pool of compounds for these biotransformations. With accurate mass acting as a highly selective filter we could monitor hundreds of compounds across multiple samples, enabling advanced comparative studies and trend analyses. Initially, we were interested in comparing the wine of the same origin (area, label) but harvested in different years.

Our differential analysis of the Les Charmes de Kirwan, Margaux, contrasted wine from production years 2003 and 2005 using SIEVE software. The features (peaks) were filtered for their statistical significance (pValue < 0.001) and significant change defined as a minimum 2-fold concentration difference between the two harvest years (Figure 1). We observed 75 individual compounds which showed at least 2-fold higher content in year 2005 compared to year 2003 (blue shaded area in Figure 1). Kaempferol and quercetin concentration increased 25- and 8-fold, respectively, in year 2005 compared to 2003 (Figure 2). On the other hand, there were 36 other compounds whose concentration in the 2005 sample was significantly less than in the 2003 sample (red shaded area in Figure 1). Some flavonoids (myricetin) showed no change in concentration between the two harvest years.

Total anti-oxidant status refers to overall antioxidant properties of wine, and can be largely ascribed to a group of compounds comprising vanillic acid, *trans*-polydatin, catechin, *m*-coumaric acid, epicatechin, quercetin, *cis*-polydatin and *trans*-resveratrol.[12] In our analysis we detected vanillic acid, (epi)catechin, coumaric acid, and quercetin. When compared to wine produced in 2003, the wine produced in 2005 contained 50, 40 and 20% less coumaric acid, vanillic acid and (epi)catechin, respectively, while the amount of quercetin increased 8-fold (Table 1).

Calc *m/z*	Formula MW	Name	Change 24h/0h	Change 2003/2005
165.0546	$C_9H_8O_3$	Coumaric acid	0.32	0.51
169.0495	$C_8H_8O_4$	Vanillic acid	0.40	0.61
199.0601	$C_9H_{10}O_5$	Syringic acid	0.59	0.92
391.1387	$C_{20}H_{22}O_8$	Polydatin	Not found	Not found
229.0859	$C_{14}H_{12}O_3$	Resveratrol	Not found	Not found
291.0863	$C_{15}H_{14}O_6$	(Epi)catechin	0.66	0.82
303.0499	$C_{15}H_{10}O_7$	Quercetin	0.78	7.96
319.0448	$C_{15}H_{10}O_8$	Myricetin	0.69	1.00
287.0550	$C_{15}H_{10}O_6$	Kaempferol	1.00	20.96

Table 1: Overview of some compounds of interest and the changes in their content between year 2003 and 2005 (column **Change 2003/2005**), and after 24 hours following exposure to air (column **Change 24h/0h**). The compounds highlighted are the major contributors to the total anti-oxidant status.[12]

The remarkable difference in the content of quercetin between the two harvest years is interesting. Quercetin is one of the most abundant natural flavonoids found in fruits, vegetables and wine.

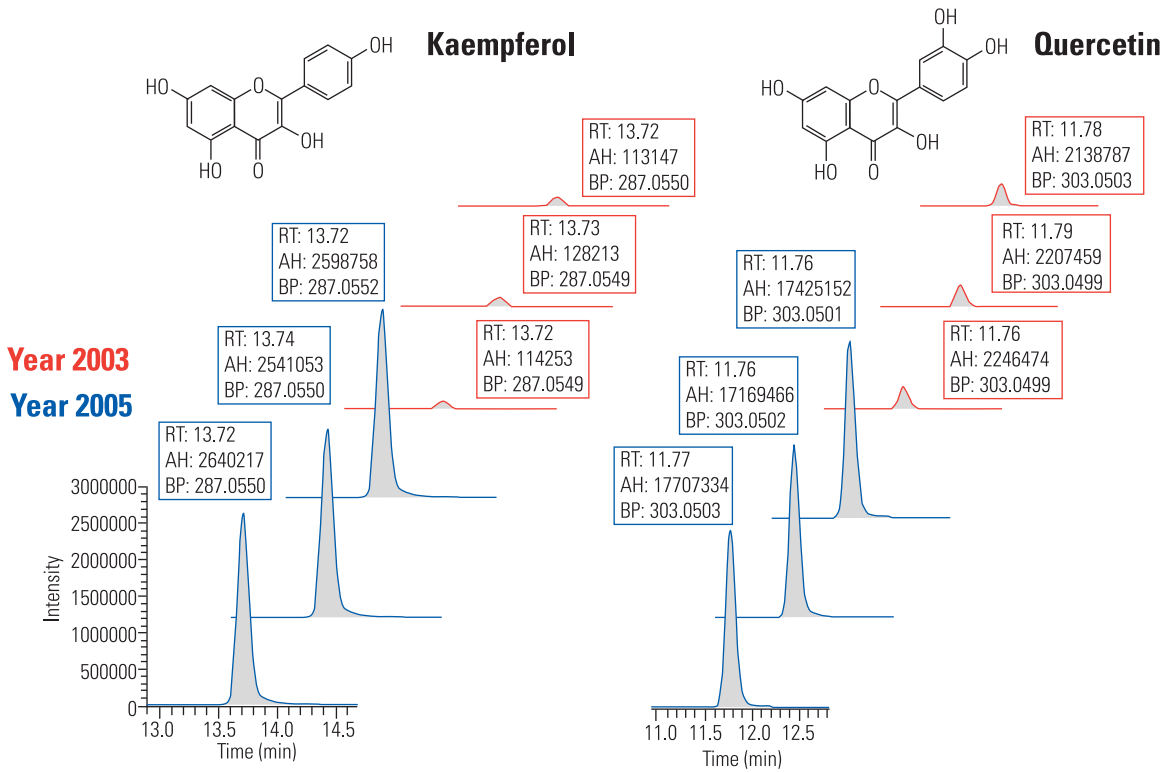

Figure 2: Extracted ion chromatogram for kaempferol and quercetin (left and right pane, respectively; 3 injections each) shows remarkable difference in concentration of these compound in wine harvested in year 2005 (blue trace) and 2003 (red trace). The mass deviation did not exceed 0.7 ppm for kaempherol (calculated *m/z* = 287.0550) and 1.3 ppm for quercetin (calculated *m/z* = 303.0499). Note the reproducibility of the retention time (RT) values and peak height calculations (AH) for 3 replicate injections.

At present, labeling requirements for red wine are far from comprehensive, basically limited to listing the total alcohol content and the comment that it contains sulfites. Including more specific information about compounds with strong anti-oxidant properties would improve a general public awareness and be helpful in the current climate of debate on healthy balanced diet. A fast but highly informative analysis of wines as described herein can thus help maintain consistency and quality, and provide useful information about product's nutritional value.

Reliable accurate mass measurements over a broad dynamic range of concentration are helpful for unambiguous identification of compounds of interest. The mass deviation of our measurements did not exceed 2 ppm using external calibration. Such an accuracy supported by reliably measured isotope abundancies in the LTQ Orbitrap XL enabled a confident assignment of elemental composition to individual peaks.

For confident identification of a compound, the elemental composition suggestions based on mass accuracy need to be complemented with the evidence from the fragmentation spectra. Our method was set up to collect higher energy collision dissociation (HCD) spectra. On average 700 such spectra were collected during each 20-minute LC-MS run. The MS/MS spectrum acquired in the multipole collision cell of the LTQ Orbitrap XL serves for confirming identity of a known compound or even determining identity of an unknown. Such an approach was demonstrated for the analysis of antioxidant compounds in olive oil.[13] Rich fragmentation, accurate mass measurement of both parent and fragment ions, and spectrum interpretation provided by Mass Frontier software were all crucial for this challenging task (Figure 3).

The anti-oxidant properties of wine are clearly beneficial to a consumer. On the other hand, wines with higher polyphenolic concentration are more susceptible to oxidation. We were interested to observe a trend of changes in the wine samples over the period of 24 hours after opening the bottle. The groups of samples from time points 0, 1, 5, and 24 hours (triplicate injections) were processed with SIEVE and further subjected to principal component analysis. The progressive changes caused by exposure to air are well observable and statistically significant (Figure 4).

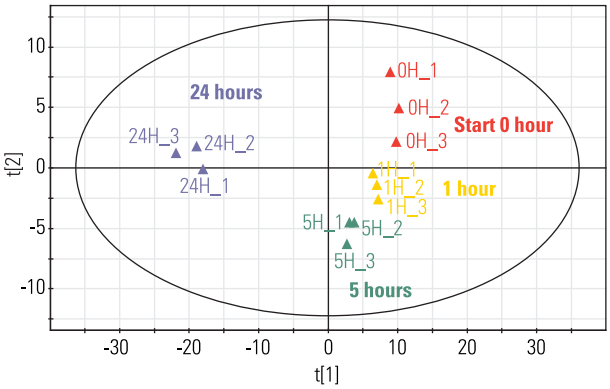

Figure 4: Wine sampled in triplicate at 0, 1, 5, and 24 hours after exposure to air. The sample groups are easily separated by the first two principal components.

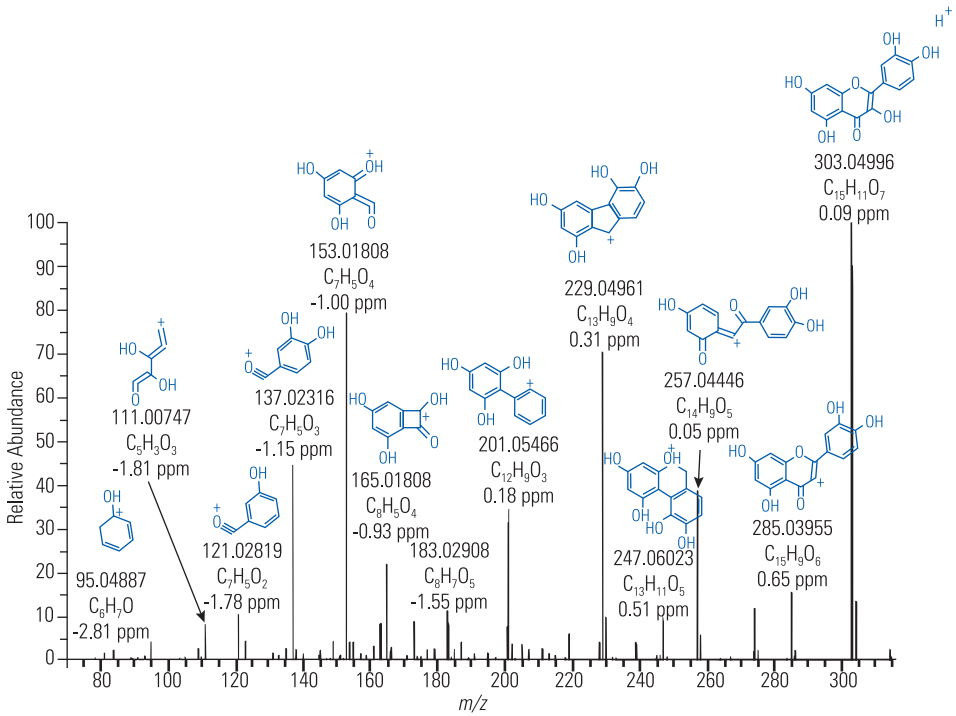

Figure 3: Confirmation and structural characterization of quercetin. Assignment of fragments in HCD spectrum using the Mass Frontier software relying on its extensive database of fragmentation mechanisms.

At this point, a potential effect of evaporation of more volatile constituents of wine has to be considered. In general, the partial pressure for compounds with molecular weight 300 and higher is considered negligible – such compounds should not be lost to evaporation at room temperature. Kaempferol (MW 286), (epi)catechin (MW 290), myricetin (MW 318) and quercetin (MW 302) would fall into such a category. Kaempferol showed no change over this period. Thus the decrease of 20% for quercetin and 30% for (epi)catechin and myricetin observed over the period of 24 hours following the bottle opening could be confidently ascribed to oxidation. For coumaric (MW 164), vanillic (MW 168) and syringic (MW 198) acids we observed a more pronounced drop in concentration (60, 70 and 40%, respectively) after 24 hours following the exposure to air (Figure 5). It might prove difficult, however, to distinguish between the effect of evaporation and oxidation under the employed experimental conditions.

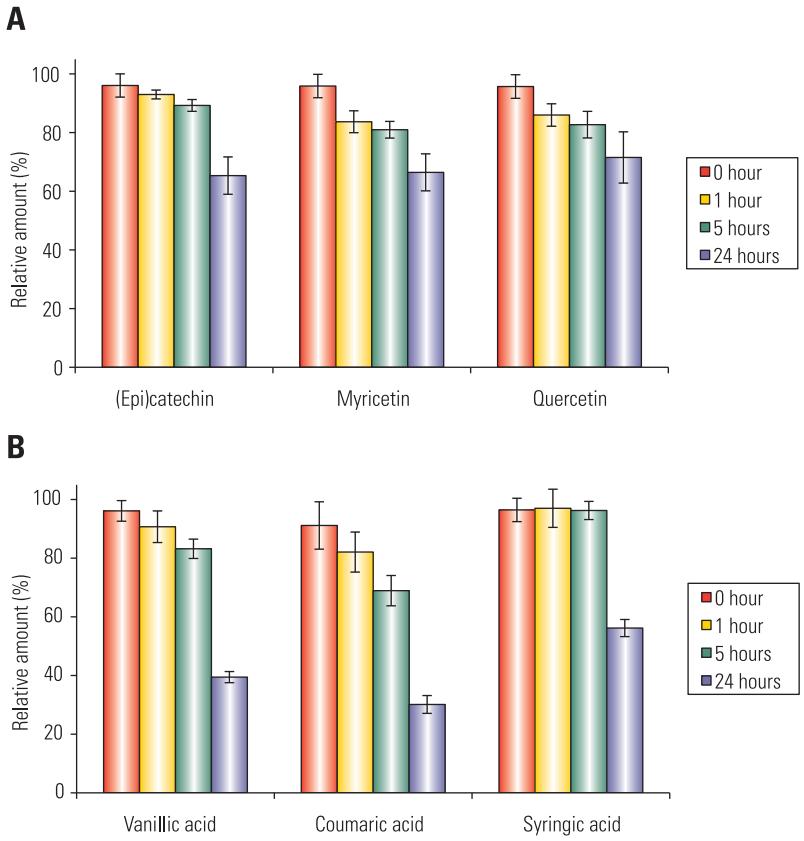

Figure 5: Changes over a 24-hour period of air exposure. The amount of a given compound at time 0 h defined as 100%. Panel A shows decrease in content of higher molecular weight compounds such as (epi)catechin (MW 290), myricetin (MW 318) and quercetin (MW 302). Lower molecular weight components including coumaric (MW 164), vanillic (MW 168) and syringic (MW 198) acid display much higher rate of disappearance (panel B). Error bars show the standard deviation for three repetitive analyses of samples at each time point.

Conclusions

As consumers are becoming increasingly aware of the harmful as well as helpful content of what they eat and drink, modern powerful analytical tools will undoubtedly play a crucial role to supply that information more accurately and quickly. Albeit a very complex mixture, red wine is perfectly suitable for mass spectrometric supported by SIEVE differential expression software. Such 'fingerprinting' analysis can be applied in quality control and process monitoring, and for highlighting relevant nutritional value to consumers.

- U-HPLC affords fast analysis times while maintaining very high chromatographic resolution (peak width 7 seconds at half height).
- The mass deviation of the LTQ Orbitrap XL measurements was always smaller than 2 ppm using external calibration up to one day old.
- Higher collision energy dissociation MS/MS spectra confirm the identity and structure of compounds in complex mixtures.
- Accurate mass measurements also significantly improve the precision of quantitation by eliminating nearly isobaric interferences. This is a particularly important aspect for complex mixture analyses, which red wine undoubtedly is.
- The methodology described here is extremely robust, allowing for an uninterrupted analysis of 24 untreated red wine samples (continued analysis over an 8-hour period).

Acknowledgements

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Application Note: 420

Metabolomic Analysis of Green and Black Tea Extracts Using an LTQ Orbitrap XL Hybrid Linear Ion Trap Mass Spectrometer

Donna L. Wilson and Charles Yang; Thermo Fisher Scientific, San Jose, CA, USA

Key Words

- Accela™ High Speed UHPLC
- SIEVE™ Software
- Higher Energy Collisional Dissociation (HCD)
- Mass Frontier™ Software
- Natural Product Analysis

Overview

Purpose: To show a complete analytical metabolomic workflow including (1) data acquisition using a high resolution accurate mass instrument that is equipped with a Higher Energy Collisional Dissociation (HCD) cell and coupled to a high pressure LC (Figure 1), (2) metabolite differential abundance analysis, and (3) structural elucidation of relevant metabolites using accurate mass and HCD fragmentation information to highlight the component differences between green and black tea.

Methods: Green and black tea extracts were analyzed using an LTQ Orbitrap XL™ with an HCD cell. Chromatography was performed using an Accela High Speed LC equipped with a 2.1 mm ID Hypersil GOLD™ column packed with 1.9 µm particles. Data Dependent™ analysis was performed using an LTQ Orbitrap XL with full scan data acquired at a resolving power of 30,000 and MS^n data acquired at a resolving power of 7500 following HCD fragmentation.

Results: The study included a comparative analysis of green and black tea using differential analysis software to identify compositional variations between the two tea samples. Using a UHPLC coupled with a small particle column afforded a fast analysis time while maintaining very high chromatographic resolution. The high mass accuracy data (better than 3 ppm with external calibration) was used to determine elemental composition and for tentative identification of compounds via database searching. HCD fragmentation facilitated structural identification and confirmation. This was demonstrated with the example of epigallocatechin gallate (EGCG).

Introduction

Metabolomics, the comprehensive and quantitative analysis of wide arrays of metabolites in biological samples, marks promising new research territory. The numerous analytes in these samples have diverse chemistries and polarities. In addition, metabolites occur at a range of concentrations within a particular sample. Consequently, comprehensive metabolomics investigations create many analytical challenges that can be addressed using LC-MS/MS.

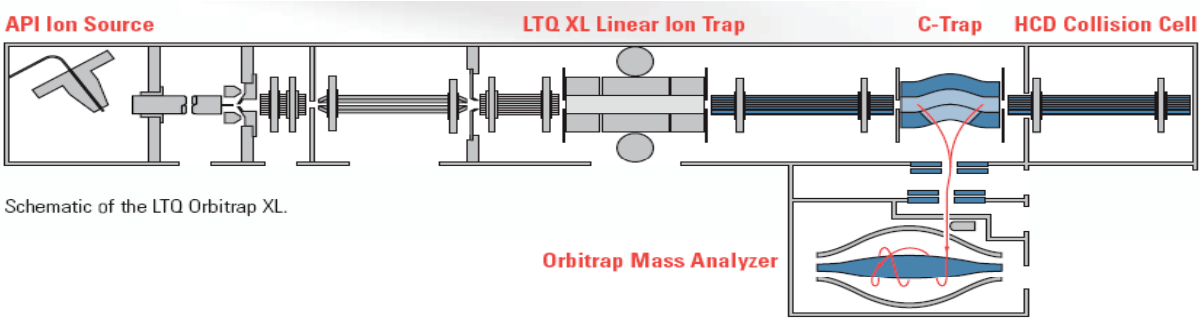
Tea contains a wide range of components including vitamins, amino acids, and polyphenols, many of which are structurally similar and may differ only in the type and location of a side chain. The use of high resolution chromatography is essential for the separation of such a complex mixture. Furthermore, acquisition of accurate mass data in both full scan and MS^n modes enables complete structural characterization.

Here, we highlight an untargeted metabolomic workflow from data acquisition through metabolite ID. The study included differential and structural characterization of polyphenolic catechin (flavan-3-ol) derivatives and theaflavin components of green tea and black tea.

Methods

Samples

Green tea and black tea aqueous extracts were examined without any pre-treatment. Each sample was analyzed in quadruplicate.



Schematic of the LTQ Orbitrap XL.

Figure 1: Schematic of the LTQ Orbitrap XL mass spectrometer with an HCD collision cell. The LTQ Orbitrap XL features an HCD collision cell to provide additional flexibility for low level components in complex mixtures. Ions can be selected in the linear ion trap and fragmented either in the ion trap (CID) or in the HCD collision cell. For HCD, ions are passed through the C-trap into the gas-filled collision cell, providing high sensitivity and high signal-to-noise fragmentation.

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Chromatography Conditions

Chromatographic separation was performed using the Accela UHPLC system (Thermo Fisher Scientific, San Jose, CA). The LC conditions were as follows:

Column: Hypersil GOLD, 100 × 2.1 mm, 1.9 µm particle size (Thermo Fisher Scientific, Bellefonte, PA)

Mobile phase: (A) water with 0.1% formic acid; (B) acetonitrile with 0.1% formic acid

Flow rate: 500 µL/min

Injection volume: 10 µL

Gradient: Linear gradient of 100%–1% A over 20 minutes

Mass Spectrometry Conditions

MS analysis was carried out using an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The MS conditions were as follows:

Positive electrospray ion source voltage: 5.0 kV

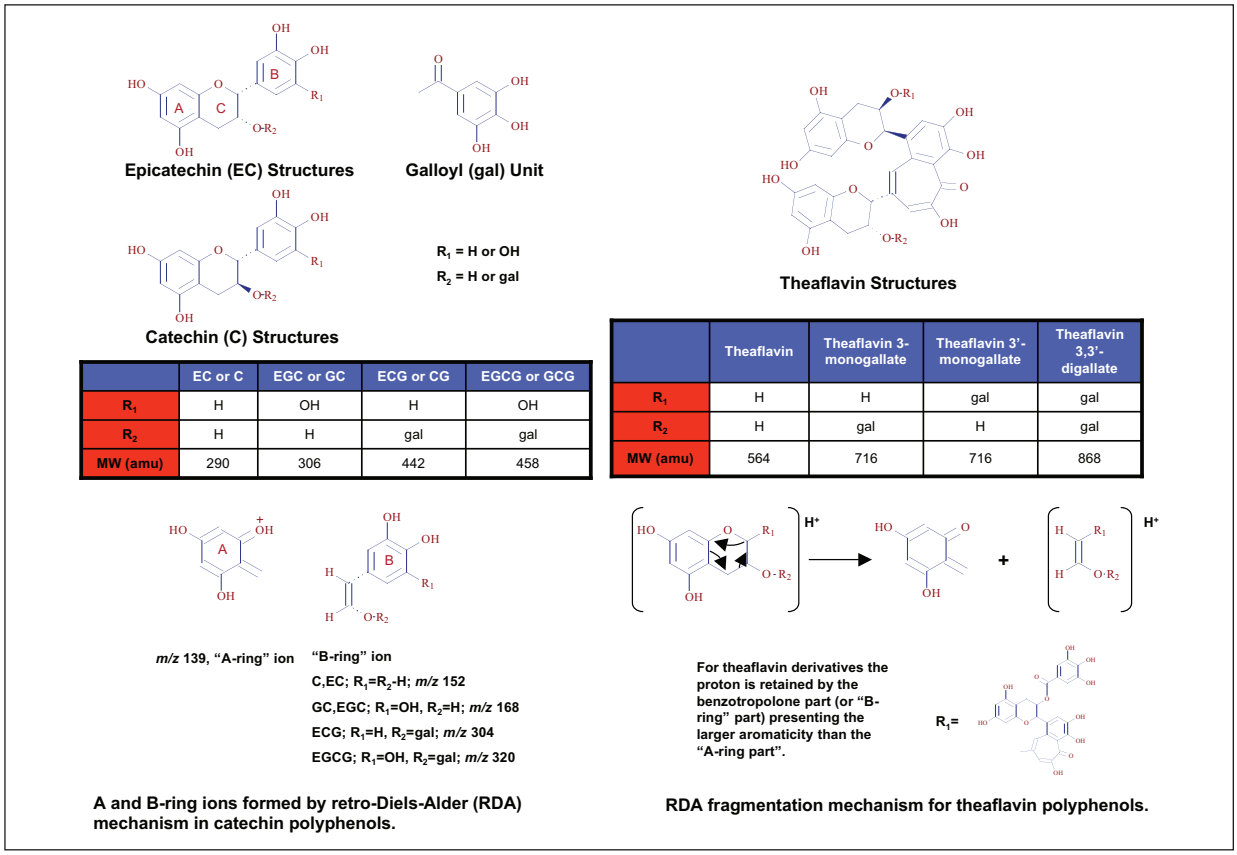
All methods: Full scan MS in the Orbitrap with a mass resolution of 30,000. Data Dependent MS/MS in the Orbitrap on the top three most intense ions from the full scan at a mass resolution of 7500.

Results

Considerable interest has developed in the potential health benefits of teas, particularly in the antioxidant and other properties of some of the polyphenolic catechins and theaflavins (Figure 2). The analysis described here focused on detection, relative quantitation, and identification of these low molecular weight components in green and black tea samples.

The HPLC separation of tea samples shows excellent peak separation and low noise, with most components eluting in less than 10 min. High resolution full scan spectra were acquired at a mass accuracy of better than 3 ppm.

After data acquisition, SIEVE software was used to determine statistically significant differences in the metabolite profiles of green and black tea samples (Figure 3). By comparing peak intensities between the two chromatographically aligned samples, metabolites present at different levels in the two teas were identified.



	EC or C	EGC or GC	ECG or CG	EGCG or GCG
R_1	H	OH	H	OH
R_2	H	H	gal	gal
MW (amu)	290	306	442	458

	Theaflavin	Theaflavin 3-monogallate	Theaflavin 3'-monogallate	Theaflavin 3,3'-digallate
R_1	H	H	gal	gal
R_2	H	gal	H	gal
MW (amu)	564	716	716	868

Figure 2: Three types of tea are produced from *Camellia sinensis* leaves: green tea (nonfermented), oolong tea (semi-fermented), and black tea (fermented). Catechins are polyphenolic antioxidant plant metabolites of the class flavanoids called flavan-3-ols and are highly present in tea plants. Fermentation induces enzymatic oxidation of flavan-3-ols and leads to the formation of two major pigments in black tea, theaflavins (TFs) and thearubigns (TRs). Catechins are expected to be more abundant in green tea and theaflavins more abundant in black tea. The proposed fragmentation pathway for these compounds proceeds via a Retro-Diels-Alder rearrangement as outlined here.

After differentially abundant metabolites of interest were detected, the accurate mass and the MS^n data were used for structural identification. The elemental formula, as determined by the accurate mass data, and the accurate mass value itself were used for metabolite database searching. The EGCG metabolite was tentatively assigned using this combination of tools.

Further metabolite characterization was accomplished using MS^n spectra and Mass Frontier software. Mass Frontier allowed confident metabolite identification using its comprehensive spectral library and predictive fragmentation algorithms to facilitate structural elucidation (Figure 4). The compounds in Figure 3 were identified using this workflow.

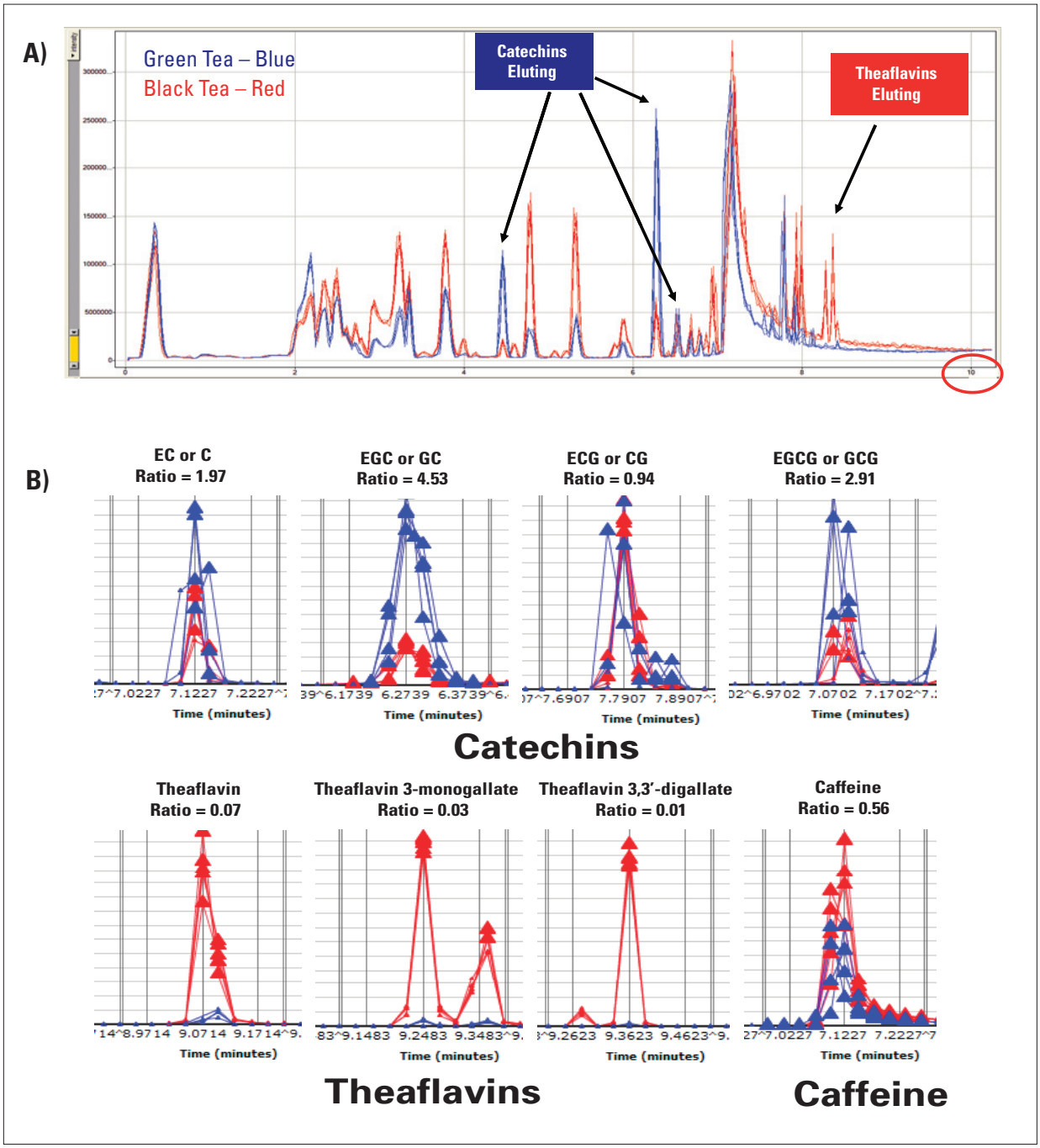


Figure 3: Differential metabolite abundance analysis with SIEVE software. A) Chromatographic alignment of the various LC sample traces is the first step in the SIEVE process. Differences between the green tea samples (Blue) and the black tea samples (Red) can be identified. The Accela UHPLC provided highly resolved chromatographic peaks and high signal-to-noise ratios. B) After alignment, the corresponding peak intensities are compared for green tea (Blue) and black tea (Red). The relative abundances of several compounds of interest are shown with their abundance ratios. These metabolites were identified using a combination of accurate mass database searching and MS^n spectra interpretation via Mass Frontier software.

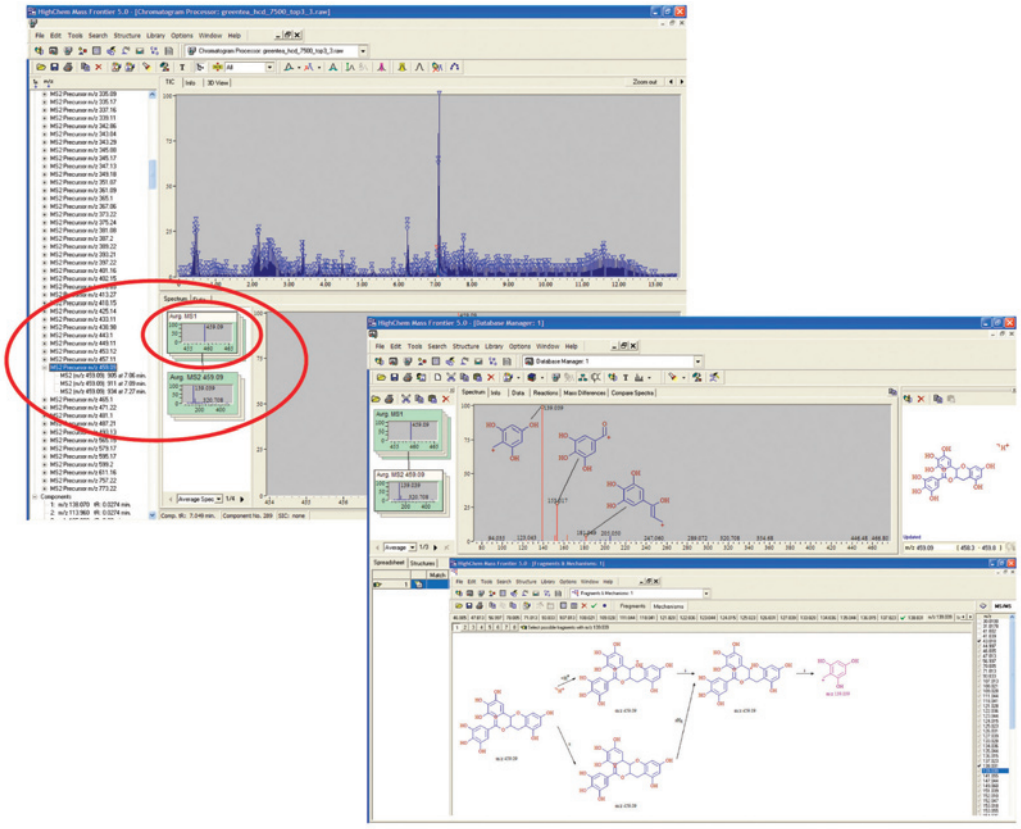


Figure 4: A metabolite of interest from Figure 3 was chosen for further characterization. This ion was present at levels ~3 times that of black tea and was identified as the potent antioxidant, EGCG. Accurate mass was used to identify the metabolite via database searching, and Mass Frontier software was used to confirm the EGCG identification by using the fragmentation spectra of the parent ion.

Conclusions

The analytical metabolomic workflow described here encompasses data acquisition, discovery of differentially abundant metabolites, and metabolite identification. The LTQ Orbitrap XL coupled to an Accela U-HPLC system afforded fast analysis times while maintaining high chromatographic resolution. Accurate mass measurements increased the confidence in elemental composition assignments and ultimately metabolite identification. SIEVE differential analysis software enabled large-scale evaluation of multiple complex LC-MS data and comparison of metabolite profiles between green and black tea samples. The spectral database and fragmentation algorithms of Mass Frontier software facilitated structural assignments of metabolites of interest utilizing MS^n fragmentation spectra.

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Measuring Phytosterols in Health Supplements by LC/MS

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Overview

Purpose: Develop a method for the extraction of phytosterols from health supplements. A liquid chromatography-mass spectrometry (LC/MS) method for analysis is used to demonstrate linear response and quantitate extraction results.

Methods: Saponification of the supplements followed by liquid-liquid extraction (LLE) separated the analytes from the matrix. Liquid chromatography along with mass selective detection was used to quantitate individual components.

Results: Good extraction efficiency combined with linear calibration gave results that were in good agreement with dose levels claimed by supplement manufacturers.

Introduction

Plant sterols, phytosterols, are claimed to help lower cholesterol levels in humans. In the year 2000, the United States Food & Drug Administration (US FDA) approved a health claim relating phytosterol ester or phytostanol ester consumption to reduced risk for coronary heart disease.[1] Thus, there is increased interest in qualitative and quantitative analysis of phytosterols in food products. Phytosterols are typically measured by gas chromatography after derivatization.[2] In this study, phytosterols were injected onto an LC-MS for direct analysis.

This application demonstrates the use of MS detection with LC for determination of several phytosterols and their extraction from some commercial health supplements. To separate the analytes from the matrix, health supplement pills were saponified in ethanolic potassium hydroxide. After the pill was completely dissolved, the solution was neutralized with acetic acid. Hexane was then used to extract the target compounds. The extract solvent was evaporated. Then samples were reconstituted and diluted to an appropriate concentration range for analysis. Chromatographic separation was optimized, but mass selectivity was required to accurately quantitate the composition of the supplements. Linear calibration and measurements for the supplements are presented.

Methods

Supplement extraction: Health supplement pills were obtained locally from a vitamin store. Each pill was placed in a 50 mL centrifuge tube and 10 mL of 2M ethanolic potassium hydroxide was added. Samples were then sonicated for 30 minutes. After each supplement was completely dissolved, an equal volume of 2M acetic acid was added to neutralize the solution. Subsequently 5 mL of Hexane was added to each centrifuge tube and the samples spun at 3,000 G for 10 minutes. The supernatant was decanted and saved while another 5 mL of hexane was added for a second extraction. The extraction was done 3 times. Each time, the supernatant was separated and all three combined and then evaporated to dryness. Samples were reconstituted with 5 mL of hexane and sonicated briefly to ensure complete dissolving. Further dilution was required to bring the concentrations into range of the calibration. Thus, a 20-fold dilution was used to prepare a 100x sample and a subsequent 10-fold dilution to produce a 1000x dilution. Extracts were stored at 5 ºC.

Reagent and Chemicals: Stock solutions at 1000 ppm for the six sterols were made up in hexane. Cholesterol (p/n C8667, CAS 57-88-5), Campesterol (p/n C5157, 474-62-4), Brassicasterol (B4936, 474-67-9), Lupeol (p/n L5632, 545-47-1) and B-Sitosterol (p/n S1270, 83-46-5) were obtained from Aldrich.

A working stock solution of the combined six phytosterols was made by combining the above stock solutions to achieve a 100 ppm primary dilution stock. Sequential dilutions of this stock were used to prepare calibration standards at concentrations of 0.5, 1, 2, 5, 10, 20, 50 and 100 part-per-million (ppm or ng/mL).

Acetonitrile (HPLC grade, AH015-4) and methanol (230-4) were obtained from Burdick & Jackson. Hexane was purchased from Aldrich (p/n 320315)

Ethanolic potassium hydroxide was made by mixing 15 mL of 45% w/w KOH concentrate (Fisher, SP236) with Ethanol (VWR, BDH 1160) to make a volume of 100 mL. An amber bottle was used to minimize light exposure and the solution discarded if not used in two weeks.

Chromatography

A Thermo Scientific Dionex UltiMate 3000 Rapid Separation LC (RSLC) system was used. This system included a LPG-3600 Ternary Pump, WPS-3000T Autosampler, and a FLM-3100 Column Oven.

A Thermo Scientific Acclaim C30 column was used to separate the compounds under isocratic conditions. The column was thermostated at 30 ºC. The solvent mixture was held constant at 50:50 MeOH/ACN. The autosampler delivered a 5 µL injection while the flow rate was 0.35 mL/min. A retention time was established for each compound. The divert valve directed flow from the column to waste or to the MS for analysis as described below. Total run time was 12 minutes, with all peaks of interest eluting between 6 and 11 minutes.

Divert Valve (to MS)

Time (min)	Position
0.0	to waste
4.5	to MS
12.0	to waste

The divert valve was used for the first 4.5 minutes, shunting the LC flow to waste to allow impurities from the extraction that were not retained on the column to bypass the MS. The valve was then switched to direct flow to the MS for analysis. A Thermo Scientific Dionex AXP Auxiliary Pump provided a continuous stream of liquid flowing through the MS source so that it would remain wet at all times.

Mass Spectrometry

A Thermo Scientific MSQ Plus single quadrupole mass spectrometer was used for analysis. The atmospheric-pressure chemical ionization (APCI) interface proved to be most effective at ionizing the relatively neutral compounds. Selectivity is inherently present due to the difference in *m/z* ratio of the analytes serving to reduce potential interferences that might be present in the extracted samples. Sensitivity is greatly enhanced through the use of the selective ion monitoring (SIM) scan function. Water loss from the protonated molecular ion was the most strongly observed species for all the analytes. Thus, the SIM scans represent $[M+H-H_2O]^+$, or a target m/z of the pseudo-molecular ion minus water. Details of the SIM scan parameters are detailed in Table 1.

Data Analysis

Integrated control of the LC and MS hardware was accomplished through the Thermo Scientific Dionex Chromeleon Chromatography software (version 6.8 SR10). This software also provided tools for data acquisition, processing and report generation.

TABLE 1. Table of SIM scan parameters*

Analyte	t_R (min)	m/z	Dwell time (s)	Cone Voltage (V)
Cholesterol	8.0	369.2	0.2	50
Brassicasterol	7.5	381.2	0.2	45
Campesterol	9.0	383.2	0.2	50
Stigmasterol	9.0	395.3	0.2	50
B-Sitosterol	10.2	397.3	0.2	40
Lupeol	6.8	409.4	0.2	40

* The optimum settings and responses may vary on different instruments, and thus optimization of the MSQ Plus™ mass spectrometer source conditions and acquisition parameters is highly recommended for best results.

Results

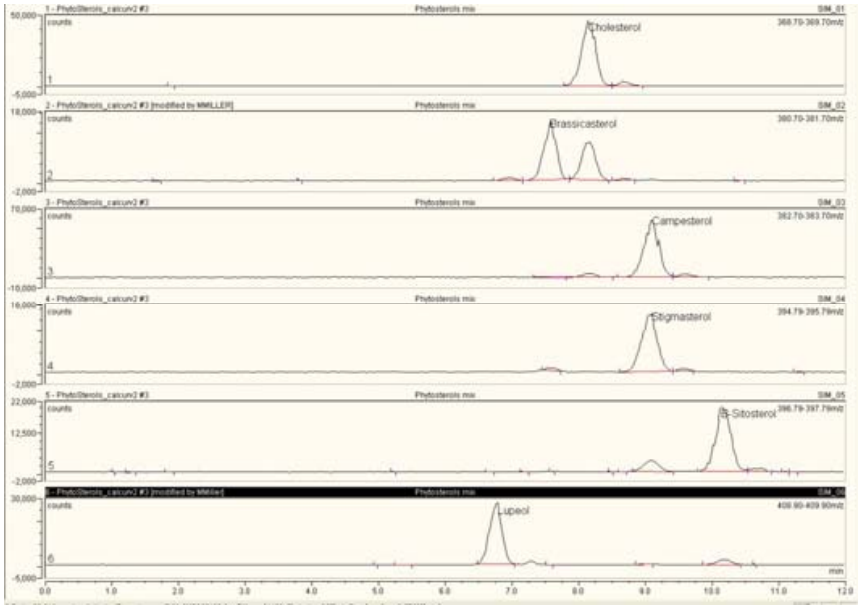
This method was used to quantify the target analytes in clean water as well as the recovery of extractions from health supplement pills

Calibration curves were generated from standards with concentrations from 0.5 to 50 ppm. All compounds showed a correlation coefficient (r^2) greater than 0.9997. Calibration ranges were from 0.5 to 100 ppm for a 5 μL injection, giving a load range of 2.5 to 500 μg on column. Results are summarized in Table 2. A typical separation is shown in the chromatogram in Figure 1.

Table 2. Calibration and Coefficient of Determination

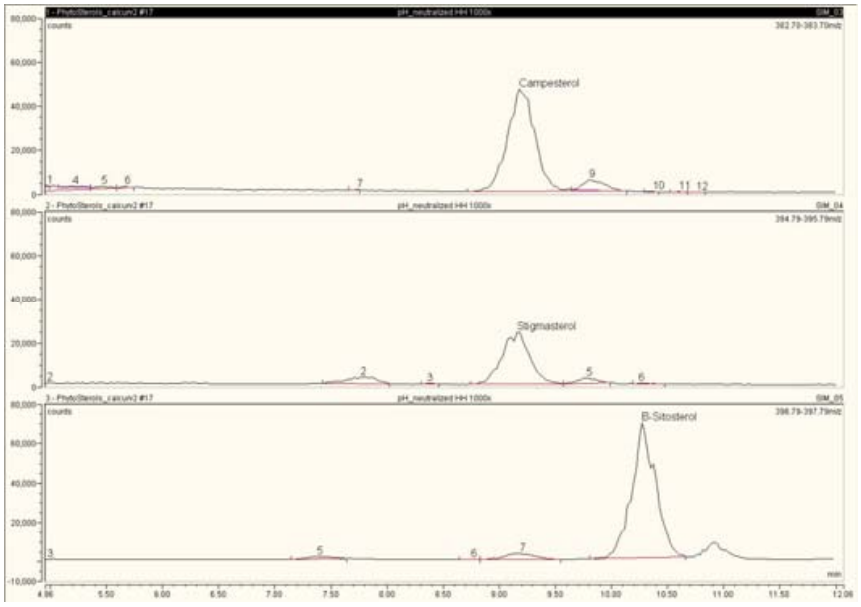
Analyte	Calibration Range (ppm)	Fitting	r^2
Cholesterol	0.5-100	Linear	0.9997
Brassicasterol	0.5-100	Linear	0.9997
Campesterol	0.5-100	Linear	0.9999
Stigmasterol	0.5-100	Linear	0.9999
B-Sitosterol	0.5-100	Linear	0.9999
Lupeol	0.5-100	Linear	0.9999

FIGURE 1. Separation of analytes in clean standard



Two health supplements were analyzed for recovery and compared with labeled amount. Measured recoveries for total amount of phytosterols were 80% for one supplement and 88% for the other. Figure 2 shows one supplement and the three detected phytosterols present after extraction.

FIGURE 2. Chromatogram of extracted health supplement after 1000x dilution



Conclusion

This application demonstrates the use the Acclaim™ C30 column coupled with the UltiMate™ 3000 HPLC and MSQ Plus™ detector to separate and quantitate the phytosterols in this method. Use of MS detection allows confirmation of analyte identity. Quantitation showed linear response for the analytes. This shows the capability of using the LC/MS combination to analyze nutraceutical health supplements for phytosterol content.

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Rapid Screening of Dietary Supplements with Direct Analysis ID-CUBE Coupled to an Exactive Mass Spectrometer

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Application Note 565

Key Words
Exactive, DART, ID-CUBE, Screening, Dietary Supplements, Herbal

Introduction

In Westernized cultures, dietary supplements have been gaining popularity in recent years. In China, traditional herbal supplements have been used for millennia. In both cases, the threat of adulteration and need for further characterization of ingredients have been driving forces behind the need for increased screening. This note presents a new approach to the rapid screening of a product marketed as an herbal weight loss aid and the fingerprint characterization of omega fatty acid dietary supplements derived from marine and flaxseed oils.

The Direct Analysis in Real Time (DART®) ionization source,[1] as well as other direct ambient ionization techniques such as Desorption Electrospray Ionization (DESI)[2] and the Atmospheric Solids Analysis Probe (ASAP®),[3] have gained momentum in recent years as more-robust and higher-throughput ionization techniques for mass spectrometry (MS) in routine markets. None of these direct ionization techniques incorporate online sample separation. As a result, a vast number of ions can be generated from what are often complex samples. Thus there is great benefit to coupling DART and other direct ambient ionization sources with high-resolution mass spectrometers that can distinguish ions of interest from those of the matrix and background interferences. Thermo Scientific Orbitrap mass analyzer technology with >100,000 mass resolution (full width at half maximum – FWHM – at *m/z* 200) and <5 ppm mass accuracy (RMS, with external mass calibration) easily achieves the levels needed for this task.

The ID-CUBE® is a next-generation DART ion source that instantaneously desorbs and ionizes analytes off standardized sample cards called OpenSpot® cards. By resistively heating the stainless steel mesh sampling surface of the OpenSpot card, the source desorbs the analytes directly into the reactive helium gas stream where they are ionized via the DART atmospheric pressure chemical ionization (APCI) processes.[1, 4-7]

The ID-CUBE can directly sample both liquids and powders. It fully integrates with the mass spectrometer without the need for stand-alone software control. The overall performance and robustness of the ID-CUBE/Orbitrap™ mass analyzer combination is demonstrated here through rapid screening of dietary supplements and an herbal weight loss aid.

Experimental

ID-CUBE/Exactive MS

The ID-CUBE ionization source (Figures 1-3), was coupled to a Thermo Scientific Exactive benchtop Orbitrap mass spectrometer using a Vapur® gas ion separator interface[8]. The ID-CUBE discharge voltage was controlled through Thermo Scientific Xcalibur software by setting the ion source spray voltage to 1.0 kV and the sheath, auxiliary and sweep gases to zero for all ID-CUBE analyses. The Exactive™ MS was operated in positive and negative ion modes with the following scan and inlet parameters:

Number of microscans:	1
Maximum inject time:	250 ms
Automatic gain control (AGC) target:	1,000,000
Capillary temperature:	200 °C
Capillary voltage:	+25/-50 V
Tube lens voltage:	+/-120 V
Skimmer voltage:	+/-25 V

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Figure 1. ID-CUBE direct analysis in real time (DART) ionization source coupled with an Exactive MS

External mass calibration was performed at the beginning of the week in which the analyses took place using positive and negative ion calibration solutions prepared according to the protocol in the Exactive MS operating manual. The calibration was performed by direct infusion using the heated electrospray (HESI) Thermo Scientific Ion Max source. The resolving power setting for the Exactive MS was set at 50,000 FWHM (at *m/z* 200) resulting in 2 scans per second.

The helium gas flow for the ID-CUBE was set at 2 SCFH (standard cubic feet per hour), corresponding to 1.14 L/min. The ID-CUBE source was operated with OpenSpot sample cards that consisted of a small piece of metal mesh with a narrowed sample spotting area. The OpenSpot cards were inserted into the slot on the top of the ID-CUBE source (Figure 2) and the heating setting was selected on the ID-CUBE switch box. All analytes were thermally profiled using all three desorption settings of Low (180 °C), Medium (360 °C) and High (580 °C) before running replicate samples. Data acquisition was set up through an Xcalibur™ software sequence. The ID-CUBE source triggered the mass spectrometer data acquisition via a contact closure.

Figure 2. OpenSpot sample card and introduction to the ID-CUBE ion source

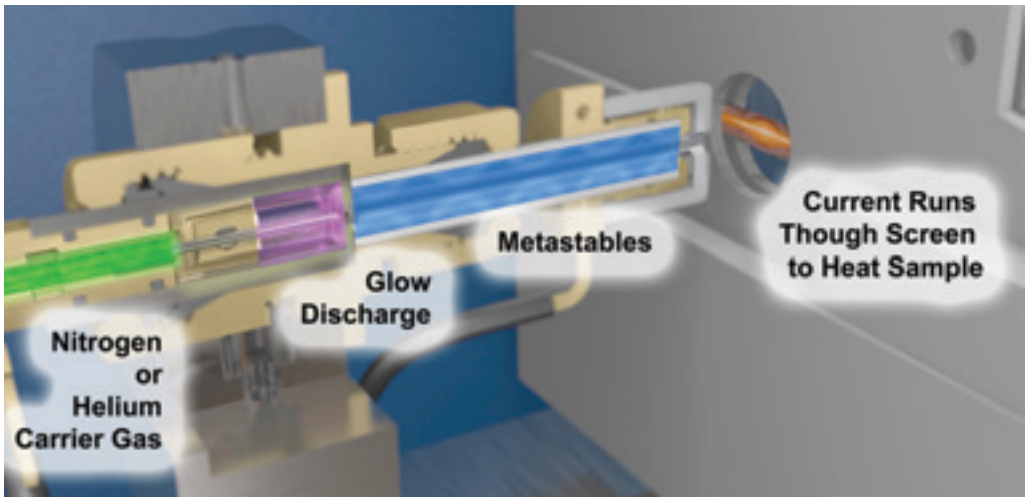

Figure 3. Schematic diagram of the ID-CUBE ambient ionization source

Analysis of Counterfeit Herbal Weight Loss Aid

Pai You Guo is a commercial Chinese herbal weight loss aid. In 2009, Pai You Guo was withdrawn from the market by its manufacturer[9] after FDA investigation determined that it contained sibutramine, an FDA-regulated appetite suppressant used for weight loss, as well as other unapproved compounds. Sibutramine itself was subsequently withdrawn from the market in the European Union, United States, and other countries. In 2010 and 2011, articles in the Boston Globe newspaper[10,11] indicated that Pai You Guo was still available through stores and over the Internet and was still being used by consumers.

For this research, Pai You Guo was purchased online in August 2011 from a vendor based in the United States. The packaging for the Pai You Guo did not state anywhere that sibutramine could be among the ingredients of the capsules.

The Pai You Guo capsules contained a very fine powder and were twisted open to directly sample the powder. Using a closed end melting point glass capillary tube, a very small amount of the powder was pressed onto the end of the glass tip and this material was scraped across the sampling area of an OpenSpot card as shown in Figure 4. The powder was thermally profiled and a heating setting of Medium was selected. For comparison, a small amount of the capsule powder was also dissolved in acetonitrile (ACN) and 5-µL aliquots were sampled from the OpenSpot cards.

Figure 4. OpenSpot cards used for liquid and solid sample application

Analysis of Omega Fatty Acid Dietary Supplements

For this research, several omega fatty acid dietary supplements were purchased locally. The omega-3, 6 and 9 fatty acids were compared between two fish oil dietary supplements (Nature Made and Nature's Bounty), MegaRed krill oil, and Nature Made triple omega dietary supplement, which contained a mixture of fish oil, safflower oil and flaxseed oil.

A similar comparison was conducted for the fingerprint fatty acid components of several flaxseed oils including Nature Made triple omega, Nature Made flax oil and Target Origin flax oil.

Preparation of these oils for ID-CUBE analysis consisted of performing a 2% dilution in toluene and spotting 5 µL of the diluted solutions directly onto an OpenSpot sample card.

Results and Discussion

Herbal Weight Loss Aid

Of the 30 capsules in the Pai You Guo package, all five capsules sampled tested positive for the presence of sibutramine. Figure 5 shows the spectra obtained from the ID-CUBE direct analysis of the capsule powder and analysis of a saturated solution of the powder. The top spectrum in Figure 5 displays the simulated $[M+H]^+$ spectrum for the elemental composition $C_{17}H_{26}NCl$ for sibutramine. The signal from the capsule powder sampled directly for the $[M+H]^+$ ion at *m/z* 280.18204, with mass accuracy of -2.2 ppm dominated the spectrum with all other peak intensities less than 15%. The capsule contents sampled as a saturated solution generated more intense signal, approximately an order of magnitude greater than the direct powder analysis because a greater amount of the capsule material was sampled. The mass accuracy reported for the analysis of the saturated solution was -2.7 ppm.

For further confirmation that sibutramine was detected in the Pai You Guo capsules, the saturated solution was subjected to an all-ion fragmentation (AIF) experiment. The higher-energy collisional dissociation (HCD) cell was used at 20 eV to fragment all ions without precursor ion isolation. The MS1 full-scan spectrum was dominated by the $[M+H]^+$ ions for sibutramine and, as a result of applying the AIF, a relatively simple fragmentation spectrum was recorded as shown in Figure 6. The major fragment ion C_7H_6Cl at *m/z* 125.01518 with mass accuracy of -0.6 ppm, as well as a minor fragment C_8H_8Cl at *m/z* 139.03071 with mass accuracy of -1.3 ppm, maintained the chlorine isotope pattern and resulted from losses of $C_{10}H_{21}N$ and $C_9H_{19}N$ respectively.

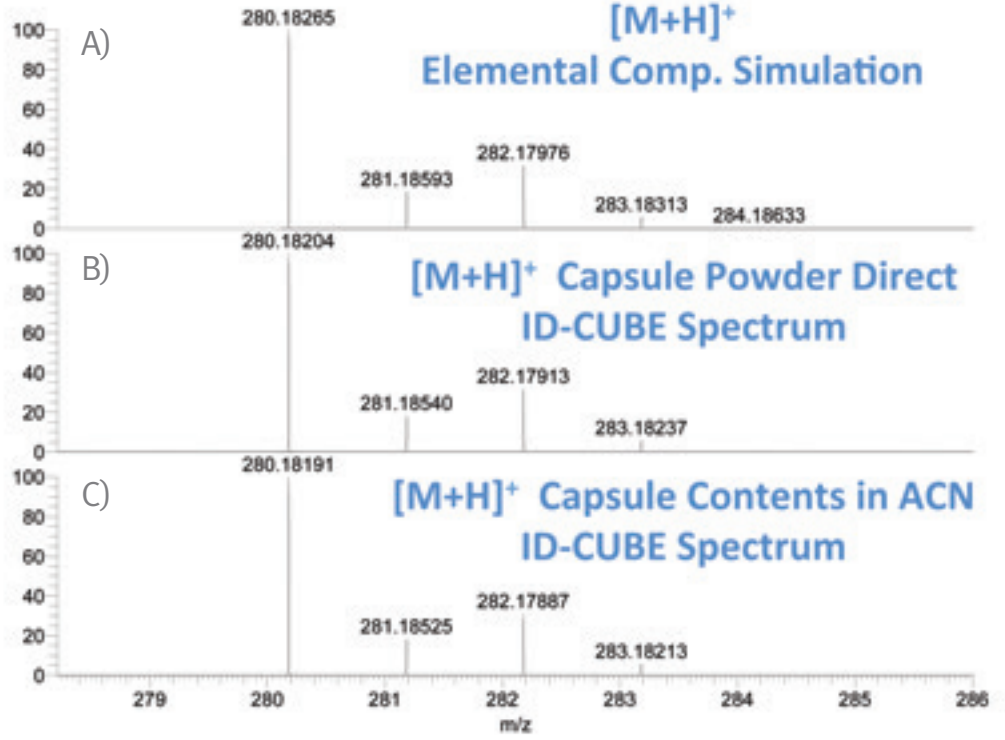

Figure 5. Mass spectra for the analysis of Pai You Guo herbal weight loss aid.

A Elemental composition simulation for sibutramine $[M+H]^+$ at *m/z* 280.18265.

B Direct analysis of the Pai You Guo capsule powder, delta ppm of -2.2 ppm.

C Analysis of 5 µL of a saturated solution in acetonitrile (ACN), delta ppm of -2.7 ppm.

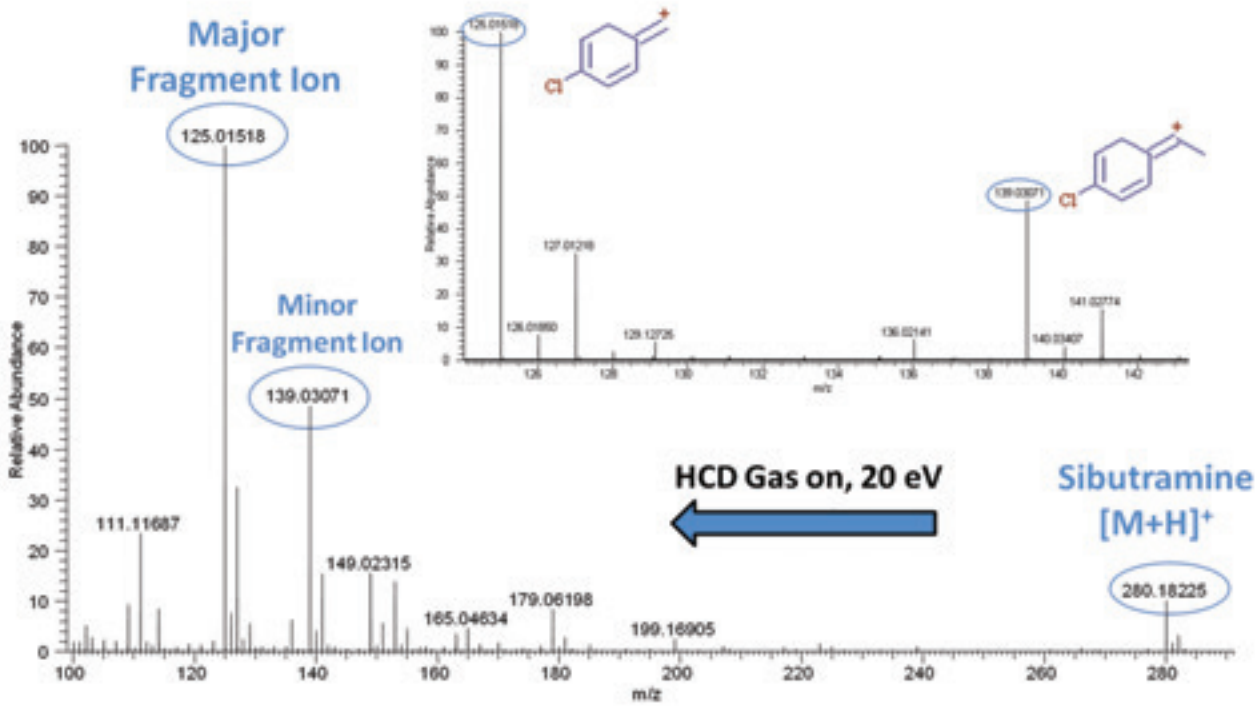

Figure 6. HCD experiment on Pai You Guo capsule content. Sibutramine $[M+H]^+$ ions ($C_{17}H_{27}NCl$) was fragmented with HCD gas on and 20 eV producing a major fragment C_7H_6Cl at *m/z* 125.01518 and a minor fragment C_8H_8Cl at *m/z* 139.03071.

Omega Fatty Acid Dietary Supplements

Another area of great interest with regard to dietary supplements is the use of marine-and plant-based oils as a source of essential omega fatty acids. Competing products in marine oil dietary supplements, namely fish oils, are being challenged by the manufacturers of krill-based oils. Krill oil dietary supplement manufacturers position their products as being superior to fish oil products for the relative amounts omega-3 essential fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These experiments profiled two fish oil dietary supplements, one plant and fish omega-3 mixture, and one krill oil omega-3 dietary supplement. The oils were not sampled directly from the supplement capsules because the concentrations are extremely high. A 2% dilution in toluene permitted a thin coating of material to be evenly applied onto the mesh of the OpenSpot sample card. The ID-CUBE heating setting was optimized in less than one minute and all of the samples were analyzed on a Medium heating setting. Sample analysis time per OpenSpot card was 10 seconds.

Figure 7 displays the results of the ID-CUBE analysis of diluted oil supplements where all four supplements contained varying levels of DHA and oleic acid. The average area (n=5) for EPA was normalized to 1, yielding relative ratios for DHA and oleic acid. The two fish oils contained the most DHA per capsule and the Nature's Made Triple Omega supplement, which was a mixture of plant and fish oil, contained nearly the same level of DHA as the krill oil per capsule. Reproducibility RSD values for replicates of five for EPA was 9% +/- 1%, normalizing the signal to oleic acid signal. For the less intense DHA signal, reproducibility for replicates of five was 19% +/- 3%.

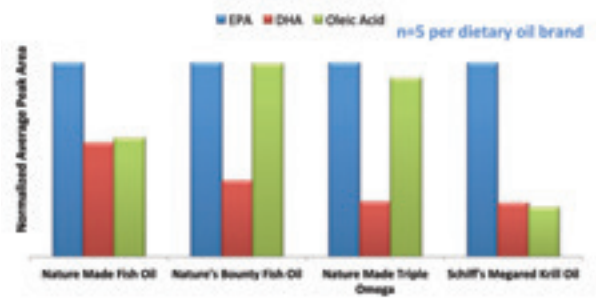

Figure 7. Analysis of marine oil samples monitoring omega-3 essential fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) as well as omega-9 oleic acid. All of the oils were sampled as a 2% solution in toluene and EPA area was normalized to 1, n = 5.

Figure 8 focuses on plant-based essential omega fatty acids α-linolenic and linoleic acids in flaxseed oils and the triple omega oil mixture. Flaxseed oil is one of the richest sources of α-linolenic acid. The name brand flaxseed oil supplement by Nature Made analyzed against the store brand Target Origin flaxseed oil yielded nearly identical ratios of α-linolenic and linoleic acids. The triple omega oil mixture contained roughly half the amount of α-linolenic as the pure flaxseed oil supplements and the linoleic acid level was nearly the same per capsule. Figure 9 shows an example mass spectrum of Omega-3, 6 and 9 fatty acids from Nature Made Triple Omega dietary supplement containing flaxseed, safflower and fish oil product.

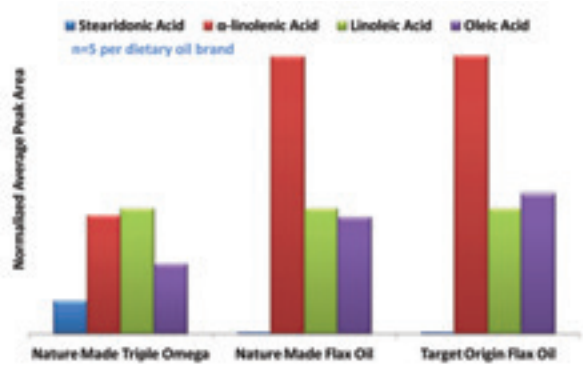

Figure 8. Omega-3, 6 and 9 fatty acid analysis of three flaxseed oil products. All of the oils were sampled as a 2% solution in toluene, n = 5.

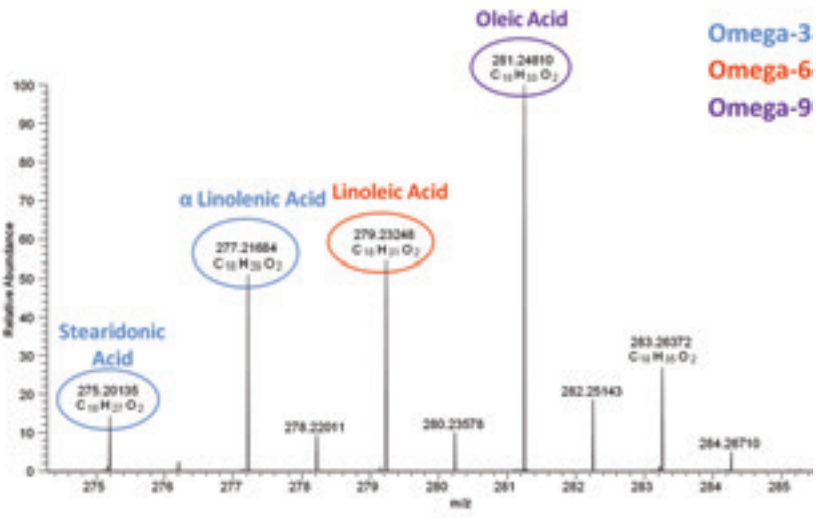

Figure 9. Mass spectrum of Omega-3, 6 and 9 fatty acids from Nature Made Triple Omega dietary supplement containing flaxseed, safflower and fish oil product

Conclusions

The combination of the ID-CUBE coupled to the Exactive high-resolution MS proved to be a rapid and robust tool for screening and fingerprint characterization. The Orbitrap platform provided extremely fast accurate-mass measurements of <3 ppm with external mass calibration for all of the analyses, and switching between the ESI source and the ID-CUBE source was very smooth, taking less than 3 minutes total. Optimization of the heating setting was achieved in less than one minute for rapid heating of the sample. Using the standardized OpenSpot sample cards, a single sample analysis could be completed in 10 seconds per card. Both liquids and powders were easily analyzed using the ID-CUBE source as shown in the experiments detecting sibutramine in Pai You Guo herbal weight loss aid. Dietary supplements could be quickly fingerprinted and characterized for quality control testing.

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Application Note 20675

LC-MS/MS Method for the Rapid Analysis of Five Artificial Sweeteners Using a Core Enhanced Technology Column

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Key Words

Accucore RP-MS, Core Enhanced Technology, solid core, sweeteners, acesulfame potassium, saccharin, sodium cyclamate, aspartame, sucralose

Abstract

This application note demonstrates the use of the Thermo Scientific™ Accucore™ RP-MS HPLC column for the rapid analysis of five artificial sweeteners.

Introduction

Artificial sweeteners are used as a sugar substitute in food and beverage products such as cereal bars and soft drinks. Their use is becoming more popular as consumers are increasingly concerned about obesity and dental decay caused from consuming natural sugars. In addition, artificial sweeteners are ideal for use by people suffering from diabetes.

Acesulfame potassium, saccharin, aspartame, and sucralose are all commonly used artificial sweeteners that have been approved for use by the United States Food and Drug Administration (FDA). Sodium cyclamate has been banned in the United States, although it is approved for use in many other countries, including the UK.

As can be seen in Figures 1 to 5 some of the sweeteners do not have a UV chromophore; therefore, detection at the required limits using HPLC-UV can be a challenge. This application demonstrates an alternative approach using LC-MS/MS with an Accucore RP-MS column.

Accucore HPLC columns use Core Enhanced Technology™ to facilitate fast and high efficiency separations. The 2.6 µm diameter particles are not totally porous, but instead have a solid core and a porous outer layer. The optimized phase bonding creates a series of high coverage, robust phases. Accucore RP-MS column uses an optimized alkyl chain length for more effective coverage of the silica surface. This coverage results in a significant reduction in secondary interactions and thus highly efficient peaks with very low tailing. The tightly controlled 2.6 µm diameter of Accucore particles results in much lower backpressures than typically seen with sub-2 µm materials.

Experimental Details

Consumables	Part Number
Fisher Scientific™ LC-MS grade water	W/0112/17
Fisher Scientific LC-MS grade acetonitrile	A/0638/17
Fisher Scientific LC-MS grade formic acid	A/3295/PB05

Acesulfame potassium, saccharin, sodium cyclamate, aspartame, and sucralose were purchased from Sigma-Aldrich®

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Sample Preparation

The sample was prepared to contain 200 ng/mL of acesulfame potassium, saccharin, sodium cyclamate, aspartame, and sucralose in water.

Separation Conditions		Part Number
Instrumentation:	Thermo Scientific Dionex™ UltiMate™ 3000 RS system consisting of an HPG-3200SD high pressure gradient pump, WPS-3000 RS thermostatted split-loop autosampler, and TCC-3000 RS column thermostat	
Column:	Accucore RP-MS 2.6 µm, 50 x 2.1 mm	17626-052130
Mobile phase A:	Water + 0.1% formic acid	
Mobile phase B:	Acetonitrile + 0.1% formic acid	
Gradient:	Time (minutes)	%B
	0.0	5
	0.10	5
	1.00	95
	1.25	95
	1.26	5
	2.00	5
Flow rate:	1 mL/min	
Column temperature:	40 °C	
Injection volume:	10 µL	
Weak injection wash solvent:	Acetonitrile / water (20:80 v/v)	
Strong injection wash solvent:	Acetonitrile / acetone / isopropranol (45:45:10 v/v/v)	

MS Conditions	
Instrumentation:	Thermo Scientific TSQ Vantage™ MS
Ionization conditions:	HESI-II
Polarity:	Negative
Spray voltage (V):	4000
Vaporizer temp (°C):	450
Sheath gas pressure (Arbitrary units):	40
Aux gas pressure (Arbitrary units):	20
Capillary temp (°C):	300
Collision pressure (mTorr):	1.5
Cycle time (s):	0.02
Q1 (peak width):	0.7
Q3 (peak width):	0.7

Compound	Sucralose	Sodium cyclamate	Acesulfame potassium	Saccharin	Aspartame
Precursor ion (*m/z*)	395.1	178.1	162.0	182.1	293.2
Product ion (*m/z*)	359.1	79.9	82.0	42.0	261.1
Collision energy (eV)	15	28	16	21	14
S-lens (RF voltage)	120	78	56	79	87

Table 1: Transition details

Data Processing

Software:	Thermo Scientific LC QUAN™

Results

All five sweeteners were analyzed in less than 1 minute using an Accucore RP-MS column as shown in Figure 6. The retention factor of the first peak was approximately 2.5. Chromatographic baseline resolution was not needed as positive identification of each individual sweetener was achieved using MS/MS detection. Table 2 shows the %RSD of the response for all five compounds with less than 5% RSD illustrating excellent precision.

Compound	Retention Time (min)	Response %RSD
Acesulfame potassium	0.30	3.8
Saccharin	0.55	3.7
Sodium cyclamate	0.61	4.0
Aspartame	0.76	4.6
Sucralose	0.76	3.8

Table 2: Precision data calculated from 6 replicate injections

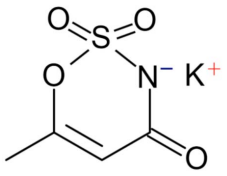

Figure 1: Structure of acesulfame potassium

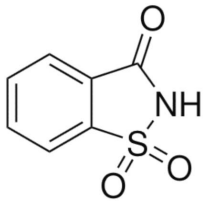

Figure 2: Structure of saccharin

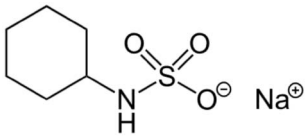

Figure 3: Structure of sodium cyclamate

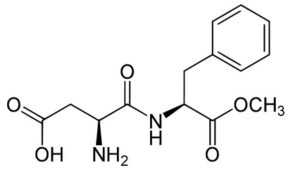

Figure 4: Structure of aspartame

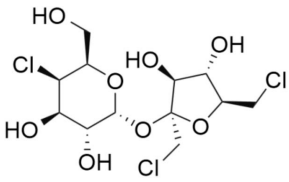

Figure 5: Structure of sucralose

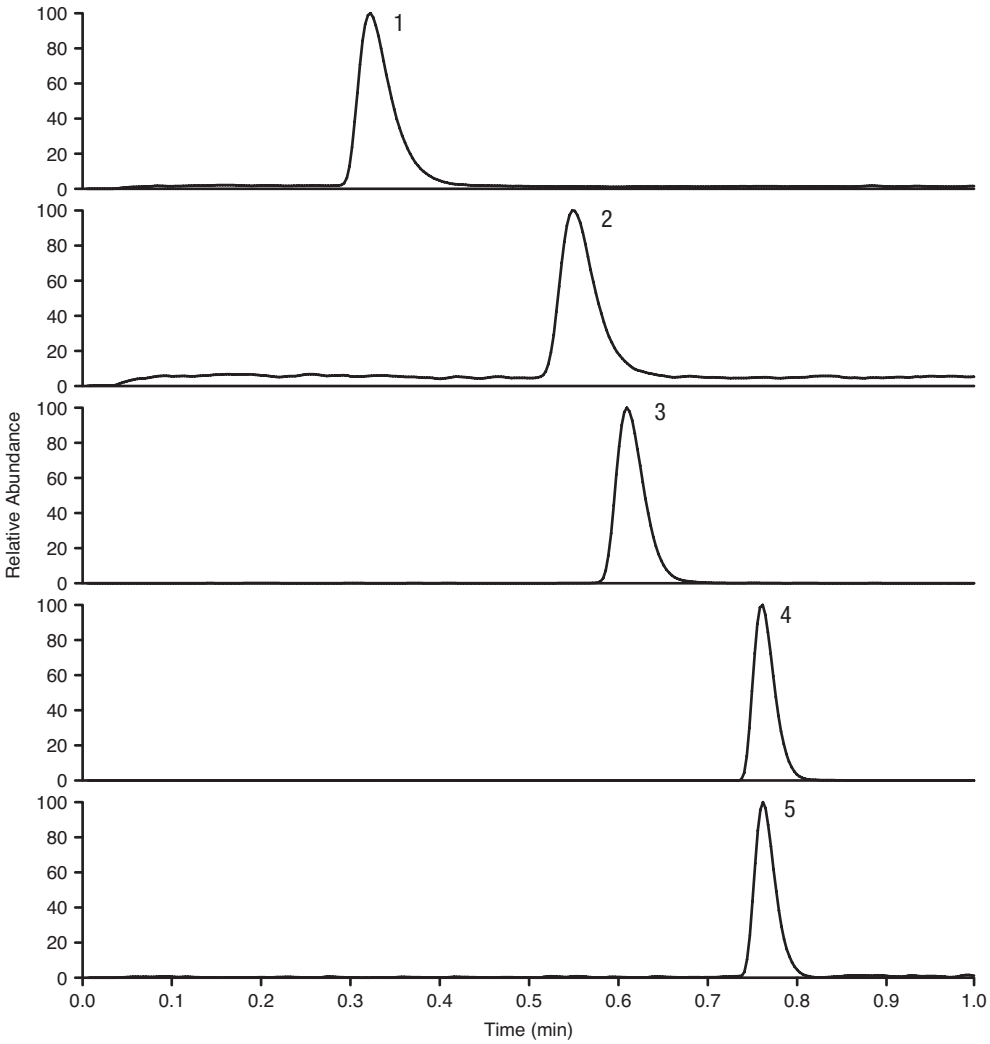

Figure 6: Selected ion chromatograms of acesulfame potassium (1), saccharin (2), sodium cyclamate (3), aspartame (4), and sucralose (5) at 200 ng/mL

Conclusion

This application demonstrates the successful LC-MS/MS analysis of five artificial sweeteners in a run time under 1 minute using an Accucore RP-MS column.

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Measurement of Vitamin D in Milk and Infant Formulas Using Automated Online Sample Preparation with Liquid Chromatography/ Tandem Mass Spectrometry

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Application Note 568

Key Words
Transcend TLX-1, TurboFlow technology, TSQ Access MAX, Vitamin D, Infant formula, Milk

Goal
To develop a rapid and sensitive automated online sample preparation LC-MS/MS method to measure vitamin D in infant formula.

Introduction
Vitamin D, including vitamin D_2 and D_3 (Figure 1), is essential to calcium absorption and bone health. Current studies suggest vitamin D could help prevent a wide range of disorders.[1] Although vitamin D can be synthesized by the human body from exposure to sunlight, this process can be impaired by many factors.[2] Therefore, fortification of vitamin D in some dietary sources becomes necessary. The Institute of Medicine (IOM) of the National Academies recommends an average vitamin D intake of 400 IU/day (10 µg/day) vitamin D for infants 12 months of age and younger. This recommendation increases to 600 IU/day for older children and adults. The intake of excessive amounts of vitamin D can, however, lead to a potentially serious condition, vitamin D toxicity, also called hypervitaminosis D. It is thus critical to measure the amount of vitamin D in foods, occurring both naturally and from fortification.

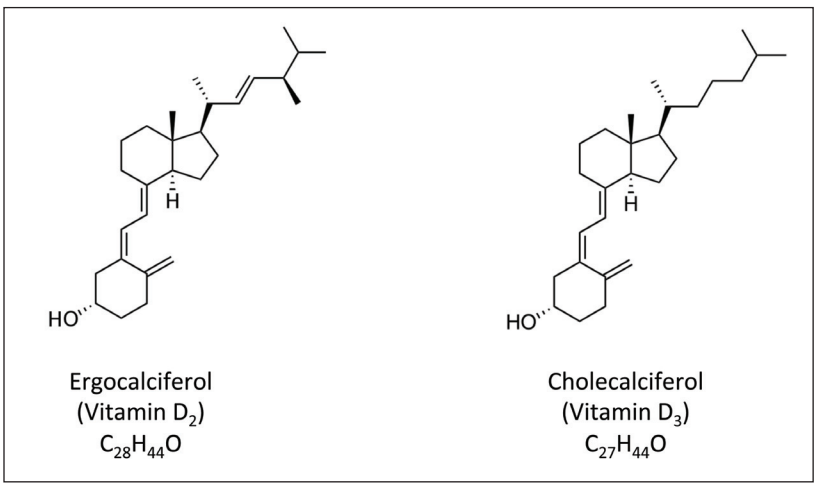

Figure 1. Chemical structures of vitamin D

The level of vitamin D in food has been determined using gas chromatography (GC)[3], high-performance liquid chromatography (HPLC)[4], and other physicochemical methods. The use of liquid chromatography/tandem mass spectrometry (LC-MS/MS) has gained popularity in vitamin D analysis due to its selectivity, specificity, and reliability. Traditional sample preparation for LC-MS/MS analysis of vitamin D can be time and labor intensive, often involving saponification, liquid extraction, solvent evaporation, manual solid phase extraction, and pre-concentration. In addition, thermal isomerization of vitamin D to previtamin D can occur during saponification, which usually complicates LC/MS analysis.

In this application note, we describe an easy and comprehensive LC-MS/MS method using a Thermo Scientific Transcend TLX-1 system powered by Thermo Scientific TurboFlow technology to measure vitamin D levels in infant formula. TurboFlow™ technology eliminates most traditional sample preparation steps.

Experimental

Stock Solutions
Vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) were purchased from Sigma (St Louis, MO). The internal standards (IS), vitamin D_2-[$^2H_3$] and vitamin D_3-[$^2H_3$] were obtained from IsoSciences (King of Prussia, PA). A stock mixture was prepared in methanol at 10 µg/mL for both analytes.

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Matrix Standard Curve

Due to the difficulty obtaining infant formula without vitamin D fortification, low-fat half and half (HH), a blend of milk and cream purchased from a local food store, was used as a blank matrix in which to prepare a standard curve. Five grams of HH spiked with internal standards (IS) were extracted using 5 mL of ethanol followed by 5 minutes of ultrasonication. Methanol was added to bring the total volume to 15 mL. The resultant solution was then transferred to multiple 2 mL polypropolyene tubes and centrifuged at 13000 RPM (at 25 °C) for 10 minutes. The supernatant was used to prepare the matrix calibrators and QC samples. The matrix calibrant concentrations ranged from 0.5 ng/mL to 100 ng/mL. The final IS concentrations were 25 ng/mL and 10 ng/mL for vitamin D_2 and D_3, respectively. Each milliliter of supernatant corresponds to 0.33 g HH as the unit conversion.

Infant Formula Sample Preparation

Solid Powder

Canned infant formula powders from various manufacturers were obtained from a local food store. The infant formula solution was prepared by adding 5 mL of water to 5 g of infant formula powder, vortexed, and then brought to a total final volume of 10 mL by adding more water. Two milliliters of this solution was transferred into a new 50 mL centrifuge tube. The extraction was performed using 5 mL each of ethanol and methanol and followed by 10 minutes of ultrasonication. Centrifugation was performed at 7500 RPM (at 25 °C) for 10 minutes to separate the precipitated solids from the supernatant. The supernatant was filtered through a 0.45 µm membrane filter.

Liquid Formula

Liquid infant formula was also obtained from a local food store. Fifteen milliliters of liquid formula were extracted using 5 mL each of ethanol and methanol followed by vortexing for 1 minute. This mixture was then centrifuged at 7500 RPM (at 25 °C) for 10 minutes to separate the precipitated solids from the supernatant. One-half milliliter of supernatant was transferred into a 2 mL polypropolyene tube and 0.5 mL of methanol was added, followed by centrifugation at 13000 RPM (at 25 °C) for 10 minutes. The supernatant was then filtered through a 0.45 µm membrane filter.

LC/MS Method

TurboFlow Method Parameters

Column:	TurboFlow C18-P XL 0.5 x 50 mm
Injection Volume:	50 µL
Solvent A:	0.1% formic acid in water
Solvent B:	0.1% formic acid in methanol
Solvent D:	1:1:1 acetonitrile isopropyl alcohol : acetone (v:v:v)

HPLC Method Parameters

Analytical Column:	Thermo Scientific Hypersil GOLD 2.1 x 50 mm, 3 µm
Solvent A:	0.1% formic acid in water
Solvent B:	0.1% formic acid in methanol

Mass Spectrometer Parameters

MS:	Thermo Scientific TSQ Access MAX triple stage quadrupole
Polarity:	Positive mode
MS Ionization Source:	Atmospheric-pressure chemical ionization (APCI)
Discharge Current:	3.5 KV
Sheath Gas Pressure (N_2):	40 arbitrary units
Auxiliary Gas Pressure (N_2):	15 arbitrary units
Vaporizer Temperature:	375 °C
Capillary Temperature:	310 °C
Collision Gas Pressure:	1.5 mTorr
Q1 Peak Width:	0.7 Da
Q3 Peak Width:	0.7 Da

The LC method schematic view in Thermo Scientific Aria operating software (OS) is shown in Figure 2. The fragment ions and corresponding mass spectrometer parameters are listed in Table 1. For vitamin D_2, the product ion *m/z* 107 was selected as quantifier due to less interference and relatively high intensity. The entire experiment was controlled by Aria™ OS version 1.6.3. The data were processed using Thermo Scientific TraceFinder software version 1.1.

Step	Start	Sec	Flow	Grad	%A	%B	%C	%D	Tee	Loop	Flow	Grad	%A	%B
1	0.00	30	2.00	Step	20.0	80.0	-	-	====	out	1.00	Step	98.0	2.0
2	0.50	120	0.15	Step	100.0	-	-	-	T	in	0.90	Step	98.0	2.0
3	2.50	20	2.00	Step	-	-	-	100.0	====	out	1.00	Ramp	40.0	60.0
4	2.83	20	2.00	Step	-	100.0	-	-	====	out	1.00	Ramp	20.0	80.0
5	3.17	60	2.00	Step	-	-	-	100.0	====	in	1.00	Ramp	10.0	90.0
6	4.17	90	2.00	Step	-	100.0	-	-	====	out	1.00	Ramp	-	100.0
7	5.67	30	2.00	Step	-	-	-	100.0	====	in	1.00	Step	-	100.0
8	6.17	30	2.00	Step	-	100.0	-	-	====	in	1.00	Step	-	100.0
9	6.67	240	2.00	Step	20.0	80.0	-	-	====	out	1.00	Step	98.0	2.0

Figure 2. TurboFlow method schematic diagram as viewed in Aria OS software

Table 1. Fragments of test compounds from Quantum Access MAX MS

Vitamin	Parent ion (*m/z*)	Quantifier (*m/z*)	Tube Lens (V)	Collision Energy (V)	Qualifier (*m/z*)	Tube Lens (V)	Collision Energy (V)
Vitamin D_2	397.3	107.1	87	29	159.2	87	22
Vitamin D_2-[$^2H_3$]	400.3	107.1	90	32	-	-	-
Vitamin D_3	385.3	259.2	106	15	91.1	106	53
Vitamin D_3-[$^2H_3$]	388.3	259.2	76	15	-	-	-

Results and Discussion

Prior to determining the calibration curve, the matrix blank was screened to ensure there was no detectable vitamin D. Figure 3 shows the representative chromatograms of both analytes at 10 ng/mL (3.0 µg/100 g), indicating good signal intensity and chromatographic peak shape. Matrix-matched calibration standards showed linear response of two orders of magnitude ($r^2 > 0.99$) for both analytes as shown in Figure 4. The limits of quantitation (LOQ) were determined at 1.0 ng/mL(0.3 µg/100 g) and 0.5 ng/mL (0.15 µg/100 g) for vitamin D_2 and D_3, respectively. All coefficients of variation (CV) (n=3) were less than 20% for the LLOQ and less than 15% for all other points of the curve.

The results of an analysis of a series of actual infant formula samples are presented in Table 2. Since none of the product labels mentioned the presence of vitamin D_2, only vitamin D_3 was measured. Vitamin D_3-[$^2H_3$] was used to minimize the impact of matrix interference. Comparing the claimed and experimentally obtained values of vitamin D, less than 15% difference was observed for all samples. These results are satisfactory considering the vitamin D content tends to decrease with time and changes in storage conditions[5]. Excellent analytical reproducibility was demonstrated with CVs (n=6) of no more than 11% for 5 tested samples. It should be noted that since the results obtained from real samples were calculated against the standard calibration curve from HH matrix, some deviation may occur. Overall, the current LC-MS/MS strategy was able to detect vitamin D present in the actual samples, proving to be a fast and efficient screening tool.

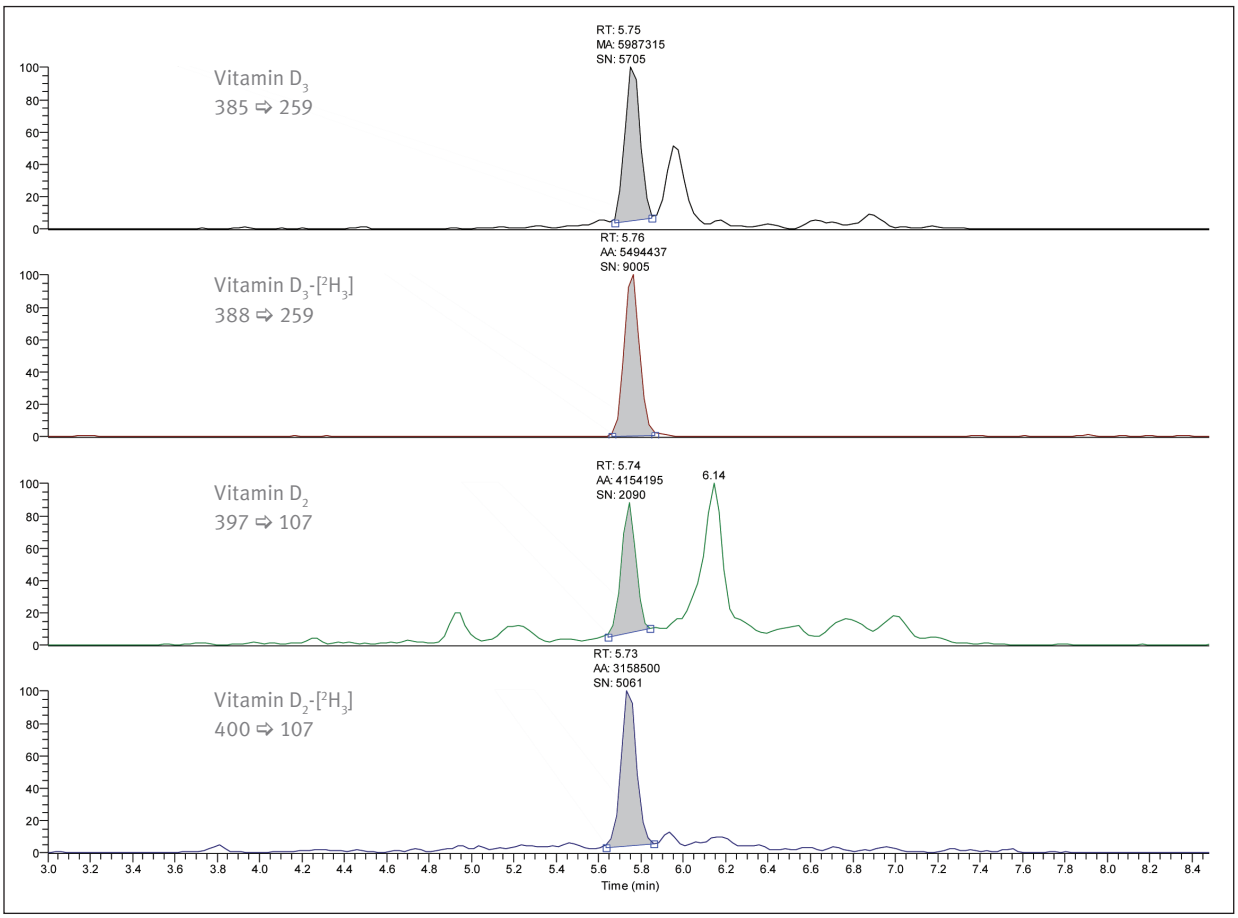

Figure 3. Example chromatogram of 10 ng/mL calibration standard

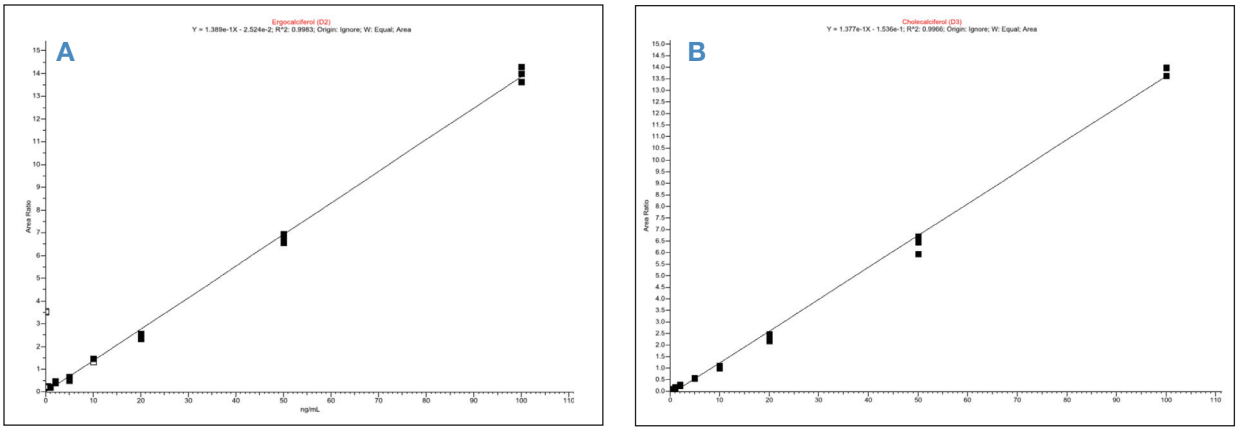

Figure 4. Linear regression curve of vitamin D_2 (A) and D_3 (B) standards based on area ratio with individual internal standards

Table 2. Results obtained for vitamin D in real infant formula samples

Sample Number	Powder or liquid	Form of vitamin D on label	Claimed (IU/5 fl oz)	LC-MS/MS (ng/mL)	Calculated (IU/5 fl oz)	Coefficient of variation (n=6) (%)
1	Powder	D_3	75	10.4	67.8	7.8
2	Powder	D_3	60	8.2	54.1	7.9
3	Powder	D_3	50	6.6	44.6	8.7
4	Powder (Soy-based)	D_3	60	8.3	55.4	6.9
5	Liquid	D_3	45	3.3	40.7	11.0

Conclusion

A quick, automated online sample preparation LC-MS/MS method has been developed that is sensitive enough to measure vitamin D content in infant formula. Because this method eliminates the saponification step, no toxic or environmentally unfriendly solvents were used in sample preparation. Good recoveries were achieved for both powdered and liquid infant formula. The sample throughput can be improved by multiplexing the two methods on different LC channels using a Transcend™ TLX-2 (or TLX-4) system. Future work will focus on the application of this methodology to other food matrices and references.

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