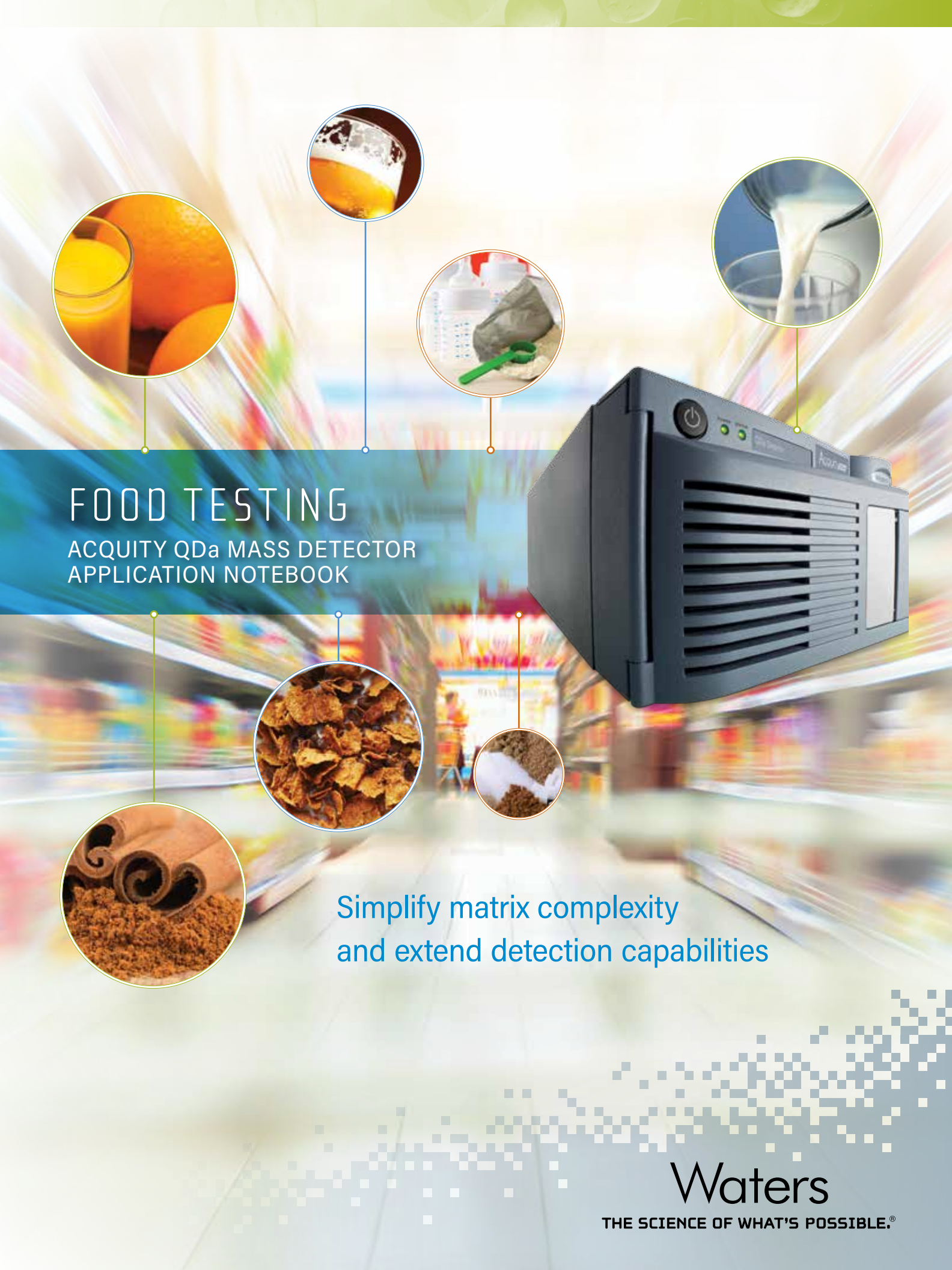

FOOD TESTING

ACQUITY QDa MASS DETECTOR APPLICATION NOTEBOOK

Simplify matrix complexity
and extend detection capabilities

Waters

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Rapid, reliable, and cost-effective methods are required by food manufacturers and ingredient suppliers in order to verify product consistency and ensure that label claims are met.

LC-MS offers the opportunity to consolidate methods along with the ability to improve detector selectivity and reduce limits of quantification.

In order to offer laboratories the opportunity to capture the benefits of mass detection without the challenges associated with the adoption of mass spectrometers, recent advances in technology have focused on improving instrument usability and robustness.

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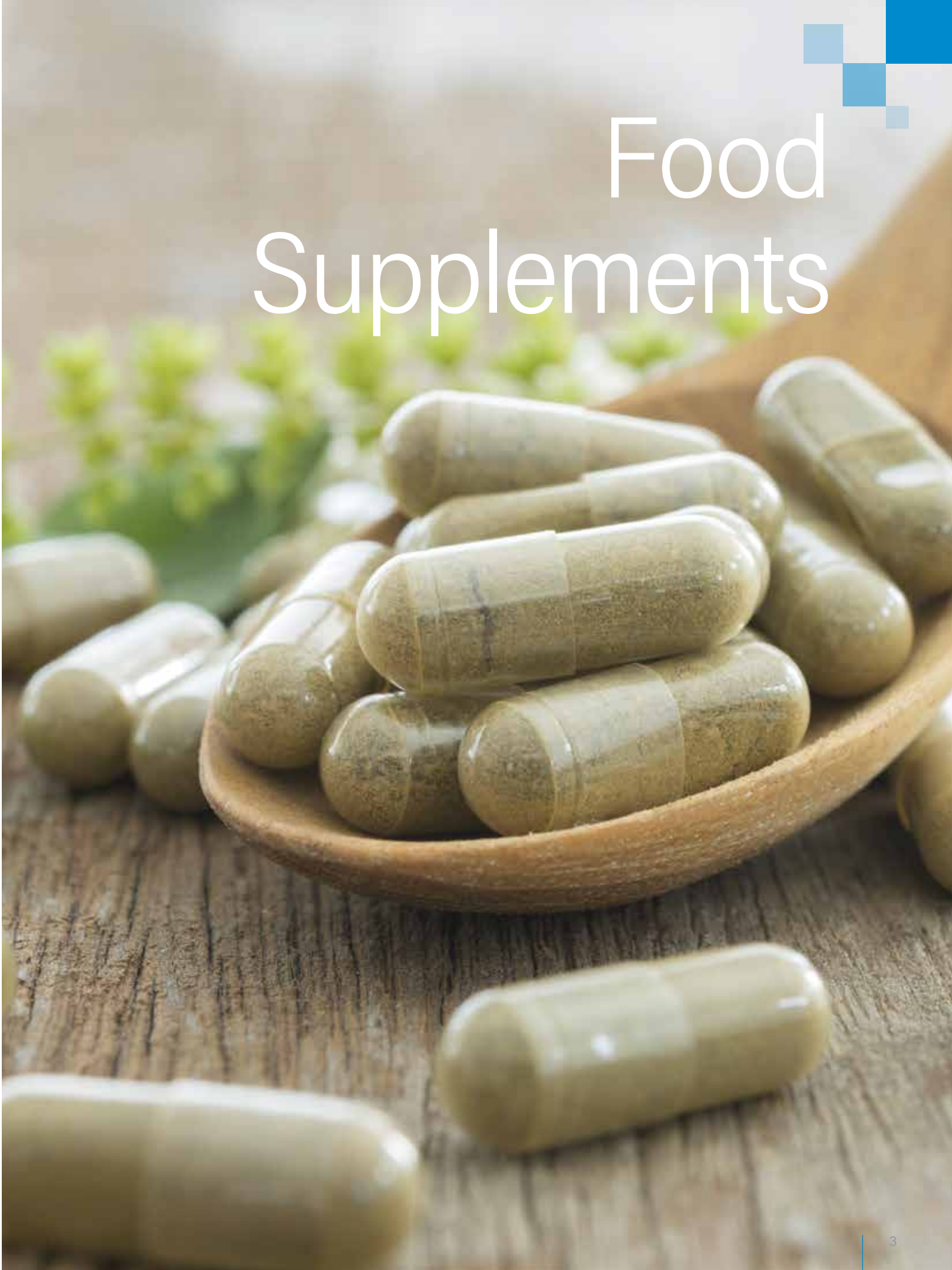
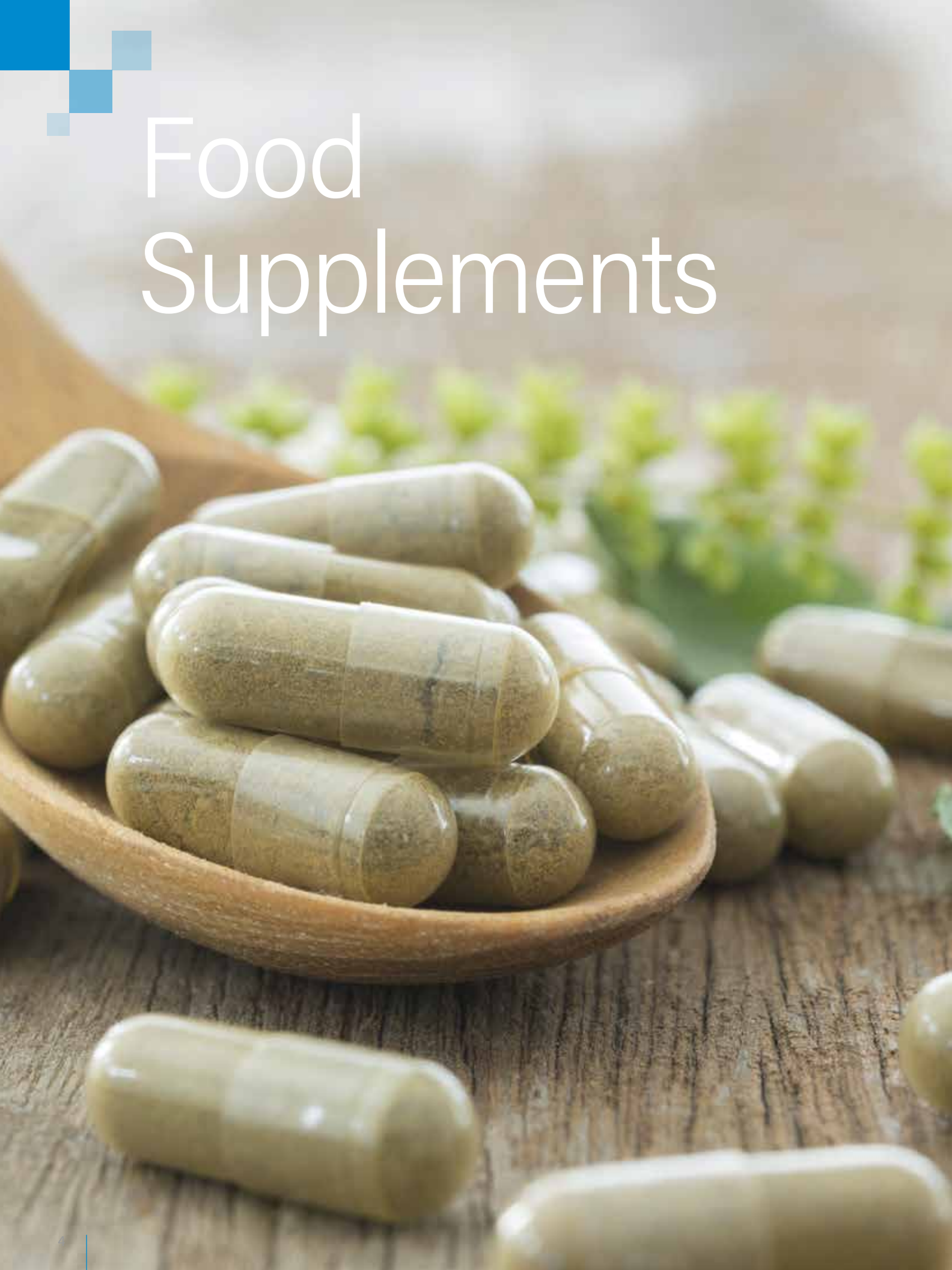
Food Supplements



Food Supplements



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Fast Analysis of Isoflavones in Dietary Supplements – Benefits of Mass Detection in Method Transfer and Sample Analysis

Jinchuan Yang, Mark Benvenuti, and Gareth Cleland
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Reduced method development and transfer times
- Improved data quality and simplified peak detection with the ACQUITY® QDa® Mass Detector
- Minimize false peak assignments and co-elution errors in routine analyses.

WATERS SOLUTIONS

ACQUITY Arc™ UHPLC System

ACQUITY QDa Mass Detector

2998 Photodiode Array (PDA) Detector

CORTECS® C_{18} Column

Empower® 3 CDS Software

KEYWORDS

Isoflavones, dietary supplement, mass detection, soy, Glycine max, red clover, Trifolium pretense, Kudzu, Pueraria lobata, daidzein, glycitein, genistein

INTRODUCTION

LC method development is always a challenging task, especially for dietary supplements from plant sources, as the target compounds are often structurally similar to each other, and the standards are not always commercially available. The UV/Vis detection method may not provide adequate detection selectivity when needed, which makes the method development and method transfer process time consuming.

Recently, an industry standard isoflavone method[1] was successfully transferred onto a Waters® ACQUITY Arc UHPLC System equipped with a 2998 PDA Detector.[2] The analysis time per injection was shortened from 74 minutes to 18 minutes. During the method transfer, an ACQUITY QDa Mass Detector was simultaneously used, which helped to shorten the method development and transfer time.

The ACQUITY QDa Mass Detector is a single quadruple mass spectrometer (MS) detector designed for routine analysis. It comes factory pre-optimized, so little MS expertise is required to operate it. The QDa is ready to operate minutes after pressing the power button. It fits neatly into Waters Alliance® HPLC, ACQUITY Arc UHPLC, and ACQUITY UPLC® stacks, and it can be controlled by either Empower CDS or MassLynx MS software.

This application note demonstrates the benefits of the ACQUITY QDa Detector for method transfer and routine analysis of isoflavones in dietary supplements. The focus of this application note is on the qualitative data analysis. The benefits for the quantitative data analysis will be evaluated in a future application note.[3] The structures of isoflavones analyzed in this study are in Figure 1.

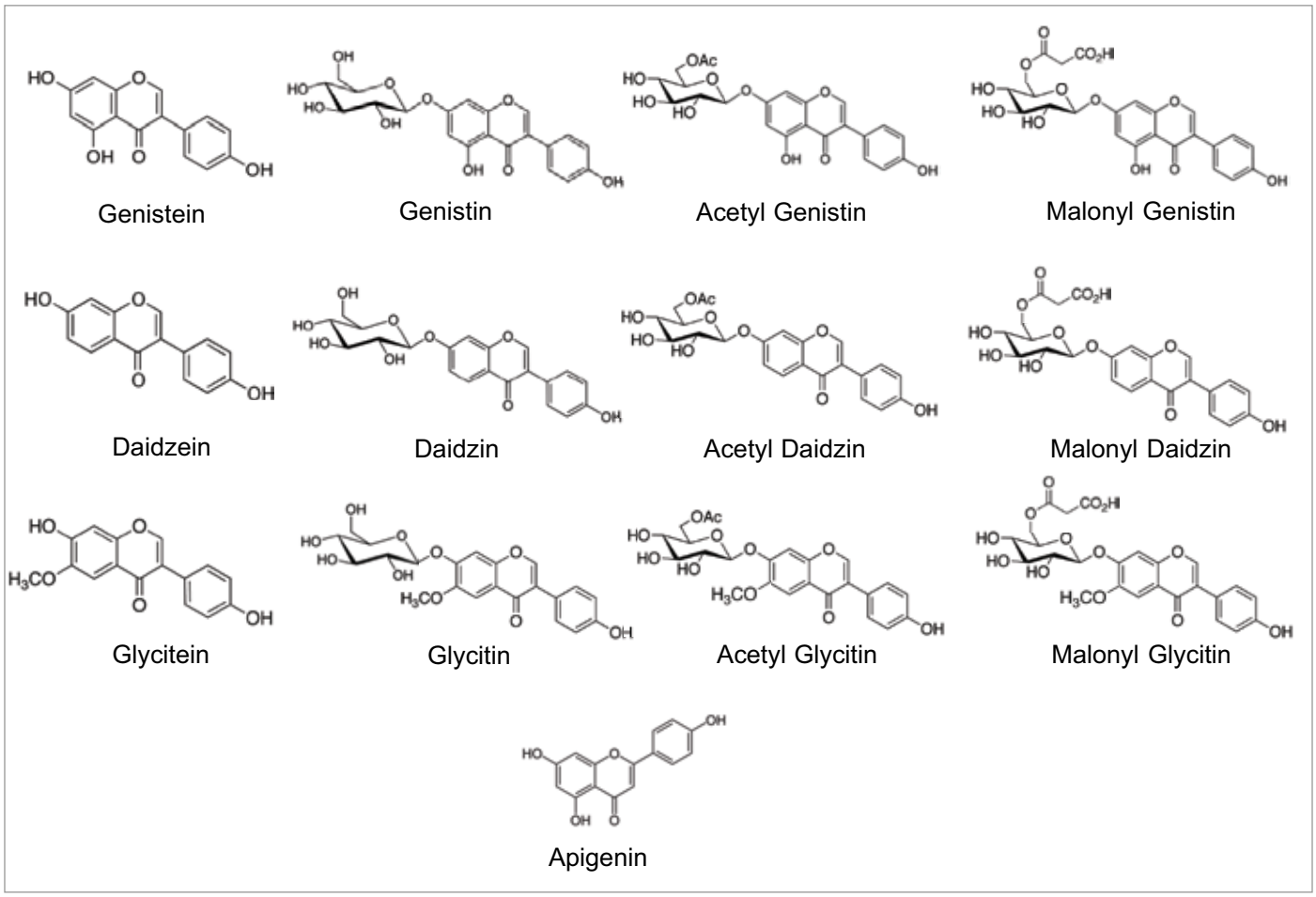

Figure 1. Structures of isoflavones in this study.

EXPERIMENTAL

Sample preparation

The standards: daidzin, glycitin, genistin, daidzein, glycitein, genistein, and apigenin, were purchased from ChromaDex (Irvine, CA) and INDOFINE Chemical (Hillsborough Township, NJ). Defatted powdered Soy RS was purchased from US Pharmacopeia (Rockville, MD). NIST SRM 3238 was purchased from NIST (Gaithersburg, MD). Isoflavone dietary supplement samples from major brands were purchased online.

The standard and sample solutions were prepared the same way as in the USP isoflavone method.[1] Sample solutions were further diluted with acetonitrile/water mixture (2/3 by volume) to various levels to fit into the calibration range (Table 2). The concentration of the internal standard was maintained at 4 ppm.

UHPLC conditions

UHPLC system:	ACQUITY Arc
Detector:	2998 PDA
Software:	Empower 3
Column:	CORTECS C18 2.7 µm, 3.0 x 100 mm
Column temp.:	30 °C
Mobile phase A:	Water with 0.1% formic acid
Mobile phase B:	Acetonitrile with 0.1% formic acid
Injection volume:	2.0 µL
Flow rate:	1.08 mL/min
Run time:	18.0 min
UV detection:	260 nm
UV resolution:	1.2 nm

Elution gradient:

	Time (min)	Flow rate (mL/min)	%A	Curve
1	Initial	1.08	90	6
2	14.40	1.08	70	6
3	14.50	1.08	10	6
4	15.20	1.08	10	6
5	15.40	1.08	90	6
6	18.00	1.08	90	6

MS conditions

MS system:	ACQUITY QDa (Performance)
Ionization mode:	ESI+
Capillary voltage:	0.8 kV
Cone voltage:	15 V
Probe temp.:	600 °C
SIR masses:	(Table 1)

Table 1. Masses of isoflavone molecular ions.

Analyte	SIR mass (Daltons)
Daidzin	417.1
Glycitin	447.0
Genistin	433.1
Daidzein	254.9
Glycitein	285.0
Genistein	270.9
Apigenin (IS)	270.9
Malonyl Daidzin	503.4
Malonyl Glycitin	533.1
Acetyl Daidzin	459.1
Acetyl Glycitin	489.0
Malonyl Genistin	519.0
Acetyl Genistin	475.1

RESULTS AND DISCUSSION

USE OF MASS DETECTION

Little MS expertise is required to operate the ACQUITY QDa Detector. In this study, the factory default instrument parameters were used without any modification. The only change that was made to facilitate the MS detection was that the mobile phase additive was changed from phosphoric acid to formic acid. The mass spectra of the seven isoflavone standards (daidzin, glycitin, genistin, daidzein, glycitein, genistein, and apigenin) were obtained under the UHPLC conditions (see Experimental section), and it was confirmed that their molecular ions were the most intense ions. Individual standards of the acetyl and the malonyl isoflavones, acetyl daidzin, acetyl glycitin, acetyl genistin, malonyl daidzin, malonyl glycitin, and malonyl genistin, were not commercially available. Their mass spectra were obtained from the USP reference materials (USP defatted powdered soy RS), which contained all the acetyl and the malonyl isoflavones. The molecular ions of these compounds are listed in Table 1 and were used in the MS SIR setup.

BENEFITS OF MS – CONFIRMATION OF PEAK ASSIGNMENTS

MS greatly simplifies the peak identification. The original USP method uses a pattern matching method and a reference material (USP defatted powdered soy RS) for the acetyl and the malonyl isoflavones peak identification in UV chromatograms, since these standards are not commercially available. Figure 2 shows the UV and the SIR chromatograms of defatted soy under the final optimized LC conditions. The SIR chromatograms of each compound are free of interference, which makes peak identification easier.

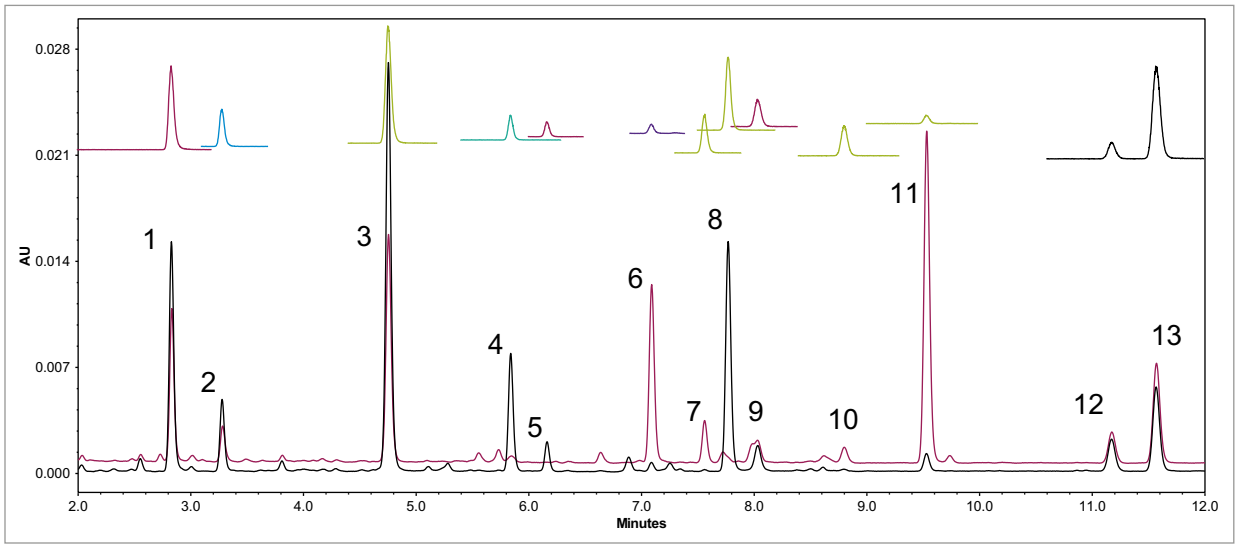

Figure 2. UV chromatograms of unheated defatted soy (black, the bottom trace) and heated defatted soy (red trace next to the bottom one), and the corresponding mass detector SIR traces for every compound. Peak ID: 1. Daidzin; 2. Glycitin; 3. Genistin; 4. Malonyl Daidzin; 5. Malonyl Glycitin; 6. Acetyl Daidzin; 7. Acetyl Glycitin; 8. Malonyl Genistin; 9.Daidzein; 10. Glycitein; 11. Acetyl genistin; 12. Genistein; and 13. Apigenin.

Figure 3 demonstrates the major advantage of MS data over UV/Vis spectroscopy data in confirming peak identifications. Figure 3 was obtained on a CORTECS C_{18} Column (2.7 µm, 3 x 100 mm), p/n 186007372, using an initial 18 minutes gradient elution program, which was converted from the USP method directly prior to further optimization.[2] The SIR chromatograms of the acetyl glycitin and the malonyl genistin clearly show a co-elution. It was difficult to discern this co-elution issue from the UV (260 nm) chromatogram. Even with the PDA peak purity test (Figure 4), it was difficult since the UV spectra across the peak were identical to the peak apex UV spectrum. The ACQUITY QDa data made the co-elution issue apparent during the early stages of the method transfer, which prevented future issues or an inappropriately transferred method.

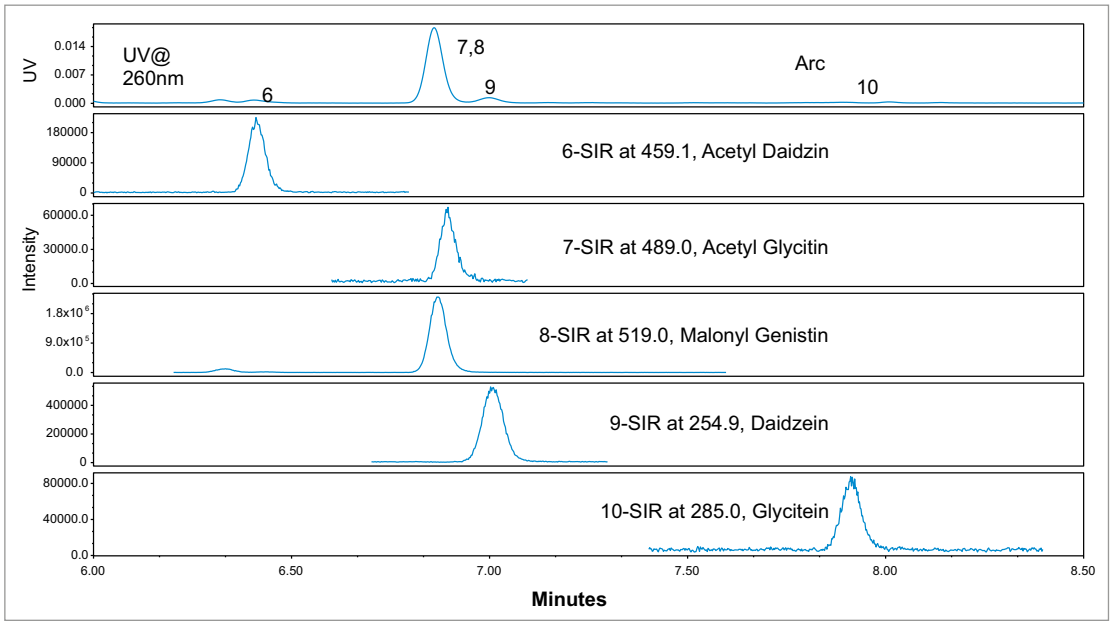

Figure 3. Sections of the UV and the SIR chromatograms of USP defatted powdered soy RS under the initial LC conditions without optimization. The LC conditions were converted from the USP method using the ACQUITY Column Calculator. Co-elution between Acetyl Glycitin and Malonyl Genistin was obvious in the SIR chromatograms.

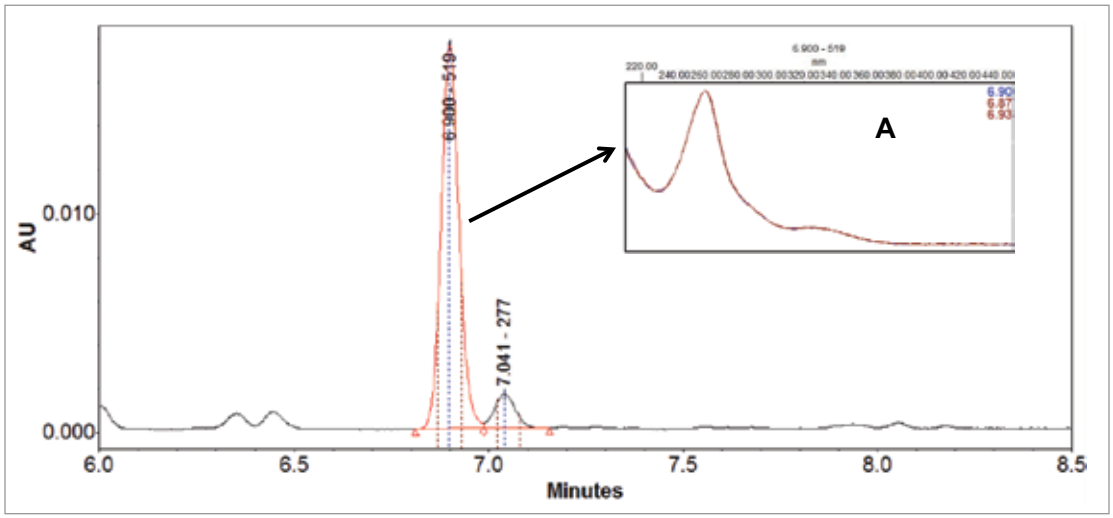

Figure 4. PDA peak purity test on the co-eluting UV peak of Acetyl Glycitin and Malonyl Genistin (6.900 min.). (A) is an overlay of the UV/Vis spectra at front, apex, and back of the 6.9 min UV peak (dotted lines). (A) shows no indication that impurity or coelution exists. Conditions are the same as in Figure 3.

BENEFITS OF MS – FASTER METHOD TRANSFER

The effects of various LC conditions (column temperature and flow rate) on the isoflavones separation were evaluated to address the co-elution observed in Figure 3. Injection of one USP defatted powdered soy RS was enough to generate SIR chromatograms of all compounds for one particular LC condition, while injections of multiple standards and the reference material would be necessary for one particular LC condition if only UV (or PDA) were available. The use of the ACQUITY QDa Mass Detector reduced the number of injections that were needed for the investigation of the effects of each particular LC condition on the separation of critical compounds, and shortened the overall investigation time. Figure 5 shows the separation of the critical compounds under the final optimized conditions.

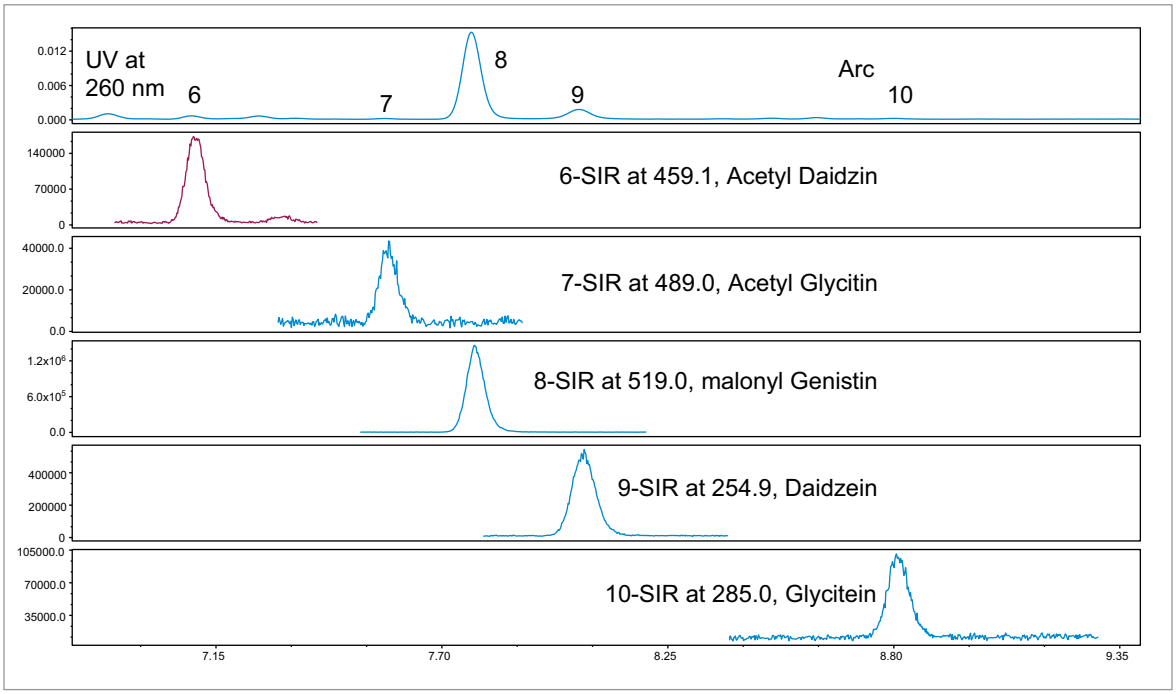

Figure 5. Sections of the UV and the SIR chromatograms of USP defatted powdered soy RS under the final optimized LC conditions. Co-elution between Acetyl Glycitin and Malonyl Genistin was solved (See UHPLC on page 3.)

BENEFITS OF MS – REDUCE ERRORS IN ROUTINE ANALYSIS

The ACQUITY QDa Mass Detector can also play an important role in routine sample analysis. Dietary supplements from plant sources often contain complex chemical matrices. The unknown matrix could interfere with the targeted compounds, and could cause error in quantitation, or even false peak assignment. Figure 6 shows the UV chromatogram of two partially co-eluting peaks for a dietary supplement sample. One of these peaks should be genistein, but it was hard to assign a peak ID because their RTs were both very close to the reference RT of genistein. The PDA data was not helpful in determining which one was genistein peak as their UV/Vis spectra were the same. Peak assignment was facile using data from the ACQUITY QDa Detector. The SIR channel of genistein (270.9 Dalton) clearly showed that the later eluting peak was genistein. This peak assignment and its purity were further confirmed and checked by inspecting the mass spectra extracted from the MS scan data at the front, the apex, and the back of the peak. Thus, the chance of false peak assignment or possible co-elution was greatly reduced.

Furthermore, Figure 6 shows how easy and simple it is to use the SIR chromatograms for the quantitation of isoflavone contents. In the UV chromatogram, one has to use a deconvolution technique to minimize the error from the interfering compound. By contrast, when using SIR chromatograms of isoflavones, there was much less chance of interference. The benefits of using MS in quantitation for isoflavone analysis will be further discussed in a separate application note.[3]

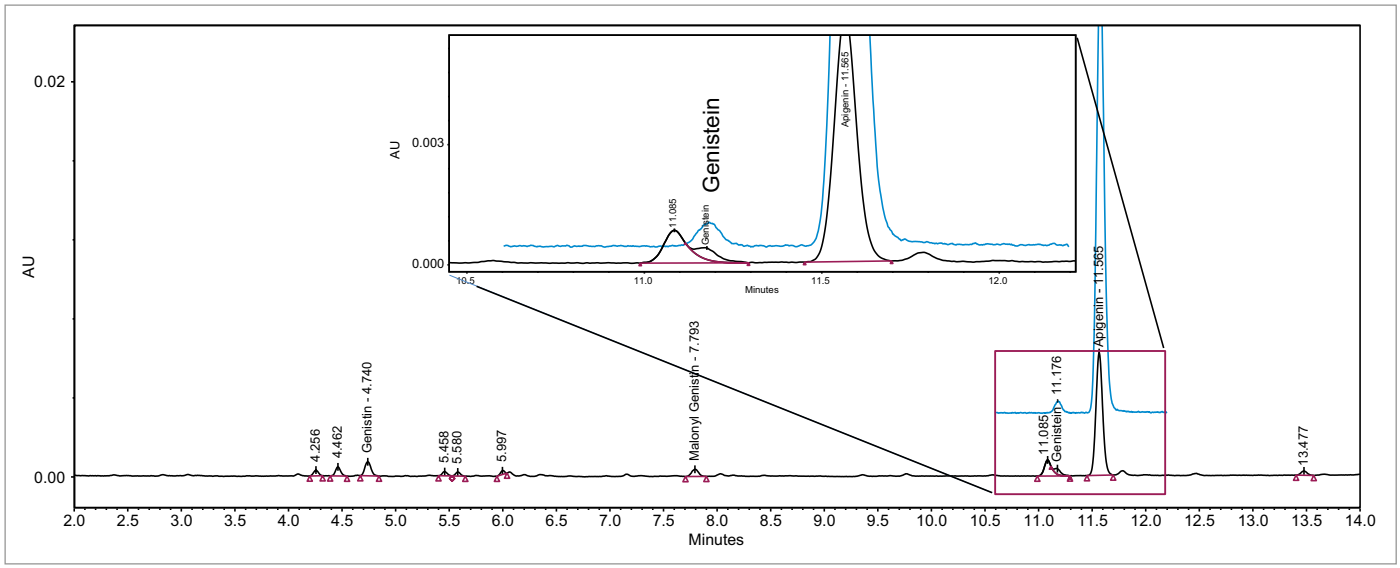

Figure 6. UV and SIR chromatograms of an unknown dietary supplement sample. The Genistein peak was identified with the help of its SIR (270.9 Dalton) chromatogram. Apigenin molecular ion has the same mass of 270.9 Daltons. The interfering peak (11.085 min) has the same UV/Vis spectrum as the genistein (not shown).

CONCLUSIONS

The MS data, simultaneously generated during the isoflavone analysis brought ease and high confidence in peak identification, and facilitated fast method transfer of the USP isoflavones method onto a Waters ACQUITY Arc UHPLC System with a 2998 PDA Detector and ACQUITY QDa Mass Detector. In comparison to PDA detection, the addition of mass detection provided an increased capability for discerning co-elution issues and confirming peak identifications.

References

1. USP Monograph. Powdered Soy Isoflavones Extract, USP39–NF34 S1 [6841], The United States Pharmacopeial Convention.
2. J Yang, M Benvenuti, and G Cleland. Fast Analysis of Isoflavones in Dietary Supplements – USP Method Transfer on a UHPLC System with Mass Detection, Waters Application Note, no. 720005858en; 2016.
3. J Yang, M Benvenuti, and G Cleland. Fast Analysis of Isoflavones in Dietary Supplements: A Comparison of Mass Detection with UV Detection, Waters Application Note.

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Fast Analysis of Isoflavones in Dietary Supplements – USP Method Transfer onto a UHPLC System

Jinchuan Yang, Mark Benvenuti, and Gareth Cleland
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Reduce analysis time of isoflavones from 74 min to 18 min.
- Easily transfer the standard USP isoflavones method to UHPLC with the aid of mass detection.
- Confirm peak identities more confidently using mass detection.

WATERS SOLUTIONS

ACQUITY Arc™ UHPLC System

ACQUITY® QDa® Mass Detector

2998 Photodiode Array (PDA) Detector

CORTECS® C_{18} Column

Empower® 3 CDS Software

KEYWORDS

Isoflavones, dietary supplement, mass detection, soy, *Glycine max*, red clover, *Trifolium pretense*, Kudzu, *Pueraria lobata*, daidzein, glycitein, genistein

INTRODUCTION

Isoflavones are found primarily in plants of soy (*Glycine max*), red clover (*Trifolium pretense*), and Kudzu (*Pueraria lobata*). The 12 major isoflavones found in these plants are daidzein, glycitein, genistein, and their respective glucoside and malonyl- and acetyl- glucoside derivatives. The structures of 12 isoflavones and an internal standard, apigenin, are shown in Figure 1. These hormone-like compounds are often used in remedies to reduce menopausal and post-menopausal symptoms. They are even associated with low breast cancer rate in Asia and the retarded progression of Alzheimer's disease.

Standard methods for isoflavones in dietary supplements have been established by organizations such as USP[1] and AOAC.[2] These methods use reversed-phase LC with C_{18} columns and ultraviolet and visible light (UV-Vis) spectroscopy for separation and quantitation. Because of the close structural similarity of these compounds, the chromatographic run times of these methods are typically over 70 minutes long. It is highly desirable to develop a more rapid isoflavone analysis method.

This application note demonstrates the transfer of the USP method onto Waters® ACQUITY Arc UHPLC System. The analysis time with the ACQUITY Arc System is only 18 minutes, including column wash and equilibration. Waters ACQUITY QDa Mass Detector was used to expedite the method transfer described in this study. The benefits of mass detection in peak identification and method optimization are also highlighted.

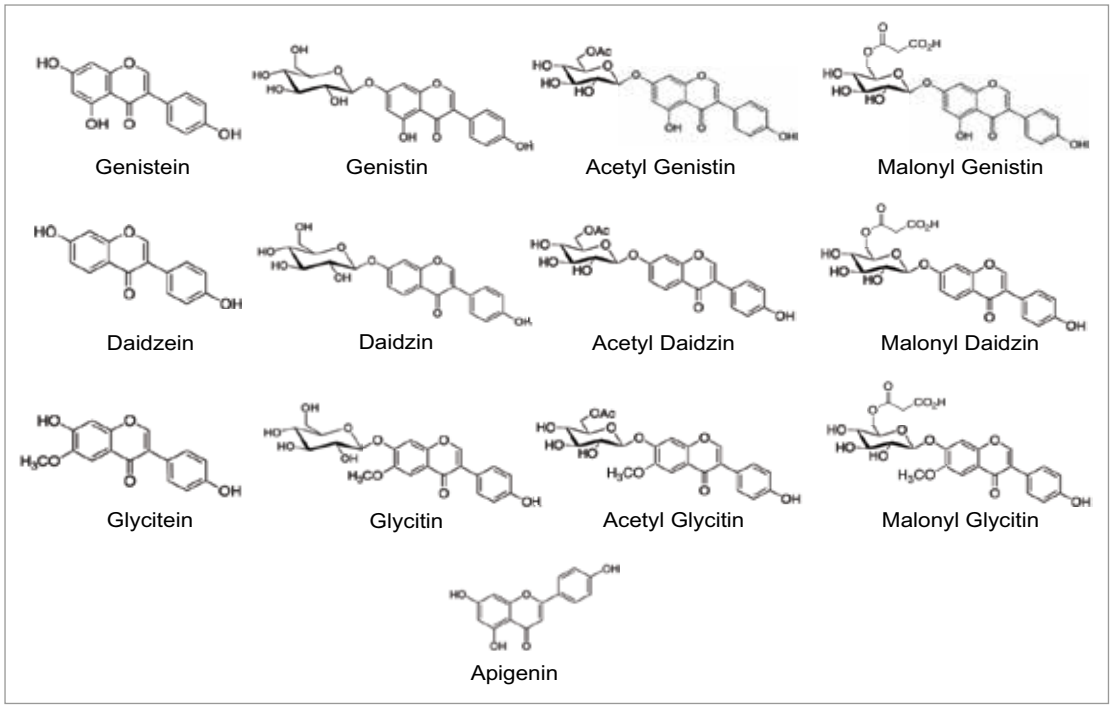

Figure 1. Structures of isoflavones in this study.

EXPERIMENTAL

Sample preparation

The standards, daidzin, glycitin, genistin, daidzein, glycitein, genistein, and apigenin, were purchased from ChromaDex (Irvine, CA) and INDOFINE Chemical (Hillsborough Township, NJ). Defatted powdered Soy RS was purchased from US Pharmacopeia (Rockville, MD). NIST SRM 3238 was purchased from NIST (Gaithersburg, MD). Isoflavone dietary supplement samples from major brands were purchased from online retail stores.

The standard and sample solutions were prepared the same way as in the USP isoflavone method.[1] Sample solutions were further diluted with an acetonitrile water mixture (2/3 by volume) to various levels to fit the calibration range. The concentration of internal standard was always kept constant at 4 ppm.

UHPLC conditions

UHPLC system:	ACQUITY Arc
Detector:	2998 PDA
Software:	Empower 3
Column:	CORTECS C_{18} 2.7 µm, 3.0 x 100 mm
Column temp.:	30 °C
Mobile phase A:	Water with 0.1% formic acid
Mobile phase B:	Acetonitrile with 0.1% formic acid
Injection volume:	2.0 µL
Flow rate:	1.08 mL/min
Run time:	18.0 min
UV detection:	260 nm
UV resolution:	1.2 nm

Elution gradient

	Time (min)	Flow rate (mL/min)	%A	Curve
1	Initial	1.08	90	6
2	14.40	1.08	70	6
3	14.50	1.08	10	6
4	15.20	1.08	10	6
5	15.40	1.08	90	6
6	18.00	1.08	90	6

MS conditions

MS system:	ACQUITY QDa (Performance)
Ionization mode:	ESI^+
Capillary voltage:	0.8 kV
Cone voltage:	15 V
Probe temp.:	600 °C
SIR masses:	

Table 1. Masses of isoflavone molecular ions.

Analyte	SIR mass (Daltons)
Daidzin	417.1
Glycitin	447.0
Genistin	433.1
Daidzein	254.9
Glycitein	285.0
Genistein	270.9
Apigenin (IS)	270.9
Malonyl Daidzin	503.4
Malonyl Glycitin	533.1
Acetyl Daidzin	459.1
Acetyl Glycitin	489.0
Malonyl Genistin	519.0
Acetyl Genistin	475.1

RESULTS AND DISCUSSION

USP METHOD TRANSFER AND OPTIMIZATION ONTO ACQUITY ARC UHPLC

The USP method (isoflavones powder extract)[1] was transferred to an ACQUITY Arc UHPLC System with a CORTECS C_{18} Column (2.7 µm, 3 x 100 mm), p/n 186007372. The CORTECS Column's 2.7 µm packing material is solid-core particle, which provides higher separation efficiency and lower back pressure than a fully porous particle column of equivalent particle size. The USP method gradient elution program was converted to a new gradient elution program using Waters ACQUITY UPLC Column Calculator.[3] The column parameters in the USP method (5 µm, 3.0 x 250 mm) and the parameters of the CORTECS C_{18} Column (2.7 µm, 3.0 x 100 mm), as well as the USP method's 74 minutes gradient elution program were entered in the column calculator, and a 18 minutes gradient elution program that is equivalent to the USP method was calculated. The mobile phase additive was changed from 0.05% phosphoric acid to 0.1% formic acid, which is a more MS friendly additive.

The factory default ACQUITY QDa Detector instrument parameters were used without any modification. The column temperature of 40 °C was tested, but was later optimized to 30 °C to meet the USP suitability criteria on peak resolution. Figure 2 shows the chromatograms of the USP defatted powdered soy RS, unheated and heated, and their Single Ion Recording (SIR) traces that were obtained using the ACQUITY QDa Mass Detector. Mass detection was used to confirm the peak identities.

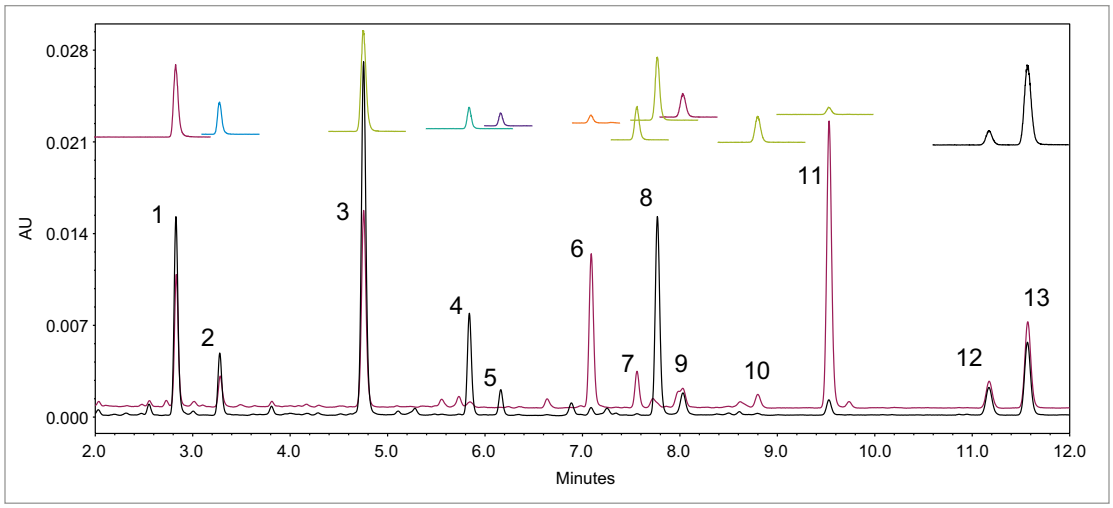

Figure 2. UV chromatograms of unheated defatted soy (black, the bottom trace) and heated defatted soy (red trace next to the bottom), and the corresponding mass detector SIR traces for every compound. Peak ID: 1. Daidzin; 2. Glycitin; 3. Genistin; 4. Malonyl Daidzin; 5. Malonyl Glycitin; 6. Acetyl Daidzin; 7. Acetyl Glycitin; 8. Malonyl Genistin; 9.Daidzein; 10. Glycitein; 11. Acetyl Genistin; 12. Genistein; 13. Apigenin.

METHOD TRANSFER AND OPTIMIZATION USING MASS DETECTION

Since the acetyl and malonyl isoflavone standards were not commercially available, the peak assignment of these compounds were carried out using a reference material and a pattern matching method as described in the USP standard. Heat treatment of defatted soy (DFS) can convert the malonyl forms to the acetyl forms. By comparing the chromatograms of the unheated DFS and the heated DFS and their reference chromatograms, one can assign the peak IDs to those acetyl and malonyl isoflavones. However, this pattern matching approach is not reliable, especially when LC conditions, such as the column, the mobile phase additives, or the LC system are changed. The ACQUITY QDa detects the ions that are formed in electrospray ionization (ESI) at unit mass resolution (0.7 Da). Table 1 lists the molecular ions of these isoflavones. Using mass detection, we were able to selectively detect these compounds and eliminate any possible interference from closely eluting compounds.

Genistein and apigenin have the same mass, but their peaks were well separated by chromatography (see Figure 2). The addition of mass detection (ACQUITY QDa Detector) enabled unambiguous assignment of peak IDs to the correct acetyl and malonyl isoflavones without resorting to individual standards.

The ACQUITY QDa Detector also sped up the method optimization because the selective detection of every compound allowed us to monitor the retention time (RT) changes of all compounds simultaneously with high confidence. This greatly saved the number of injections in method optimization. More details on how the QDa detector benefits the method transfer are discussed in a separate application note.[4]

METHOD PERFORMANCE CHARACTERISTICS

Table 2 shows the UV calibration results of the standards. Apigenin was used as the internal standard for calibration and quantitation of all compounds. The square of the correlation coefficients (R^2) between the relative responses (peak area ratio) and the concentration of standards in solutions (ppm) for all compounds were better than 0.999. The retention time relative standard deviations for all compounds were less than 0.12%.

Table 2. Isoflavones retention times and their relative standard deviation, calibration equations, R^2, and linear ranges.

Analyte	RT		Equation	R^2	Range (ppm)
	Min	RSD (%)			
Daidzin	2.81	0.12	$Y = 2.09x10^{-1} X + 3.09x10^{-3}$	0.9998	0.075–10
Glycitin	3.26	0.09	$Y = 2.42x10^{-1} X + 5.43x10^{-3}$	0.9998	0.075–10
Genistin	4.75	0.05	$Y = 4.53x10^{-1} X + 9.43x10^{-3}$	0.9998	0.05–10
Daidzein	8.03	0.03	$Y = 2.60x10^{-1} X + 4.65x10^{-3}$	0.9998	0.1–10
Glycitein	8.80	0.03	$Y = 4.96x10^{-1} X + 1.07x10^{-3}$	0.9998	0.05–10
Genistein	11.17	0.02	$Y = 4.91x10^{-1} X + 9.73x10^{-3}$	0.9998	0.05–10

Table 3 shows the isoflavone results for the NIST 3238 SRM and the comparison to its certified and reference values. A relative difference of <11% was obtained for the genistin, glycitin, daidzin, genistein, and glycitein. The result of daidzein was 15% higher than the NIST value. A literature search found that a high daidzein value was also reported elsewhere.[5] The accuracy for the daidzein, genistein, and glycitein were also evaluated by a spiking experiment (Table 4). Recoveries of 98% to 101% were obtained for these compounds.

Table 3. Comparison of determined isoflavone values to the certified and reference values of NIST 3238 SRM.

	NIST value (mg/kg)	Determined value (mg/kg)	Relative difference
Genistin	12700±530	11450	-10%
Glycitin	3760±180	3355	-11%
Daidzin	13400±2400	14700	10%
Daidzein	241±5	277	15%
Genistein	108±10	99	-8%
Glycitein	211±5	195	-8%

Table 4. Recovery results from a spiking experiment.

	Original value (mg/kg)	Spiked level (mg/kg)	Determined value (mg/kg)	Recovery (%)
Daidzein	259	499	751	98%
Genistein	41	499	546	101%
Glycitein	68	499	557	98%

ANALYSIS OF ISOFLAVONE DIETARY SUPPLEMENTS

The isoflavone content in four isoflavone dietary supplement samples were measured by the fast 18-minute UHPLC-UV method described above. Sample types included tablets, capsules, and soy powder. The USP calibration and quantitation protocols[1] were followed in the data processing.

The same conversion factors for the acetyl and malonyl derivatives that are specified in the USP HPLC-UV method were used in the analyses. Table 5 shows the determined individual and the total isoflavones, as well as the label claimed total isoflavone contents. For easy comparison, the label claim values were converted to concentration (mg/kg). Two of the three samples (C and D) showed good agreement between the determined values and their label claim values, while one sample (B) contained much less measured total isoflavone content then claimed on its label. The reason for such low total isoflavone content is unknown.

Table 5. Isoflavone contents in dietary supplements and their label claim values.

Sample (mg/kg)	B		C		D		E	
	Mean	%RSD	Mean	%RSD	Mean	%RSD	Mean	%RSD
Daidzin	3074	1.8	12540	0.8	442	8.4	0	
Glycitin	693	0.2	4683	0.2	0		0	
Genistin	335	2.0	1786	0.8	730	1.9	303	3.0
Malonyl Daidzin	0		0		0		0	
Acetyl Daidzin	1580	1.5	7222	0.5	0		0	
Acetyl Glycitin	390	2.0	2083	2.6	0		0	
Malonyl Genistin	0		0		354	2.8	143	7.4
Daidzein	6266	0.8	506	0.9	0		0	
Glycitein	0		224	1.8	0		0	
Acetyl Genistin	162	1.9	992	1.7	0		0	
Genistin	0		0		0		159	10.1
Total Isoflavones	12,543		30,006		1,527		605	
Label Claim Value	>31,250		>25,000		867 to 2,600		no claim	

CONCLUSIONS

The USP method for isoflavones was successfully transferred to an ACQUITY Arc UHPLC System with a 2998 PDA Detector. The total analysis time per injection on the UHPLC system was 18 minutes, which was significantly shorter than the 74 minutes for the USP method. This corresponds to a three times increase in the analysis throughput, and a 75% cost savings for solvents used. The ACQUITY QDa Mass Detector provided excellent detection selectivity, which is a great asset in method transfer and development, as well as in the isoflavone analysis of unknowns and challenging samples where potential interference risk is high. Analysis of isoflavones in three dietary supplements showed compliance in the label claims for two samples. Low isoflavone content was found in one of the three tested samples for unknown reason.

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Selective Quantitative Determination of Water Soluble Vitamins in Various Food Matrices Using the ACQUITY UPLC H-Class System and ACQUITY QDa Mass Detector

Mark E. Benvenuti, Dimple Shah, and Jennifer A. Burgess
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Selectivity of mass detection ensures unambiguous detection of vitamins at low levels, enabling simpler sample preparation protocols with dilution of sample extracts.
- The ACQUITY® QDa® Mass Detector has been designed for integration with UPLC® and UHPLC systems to provide robust reliable orthogonal detection to UV spectroscopy allowing new users to quickly take advantage of the additional selectivity of mass detection.
- The ACQUITY QDa Mass Detector can be incorporated into existing liquid chromatography workflows in order to vastly increase the speed and selectivity over other LC detectors.

WATERS SOLUTIONS

ACQUITY UPLC® H-Class System

ACQUITY QDa Mass Detector

ACQUITY UPLC HSS T3 Column

KEYWORDS

WSV, vitamins, QDa, mass detection, B vitamins, vitamin C

INTRODUCTION

Many food and beverage products are routinely fortified with vitamins to enhance their nutritional value and to help address any deficiencies in dietary requirements. In order to meet legal requirements, manufacturers must label their products according to the regulations of the country in which the product is consumed. Examples of these regulations include European Commission (EC) 1925/2006 on the addition of vitamins and minerals, and Title 21 Code of Federal Regulations (C.F.R.), Part 101 on food labeling in the United States.

Rapid, reliable, and cost-effective methods are required by food manufacturers and ingredient suppliers in order to verify product consistency and ensure that label claims are met. This can be a challenging task with the combination of complex matrices and low fortification levels of some vitamins. In addition, many of the methods currently employed stipulate that the vitamins are either analyzed separately, or in small groups. Established techniques include microbiological assays, colorimetric and fluorimetric analysis, titrimetric procedures and HPLC methodologies.[1] LC-MS offers the opportunity to consolidate methods along with the ability to improve detector selectivity and reduce limits of quantification. Waters® ACQUITY QDa Mass Detector offers laboratories the opportunity to capture the benefits of mass detection without the challenges associated with the adoption of mass spectrometers.

In this application note, 12 water soluble vitamins (WSVs) were analyzed in dietary supplements and beverage samples using the ACQUITY UPLC H-Class System with the ACQUITY QDa Mass Detector.

EXPERIMENTAL

Table 1 lists the water soluble vitamins included in this study along with the observed retention times, single ion recording (SIR) *m/z*, and cone voltage.

Table 1. Retention times, SIR channels, and cone voltages for the water soluble vitamins studied.

Analyte	RT (min)	SIR *m/z*	Cone voltage (V)
Ascorbic acid (C)	0.91	177	2
Thiamine (B1)	1.01	265	5
Nicotinic acid (B3)	1.27	124	15
Pyridoxal (B6)	1.75	168	5
Nicotinamide (B3)	2.48	123	15
Pyridoxine (B6)	2.50	170	10
Ca_Pantothenate (B5)	5.88	242	15
Cyanocobalamin (B12)	7.17	678	2
Folic acid (B9)	7.22	442	5
B2-5-Phosphate	7.35	457	5
Biotin (B7)	7.50	245	10
Riboflavin (B2)	7.74	377	15

Table 2. Standards concentrations for B vitamins and vitamin C.

Standard	Individual B complex vitamins (mg/L)	Vitamin C (mg/L)
1	1.00	50.0
2	0.75	37.5
3	0.50	25.0
4	0.25	12.5
5	0.10	5.0
6	0.075	3.75
7	0.050	2.5
8	0.025	1.25
9	0.010	0.5
10	0.005	0.25
11	0.001	0.05

Standard preparation

Individual 1 mg/mL WSV stocks were prepared in water. In the case of vitamins B2, B7, and B9, 200 µL of 1 N NaOH were added to affect dissolution. Vitamin C was dissolved in a low pH acetate buffer to enhance stability. From these individual stocks, a mixed stock was prepared by adding 1.25 mL of the vitamin C stock and 0.025 mL of the other stocks and diluting to 25 mL with water. This mixed stock (50 ppm vitamin C, 1 ppm of the other analytes) was further diluted to provide 11 individual calibration standards, listed in Table 2.

Sample preparation

A packet (8.50 g) of a powdered vitamin beverage was dissolved in 100 mL water and filtered through a 0.2-µm PVDF filter. This sample was then prepared at two additional dilution levels: 1:250 and 1:10. These three dilution levels were injected to cover the different concentrations of vitamins in this sample.

A multi-vitamin supplement tablet was crushed using a mortar and pestle. The powder (1.34 g) was quantitatively transferred to a beaker to which 100 mL water was added. This mixture was sonicated for 15 minutes then stirred and filtered through a 0.2-µm PVDF filter. Three additional dilutions of this sample were prepared in water: 1:1000, 1:100, and 1:20. These dilutions and the initial dissolved tablet solution (undiluted) were analyzed in order to cover the different concentrations of vitamins in this sample.

Two different vitamin water samples were prepared by diluting 1:20 with water and filtering through a 0.2-µm PVDF filter.

EXPERIMENTAL

UPLC conditions

UPLC system:	ACQUITY UPLC H-Class
Run time:	17.5 min
Column:	ACQUITY UPLC HSS T3 1.8 µm, 2.1 x 100 mm (p/n 186003539)
Column temp.:	30 °C
Mobile phase A:	10 mM ammonium formate, 0.1% formic acid in water
Mobile phase B:	10 mM ammonium formate, 0.1% formic acid in methanol
Injection volume:	5 µL

	Time (min)	Flow rate (mL/min)	%A	%B
1.	Initial	0.45	99	1
2.	3.0	0.45	99	1
3.	3.1	0.45	95	5
4.	5.1	0.45	80	20
5.	7.1	0.45	2	98
6.	9.0	0.45	2	98
7.	9.1	0.45	99	1
8	17.5	0.45	99	1

Table 3. UPLC gradient for the separation of water soluble vitamins.

Detector conditions

Detector 1:	ACQUITY UPLC PDA
Wavelength:	Scanning 210 to 400 nm; Analog channel at 270 nm
Scan rate:	10 pts/sec
Detector 2:	ACQUITY QDa
Ionization mode:	ESI+
Run time:	8.0 min
Probe temp.:	600 °C
Capillary voltage:	0.8 kV
Mass range:	*m/z* 50 to 800 (centroid) and select SIRs*
Sampling freq.:	5 Hz
Cone voltage:	Full scan data: 15 V

*See Table 1 for cone voltage of individual SIR channels.

SIR *m/z* were assigned based on previous work.[2]

RESULTS AND DISCUSSION

A chromatogram showing an overlay of all 12 water soluble vitamins used in this study is shown in Figure 1, where all compounds eluted within eight minutes. Using this method, there were two co-eluting pairs (nicotinamide and pyridoxine at ~2.5 minutes and cyanocobalamin and folic acid at ~7.25 minutes). The use of mass detection means that it is no longer necessary to ensure baseline separation of all the analytes. The discrimination offered with mass detection means that these compounds can be accurately measured using their mass-to-charge ratio (*m/z*). This is demonstrated in Figure 2 where the linearity of selected vitamins are shown, including vitamins that co-eluted. Figure 2D and 2F show the calibration curves of folic acid (*m/z* 442) and cyanocobalamin (*m/z* 678), respectively. The selectivity offered with mass detection means that these compounds can be determined quantitatively, even though they co-elute. Figure 2 also shows example calibration curves of vitamins that can be challenging to analyze by UV. For example, biotin (Figure 2A) and calcium pantothenate (Figure 2H) are vitamins that show low responses using UV detection. Those compounds are often analyzed at low wavelengths to obtain a sufficiently sensitive response.[3] At such low wavelengths, the specificity of the analysis may be compromised. Mass detection ensures that the analysis is both specific and sensitive.

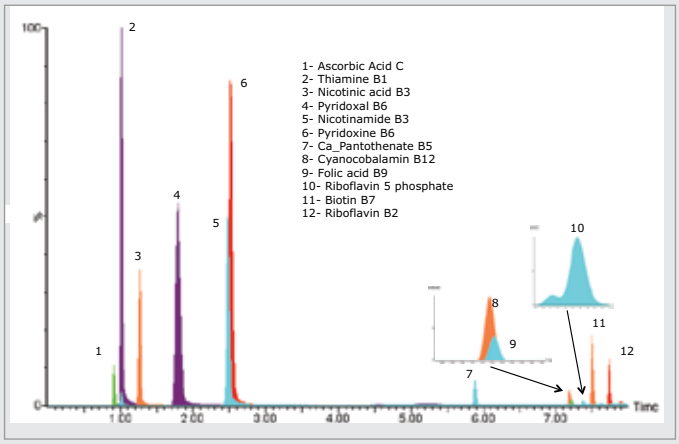

Figure 1. Overlay of SIR chromatograms of 12 water soluble vitamins separated in eight minutes.

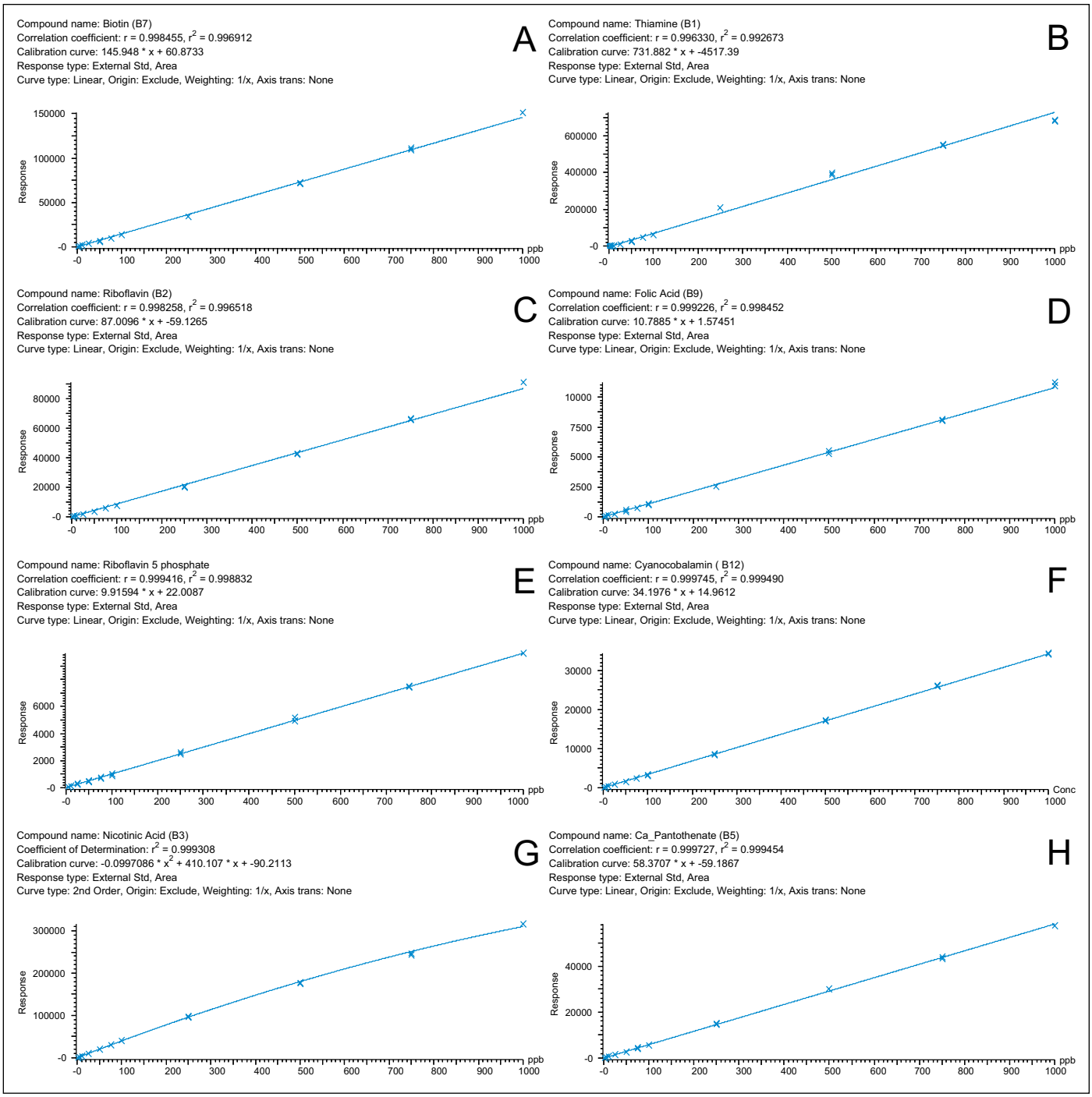

Figure 2. Calibration curves for selected water soluble vitamins.

Mass detection offers the opportunity to detect vitamins at lower levels than can be achieved with UV detection. In Figure 3, the SIR chromatograms of vitamins pyridoxine, pyridoxal, nicotinic acid, and nicotinamide at 5 ppb (5 µg/L) are shown, along with the UV chromatogram (Figure 3A, 270 nm). As shown in Figure 3A, the vitamins could not be detected by UV at this level. The lower limits of quantification that can be achieved with mass detection is important for the quantification of vitamins at low levels. Improved sensitivity also helps to deal with the wide variety of matrices that are encountered by allowing sample extracts to be diluted. In this work, vitamin supplements and drinks were analyzed simply by diluting the sample (in the case of a tablet, an initial step to crush the tablet was required).

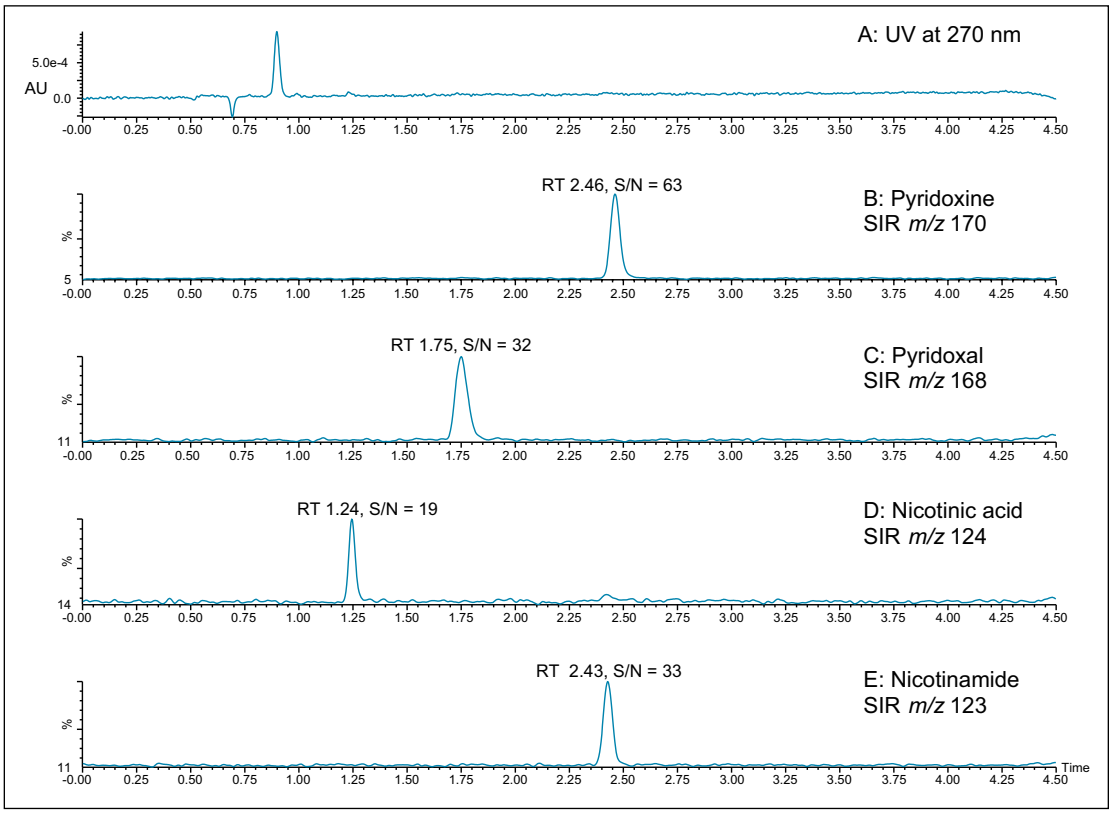

Figure 3. UV chromatogram at 270 nm and SIR chromatograms of a standard mix of vitamins at 5 µg/L. SIR channels for four vitamins are shown. B: pyridoxine, C: pyridoxal; D: nicotinic acid; and E: nicotinamide.

Figure 4 shows the detection of vitamin B5 (calcium pantothenate) in two vitamin water samples. As shown in the UV chromatogram, vitamin B5 could not be detected by UV without additional sample preparation. Vitamin B1 (thiamine) is another vitamin that is difficult to detect using UV. Figure 5 shows an example of the detection of vitamin B1 and vitamin C (ascorbic acid) in a diluted powdered vitamin beverage. Although vitamin C could be detected in the UV chromatogram, vitamin B1 was not detected. Vitamin B1 however, was clearly detected using SIR with the ACQUITY QDa Mass Detector, as shown in Figure 5A.

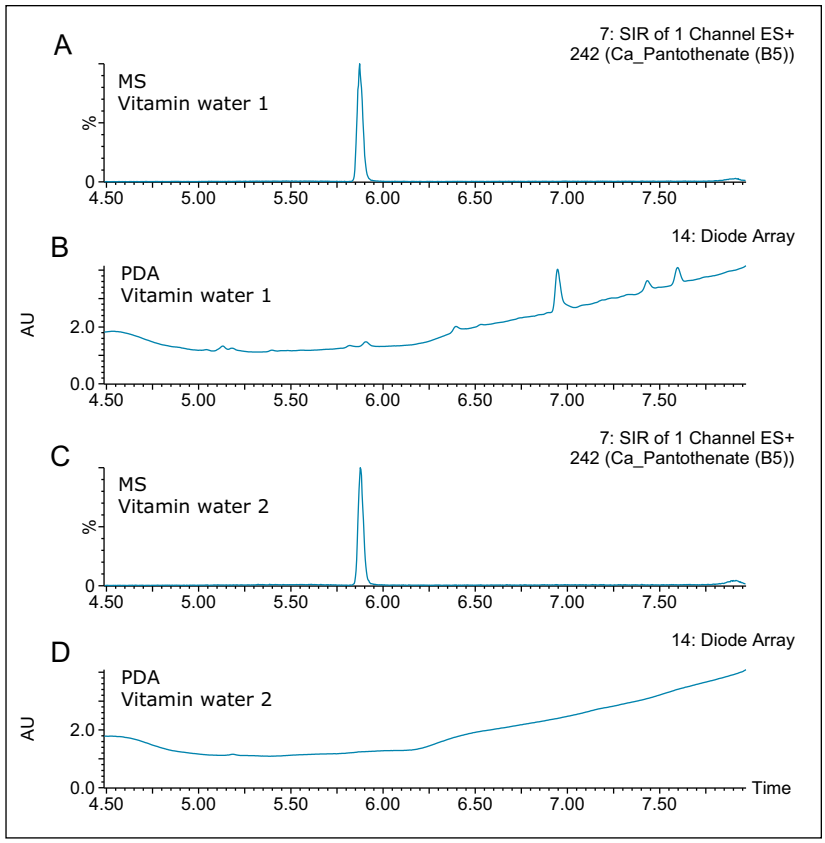

Figure 4. Detection of vitamin B5 in two different vitamin water samples. The peak at 5.9 minutes shows excellent signal-to-noise using mass detection (A and C) but cannot be detected using UV (B and D).

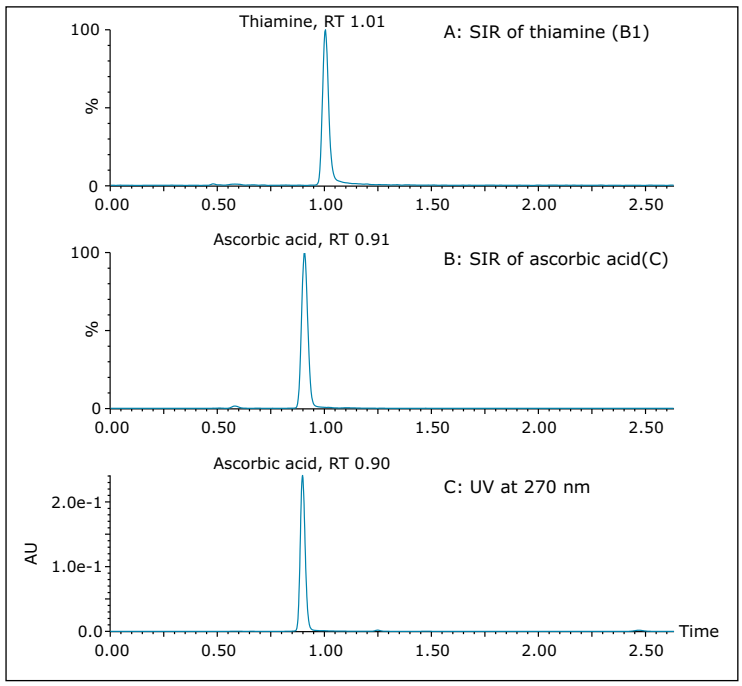

Figure 5. Chromatograms from a 1:250 dilution of a powdered vitamin beverage. A: SIR of vitamin B1 (thiamine), B: SIR of vitamin C (ascorbic acid), C: UV at 270nm; vitamin C was detected in the UV trace while vitamin B1 was not detected by UV.

Cyanocobalamin is a WSV that is fortified at very low levels in supplements and foods and it traditionally requires separate methodologies for its quantification. Two-dimensional chromatography is a routine strategy for the detection of this vitamin.[4] Figure 6 shows an example of cyanocobalamin detected in the multi-vitamin supplement tablet using the UPLC-MS method presented here. At this level, no peak was apparent in the UV chromatogram (Figure 6B). Mass detection offers the ability to detect vitamin B12 using the same method used to detect vitamins that are fortified at much higher levels. The ACQUITY QDa Detector, which can easily be incorporated into existing LC workflows, offers an easier-to-use method than existing multi-dimensional methods.

Another challenge that is encountered in vitamin analysis is the wide range of concentrations at which the vitamins are fortified. For the example of the multi-vitamin supplement in tablet form that was used in this study, the label stated that the B vitamins ranged from 6 µg for vitamin B12 (cyanocobalamin) to 16 mg for B3 labeled as niacin (nicotinic acid), with other B vitamins within that range. In this work, the same LC-MS method was used for the analysis of all the vitamins, with different dilution factors of the initial extraction in order to account for the different vitamin levels. Figure 7 shows chromatograms from the analysis of the multi-vitamin tablet. Figures 7A and 7B show the SIR channels of riboflavin (B2) in the 1:100 dilution of the sample and the undiluted sample, respectively. Figure 7C shows the SIR channel of cyanocobalamin (B12) in the undiluted sample. No peak was detected in the diluted sample (data not shown). The UV trace of the undiluted sample at 270 nm is shown in Figure 7D, and the riboflavin peak showed a good response for this sample. The quantified amount for riboflavin and cyanocobalamin were 12.5 ppm and 41 ppb, respectively. These amounts corresponded to 96% and 68% of the label claim of the supplement. Although the label claims were not verified for this work, nor did we undertake a recovery study, this short study demonstrated the feasibility of using the multiple dilution strategy within the calibration range specific in Table 2.

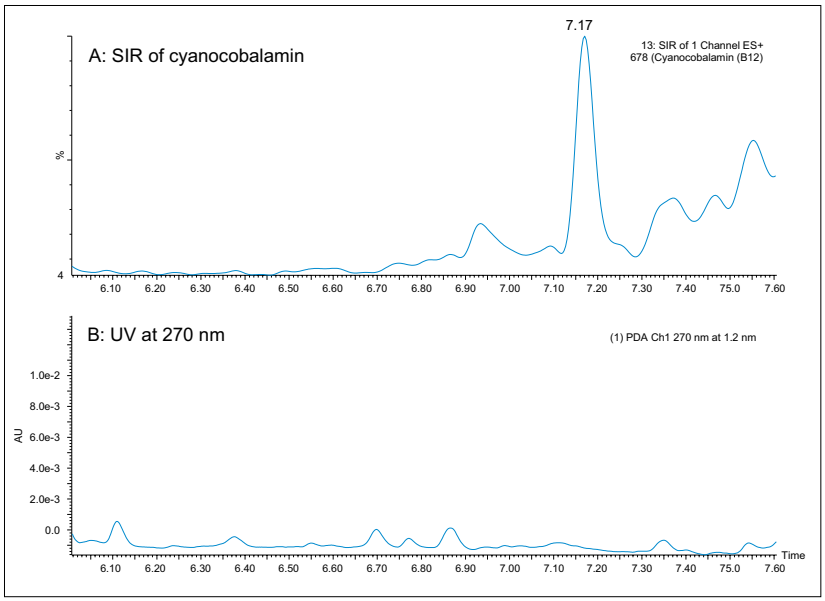

Figure 6. Detection of Vitamin B12 in a vitamin supplement tablet using mass detection (A). The level of the vitamin is below the UV detection limit (B).

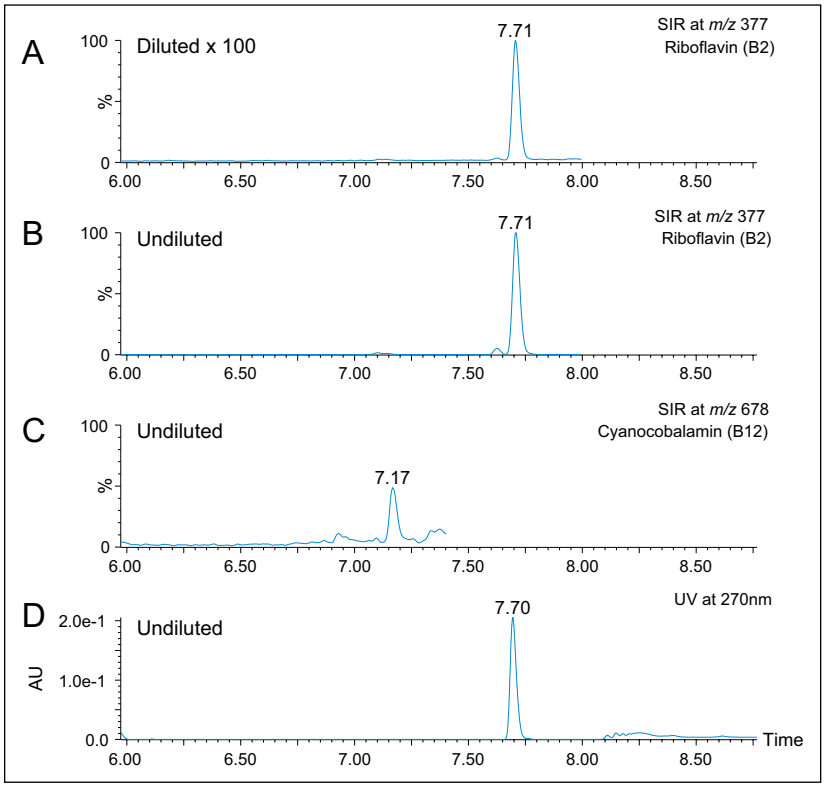

Figure 7. Detection of two B vitamins of very different concentrations in a vitamin supplement tablet. Riboflavin can clearly be detected in a 1:100 dilution of the sample extract (A), whereas B12 is only apparent in the undiluted extract (C). The response of riboflavin in the undiluted extract (B) is outside the calibrated range. At this level it can easily be detected using UV (D).

In order to assess the repeatability of the method for the B vitamins, multiple injections at different vitamin levels were assessed. Results for retention time repeatability, and peak area repeatability are shown in Tables 4 and 5, respectively. In Table 4, 10 injections of two different standards had been combined to give a total of 20 injections. Retention time stability was excellent, even for the early eluting water soluble vitamins, with all RSDs at or below 0.6%. Peak area repeatability was assessed with 10 injections at 0.025 mg/L (Table 5). For the majority of vitamins, %RSDs were well below 10%, with the exception of folic acid and riboflavin 5 phosphate, which were the lower responding analytes mentioned above. Vitamin C was excluded from this study as it is known to degrade over time.

Table 4. Repeatability of retention times for 20 injections using two different standards 0.75 mg/L (10 injections) and 0.025 mg/L (10 injections).

Analyte	%RSD for retention time
Thiamine (B1)	0.6
Nicotinic acid (B3)	0.19
Pyridoxal (B6)	0.23
Nicotinamide (B3)	0.22
Pyridoxine (B6)	0.26
Ca_Pantothenate (B5)	0.04
Cyanocobalamin (B12)	0.03
Folic acid (B9)	0.03
Riboflavin 5 phosphate	0.03
Biotin (B7)	0.02
Riboflavin (B2)	0.03

Table 5. Repeatability of B vitamins peak areas for 10 injections of a standard mix at 0.025 mg/L.

Analyte	%RSD for area
Thiamine (B1)	6.78
Nicotinic acid (B3)	2.35
Pyridoxal (B6)	2.62
Nicotinamide (B3)	2.24
Pyridoxine (B6)	2.65
Ca_Pantothenate (B5)	4.60
Cyanocobalamin (B12)	7.00
Folic acid (B9)	11.53
Riboflavin 5 phosphate	14.29
Biotin (B7)	2.77
Riboflavin (B2)	2.28

CONCLUSIONS

This work shows the capability of the ACQUITY QDa Mass Detector to accurately quantify water soluble vitamins at levels that cannot be achieved with UV. The acquisition of SIR channels allows for sensitive and selective quantification of analytes, even when co-elution occurs. This helps to remove the burden of ensuring all analytes are baseline separated and enables the detection of lower levels of vitamins.

The ACQUITY QDa Mass Detector allows new users to:

- Quantify analytes which have little or no UV response.
- Selectively quantify compounds that co-elute but have different masses.
- Consolidate water soluble vitamin methods into a single LC-MS method.
- Reduce limits of quantification in order to assess easier sample preparation strategies.
- Easily integrate into existing LC workflows, with the choice of Empower® 3 CDS or MassLynx® MS software control.
- Quickly take advantage of the ACQUITY QDa's mass detection capabilities – no special mass spectrometry knowledge required.

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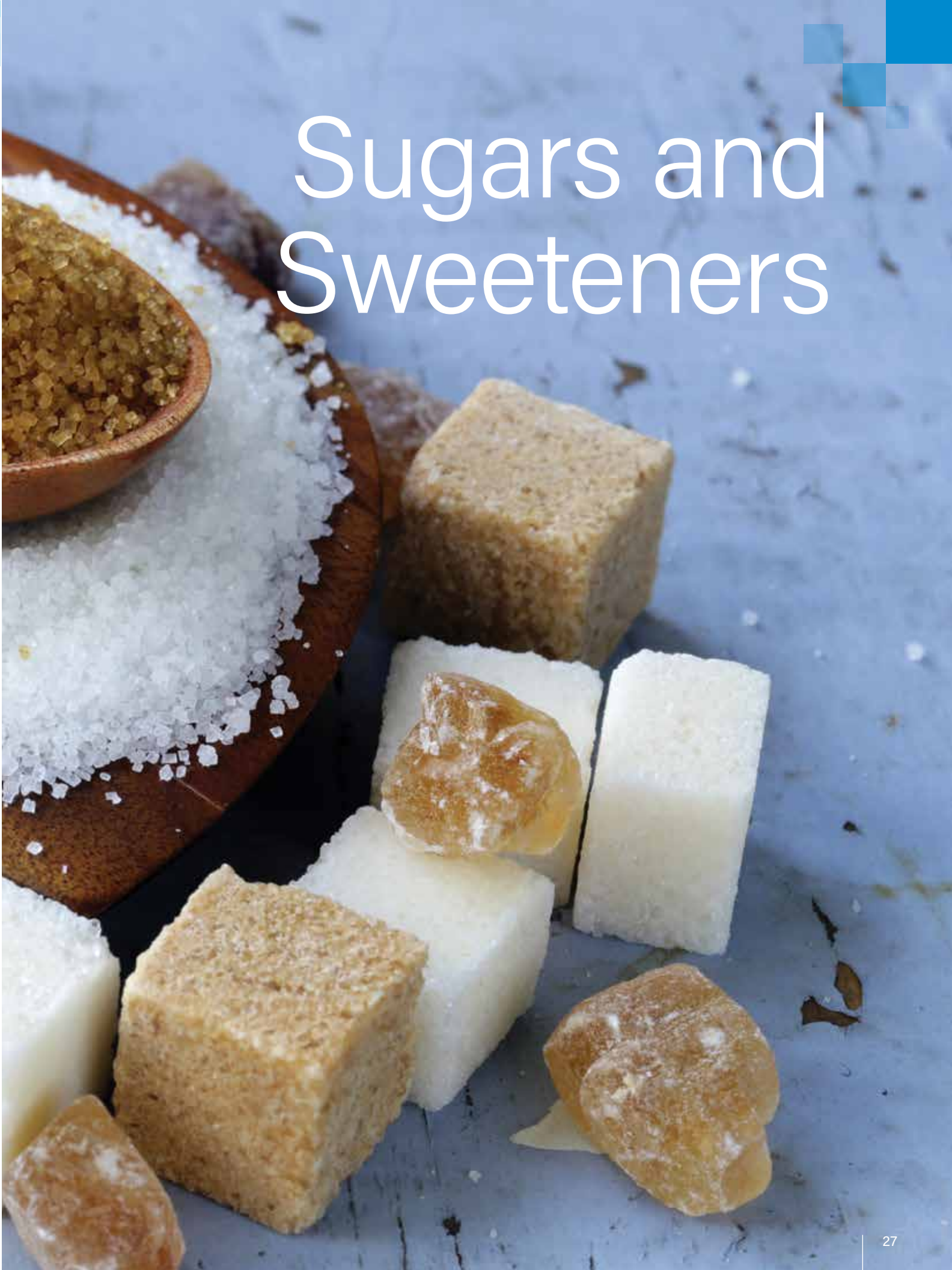
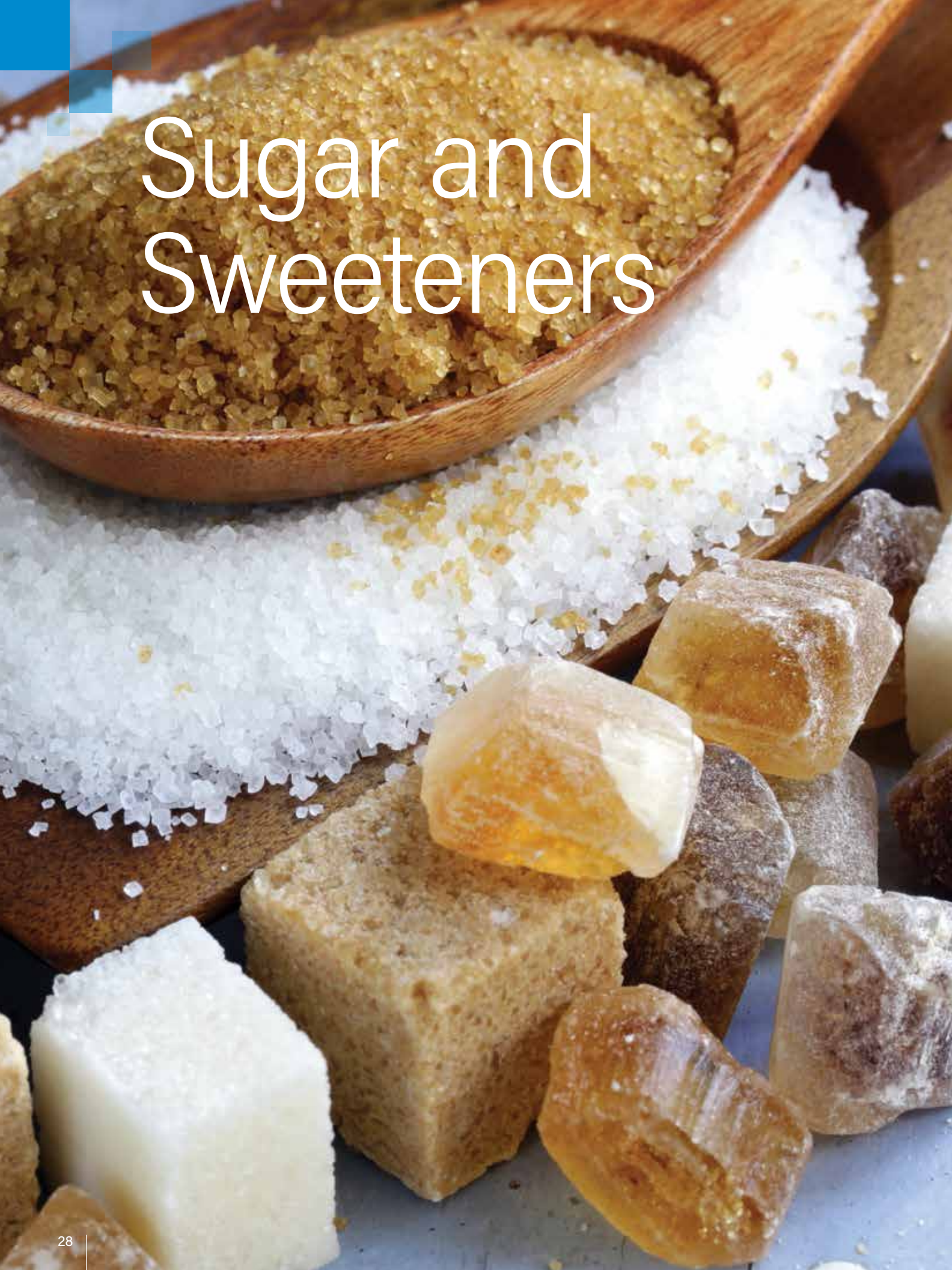
Sugars and Sweeteners



Sugar and Sweeteners



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Profiling Mono and Disaccharides in Milk and Infant Formula Using the ACQUITY Arc System and ACQUITY QDa Detector

Mark Benvenuti, Gareth Cleland, and Jennifer Burgess
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- The ACQUITY Arc™ System provides a choice of two flow paths to emulate HPLC or UHPLC separations.
- Chromatographic separation of the difficult isomeric pair, glucose and galactose.
- The ACQUITY® QDa® Detector provides complimentary detection to Refractive Index (RI) or Evaporative Light Scattering (ELS) detectors that are commonly employed for carbohydrate analysis.

WATERS SOLUTIONS

ACQUITY® Arc™ System

ACQUITY QDa®

XBridge® BEH Amide XP Column

KEYWORDS

Mass detection, infant formula, milk, carbohydrate, mono and disaccharide

INTRODUCTION

Sugars and sugar alcohols are classes of carbohydrates that are natural constituents of foods and provide important nutritional benefits. Some sugars are added to processed foods in order to enhance flavor or to mimic fresh food products. With the increasing incidence of obesity and diabetes across the developed world, the need to monitor sugar intake has grown in recent years. Consequently, there are now requirements to provide accurate information about sugar content on food product labels in order to comply with increasingly stringent regulatory demands.

The analysis of these compounds is challenging because they lack chromophores within their compound structures, and because of the close similarity among the various molecules, many of which are simple isomers of one another. Structures and formulae are shown in Figure 1. Due to its separation power, accuracy and speed of analysis, HPLC has become the method of choice for the analysis of sugars.[1] HPLC techniques typically employ RI or ELS detection. RI detection requires careful control of the mobile phase to avoid any changes during the analysis and therefore requires isocratic elution. With RI detection it is also difficult to change the mobile phase composition from one analysis to the next because the RI detector may require several hours to equilibrate when a different mobile phase composition is introduced. ELS detection is more robust for mobile phase composition changes, but ELS often does not meet the sensitivity demands for the detection of sugars in complex matrices.

An alternative gaining traction is the use of mass detection with electrospray ionization (ESI). Waters® ACQUITY QDa Detector offers the opportunity to decrease detection limits as well as the ability to obtain mass spectral information on components in the sample. The combination of chromatographic retention time and mass information can provide improved selectivity for the profiling of sugars and sugar alcohols. The ACQUITY QDa Detector is the only mass detector that has been holistically designed to be incorporated with an LC system. It fits in the LC stack, occupying the same amount of space as a PDA detector. Extensive training is not required, so users already familiar with HPLC can quickly take advantage of the improved selectivity and sensitivity that mass detection affords. In this application note we describe the use of the ACQUITY QDa Detector coupled to the ACQUITY Arc System for the profiling of sugars in milk and infant formula.

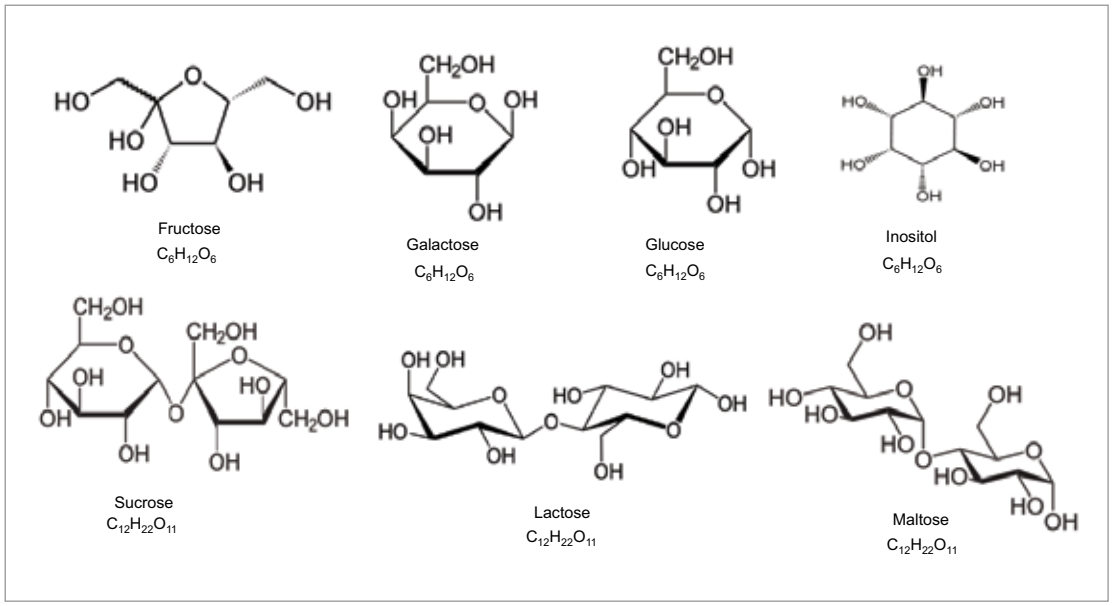

Figure 1. Structures and formulas for the sugar compounds analyzed.

Figure 2. ACQUITY Arc System shown with the PDA and ACQUITY QDa detectors.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY Arc Path 1
Run time:	30.0 min
Column:	XBridge BEH Amide XP, 2.5 µm, 3.0 × 150 mm
Column temp.:	85 °C
Mobile phase:	90:5:5 Acetonitrile-water-2-propanol with 0.05 % diethylamine and 500 ppb guanidine hydrochloride
Flow rate:	0.8 mL/min
Injection volume:	1 µL

MS conditions

MS system:	ACQUITY QDa (Performance)
Ionization mode:	ESI-
Capillary voltage:	0.8 V
Cone voltage:	5.0 V
Probe temp.:	600 °C
Acquisition rate:	2 Hz
Full scan:	100 to 500 *m/z*
Curve fit:	Quadratic, 1/x weighting
Smoothing:	Mean, Level 7
SIR $\{M+Cl^-\}^-$:	215.0 fructose, glucose, galactose, inositol 377.0 sucrose, lactose, maltose

Standard preparation

A 50 mg/L stock of the seven saccharides listed above was prepared in 1:1 acetonitrile-water. This stock was further diluted to produce nine individual levels (0.5, 1, 2, 2.5, 5, 10, 20, 25, and 50 mg/L).

Sample preparation

Samples of a non-fat dry milk powder, a dairy-based infant formula, a soy based infant formula, and a low fat milk were purchased. These were prepared based on the procedure described by Chavez-Servin et al[2] as follows:

- Add approximately 0.6 g sample to a 25 mL volumetric flask.
- Add 10 mL 1:1 ethanol-water.
- Sonicate in a water bath at 60 °C for 25 min.
- Cool, add 250 µL Carrez 1* reagent, stir for 1 min.
- add 250 µL Carrez 2** reagent, stir for 1 min.
- Add 5 mL acetonitrile, mix.
- Make to 25 mL with 1:1 ethanol-water.
- Mix well and transfer to a 50 mL centrifuge tube, allow to settle.
- Centrifuge at an rcf of 2465 g for 30 min.
- Filter supernatant through a 0.2 µm PVDF filter.
- *Carrez 1 reagent: dissolve 0.36 g $K_4[Fe(CN_6).3H_20$ in 10 mL water.
- **Carrez 2 reagent: dissolve 0.72 g $ZnSO_4.7H_2O$ in 10 mL water.

The supernatant was diluted with 1:1 water:acetonitrile, 1:500 for the analysis of sucrose and lactose, and 1:20 for the analysis of fructose, glucose, galactose and inositol. The initial dilution of 25 was included for the final quantification calculations.

RESULTS AND DISCUSSION

Figure 2 shows the ACQUITY Arc System with the ACQUITY QDa and PDA detectors. Although shown with PDA, UV detection was not used for this analysis. Figure 3 shows the SIR chromatograms of the seven saccharide standards at a 5 ppm level (standard 5) used in the study. The annotated *m/z* (215 and 377) represent the $[m+ Cl]^-$ adducts. Figure 4 shows the mass spectra for the analytes. Addition of guanidine hydrochloride to the mobile phase shifts the equilibrium to the chloride adducts, *m/z* 215 for the monosaccharides and inositol, and 377 *m/z* for the disaccharides. Note the baseline separation of galactose and glucose using this chemistry, and also that lactose eluted before maltose.

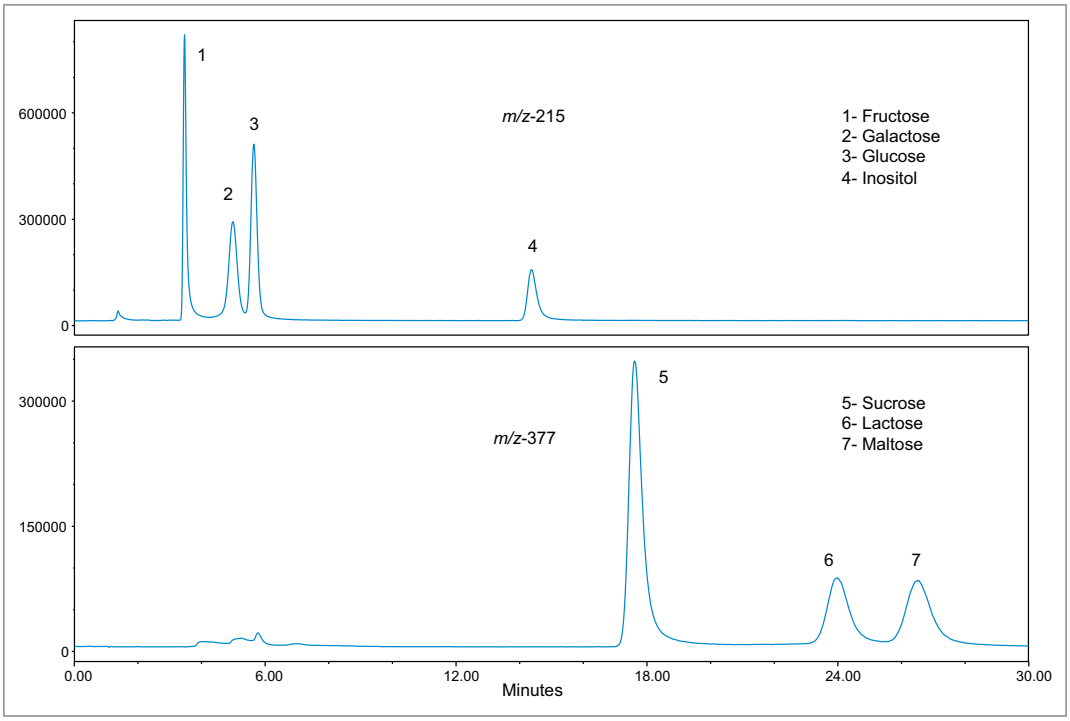

Figure 3. SIR chromatograms of the seven saccharide standards used in the study, the annotated m/z represents the $[m+ Cl]^-$ adducts.

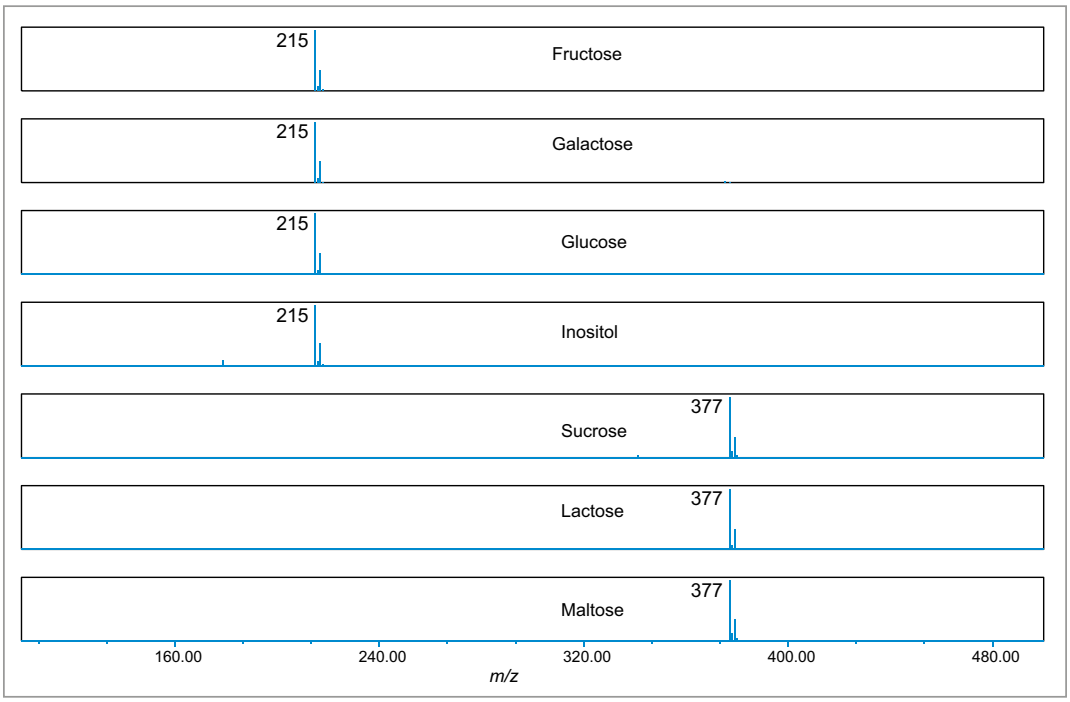

Figure 4. Mass spectral information extracted from SIRs of the seven saccharide standards. The annotated m/z represents the $[m+ Cl]^-$ adducts.

Figure 5 shows the SIR chromatograms of the saccharide standards at 5 ppm (standard 5) along with the dairy and soy based infant formulas at *m/z* 215. Note the absence of galactose in the soy based formula as would be expected. However, inositol is present in both formulations as it is an important nutrient in infant formula.[3] Inositol is highlighted in Figure 6.

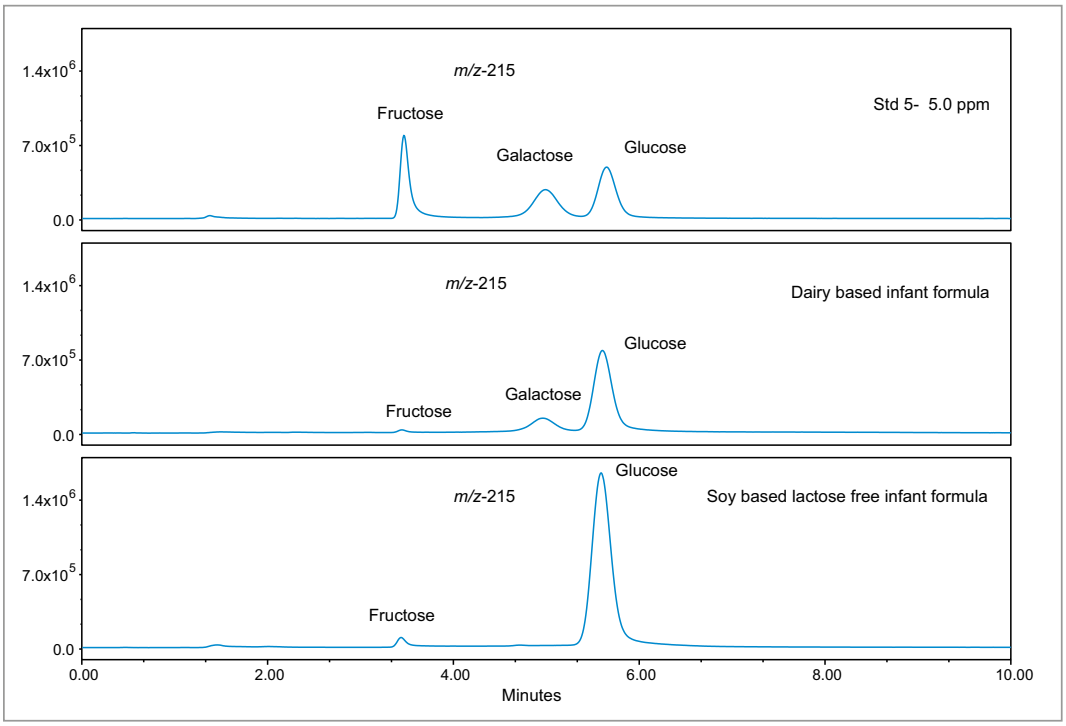

Figure 5. SIR chromatograms of Standard 5 with a dairy and soy based infant formula at m/z 215 for the saccharides fructose, galactose and glucose.

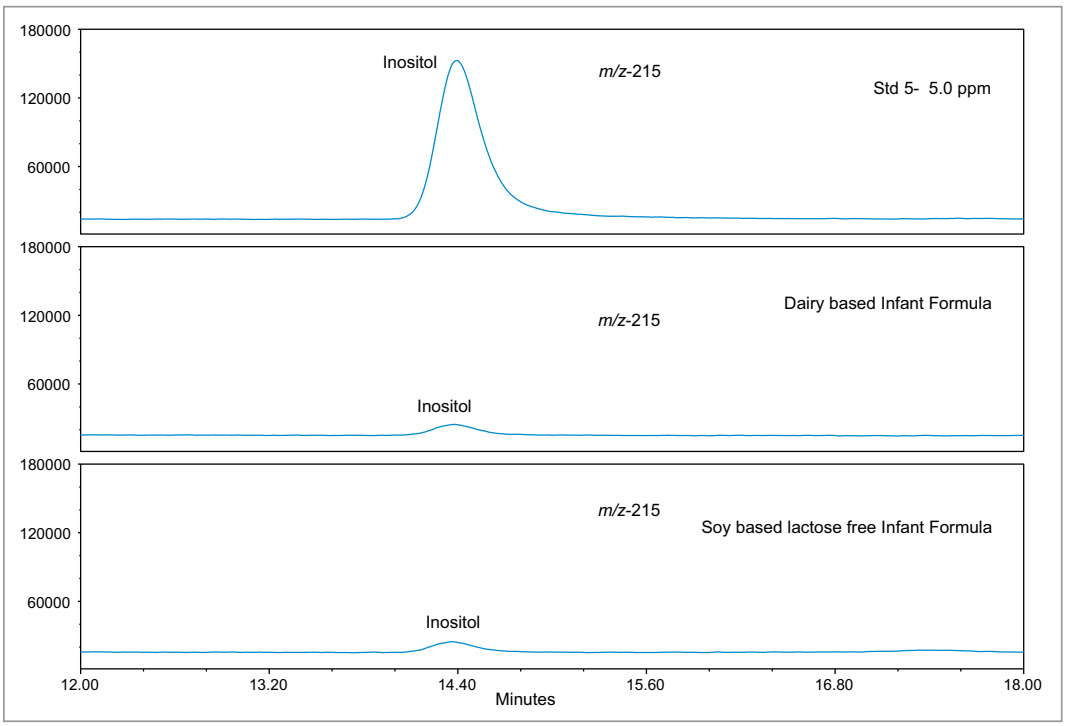

Figure 6. Annotated SIR profile of inositol in standard 5, along with a dairy and soy based infant formula.

Figure 7 shows the SIR chromatograms of the saccharide standards at 5 ppm (standard 5), along with the dairy and soy-based infant formulas at *m/z* 377. Here we see the absence of lactose in the soy formulation. However sucrose and maltose are present, which are absent in the dairy formulation. This is also to be expected as these two sugars are derived from plant based sources and should not be found in a dairy matrix unless added artificially. Figure 8 shows the calibration curves for the analytes. The regression coefficient (R^2) was >0.998 for all analytes.

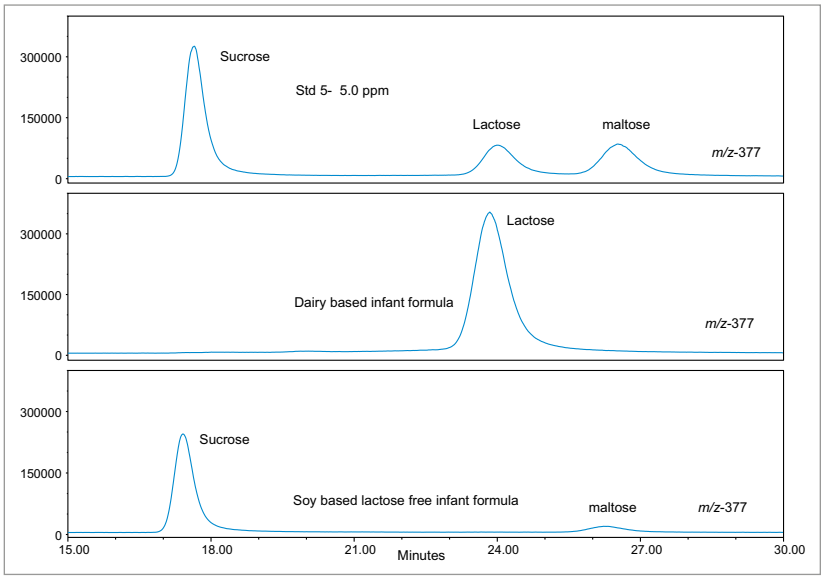

Figure 7. Annotated SIR profile of sucrose, lactose, and maltose in standard 5, along with a dairy and soy based infant formula.

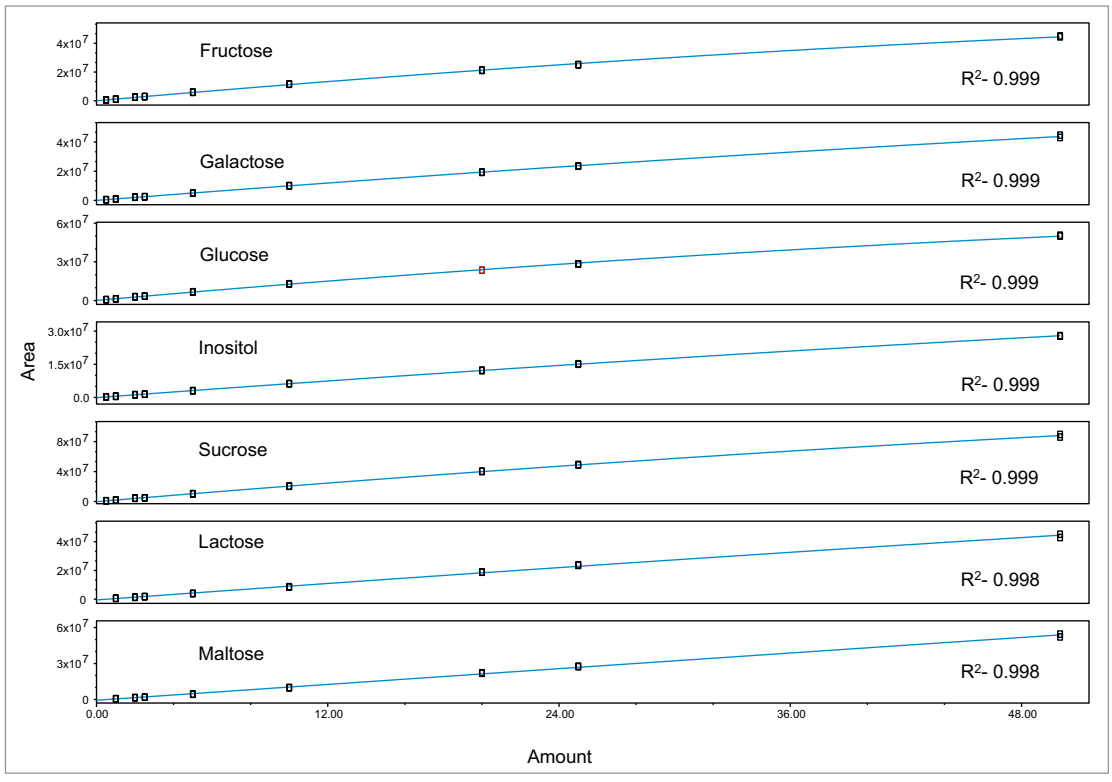

Figure 8. Calibration curves for the seven saccharide standards showing R^2 values, a quadratic fit was used.

Table 1 lists the quantitated values for the saccharides studied. The values for inositol in the dairy and soy based infant formula generally agree with the values reported by Indyk.[4] Likewise, the value for lactose in the dairy infant formula generally agrees with that reported by Ferreira.[5] Table 2 lists reproducibility data for 7 injections of the soy based infant formula. RSD's were <0.25% for retention time and 8.20% for amount.

Sample	Fructose	Galactose	Glucose	Inositol	Sucrose	Lactose	Maltose
Non Fat Dry Milk	0.04	0.837	1.295	0.389	ND	381.647	ND
Dairy Infant Formula	0.113	2.377	7.681	0.339	ND	394.996	ND
Soy Infant Formula	0.481	ND	19.579	0.359	83.705	ND	28.594
Low Fat Milk	ND	0.130	0.125	0.078	ND	42.775	ND

Table 1. Calculated concentrations from the quantification studies of the various dairy and infant formulas profiled in this study (g/kg).

Analyte	RT	% RSD	Amount	%RSD
Fructose	3.44	0.10	0.48	8.17
Glucose	5.59	0.10	19.58	3.71
Inositol	14.34	0.10	0.36	3.14
Sucrose	17.41	0.14	83.71	1.42
Maltose	26.27	0.22	28.59	2.02

Table 2. Reproducibility data for retention time (Min) and amount (g/kg) for seven injections of a soy based infant formula.

CONCLUSIONS

The analysis of carbohydrates in dairy products can be challenging because of the mix of closely related UV transparent compounds. The combination of the ACQUITY Arc System, ACQUITY QDa Detector, and the XBridge BEH Amide Column offers scientists the advanced performance expected of ACQUITY separations, high resolution, sensitivity, and improved throughput, along with a complimentary mass detector to RI and ELS that provides the additional advantages of:

- Improved analytical selectivity by combining both retention time and mass analysis for compound identification.
- Detection of UV transparent molecules using a sensitive and selective detector.
- Chromatographic separation of the difficult isobaric pair, galactose and glucose.

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4. Ibid p 401.
5. Ferreira, Op. Cit. p 227.

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Profiling and Quantification of Mono and Disaccharides and Selected Alditols in Juice, Beer, Wine, and Whiskey Using the ACQUITY Arc System with Mass Detection

Mark E. Benvenuti, Gareth Cleland, and Jennifer Burgess
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Detection of sugars at lower levels than with Refractive Index (RI) or Evaporative Light Scattering Detection (ELS).
- Minimum sample preparation required and samples can be diluted to reduce matrix effects.
- Chromatographic separation of the difficult isomer pairs sorbitol and mannitol.

WATERS SOLUTIONS

ACQUITY® Arc™ System

ACQUITY QDa® Mass Detector

XBridge® BEH Amide XP Column

Empower® 3 CDS Software

KEYWORDS

Saccharides, sugars, fructose, glucose, sucrose, maltose, mannitol, sorbitol, whiskey, carbohydrate

INTRODUCTION

Sugars and sugar alcohols are classes of carbohydrates that are important in human nutrition and natural constituents of foods. With the increasing incidence of obesity and diabetes across the developed world, interest in monitoring sugar intake has vastly increased in recent years. Consequently, there are now requirements to provide accurate information on product labeling in order to comply with increasingly stringent regulatory demands. Profiling the sugar content of products is also a useful tool in assessing product authenticity and potential adulteration.

The analysis of sugars and sugar alcohols remains a challenging application, owing to the lack of chromophores and the similarity between these molecules. Many of these sugar compounds are isomers of one another, as can be seen in Figure 1, which illustrates the formulae and structures of the compounds analyzed in this study. Due to its separation power, accuracy, and speed of analysis, HPLC has become the method of choice for the analysis of sugars. An alternative to RI and ELS detection is the use of mass detection with electrospray ionization (ESI). Mass detection is complementary to traditional detectors used for LC.

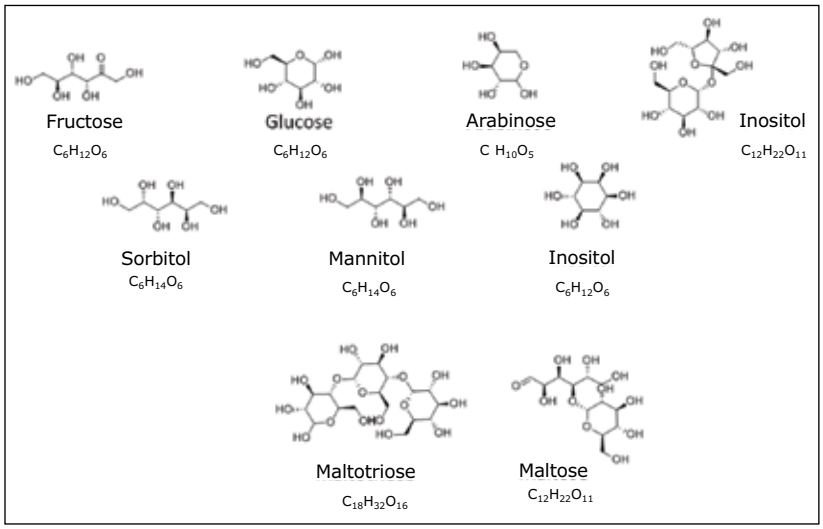

Figure 1. Structures and formulae for the sugar compounds analyzed.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY Arc
Data system:	Empower 3
Runtime:	40.0 min
Column:	XBridge® XP BEH Amide 2.5 µm, 3.0 x 150 mm
Column temp.:	85 °C
Mobile phase A:	90% acetonitrile: 5% IPA:5% water*
Mobile phase B:	80% acetonitrile: 20% water*
Flow rate:	0.8 mL/min
Injection volume:	1 µL

	Time (min)	Flow rate (mL/min)	%A	%B
1.	Initial	0.8	100	0
2.	4.5	0.8	100	0
3.	18.0	0.8	0	100
4.	25.0	0.8	0	100
5.	25.1	0.8	100	0
6.	40.0	0.8	100	0

*Both containing 500 ppb guanidine hydrochloride and 0.05% diethylamine.

MS conditions

MS system:	ACQUITY QDa (Performance mode)
Ionization mode:	ESI-
Capillary voltage:	0.8 V
Cone voltage:	5.0 V
Probe temp.:	600 °C
Acquisition rate:	2 Hz
Full scan:	50 to 800 Hz
Curve fit:	Quadratic, 1/x weighting
Smoothing:	Mean filter, Level 7

Standard preparation

A 100 mg/L stock of the nine saccharides listed above was prepared in 1:1 acetonitrile-water. This stock was further diluted to produce nine individual levels (1, 2, 4, 5, 10, 20, 40, 50, and 100 mg/L).

Sample preparation

All samples were purchased locally. The juice samples assessed included orange, apple, pineapple, pomegranate, and grape. The alcoholic beverages assessed included five beers, three lagers (one non-alcoholic), a lemon flavored beer, one hard cider, one sherry, one red wine, and four whiskeys. The beer samples were sonicated to remove carbonation. All of the samples were filtered through a 0.22 µm PVDF syringe filter and diluted in 1:1 acetonitrile-water. The dilution factors are listed in Table 1.

Sample	Dilution factor
Lager 1 and 2	5
Non-Alcoholic	500
Hard Cider	500
Lemon Flavored Beer	50
Sherry Wine	500
Red Wine	500
Whiskey	2
Orange Juice	1000
Apple Juice	1000
Pineapple Juice	1000
Pomegranate Juice	1000
Grepe Juice	2500

Table 1. Dilution factors for the "off-the-shelf" samples studied.

SIR channels:

Analyte	Formula	SIR (*m/z*) ([M+Cl]$^-$ ion)
Arabinose	$C_5H_{10}O_5$	185
Fructose	$C_6H_{12}O_6$	215
Glucose	$C_6H_{12}O_6$	215
Inositol	$C_6H_{12}O_6$	215
Sorbitol	$C_6H_{14}O_6$	217
Mannitol	$C_6H_{14}O_6$	217
Sucrose	$C_{12}H_{22}O_{11}$	377
Maltose	$C_{12}H_{22}O_{11}$	377
Maltotriose	$C_{18}H_{32}O_{16}$	539

It offers the opportunity to decrease detection limits and also to obtain mass spectral information on the components in the sample. The combination of both chromatographic retention time and mass information results in increased selectivity for the analysis of sugars and sugar alcohols. Here we show the application of the Waters® ACQUITY QDa Mass Detector coupled to the ACQUITY Arc System for the profiling and quantification of sugars in juice, wine, beer, and whiskey samples.

RESULTS AND DISCUSSION

Figure 2 shows the ACQUITY Arc System with the ACQUITY QDa Mass Detector and a PDA Detector. The PDA is shown for reference but was not used in this application. Figure 3 shows the SIR chromatograms for a mixed standard at 100 mg/L for each of the analytes listed above. Excellent separation of all of the standards was achieved. Initially, using isocratic conditions the lower mass saccharides were separated, including the difficult pair sorbitol and mannitol. After 4.5 minutes a gradient was started which allowed timely separation of the larger molecular weight saccharides in the mix.

Figure 2. ACQUITY Arc System with the PDA and ACQUITY QDa detectors.

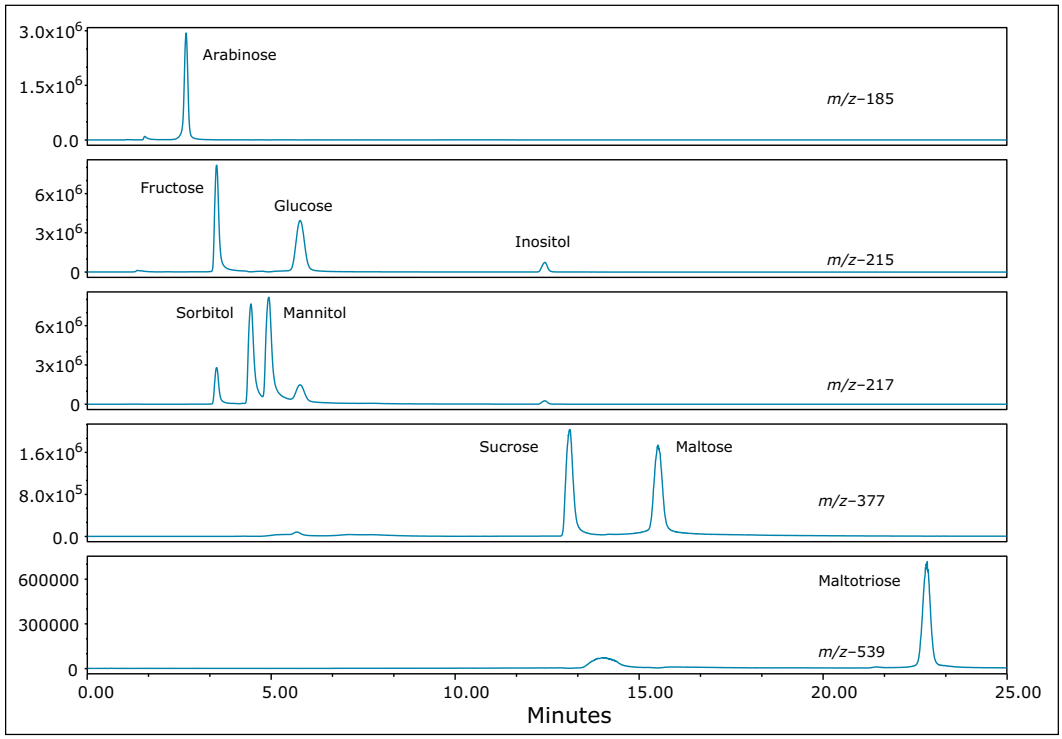

Figure 3. SIR chromatograms of the nine saccharide standards used in the study. The annnotated m/z represents the $[M+Cl]^-$ adducts.

The mass spectra extracted from the SIR of each standard is shown in Figure 4. The use of guanidine chloride in the mobile phase ensured that the compounds were driven to their chloride adduct ($[M+Cl]^-$ ion). The smaller ^{37}Cl adduct response was also present. Figure 5 shows the calibrations curves for the compounds studied. An R^2 value >0.995 was achieved for all of the analytes.

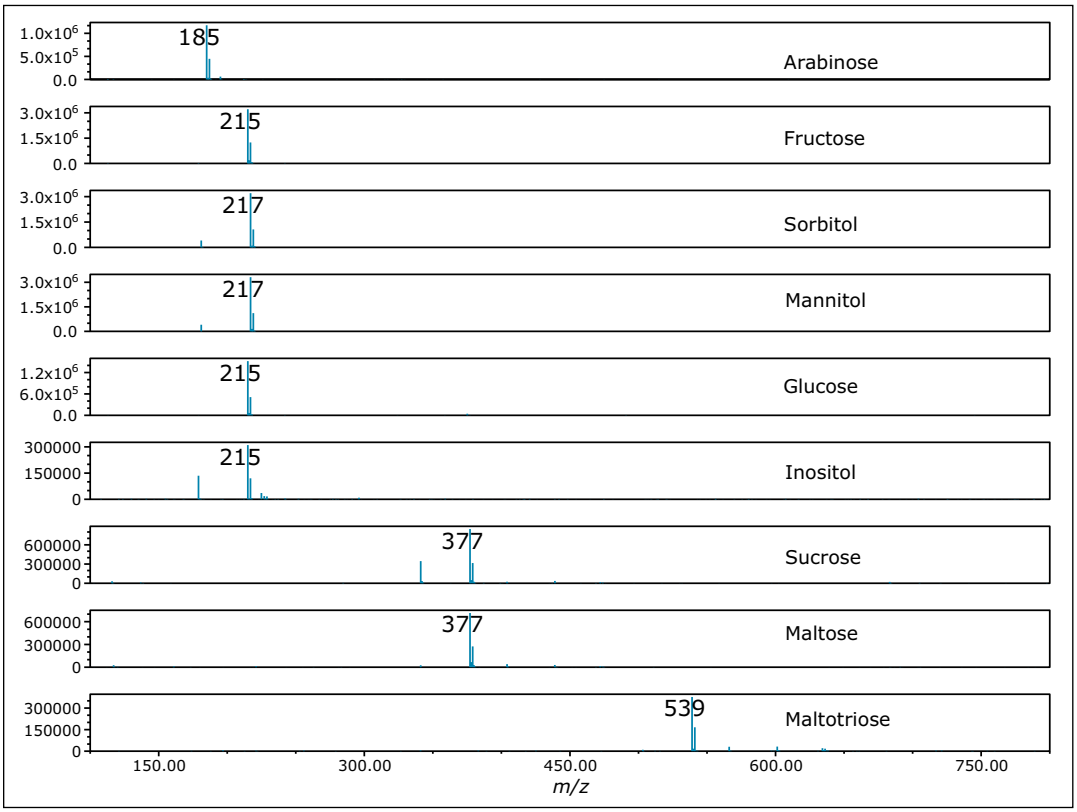

Figure 4. Mass spectral information extracted from SIRs of the nine saccharide standards. The annotated m/z represents the [M+ Cl]- adducts.

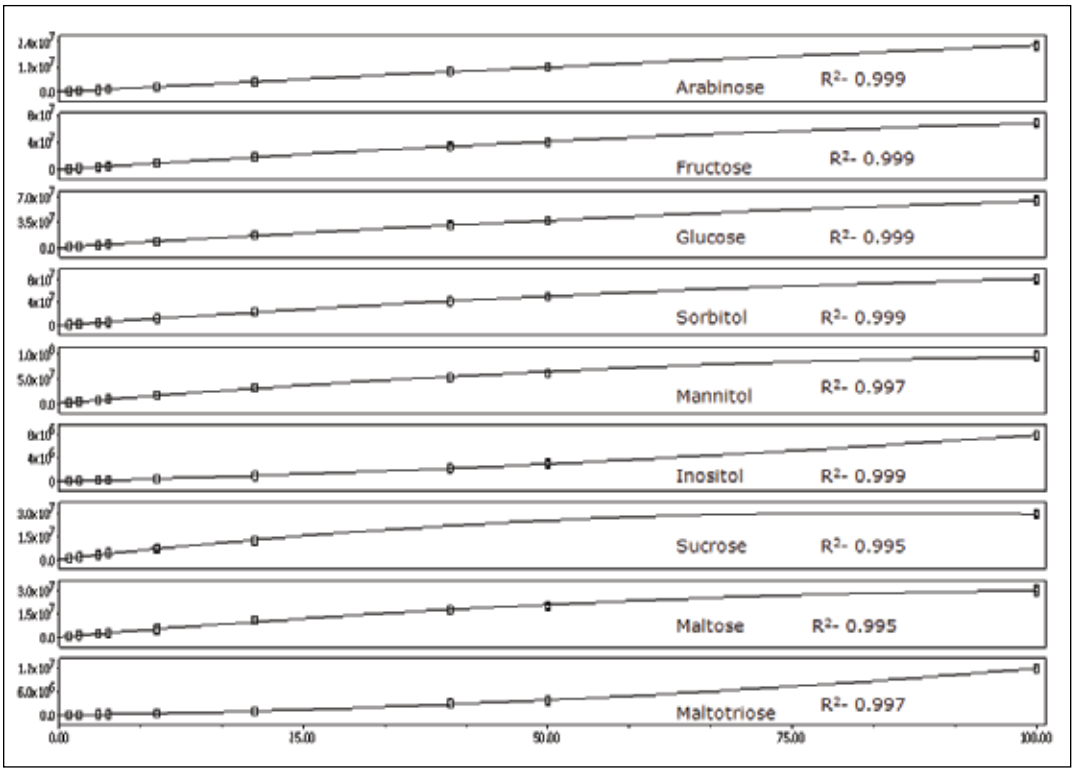

Figure 5. Calibration curves for the nine saccharide standards analyzed showing R^2 values obtained for each analyte.

Figure 6 (A–E) shows the SIR profiles of a lager beer. In Figure 6A (*m/z* 185) arabinose is present. Other peaks are also apparent, suggesting the presence of other pentose saccharides. In Figure 6B (*m/z* 215) traces of fructose and glucose can be seen. The enhanced sensitivity of the ACQUITY QDa allows improved detection of these compounds, as opposed to less sensitive methods such as Refractive Index.[1] In Figure 6C (*m/z* 217) traces of sorbitol and mannitol are present. We also saw small peaks representing the extraction of the Cl^{37} adducts of fructose and glucose, which have the same molecular weight as sorbitol and mannitol. In Figure 5D and 5E (*m/z* 377 and 539 respectively), we observed the DP2 and DP3 compounds maltose and maltotriose, along with isomers of the same mass, which would be expected for a beverage derived from grain.

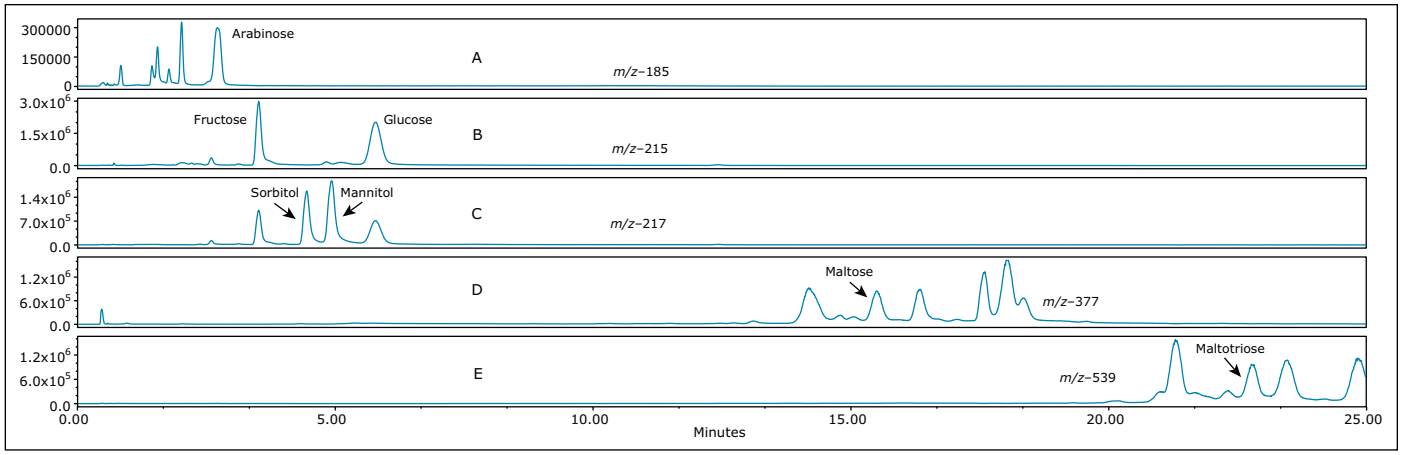

Figure 6. Annotated SIR profile of a lager beer annotated with saccharides found to be present.

A sherry wine profile is shown in Figure 7 (A–E). The main analytes found to be present in sherry are fructose and glucose (Figure 7B). A small amount of arabinose was present (Figure 7A), along with trace levels of sorbitol and mannitol (Figure 7C). Maltose was also apparent (Figure 7D). The DP3 compounds were absent (Figure 7E), as would be expected, since wine is derived from grapes rather than from grains.

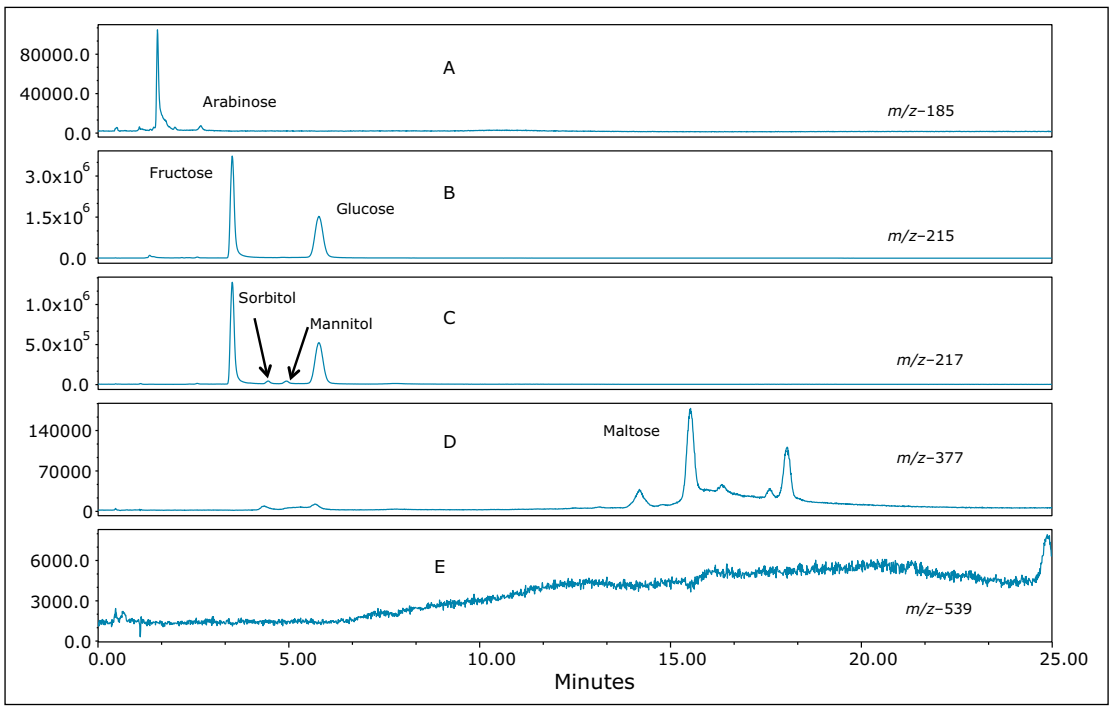

Figure 7. SIR profile of a sherry wine annotated with saccharides found to be present.

Figure 8 (A–E) shows the SIR profile of a whiskey sample. The presence of arabinose (Figure 8A), fructose and glucose (Figure 8B) was evident. Of particular interest was an unknown saccharide apparent in Figures 8B (*m/z* 215) and 8C (*m/z* 217) at retention time 4.85 minutes. Using retention time alone with an RI or ELS detector, this peak would most likely have been misidentified as mannitol. The presence of this peak at both *m/z* 215 and *m/z* 217 indicated that this component has the same mass as a monosaccharide, rather than an alditol. Mannitol does not have an ion at *m/z* 215, as can be seen in Figures 2 and 3.

Finally, the SIR chromatograms from an apple juice sample are shown in Figure 9 (A–E). The presence of arabinose, fructose, glucose, sorbitol, and sucrose are highlighted.

The quantification of various fruit juices is shown in Table 2. Fructose, glucose, and sucrose were present in the orange, apple, and pineapple juices. The amounts and ratios of sugars in these juices are similar to those reported elsewhere.[2,3] Of particular interest was the detection of sorbitol in pomegranate juice. Sorbitol is not usually present in pomegranate juice[4] and its detection could be evidence of adulteration. A second sample tested showed no sorbitol (Figure 10). The grape juice sample showed fructose, glucose, but no sucrose as expected.[2,3]

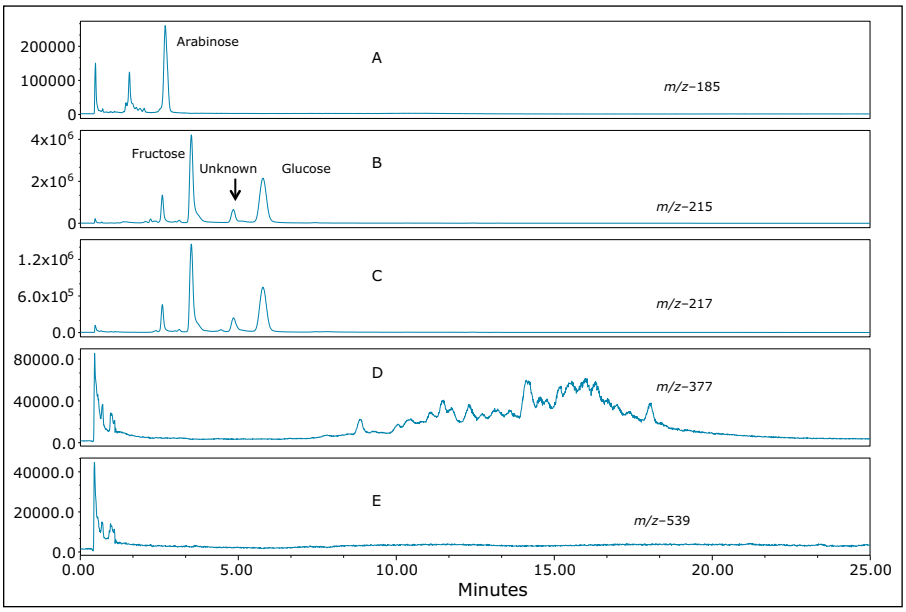

Figure 8. SIR profile of a whiskey annotated with saccharides found, and an unknown saccharide found to be present at m/z 215.

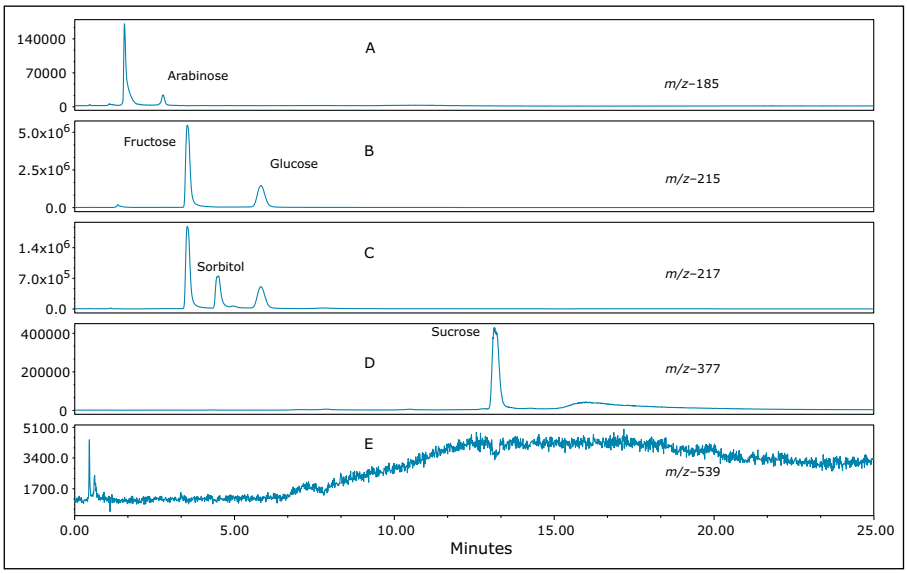

Figure 9. SIR profile of an apple juice sample annotated with saccharides found to be present.

Juice	Fructose	Glucose	Sorbitol	Mannitol	Sucrose
Orange	25.1	23.6	ND	ND	52.6
Apple	70.2	30.2	7.6	ND	10.6
Pineapple	37.4	40.6	ND	ND	41.9
Pomegranate	64.8	71.1	19.7	4.3	0.9
Grape	83.0	82.5	ND	ND	ND

Table 2. Calculated concentrations from the quantification studies of the various fruit juices profiled in this study (g/L).

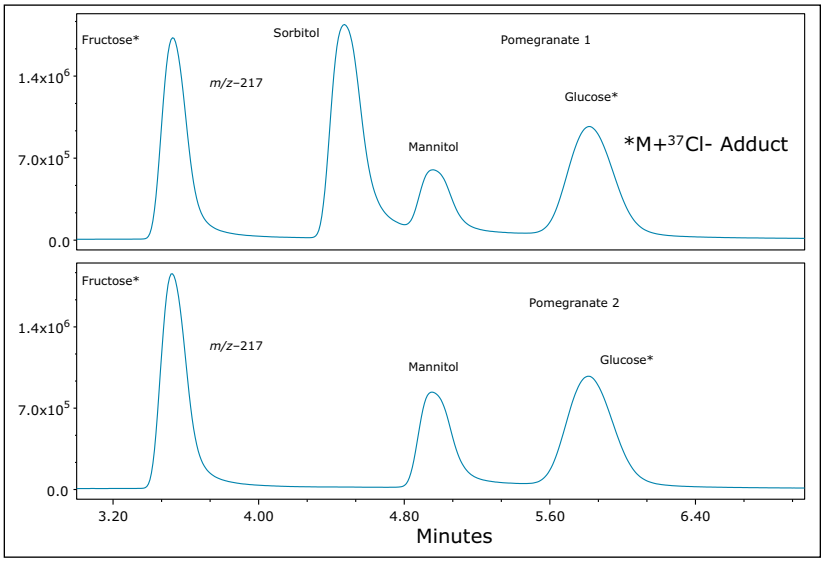

Figure 10. Zoomed-in SIR chromatograms annotated with saccharides found to be present in two pomegranate juice samples. One sample showed the presence of sorbitol, indicating a potential adulteration.

Analyte	RT	%RSD	Amount	%RSD
Fructose	3.54	0.12	25.1	1.33
Glucose	5.84	0.11	23.6	1.82
Sucrose	13.16	0.12	52.6	7.82

Table 3. Reproducibility data for retention time (min) and amount (g/L) based on seven injections of orange juice.

CONCLUSIONS

- The ACQUITY QDa Mass Detector coupled to the ACQUITY Arc System provides improved sensitivity and selectivity to analyze and quantify mono and disaccharide samples in a single injection.
- Mass detection is a viable alternative to Refractive Index (RI) or Evaporative Light Scattering (ELS) methods.
- This enhanced sensitivity allows the analysis of samples at higher dilution levels, which minimizes matrix effects.
- The combination of mass detection and chromatographic separation provides increased selectivity in identifying analytes of interest, while reducing false positives.

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A Method for the Rapid and Simultaneous Analysis of Sweeteners in Various Food Products Using the ACQUITY H-Class System and ACQUITY QDa Mass Detector

Mark Benvenuti, Dimple Shah, and Jennifer A. Burgess
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- The ACQUITY® QDa® Detector facilitates simultaneous analysis of sweeteners in single run.
- Quantification of natural and artificial non-nutritive sweeteners in less than 10 minutes.
- Mass detection provides information-rich orthogonal detection for co-eluting compounds.
- The ACQUITY QDa Mass Detector provides sensitive and selective determination of UV transparent sweeteners.

WATERS SOLUTIONS

ACQUITY UPLC® H-Class System

ACQUITY QDa Mass Detector

MassLynx® MS Software

KEYWORDS

Aspartame, saccharin, rebaudioside, stevioside, Stevia, acesulfame-K, neotame, sucralose, mass detection

INTRODUCTION

Sugars are renowned for their sweet taste and are often added to manufactured foods to enhance human perception of flavor. Due to the negative health effects of excessive consumption of sugar, alternative non-nutritive sweeteners are commonly used in food and beverage products. Examples include soft drinks, table-top sweeteners, chocolates, dairy products, and many other so-called "diet" foods. In many cases it is a combination of various sweeteners that are used to impart overall sweetness to these products. Aspartame, saccharin, acesulfame-K, neotame, and sucralose are approved artificial sweeteners by the U.S. FDA.[1] Compounds such as rebaudioside A and stevioside, which originated from the South American Stevia plant, are also becoming more popular in the U.S. as a sweetener. In 2010, the European Food Safety Authority (EFSA), approved the use of stevioside as a sweetener.

The European Union (EU) Directive 94/35/EC, along with four amendments: 96/83/EC, 2003/115/EC, 2006/52/EC, and 2009/163/EU restrict the level of sweeteners in specific types of food. The EU Commission Regulation 1129/2011 lists the maximum level of the sweeteners permissible in various food products. Hence the determination of the amount of these sweeteners in foods is also important in order to ensure consistency in product quality.

The most common method for the detection of sweeteners is HPLC coupled to a UV detector. This configuration enables the detection of some sweeteners such as acesulfame-K, aspartame, saccharin, and neotame. However cyclamate and sucralose cannot be analyzed by UV because they lack a chromophore. The ability to analyze all of these sweeteners using a single method with mass detection would be ideal. Waters has developed the ACQUITY QDa Mass Detector to allow food and beverage scientists to incorporate mass detection into their existing chromatographic workflows. The ACQUITY QDa not only allows for the detection of all sweeteners in a single run, but it also brings improved discrimination to the analysis, thereby eliminating the requirement of baseline separation of all compounds.

The combination of Waters® ACQUITY UPLC H-Class System with the ACQUITY QDa Mass Detector is extremely beneficial for food and beverage manufacturers for the identification and quantification of sweeteners in their products using a single analysis method.

In this application note, a fast, reliable and sensitive method was developed to analyze sweeteners in food and beverage samples.

EXPERIMENTAL

UPLC conditions

UPLC® system:	ACQUITY UPLC H-Class
Column:	ACQUITY UPLC HSS T3 1.8 µm, 2.1 x 100 mm
Column temp.:	40 °C
Injection volume:	2 µL
Flow rate:	0.4 mL/min
Mobile phase A:	Water + 0.1% formic acid
Mobile phase B:	Acetonitrile 0.1% formic acid
Wash solvent:	50/50 water/methanol (v/v)
Purge wash:	10/90 acetonitrile/water (v/v)
Seal wash:	10/90 acetonitrile/water

Time (min)	Flow rate (mL/min)	%A	%B	Curve
Initial	0.4	90	10	6
1.0	0.4	90	10	6
4.0	0.4	40	60	6
5.0	0.4	40	60	6
5.1	0.4	0	100	6
6.1	0.4	0	100	6
6.2	0.4	90	10	6
9.5	0.4	90	10	6

Table 1. UPLC gradient method for sweeteners analysis.

Detector conditions

Detector 1:	ACQUITY UPLC PDA
Wavelength:	Scanning 210 to 400 nm
Analog channels:	at 214 and 254 nm
Scan rate:	10 pts/sec
Detector 2:	ACQUITY QDa (Performance)
Ionization mode:	ESI-
Probe temp.:	500 °C
Capillary voltage:	0.6 kV
Sampling rate:	5 pts/sec
Acquisition type:	Full scan *m/z* 150 to 1100 (centroid)
Cone voltage:	Ramp from 5 to 40 V
SIR channels:	See Table 2

Standard preparation

Stock solutions of each of the sweeteners were prepared in water. A series of working solutions were also prepared in water with a concentration range from 0.04 to 30 mg/L.

Sample preparation

A total of seven different samples were analyzed in this work. All of the samples were purchased from local markets. The samples included two beverages (diet cola and diet tea), two table-top sweeteners, a ready-made pudding, hard candy, and diet marmalade.

The table-top sweeteners (~1 g) were dissolved in 100 mL water and further diluted at two additional levels: 1:20 and 1:10. All three dilution levels were injected to cover the different concentrations of sweeteners in this sample.

The candy (5.281 g) and pudding (1.244 g) were dissolved separately in 100 mL water and diluted 1:10. These dilution levels were injected.

Marmalade (1.432 g) was dissolved in 100 mL water and analyzed.

The beverages were filtered through a 0.22-µm PVDF filter, diluted 1:20 with water, and analyzed.

RESULTS AND DISCUSSION

The chemical structures for the eight sweeteners analyzed in this study are shown in Figure 1. These sweeteners were separated on a reversed-phase column and detected using the ACQUITY QDa Mass Detector. The retention times, along with the Single Ion Recording (SIR) mass-to-charge ratio (*m/z*), and cone voltages of these sweeteners are shown in Table 2.

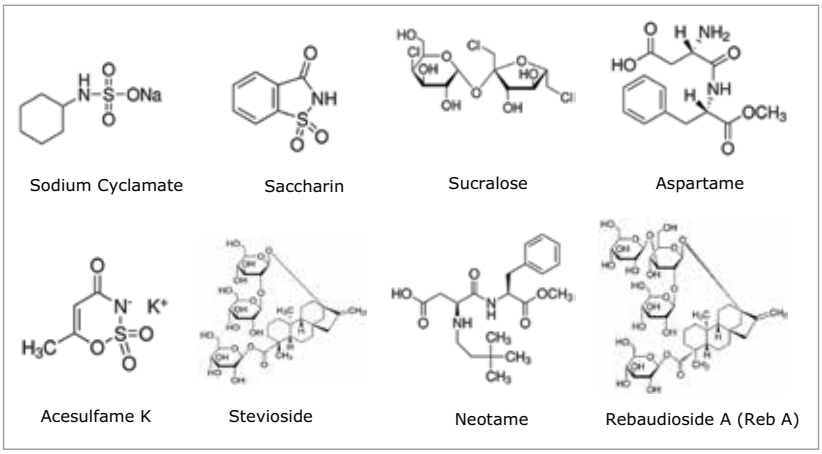

Figure 1. Chemical structures of the sweeteners analyzed.

Compounds	SIR *m/z*	Cone Voltage (V)	RT (min)
Acesulfame K	162.0	12	1.04
Aspertame	293.0	12	3.20
Cyclamate	178.0	24	1.77
Neotame	377.0	15	4.43
Rebaudioside A	965.5	28	4.11
Saccharin	182.0	24	1.34
Stevioside	803.3	24	4.14
Sucralose	395.0	20	3.20

Table 2. Retention times, SIR m/z and cone voltages for the sweeteners.

Figure 2 shows the SIR chromatograms of the individual sweetener standards in solvent. As shown in Figure 2, there are two co-eluting pairs.

Aspartame (3.20) and sucralose (3.20) eluted at the same time, while rebaudioside A (4.11) and stevioside (4.14) eluted at very nearly the same time. However, due to the different mass of aspartame (*m/z* 293) and sucralose (*m/z* 395), mass detection was able to separate and detect these compounds. Likewise, rebaudioside A (*m/z* 965.5) and stevioside (*m/z* 803.3) were co-eluting, but they were still able to be individually detected using the ACQUITY QDa Mass Detector.

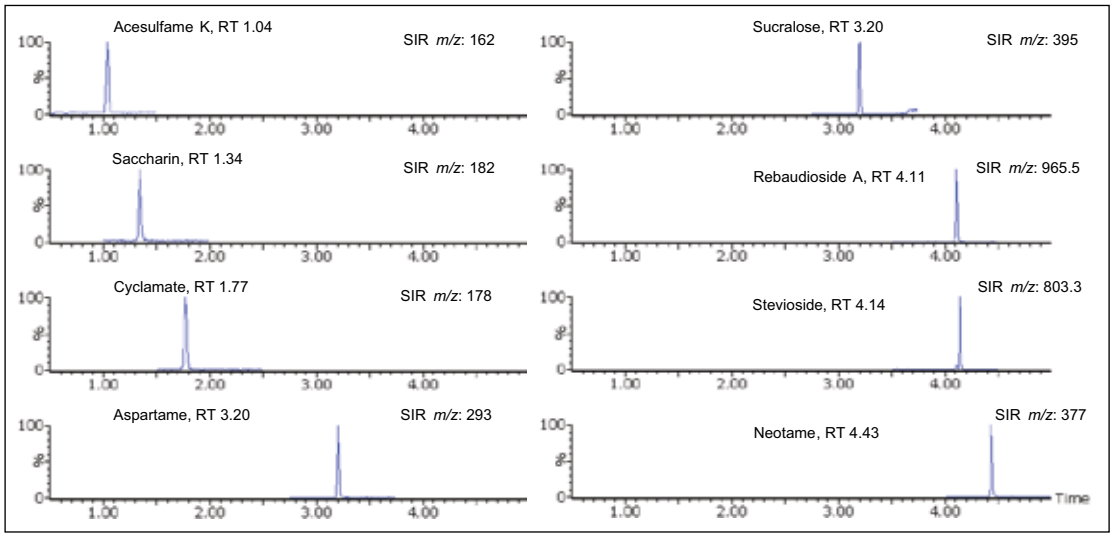

Figure 2. SIR chromatograms of all sweeteners in a solvent standard.

Example calibration curves of two of the co-eluting sweeteners are shown in Figure 3. Even though the compounds are not chromatographically resolved, mass detection offers the selectivity that enables accurate quantification results. The discrimination offered by mass detection further eliminated tedious method development and/or method optimization steps that would be necessary to attempt to obtain baseline separation of the co-eluting components.

Mass detection offers the opportunity to distinguish between sweeteners that cannot be not detected by UV due to the lack of a chromophore group in the structures. Even though cyclamate and sucralose are both UV transparent compounds, their detection can be easily achieved with the ACQUITY QDa Mass Detector. Figure 4 shows the SIR chromatograms of cyclamate (RT 1.77) and sucralose (RT 3.20), along with the UV channel at 254 nm at 5 mg/L.

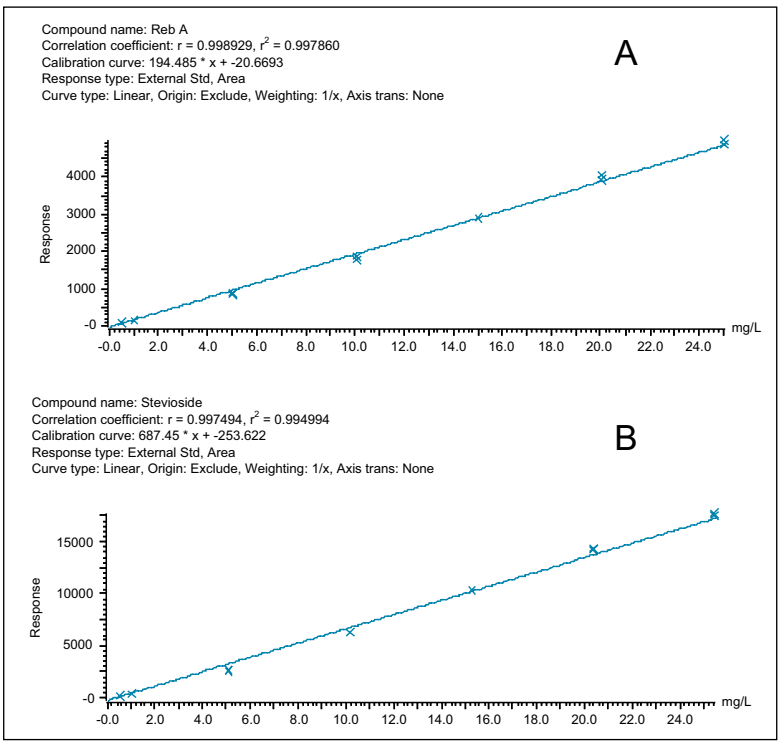

Figure 3. Calibration curve of co-eluting pairs: A. rebaudioside, and B. stevioside.

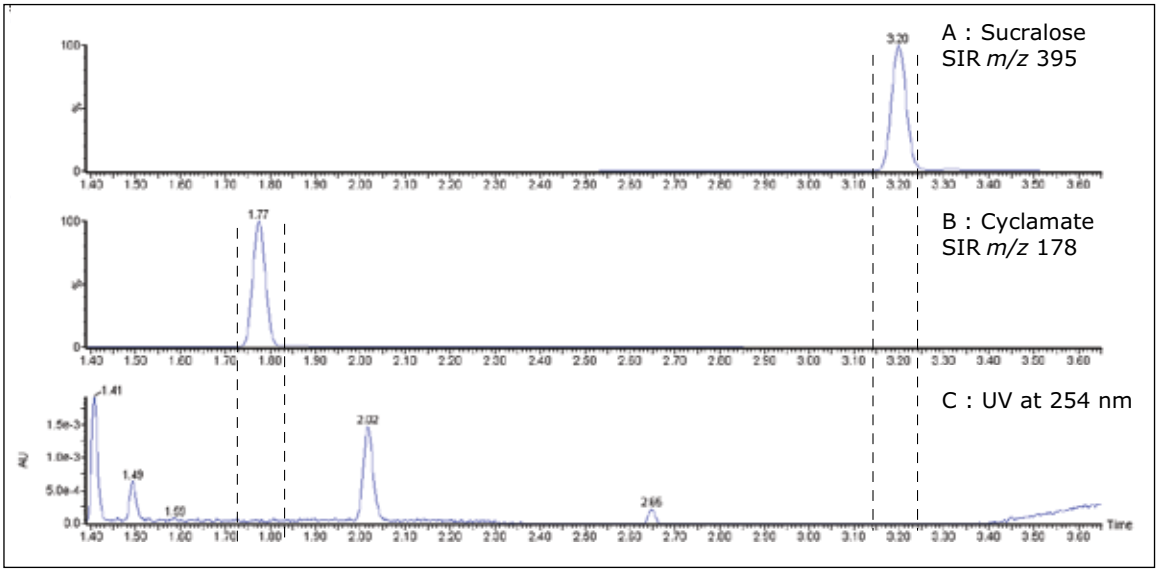

Figure 4. SIR chromatogram of A. sucralose, B. cyclamate, and C. UV chromatogram at 254 nm of a standard mix at 5 mg/L.

Calibration curves were created for the target analytes. Table 3 lists the optimum calibration range, correlation coefficient, and curve fit used for each of the compounds studied. The correlation constant (R^2) was >0.995 for all of the sweeteners.

ANALYSIS OF SAMPLES

The seven samples were prepared as previously described, and analyzed in duplicate. Table 4 shows the quantification results of all seven samples. The first table-top sweetener contained acesulfame K (98 mg/kg), aspartame (15,080 mg/kg), and saccharin (3798 mg/kg). The second table-top sweetener was found to contain rebaudioside A (3105 mg/kg) and stevioside (630 mg/kg). With the discrimination and sensitivity of mass detection it was easily apparent that both of these co-eluting sweeteners were present in the sample, even though one was present at a much lower level and was not labeled to be present. Figure 5 shows the SIR chromatograms of rebaudioside A and stevioside in a solvent standard and in the second table-top sweetener.

Aspartame was detected in the diet cola and diet tea at 30 mg/L and 489 mg/L respectively. Sucralose was found in the hard candy (145 mg/kg) and in the marmalade (297 mg/kg). The amount of sucralose found in the marmalade was within the EU Commission Regulation 1129/2011.

Analyte	Calibration range (mg/L)	Correlation coefficient (R^2)	Curve fit
Acesulfame K	0.02–2.5	0.999	Quadratic, 1/X weighting
Aspartame	0.2–25.0	0.998	Quadratic, 1/X weighting
Cyclamate	0.2–25.0	0.999	Quadratic, 1/X weighting
Neotame	0.2–25.0	0.995	Quadratic, 1/X weighting
Rebaudioside A	0.5–25.0	0.998	Linear, 1/X weighting
Saccharin	0.02–2.5.0	0.999	Quadratic, 1/X weighting
Stevioside	0.5–25.0	0.995	Linear, 1/X weighting
Sucralose	0.2–25.0	0.998	Quadratic, 1/X weighting

Table 3. Calibration range, correlation coefficient values and curve fit (R^2) for the eight sweeteners.

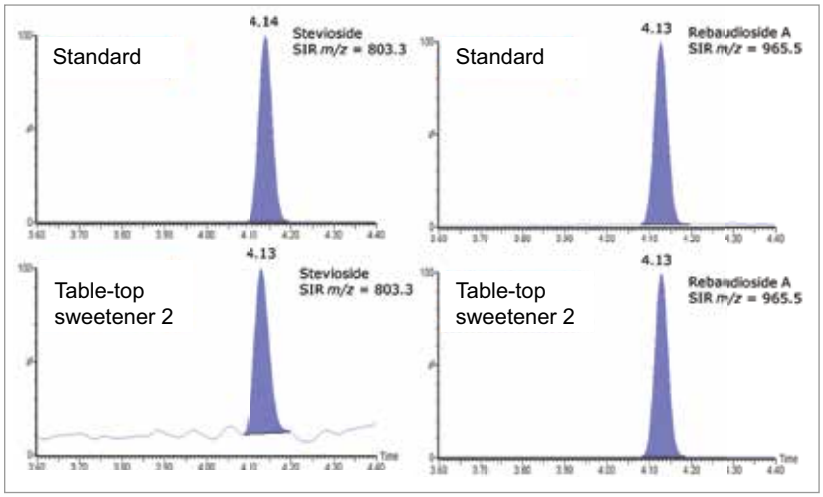

Figure 5. SIR chromatogram of stevioside and rebaudioside A in solvent and sample (table-top sweetener 2).

Sample	Acesulfame K	Aspartame	Rebaudioside A	Saccharin	Stevioside	Sucralose	Unit
Diet cola	30	480	–	–	–	–	mg/L
Candy	–	–	–	–	–	145	mg/kg
Diet tea	–	379	–	–	–	–	mg/L
Marmalade	–	–	–	–	–	297	mg/kg
Pudding	208	–	–	–	-	168	mg/kg
Table-top sweetener 1	98	15080	–	3798	–	–	mg/kg
Table-top sweetener 2	–	–	31050	–	630	–	mg/kg

Table 4. Quantification results of the seven samples tested.

CONCLUSIONS

This application note has described a method for the separation, detection, and quantification of natural and artificial non-nutritive sweeteners in less than 10 minutes using the ACQUITY UPLC H-Class System coupled with the ACQUITY QDa Mass Detector.

The ACQUITY QDa Mass Detector provides single method to analyze both UV-transparent and non-transparent sweeteners, and this method can be incorporated into existing workflows – with or without UV detection. Mass detection provides a much higher level of analyte discrimination and it does not require baseline separation of analytes with different masses. This helps to minimize method development times and removes the need for separate injections of individual standards to verify compound identifications.

Reference

1. D J Yang, B Chen. Simultaneous determination of non-nutritive sweeteners in foods by HPLC/ESI-MS. *J Agric Food Chem.* 57(8): 3022–3027, 2009.

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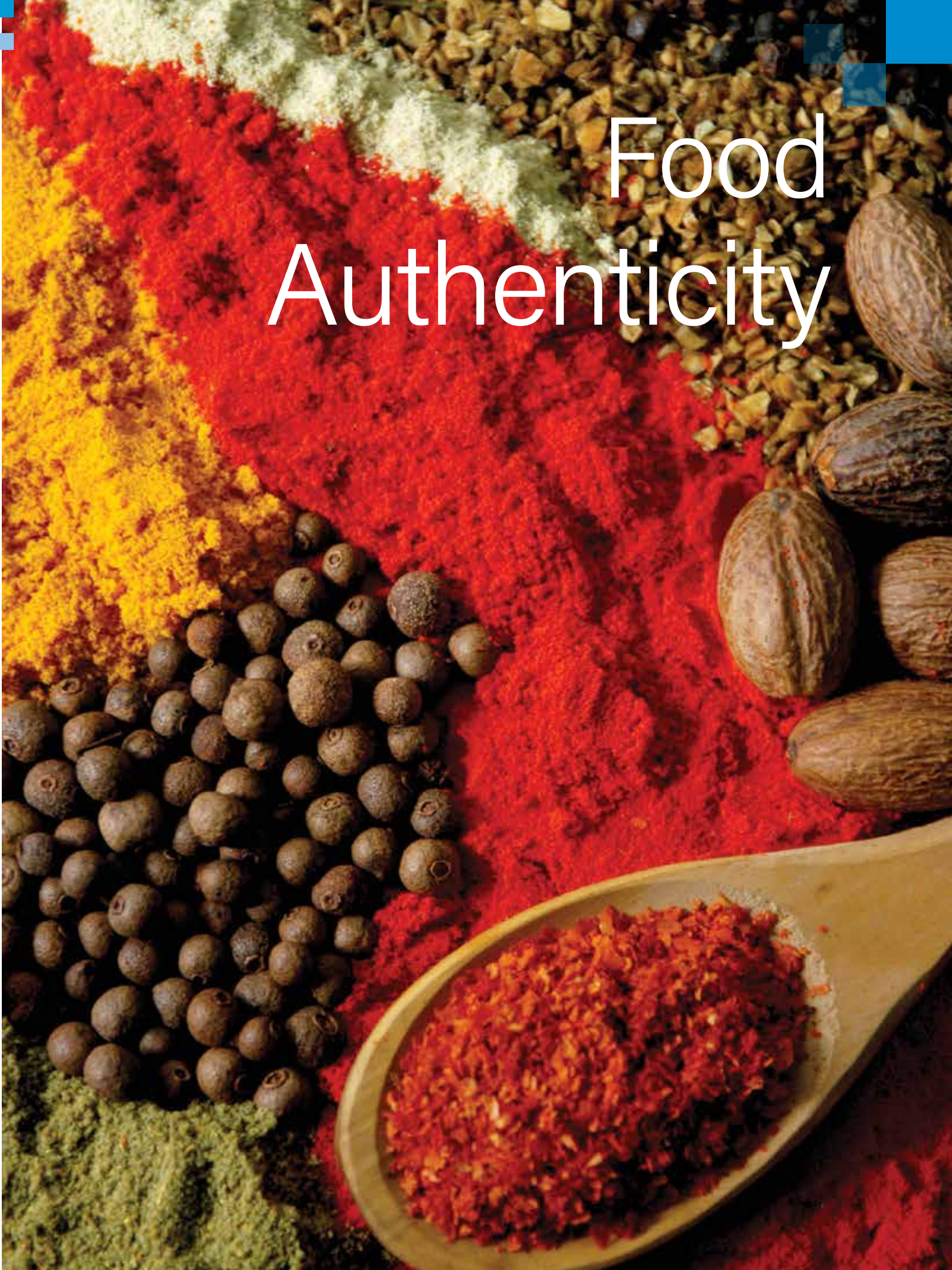
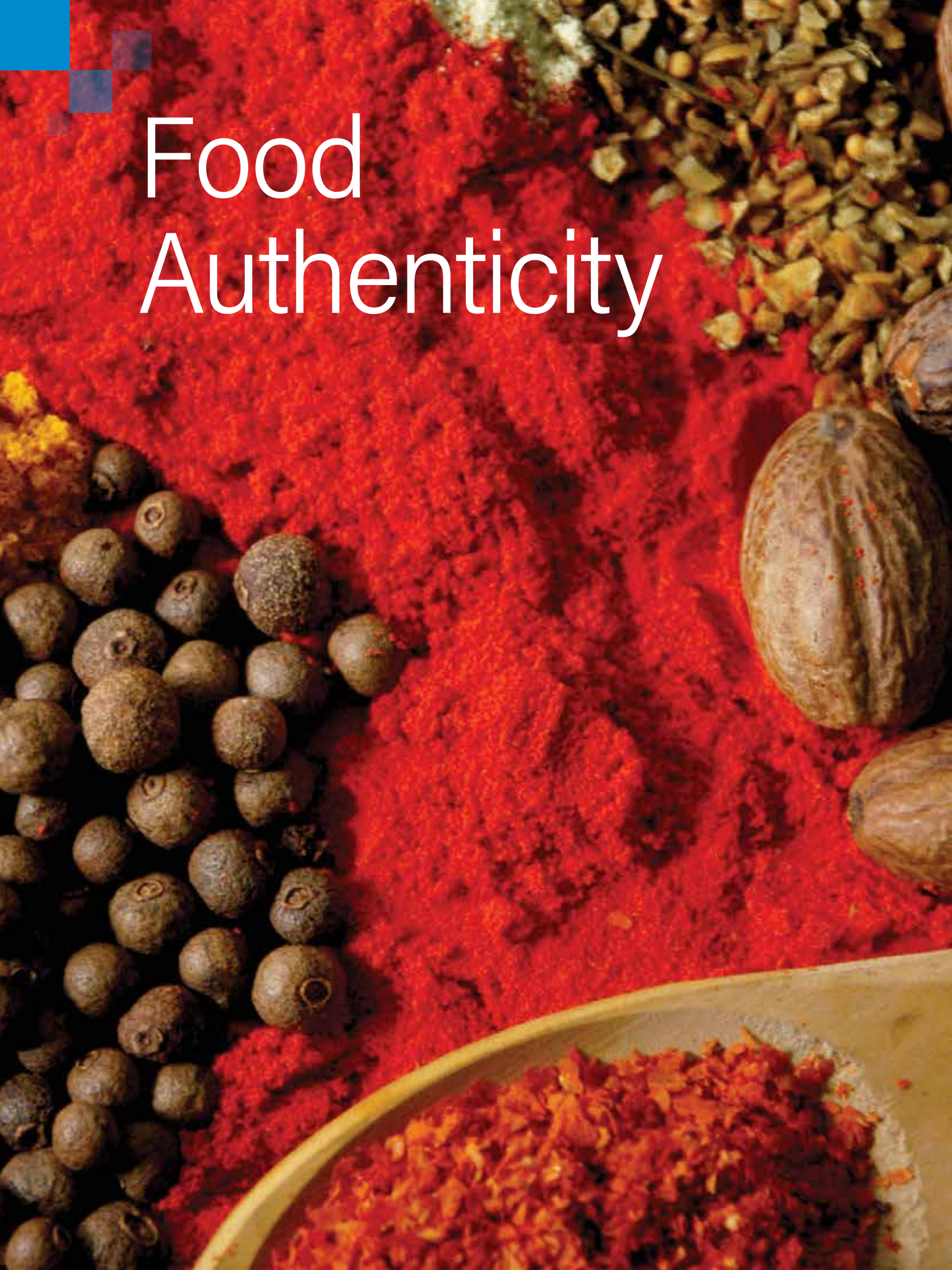

Food
Authenticity

Food Authenticity



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Rapid Profiling and Authentication of Cinnamon Samples Using Ambient Ionization (DART) and Single Quadrupole Mass Spectrometry

Kari Organtini and Gareth Cleland
Waters Corporation, Milford, MA, USA

AIM

Demonstrate the utility and ease-of-use of DART coupled to the ACQUITY® QDa® Detector for the authentication of ground cinnamon and cinnamon sticks based on *Cinnamomum* species.

APPLICATION BENEFITS

Using direct analysis of samples in combination with simple mass detection allows:

- Rapid screening
- Ambient ionization
- Little to no sample preparation
- No chromatography needed
- Ease of use
- Spice profiling

WATERS SOLUTIONS

ACQUITY QDa Detector

MassLynx® MS Software

KEYWORDS

Spices, cinnamon, *C. verum*, coumarin, *C. cassia*, *C. burmannii*, *C. loureiroi*, mass detection, authenticity, DART, ambient ionization, spice profiling, adulteration

INTRODUCTION

Cinnamon is a popular spice used for cooking and baking, plus is also used as an herbal medicine. Cinnamon is derived from the dried inner bark of the *Cinnamomum* tree. There are four species of cinnamon commercially sold for consumption: *C. verum* (Ceylon), *C. cassia* (Chinese), *C. burmannii* (Korintje), and *C. loureiroi* (Saigon). Each variety has a distinct flavor and aroma profile resulting from the essential oil in the cinnamon tree bark.

Due to its popularity, cinnamon is one of the most commonly adulterated spices on the market. *C. verum* is considered the "true cinnamon" and it is more expensive than the other varieties. This leads to adulteration of cinnamon, or even substitution with a lower priced variety.

While there are subtle taste and odor differences between the species of cinnamon, there is an important difference in chemical makeup of the cinnamon varieties. *C. verum* is known to be the only species of cinnamon to naturally contain low levels of coumarin.[1] Coumarin is a compound of concern as it is regarded as a hepatotoxic compound. It produces a sweet smell that made it a popular food additive before it was banned due to its potential toxicity. Therefore substitution of *C. verum* with *C. cassia*, *C. burmannii*, and *C. loureiroi* cinnamon can be concerning.

Typically, analysis to determine cinnamon species is performed using a variety of methods that include sample extraction and chromatographic separation steps. Direct Analysis in Real Time (DART) is an ambient ionization technique[2] that eliminates the needs for time-consuming sample preparation and chromatographic separation. In this application note, we describe a novel approach that employs DART and Waters® ACQUITY QDa Detector for the simple and rapid analysis of cinnamon samples to determine species origin. Whole stick and ground cinnamon samples were successfully analyzed using this DART-MS technique and species identifications on store bought cinnamon samples are provided.

EXPERIMENTAL

DART conditions

Ionization mode:	+
Temp.:	450 °C
Sampling speed:	1.0 mm/sec
Grid voltage:	350 V

MS conditions

MS system:	ACQUITY QDa (Performance Option)
Ionization mode:	+
Cone voltage:	5 V
Mass range:	50 to 500 amu
Acquisition speed:	2 Hz

Sample analysis

A variety of cinnamon samples were purchased from local retailers. Cinnamon of varying price points were acquired for this sample analysis. Ground cinnamon (12 samples) and whole cinnamon sticks (3 samples) were tested. By examining the labels and researching manufacturers websites, it was determined that known samples of *C. verum*, *C. cassia*, *C. burmannii*, and *C. loureiroi* were available in the ground cinnamon testing group. However, eight of the remaining ground cinnamon samples were vague on their label claims, and as a result were treated as unknown samples during the analysis.

Cinnamon sticks were broken into smaller pieces prior to analysis and held in front of the heated helium DART beam with a pair of tweezers to perform sampling. The ground cinnamon samples were introduced onto the QuickStrip cards (see Figure 1) by dipping a cotton swab into the sample and then rubbing the swab over the mesh screen of the card's sampling area. Most of the ground cinnamon fell through the screen, but enough of a residue was left behind for analysis. Sampling in this manner maintained the cleanliness of the ACQUITY QDa source.

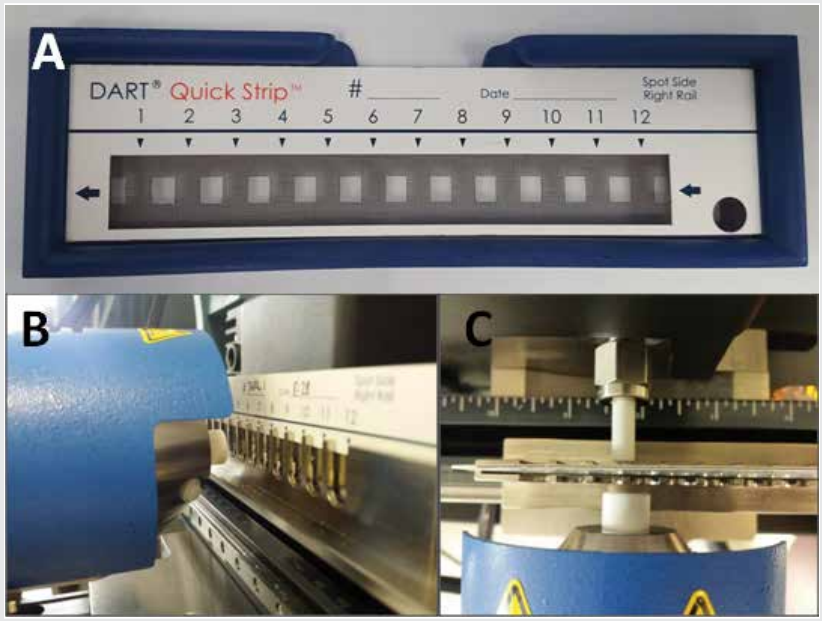

Figure 1. A. 12 spot QuickStrip card used for sampling; B. QuickStrip card automatically being moved into the heated helium ionization beam; and C. Ceramic tube pulling ions into the ACQUITY QDa source (top down look at the DART source).

RESULTS AND DISCUSSION

CINNAMON STICK ANALYSIS

Of the three cinnamon stick samples available for analysis, one was of the *C. verum* species, and two were of the *C. burmannii* species. Representative mass spectra of each species are presented in Figure 2. The spectra are not complex, but the zoomed insets of Figure 2 highlight the important features. The distinguishing feature between the two species analyzed in stick form is the ratio of cinnamaldehyde (*m/z* 133) to coumarin (*m/z* 147). *C. verum* is known to contain very small levels of coumarin, which was verified by this analysis. In contrast, *C. burmannii* is known to contain high levels of coumarin, again verified by the DART-MS analysis of the cinnamon stick.

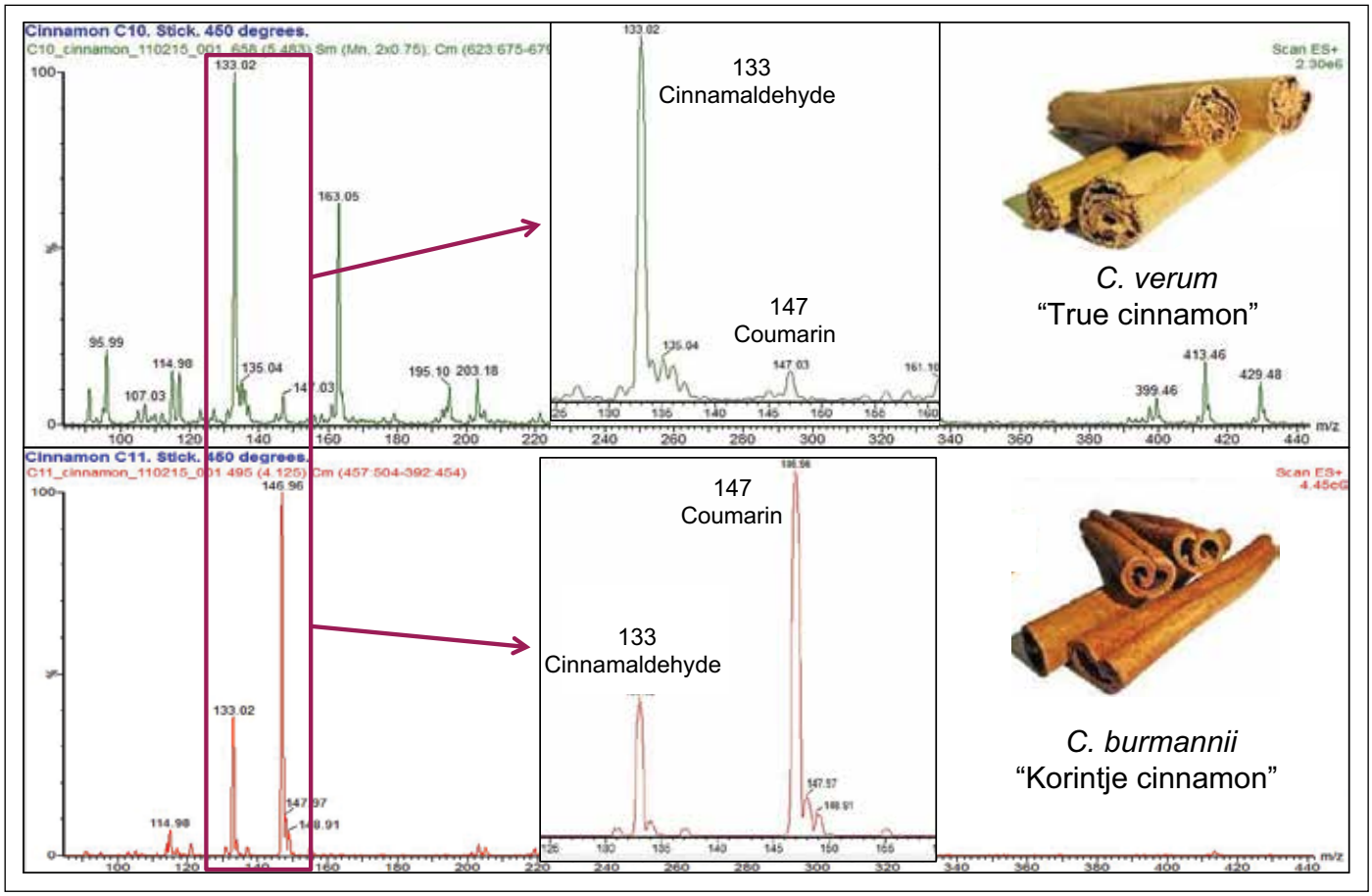

Figure 2. DART-MS analysis of whole cinnamon sticks. Mass spectrum for C. verum (top); mass spectrum for C. burmannii (bottom).

GROUND CINNAMON ANALYSIS

Analysis of ground samples of the different cinnamon species was also performed to determine if the ground spices could be distinguished by species as well. Ground spice analysis was performed by rubbing the sample onto a 12 position QuickStrip card. Analysis of the entire 12 sample card was performed in less than 5 minutes. Among the 12 ground cinnamon samples purchased, four were labeled with the derived cinnamon species. The remaining samples were treated as unknowns. Similar to the cinnamon stick analysis, the mass spectra were not complex, although there were distinguishing features present. Figure 3 shows the spectra obtained from the DART-MS analysis of the four known ground cinnamon samples. The Ceylon sample contained strong cinnamaldehyde and methyl cinnamate (*m/z* 163) peaks, but did not contain the presence of coumarin. The *C. burmannii* and *C. loureiroi* species both contained coumarin as the dominant peak, a smaller cinnamaldehyde peak, and only the *C. loureiroi* contained substantial amounts of methyl cinnamate. The *C. cassia* sample contained cinnamaldehyde as the dominant peak, and it also contained smaller levels of coumarin and methyl cinnamate. The DART-MS analysis allowed the unique characteristics for the four ground cinnamon samples to be distinguished from one another.

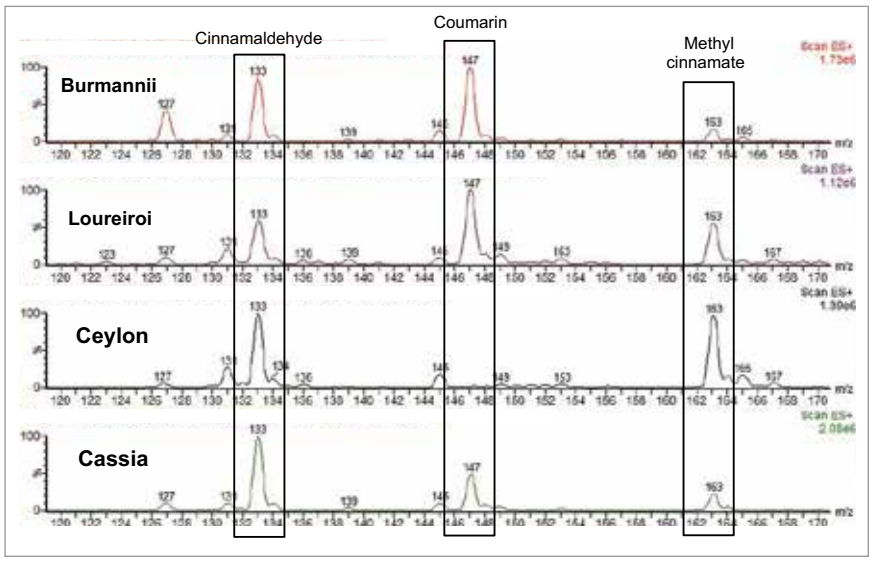

Figure 3. DART-MS analysis of four known ground cinnamon species.

Table 1 lists the species identifications made using the DART-MS analysis for all of the cinnamon samples. Of the 12 ground cinnamon samples tested, besides the sample used as the known high quality reference sample, only one other ground cinnamon indicated it may contain *C. verum* (Ceylon). This was sample C5, and it was identified as a "Ceylon mix" because the mass spectra contained large cinnamaldehyde and methyl cinnamate peaks, in agreement with Ceylon cinnamon, but it also contained a mid-sized coumarin peak. Since Ceylon cinnamon is the only species to contain high levels of methyl cinnamate, this suggests that this sample could be a mix of Ceylon cinnamon and a cheaper coumarin containing cinnamon. The species of the remainder of the ground cinnamon samples were easily identified based upon the characteristics of their mass spectra.

Sample	Form	Species Identification
C1	**ground**	*C. burmannii*
C2	ground	*C. burmannii*
C3	ground	*C. cassia*
C_4*	ground	*Ceylon*
C5	ground	*Ceylon mix*
C_6*	ground	*C. Loureiroi*
C7	ground	*C. burmannii*
C_8*	ground	*C. burmannii*
C9	ground	*C. burmannii*
C10*	stick	*Ceylon*
C11*	stick	*C. burmannii*
C12*	stick	*C. burmannii*
C13	ground	*C. cassia*
C14	ground	*C. burmannii*
C15*	ground	*C. cassia*

*Table 1. Identifications of the unknown cinnamon samples using DART-MS analysis. * Indicates that the species of the sample was known prior to analysis, as determined from manufacturer information.*

CONCLUSIONS

The cinnamon analysis demonstrated that DART coupled to the ACQUITY QDa Detector can be applied to the analysis of both whole and ground spice samples. The analysis of up to 12 samples can be performed in less than 5 minutes, providing a rapid screening technique. DART-MS analysis was also able to determine the unique characteristics between the known reference samples that could be used to easily and rapidly distinguish cinnamon species of the unknown samples. This technique could easily be utilized for authenticity or food safety applications.

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Rapid Screening of Fatty Acids in Oil Supplements Using Ambient Ionization (DART) and Mass Detection

Kari Organtini, Giuseppe Astarita, and Gareth Cleland
Waters Corporation, Milford, MA, USA

AIM

Demonstrate the utility and ease-of-use of DART coupled to single quadrupole mass detection for the identification and rapid authentication of omega-3 and omega-6 polyunsaturated fatty acids in oil supplements.

APPLICATION BENEFITS

Using direct ambient ionization of samples in combination with mass detection allows:

- Rapid screening
- Minimal sample preparation
- No chromatography needed
- Ease of use
- Perform authenticity experiments
- Sample profiling

WATERS SOLUTIONS

ACQUITY® QDa® Detector
MassLynx® MS Software

KEYWORDS

Fatty acids, PUFA, supplements, mass detection, authenticity, Direct Analysis in Real Time, DART, ambient ionization

INTRODUCTION

Polyunsaturated fatty acid (PUFA) oils are popular dietary supplements due to the many health benefits associated with their consumption. Omega-3 fatty acids have been shown to lower the risk of cardiovascular diseases and reduce inflammation, whereas high intake of omega 6 fatty acids has been linked to increased inflammation. Therefore, balancing the omega-3 to omega-6 ratio is important, but the Western diet is known to be high in omega-6 fatty acids. This imbalance promotes the use of dietary oil supplements.

Fish oils provide a source of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) omega-3 PUFAs, whereas plant based oils provide a source of alpha linolenic acid (ALA) as its omega-3 PUFA. With PUFA supplements being such a large part of the market, it is important to monitor adulteration and fatty acid sources of these supplements to protect consumers.

Typically, fatty acid analysis is performed using GC-FID instrumentation, requiring sample derivatization to the methyl or ethyl ester forms prior to analysis.[1] The derivatization process takes approximately 35 minutes, and the GC method takes close to an hour.

Direct Analysis in Real Time (DART) is an ambient ionization technique that allows sample analysis to be performed in minutes.[2] DART analysis benefits from the elimination of the need for time-consuming sample preparation and chromatography, making the analyses very rapid. Samples can be deposited on a screen that is moved through a heated ionizing helium beam in an automated fashion. Resulting ions are typically of the $[M+H]^+$ or $[M-H]^-$ nature. Coupling DART to Waters® ACQUITY QDa Detector allows the entire system to remain compact and easy to operate. This allows the DART-MS system to be operated outside a typical laboratory space if desired to generate mass spectral information.

EXPERIMENTAL

DART conditions

Ionization mode:	–
Temp.:	200 °C
Sampling speed:	0.5 mm/sec °C
Grid voltage:	-350 V

MS conditions

Ionization mode:	–
Cone voltage:	15 V
Mass range:	50 to 1000 amu in full scan mode
Sampling frequency:	2 Hz

Selected Ion Recording (SIR) *m/z* values for the deprotonated fatty acids $[M-H]^-$ are listed in Table 1.

Sample analysis

The PUFA standard mix was obtained from Cayman Chemicals. Fatty acid supplement capsules were obtained commercially and the known amounts of omega fatty acids in the capsules were determined from the nutritional facts label on the bottle. Both standards and supplements were diluted in toluene. To collect the oil from the capsules, the capsule shell was cut with a razor blade and a pipette was used to transfer the oil from the capsule into a new vial. Prior to analysis, the oil from the supplements was diluted 1:50 in toluene.

DART-MS analysis was performed by spotting 5 µL of sample onto QuickStrip cards and allowing the solvent to evaporate prior to analysis (see Figure 1). The helium beam used for ionization was heated to 200 °C, the temperature previously determined as the optimal ionization temperature for the set of fatty acids studied in these samples.

Fatty acid	Abbreviation	SIR *m/z*
Palmitic acid*	PA	255.2
Stearidonic acid	SA	275.2
Linolenic acid (alpha* and gamma isomers)	ALA/GLA	277.2
Linoleic acid*	LA	279.2
Oleic acid*	OA	281.2
Eicosapentaenoic acid*	EPA	301.2
Arachidonic acid	AA	303.2
Di-homo-γ-Linolenic acid	DGLA	305.2
Docosahexaenoic acid*	DHA	327.2
Docosapentaenoic acid	DPA	329.2
Adrenic acid	–	331.3

Table 1. Masses monitored using the ACQUITY QDa Detector in SIR mode.
** Denotes fatty acids expected to be present in the oil capsule supplements.*

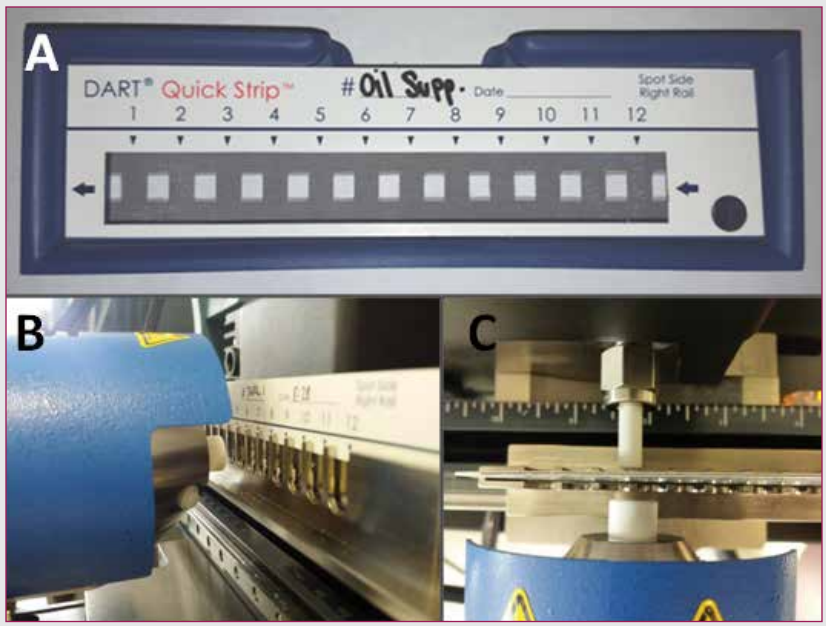

Figure 1. Images depicting A: the 12 spot QuickStrip card used for sampling; B. the QuickStrip card automatically being moved into the heated helium ionization beam; and C. the ceramic tube pulling ions into the QDa source (top down look at the DART source). The sample is applied to the QuickStrip card on the mesh screen in between each white open square.

RESULTS AND DISCUSSION

METHOD DEVELOPMENT

During method development of the DART analysis, ionization efficiency was determined to be temperature dependent. Shorter chain fatty acids preferred a lower ionization temperature compared to longer chain fatty acids. For this study, only a select range of fatty acids were stated to be present in the oil supplements tested (*m/z* range 255 to 327), so the DART method was optimized for this range of fatty acids. Response for the fatty acids in this range present in the standard mix appeared to be comparable at an ionization temperature of 200 °C, as shown in Figure 2. Stearidonic acid appeared to not have as an efficient ionization at 200 °C compared to the other PUFAs in the mix. Although stearidonic acid was in the PUFA standard mix used for method development, it was not a component of the oil supplements tested.

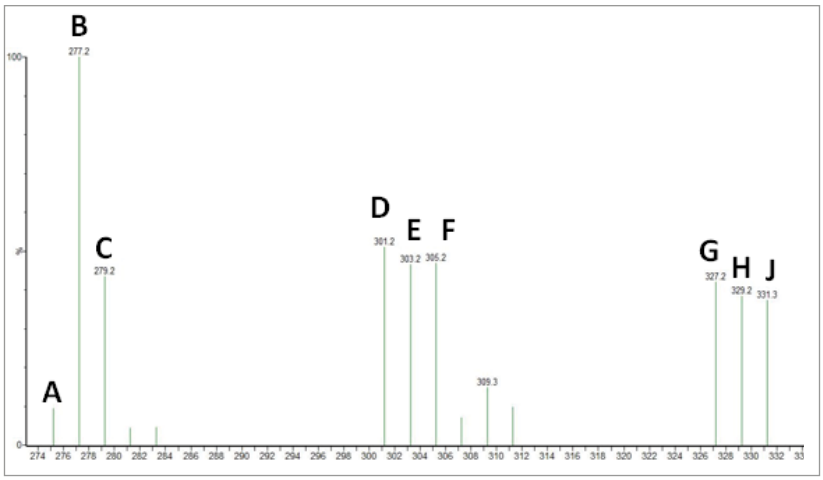

Figure 2. Mass spectrum of the fatty acid standard mix. Peaks are identified as the following: A. SA; B. ALA and GLA; C. LA; D. EPA; E. AA; F. DGLA; G. DHA; H. DPA; and J. adrenic acid. Peak B is expected to have twice the response of the other fatty acids in the mix because it represents both the alpha and gamma isomers of linolenic acid.

REPRODUCIBILITY OF METHOD

Reproducibility of the method was also tested, as shown in Figure 3. The fatty acid standard mix was spotted 10 times on a QuickStrip sampling card for replicate analysis. Figure 3 shows the extracted ion for EPA (*m/z* 301.2). Each peak represents one spot on the QuickStrip card, which have been integrated to compare the peak areas. The repeatability of the method is also demonstrated in Table 2 which compares the expected and experimental percentage of each fatty acid in the mix. With the exception of stearidonic acid, the experimentally determined percentage of the fatty acids compared favorably with the expected percentage.

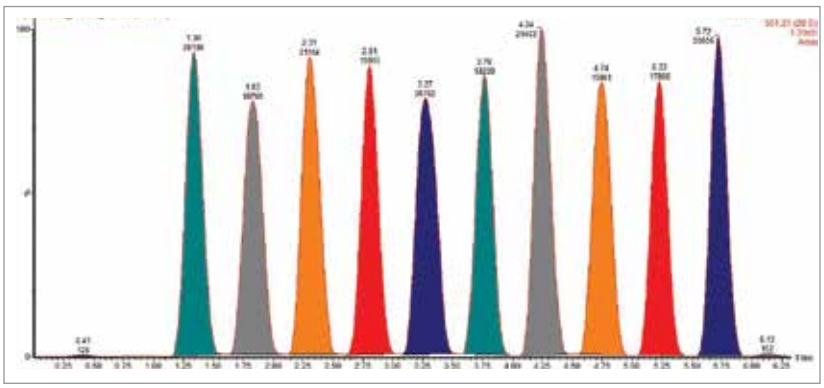

Figure 3. Chromatogram of DART-MS analysis of 10 replicate QuickStrip spots containing the same mixture of fatty acid standards demonstrating reproducibility. Showing extracted SIR for fatty acid EPA.

Fatty acid	Expected % composition	Experimental % composition
Stearidonic	**10**	**2.4 ± 0.2**
α-Linolenic/γ-Linolenic	20	20.1 ± 2.1
Linoleic	10	11.1 ± 1.1
Eicosapentaenoic	10	12.2 ± 0.6
Arachidonic	10	12.1 ± 0.9
Di-homo-γ-Linolenic	10	10.3 ± 0.7
Docosahexaenoic	10	11.8 ± 0.9
Docosapentaenoic	10	10.5 ± 0.6
Adrenic	10	9.6 ± 0.5

Table 2. Comparison of the expected and experimental (n=10) composition of fatty acids present in the standard mix.

ANALYSIS OF OIL SUPPLEMENTS

Fish, flax seed, and safflower oil supplements were analyzed using the DART-MS method developed. Each sample was analyzed in triplicate. The expected percentage of each fatty acid present in each oil supplement was determined from the nutritional facts listed on the bottle. The experimental percentage was determined based upon the peak areas from extracted SIRs of each compound. The results are detailed in Table 3. All three oil supplements compared quite well with the expected percentage of each fatty acid, with the fish oil supplement comparing the closest. It is important to keep in mind that these are commercially obtained supplements so it is not known if the true contents differ from those listed on the bottle label.

	Fish oil		Safflower oil		Flax seed oil	
	Expected %	**Experimental %**	**Expected %**	**Experimental %**	**Expected %**	**Experimental %**
EPA	60	59				
DHA	40	41				
ALA					67	57
LA			63	55	16	21
OA			22	33	16	22
PA			15	11		

Table 3. Expected and experimental (n=3) percentage of each omega fatty acid present in each oil supplement tested. Expected percentage was interpreted from the nutritional label.

Figure 4 provides a visual depiction of the fatty acid contents of the oil supplements tested. Similarly to Figure 3, each peak in the chromatograms is represented by a single spot on the QuickStrip card. The first three spots on the card were fish oil, followed by three safflower oil spots, and finally three flax seed oil spots. A blank spot was left in between each type of oil to monitor if there was any carryover between spots on the card. Each chromatogram represents the EIC of a different fatty acid. Figure 4 shows that the fatty acids indicated on the bottle label were present in each sample, but that fish oil and flax seed oil both contained an extra fatty acid. The fish oil sample indicates that oleic acid is also present in the sample, but it is not listed as a component of the supplement, and fish is not typically a source of this omega-9 fatty acid. However, the ingredients do indicate that there is soy oil present in the supplement to provide tocopherols to preserve freshness. Soy is a known source of oleic acid, and therefore could account for its presence in the supplement. The flax seed oil supplement indicates the presence of DHA, an omega-3 fatty acid that is derived from a fish source, not plant based. It is unlikely that this is a result of carryover happening from the fish oil supplement as there is no DHA present in the safflower oil that was sampled in between the fish and flax seed oils. It is unclear the source of the DHA present in the flax seed oil, but it indicates possible adulteration, or more likely, contamination during the manufacturing process.

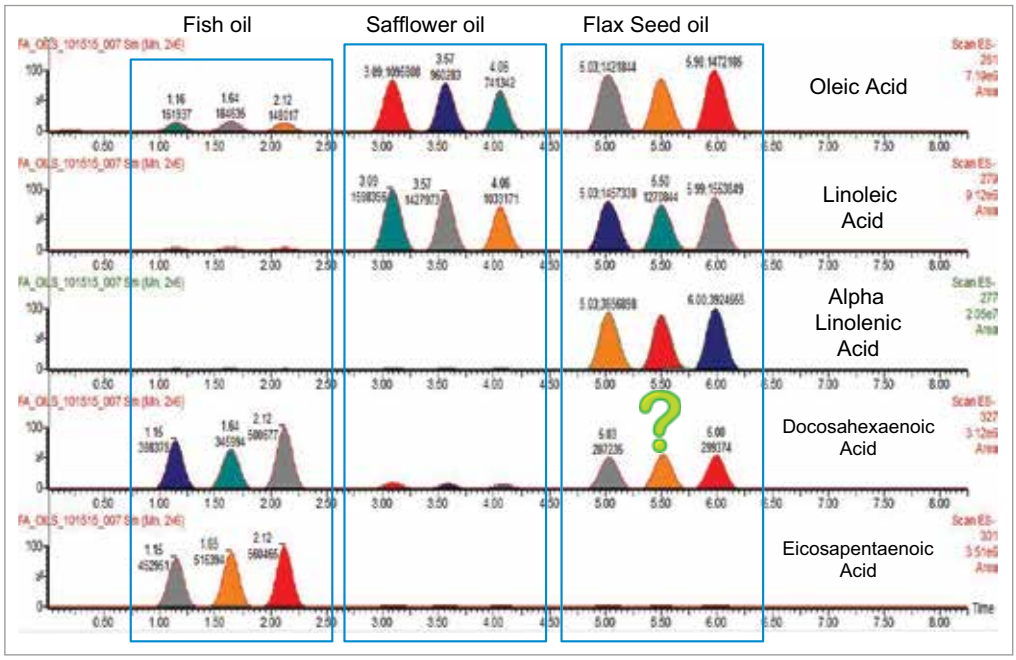

Figure 4. Extracted SIR chromatograms showing the fatty acids present in each of the oil supplement samples (n=3).

CONCLUSIONS

DART-MS analysis allowed for rapid screening of PUFA supplements with sample dilution as the only sample preparation step needed prior to analysis. Using this method, up to 12 samples can be analyzed in approximately 6 to 7 minutes. Through the use of known standards, the DART-MS method was determined to be accurate and reproducible.

The method was successfully tested on the analysis of three different fatty acid oil supplements (fish, safflower, and flax seed) to determine how the levels of each fatty acid compared to those reported on the ingredient label. The experimentally determined levels corresponded well with the reported levels in all three oil supplements. The supplement analysis also indicated an incongruity, which was the presence of DHA in the flax seed oil sample.

The DART and ACQUITY QDa mass detection system has the potential for a variety of applications in respect to analysis of oils. It is a quick and easy technique that eliminates the lengthy sample preparation needed for analysis of samples via GC-MS. Notably, DART-MS allows the system to be operated outside a typical laboratory space for quality control monitoring during manufacturing, or as a technique to rapidly identify adulterated samples on the market.

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Beverage Analysis

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Soft Drink Analysis on an ACQUITY Arc System

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Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

Soft Drink analysis using the ACQUITY® Arc™ System, along with Waters® Beverage Mobile Phase, XBridge® Phenyl Column Chemistry, and a choice of Waters Beverage Standards, or Waters Big-4 Standard concentrate provides multiple benefits such as:

- Ethanol-based, ecologically friendly mobile phase.
- Choice of a single-point calibration suitable for lower levels of analytes common to most soft drinks, or a multi-point calibration especially suited to the higher levels of caffeine found in energy drinks when saccharin and aspartame are not required.
- Dual wavelength monitoring to avoid known co-eluting compounds.
- Use of 2.5 micron particle size columns at system pressures up to 9500 psi.
- Dual flow paths to emulate HPLC or UHPLC separations.

WATERS SOLUTIONS

ACQUITY Arc System

2489 UV/Visible (UV/Vis) Detector

ACQUITY QDa® Mass Detector

XBridge Phenyl ***XP*** Column

Beverage Mobile Phase

Beverage Analysis Standard

Big-4 Calibration Stock Standard

KEYWORDS

Soft drink, LC, non-nutritive sweetener, acesulfame K, saccharin, caffeine, aspartame, benzoate, sorbate, beverage

INTRODUCTION

The soft drink market is an important revenue source for many major food and beverage producers. Such beverages include traditional carbonated soft drinks, high energy drinks, and recently popular healthy formulations such as vitamin waters and teas.

These products often contain caffeine as a stimulant, benzoate, and sorbate as preservatives; and for diet preparations, non-nutritive sweeteners such as acesulfame K, aspartame, and saccharin.

For quality control purposes the conformance of target concentrations of analytes to specified ranges is critical. In this application note, we show that the Waters ACQUITY Arc System, XBridge Phenyl ***XP*** Columns, along with Waters Beverage Mobile Phase, can separate these compounds in under 10 minutes.

Figure 1. ACQUITY Arc System.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY Arc
Runtime:	10.0 min
Column:	XBridge Phenyl ***XP*** 2.5 µm, 4.6 x 50 mm
Column temp.:	35 °C
Mobile phase:	Waters Beverage Mobile Phase
Flow rate:	1.0 mL/min
Flow path:	1 (HPLC emulation)
Injection volume:	5 µL
Detector:	2489 UV/Visible (UV/Vis)
Detection:	214, 247 nm

Standard preparation

A: Beverage analysis standard (single point)

One bottle of Waters Beverage Analysis 5 Standards Solution, p/n 186006008, was poured into one bottle of Waters Beverage Analysis Standards Solid (aspartame), p/n 186006010, and mixed until all of the aspartame was dissolved. This resulted in a standard with a concentration of 150 mg/L acesulfame K, 100 mg/L saccharin, 200 mg/L benzoate, 100 mg/L sorbate, 100 mg/L caffeine, and 500 mg/L aspartame.

B: Standard concentrate (multi-point)

Waters Big-4 Calibration Stock Standard, p/n 186007980, (1000 mg/L each of acesulfame K, benzoate, sorbate, and caffeine) was diluted in water to produce eight separate levels with concentrations listed in Table 1.

Std	Big 4 analytes (mg/L)
1	500
2	400
3	200
4	100
5	50
6	40
7	20
8	10

Table 1. Standard amounts for the Big-4 analytes: acesulfame K, benzoate, sorbate, and caffeine.

Sample preparation

Carbonated beverages were sonicated to remove carbon dioxide. All beverages were filtered through a 0.2-µm PVDF filter, then injected.

RESULTS AND DISCUSSION

Chromatograms resulting from separation of beverage standards using the ACQUITY Arc System with an XBridge Phenyl ***XP*** Column are shown in Figure 2. Depending on the requirements of the analysis, there are two different options for the standards. The first standard, shown in Figure 2A, is the Waters Beverage Standard. This standard contains (in order of elution): acesulfame K, saccharin, benzoate, sorbate, caffeine, and aspartame. Each component has a different UV absorbance spectrum so that the peak height ratios are different as a function of the wavelength. As can be seen from the chromatogram in Figure 2A, two different wavelengths can be programmed and monitored in the same run. In Figure 2A and 2B chromatograms for both 214 nm and 247 nm are shown. Figure 2B shows the chromatograms for the Big-4 Standard, which has the same analytes as the Waters Beverage Standard, with the exception of saccharin and aspartame. These two sweeteners are not required for the analysis of beverages that do not use these ingredients.

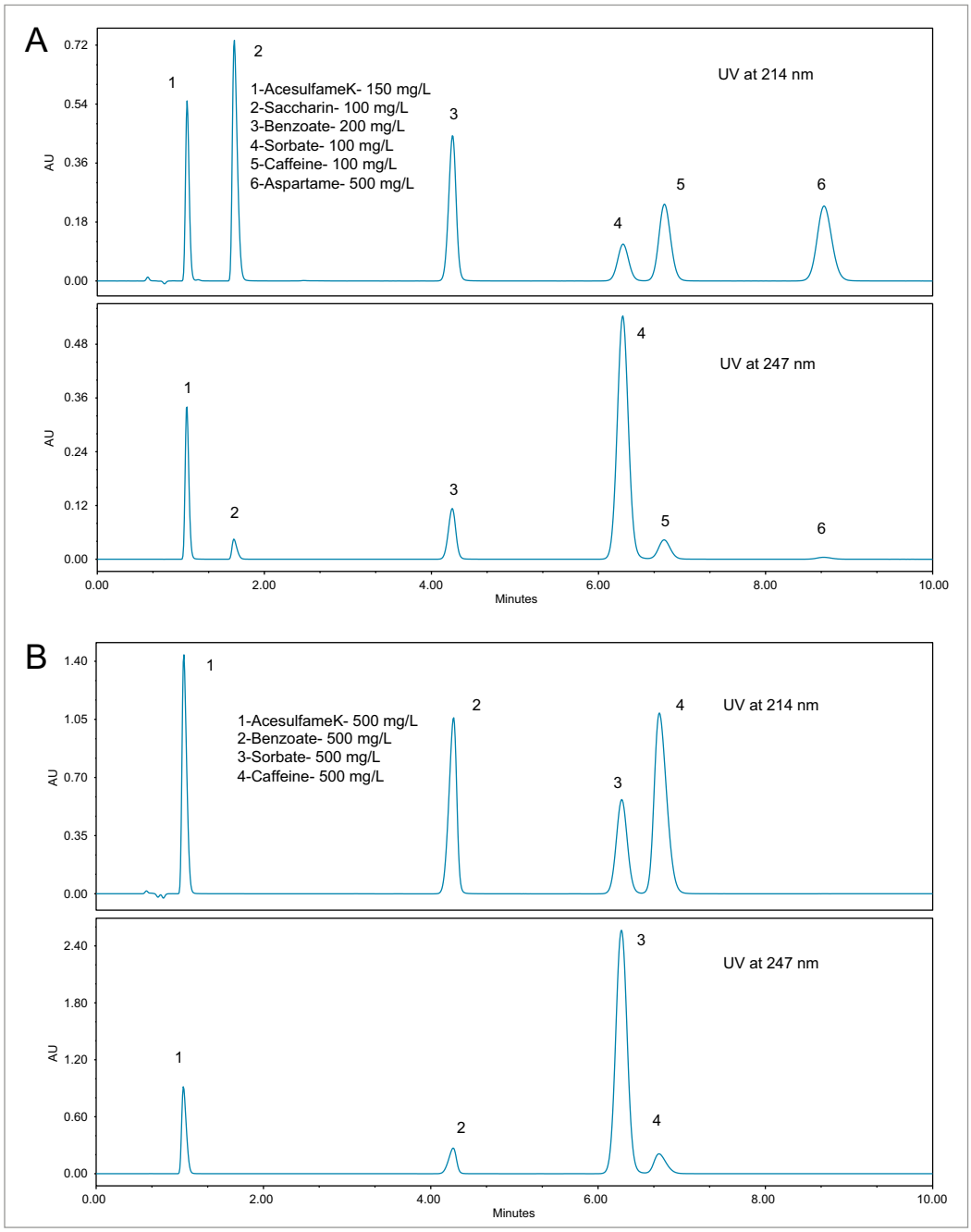

Figure 2A. Beverage Standard.
Figure 2B. Waters Big-4 Beverage Standard.

In order to assess the method with the sample types that are typically encountered, five different beverages were purchased from a local store for analysis. The resulting chromatograms from the analysis of these beverages are shown in Figures 3 to 7. The more traditional-style carbonated beverages are represented by two examples, a diet cola (Figure 3), and a lemon-lime soft drink (Figure 4). For each sample, the amounts of each analyte were calculated using both wavelengths for comparison. In Figures 3 and 4, the calculated amounts using the two different wavelengths agree within 4% for all of the analytes. A single point calibration was used to calculate the amounts shown in Figures 3 to 7.

Newer formulations of soft drinks often include ingredients or additives that have known or perceived health benefits. Examples include essential nutrients, antioxidants, and plant extracts. Figures 5, 6, and 7 show three different examples. Figure 5 displays the chromatograms for a diet vitamin water, and Figure 6 for a diet energy drink. For both of these samples, the quantification at both wavelengths is in agreement (within 2.1%) with the exception of acesulfame K. The diet lemon tea showed excellent agreement at both wavelengths (within 3.4%), even for acesulfame K.

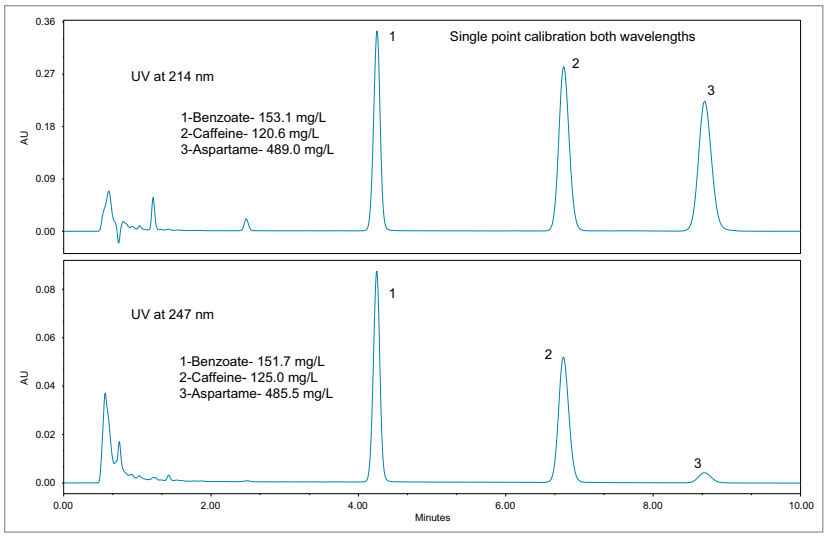

Figure 3. Diet cola chromatograms.

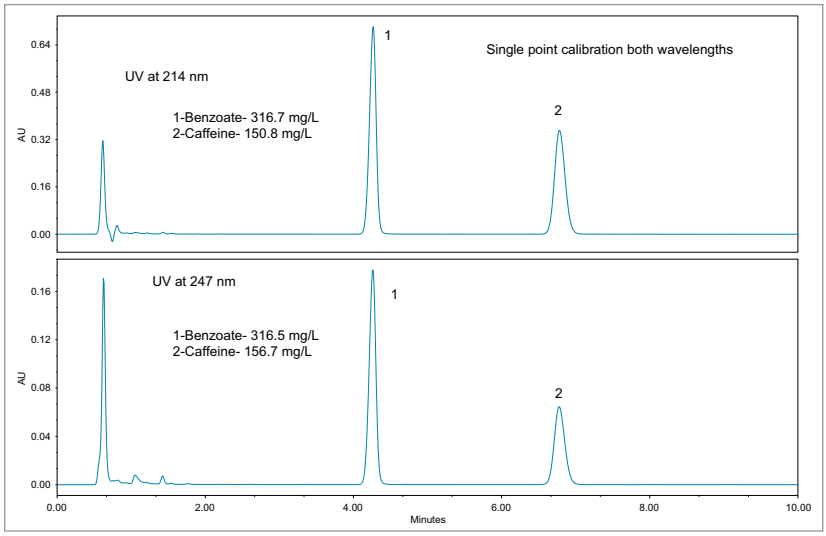

Figure 4. Lemon-lime soft drink chromatograms.

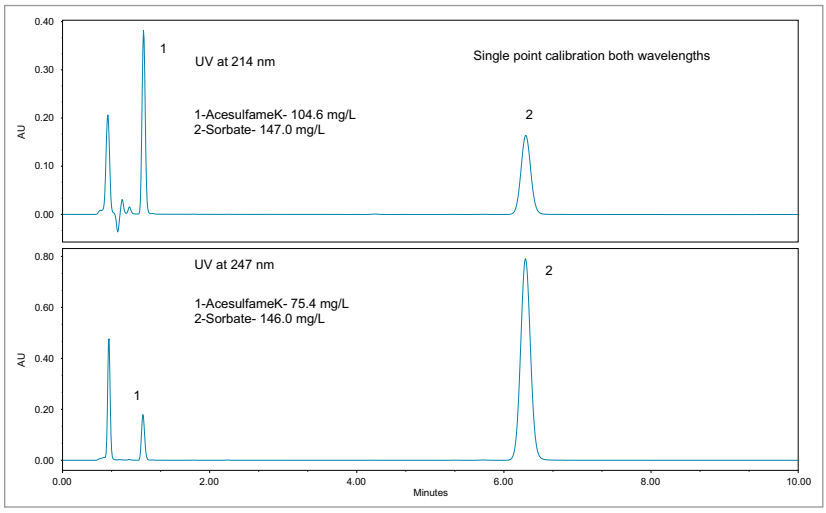

Figure 5. Diet vitamin water chromatograms.

Further investigation of the difference in quantification for acesulfame K in the diet vitamin water and diet energy drink revealed that both of these beverages contain B vitamins, where the other soft drinks did not. Using the ACQUITY QDa Detector, which enables mass analysis of the analytes, it was apparent that the B vitamins closely elute with acesulfame K. Figure 8 shows an overlay of the vitamin water chromatograms of acesulfame K at 214 nm and the individual mass-to-charge ratios of the B vitamins acquired using the ACQUITY QDa Detector. Note that vitamin B6 coelutes with acesulfame K.

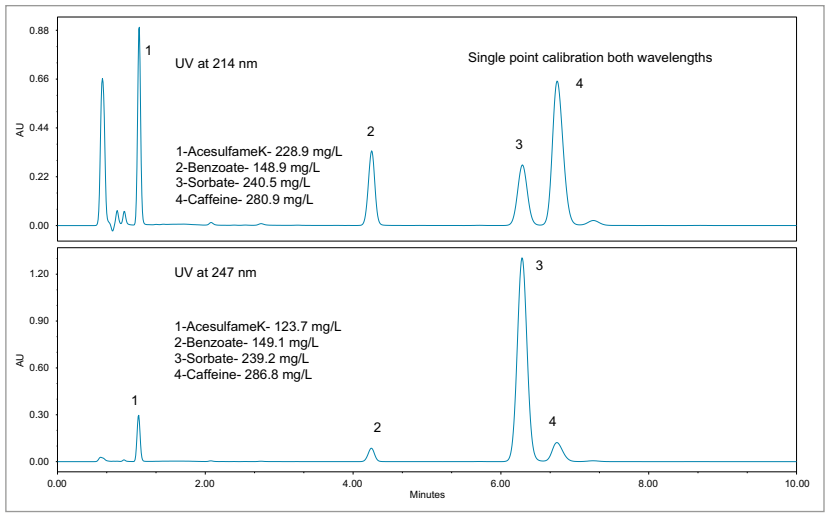

Figure 6. Diet energy drink chromatograms.

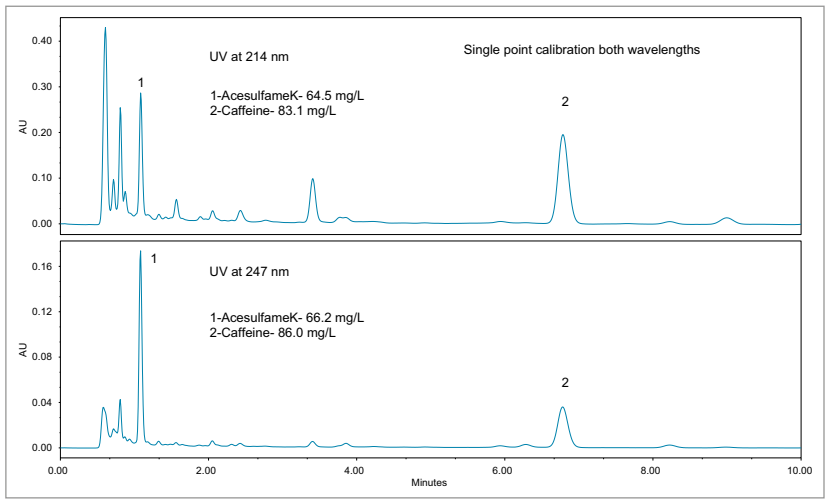

Figure 7. Diet lemon tea chromatograms.

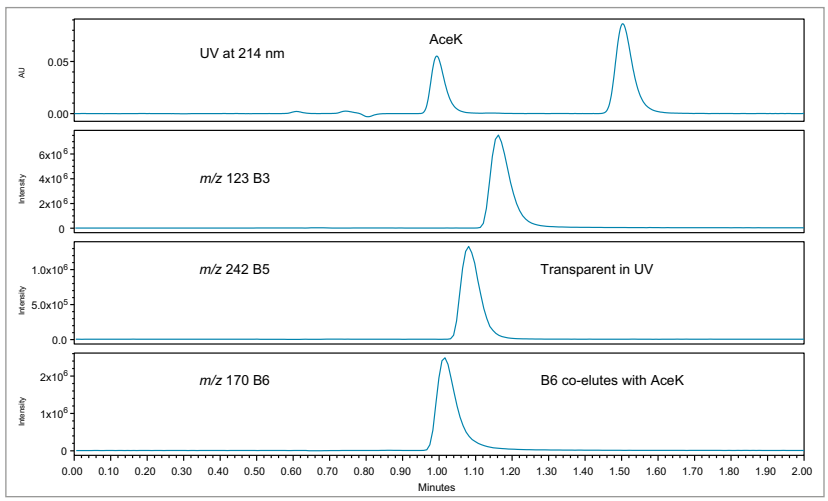

Figure 8. Overlay of UV chromatogram of Acesulfame K with SIR channels of B vitamins.

The UV spectra of vitamin B6 and acesulfame K are shown in Figures 9A and 9B, respectively. At 214 nm, which is non-specific, vitamin B6 has high absorbance and contributes to the overall response at 214 nm, along with acesulfame K. This results in over-estimation of the amount when using 214 nm. As can be seen, vitamin B6 is almost transparent at 247 nm, making this the wavelength of choice for beverages containing acesulfame K and vitamin B6.

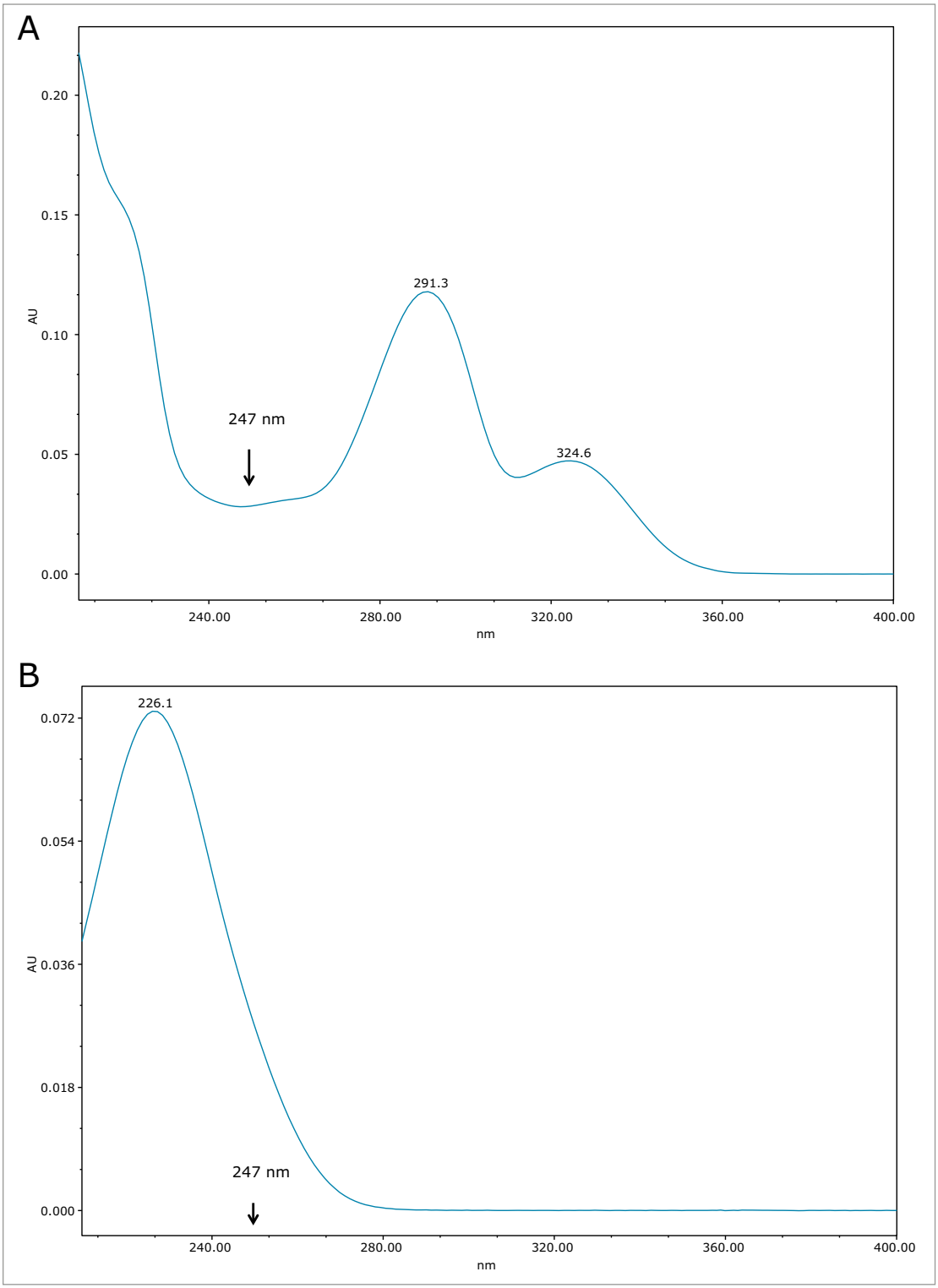

Figure 9A. UV spectrum of vitamin B6. Figure 9B. UV spectrum of acesulfame K.

The calibration curves created using the multi-point calibration from the Waters Big-4 Standard are shown in Figure 10. R^2 values were >0.999 for all compounds. Table 2 lists the retention time reproducibility for six injections of the Waters Beverage Analysis Standard. The %RSD was <0.2% for all analytes. Table 3 lists the reproducibility data for retention time and amounts for seven injections of the lemon-lime soft drink, which contained benzoate and caffeine. Quantification was made using the single-point calibration. Retention time reproducibility was 0.05% or less and the reproducibility for amount was <0.25%. Table 4 compares the quantification of the energy drink using both the single- and multi-point calibration at 247 nm to eliminate B vitamin interference.

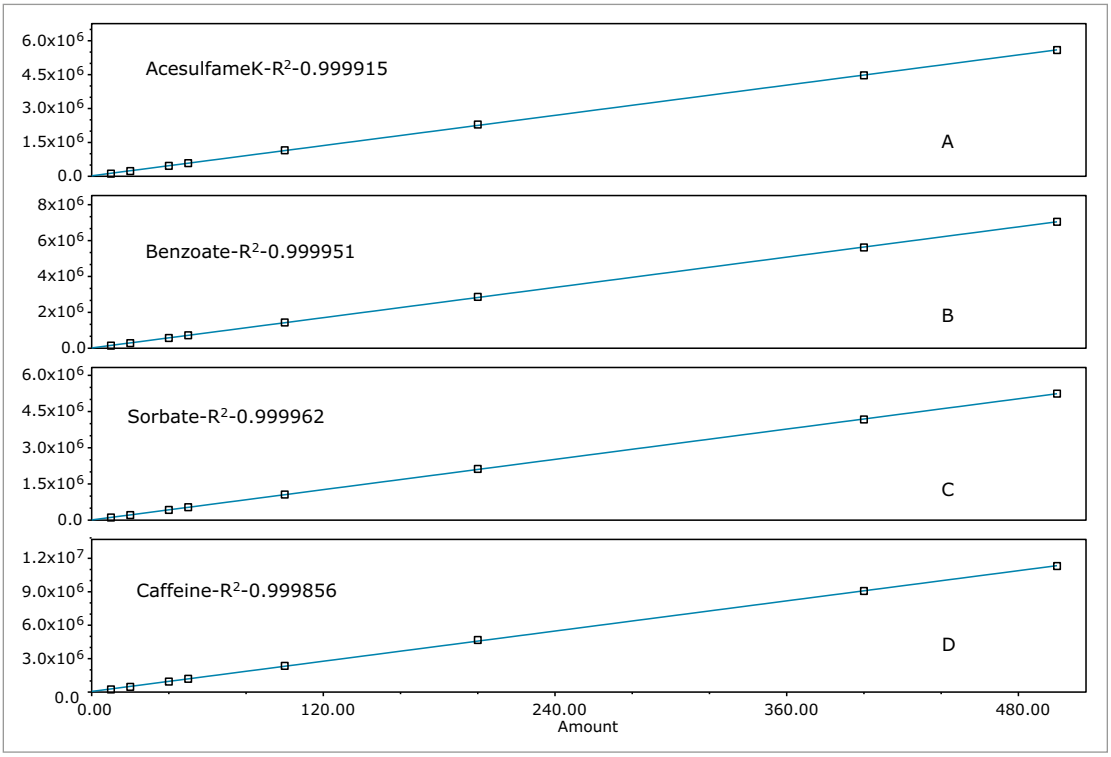

Figure 10. A–D: calibration curves of Big-4 analytes.

Analyte	RT (min)	%RSD
Acesulfame K	1.08	0.16
Saccharin	1.64	0.10
Benzoate	4.25	0.04
Sorbate	6.30	0.03
Caffeine	6.79	0.03
Aspartame	8.70	0.02

Table 2. Retention time reproducibility for six injections of Waters Beverage Standard.

Analyte	RT	%RSD	Amount	%RSD
Benzoate	4.27	0.05	316.64	0.17
Caffeine	6.78	0.04	150.80	0.21

Table 3. Retention time and reproducibility amount for seven injections of the lemon-lime soft drink.

Analyte	Single-point at 247nm	Multi-point at 247 nm	%RSD
AcesulfameK	123.7	123.9	0.24
Benzoate	149.1	148.9	0.13
Sorbate	239.2	245.1	1.24
Caffeine	286.8	288.6	0.33

Table 4. Comparison of the quantitation of the diet energy drink (mg/L) using single- and multi-point calibration at 247 nm.

CONCLUSIONS

Soft drink analysis using the ACQUITY Arc System provides a simple method for the analysis of soft drink additives. Implementation of such a procedure in a manufacturing environment has the capacity to improve overall workplace efficiency as well as:

- The choice of a single- or multi-point calibration, depending on the analytes and analyte levels required.
- Ethanol based mobile phase allows for simple, cost-effective disposal.
- Simple sample preparation requiring only sonication and filtering.
- Choice of UV wavelengths to remove vitamin B6 interference from acesulfame K quantification.

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Analysis of Flavonoids in Juices with the ACQUITY QDa Mass Detector

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APPLICATION BENEFITS

- The ACQUITY® QDa® Mass Detector provides better detection selectivity than UV/Vis or PDA detection.
- The superb selectivity offered by ACQUITY QDa allows for a simple and rapid chromatographic method without the requirement of baseline separation.
- A single sample dilution ratio in sample preparation is used for the analysis of a wide range of concentration levels.
- Mass Detection can significantly improve the throughput of flavonoids analysis with comparable accuracy and precision to the UV/Vis detector.

WATERS SOLUTIONS

ACQUITY UPLC® H-Class System

ACQUITY UPLC PDA Detector

ACQUITY QDa Mass Detector

MassLynx® MS Software

KEYWORDS

Polyphenol, flavonoids, flavanones, polymethoxylated flavones, mass detection, diosmin, didymin, sinensetin, nobiletin, tangeritin

INTRODUCTION

Flavonoids belong to a group of naturally occurring polyphenolic compounds found in many sources including citrus fruits.[1] There are two important sub groups: flavanones and flavones. Flavones are also known as polymethoxylated flavones (PMF). The most common flavanone is hesperetin from oranges; and the most common flavones are tangeretin and nobiletin in orange and tangerine peels.[2] The analysis of flavonoids in citrus species is often used for quality control and product authenticity testing because of their remarkable taste properties and variation of flavonoid profiles in the different species and varieties of citrus.[3,4] Figure 1 shows the chemical structures of the flavonoids used in this study.

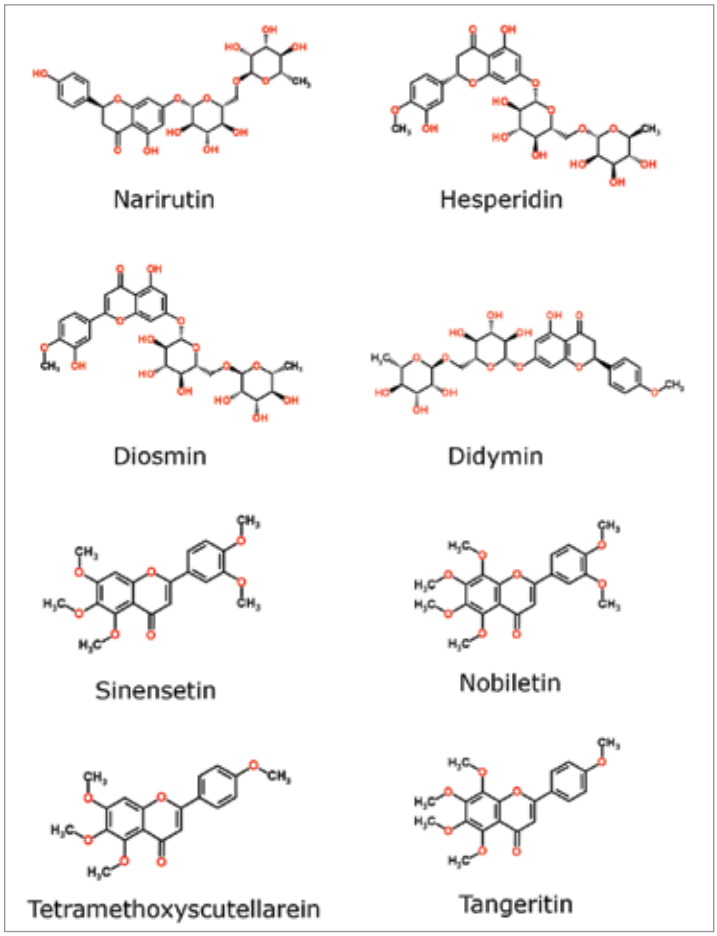

Figure 1. Chemical structures of flavone glycosides and polymethoxylated flavones.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC H-Class
Detection:	ACQUITY PDA
Column:	ACQUITY UPLC HSS T3 1.8 µm, 2.1 x 100 mm, (p/n 186003539)
Column temp.:	35 °C
Sample temp.:	4 °C
Injection volume:	2.0 µL
Flow rate:	0.6 mL/min
Mobile phase A:	0.1% Formic acid in deionized water
Mobile phase B:	0.1% Formic acid in acetonitrile
Run time:	13 min

Gradient:

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Curve
Initial	98	2	6
5.5	27	73	6
6.0	0	100	6
10.0	0	100	6
10.5	98	2	6
13.0	98	2	6

MS conditions

MS system:	ACQUITY QDa
Ionization mode:	ESI+
Acquisition:	SIR
Cone voltage:	5 V
Probe:	Default (600 °C)
Capillary:	Default (0.8 kV)
Sampling freq.:	Default (5 Hz)

Data management

Data were processed using MassLynx MS Software

The most common technique for flavonoids analysis is reversed-phase liquid chromatography with UV/Visible absorbance detection (UV/Vis) or Photodiode Array detection (PDA). PDA is preferred over UV/Vis since it can provide spectral data and facilitate peak purity checks. The challenge with PDA analysis is that flavonoids consist of dozens of structurally similar compounds, typically differing only in the degree of ring substitution, the type of substitution, and the type and degree of glycosylation. PDA detection lacks the selectivity to distinguish the subtle structural difference in flavonoids. Mass spectrometry (MS) can provide better selectivity (except isobaric compounds) and better sensitivity for flavonoids analysis than PDA detection. However, the cost and the ease of use of MS instruments can be an obstacle towards the adoption of MS for flavonoids analysis, especially for QC labs in manufacturing plants.

Waters® ACQUITY UPLC H-Class System with the ACQUITY QDa Mass Detector offers the best balance of selectivity, usability, and affordability for flavonoids analysis. The ACQUITY QDa is designed to provide mass spectral data with minimal tuning at an affordable cost. It not only extends the sample detection coverage of optical detection to compounds with no UV or fluorescence chromophore, but it also improves the selectivity of compounds of similar structures, which is very useful in flavonoids analysis.

RESULTS AND DISCUSSION

The detection of flavonoids was achieved by selective ion recording (SIR) of the molecular ions of the flavonoids of interest. Table 1 shows the monoisotopic masses of the flavonoids and their molecular ion mass-to-charge ratios (*m/z*). Most of them have different *m/z* values, except for only one pair of compounds, the sinensetin and tangeretin, which both have the same *m/z* value. The sinensetin and tangeretin can be easily separated chromatographically, so they will not interfere with each other. Figure 2 shows the chromatograms from UV (325 nm) and from ACQUITY QDa detection for a juice sample. As can be seen in Figure 2, sinensetin (peak E) and tangeretin (peak H) elute at different times. The nobiletin and the tetramethoxyscutellarein peaks partially overlap each other. The quantitation of these peaks is a challenge using UV/Vis or PDA detection, but it is not an issue with the ACQUITY QDa Detector, since these two analytes can be selectively monitored at *m/z* 403.2 and 343.1, respectively.

Table 1. Monoisotopic masses and molecular ions m/z of analytes.

Analyte	Molecular ion *m/z*	Monoisotopic mass (Da)
Narirutin	581.2	580.18
Diosmin	609.2	608.17
Hesperidin	611.2	610.19
Didymin	595.3	594.19
Sinensetin	373.1	372.12
Nobiletin	403.2	402.13
Tetramethoxyscutellarein	343.1	342.11
Tangeretin	373.1	372.12

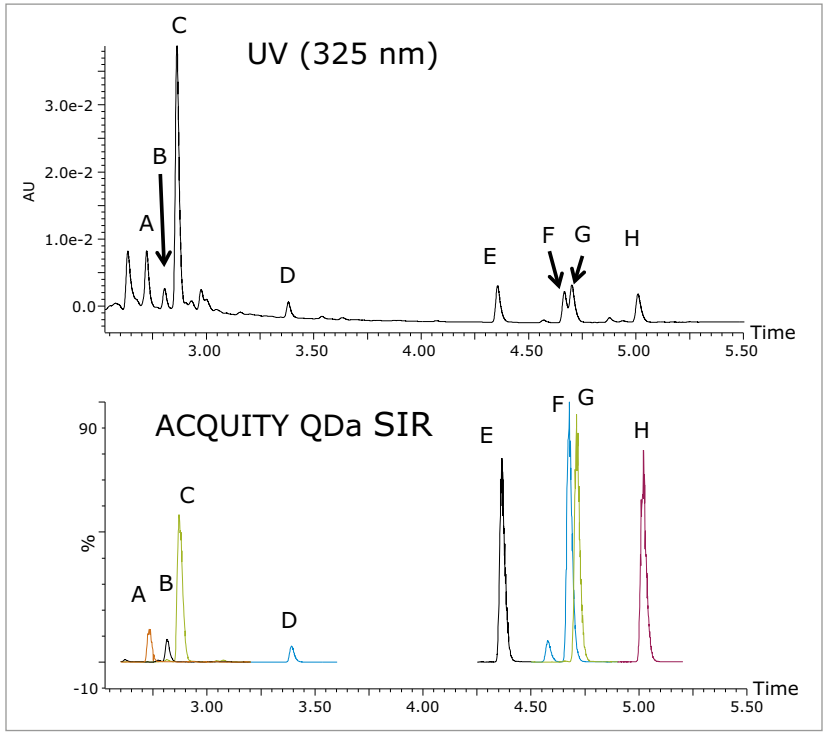

Figure 2. UV and SIR chromatograms of flavonoids in juice: A. narirutin, B. diosmin, C. hesperidin, D. didymin, E. sinensetin, F. nobiletin, G. tetramethoxyscutellarein, and H. tangeretin.

The selectivity of the ACQUITY QDa Mass Detector allows for simplified chromatographic method development work. It is acceptable to have a chromatographic method without baseline separation, as long as the compounds can be selectively detected. With UV/Vis or other optical detectors, it is required to obtain chromatographic baseline resolution for all analytes, which can be a challenge for structurally similar compounds, and it often results in longer run times. The total injection cycle time of the method presented here, including column equilibration, is 13 minutes, which is at least four times faster than existing methods. Table 2 is a summary of analysis results for a fruit juice. The analytical performance is comparable to the existing QC method.

Table 2. Accuracy and repeatability of polyphenols determination in citrus juice (n=10).

Analyte	RT (min)	Accuracy (%)	Repeatability RSD (%)
Narirutin	2.73	86	3
Diosmin	2.81	139	8
Hesperidin	2.87	82	3
Didymin	3.39	94	4
Sinensetin	4.36	103	4
Nobiletin	4.68	110	3
Tetramethoxyscutellarein	4.71	100	3
Tangeretin	5.02	112	3

CONCLUSIONS

Waters ACQUITY UPLC H-Class System with the ACQUITY QDa Mass Detector provides increased selectivity for the detection of flavonoids in citrus juices as compared to optical detectors that are typically used. The improved detection and selectivity simplify analytical method development, allowing for faster and easier chromatographic methods, as well as faster and more efficient sample preparation procedures. These improvements in turn increase lab productivity and sample throughput. In addition, the ease of use and affordable cost of the ACQUITY QDa Detector make it an essential tool for routine analyses.

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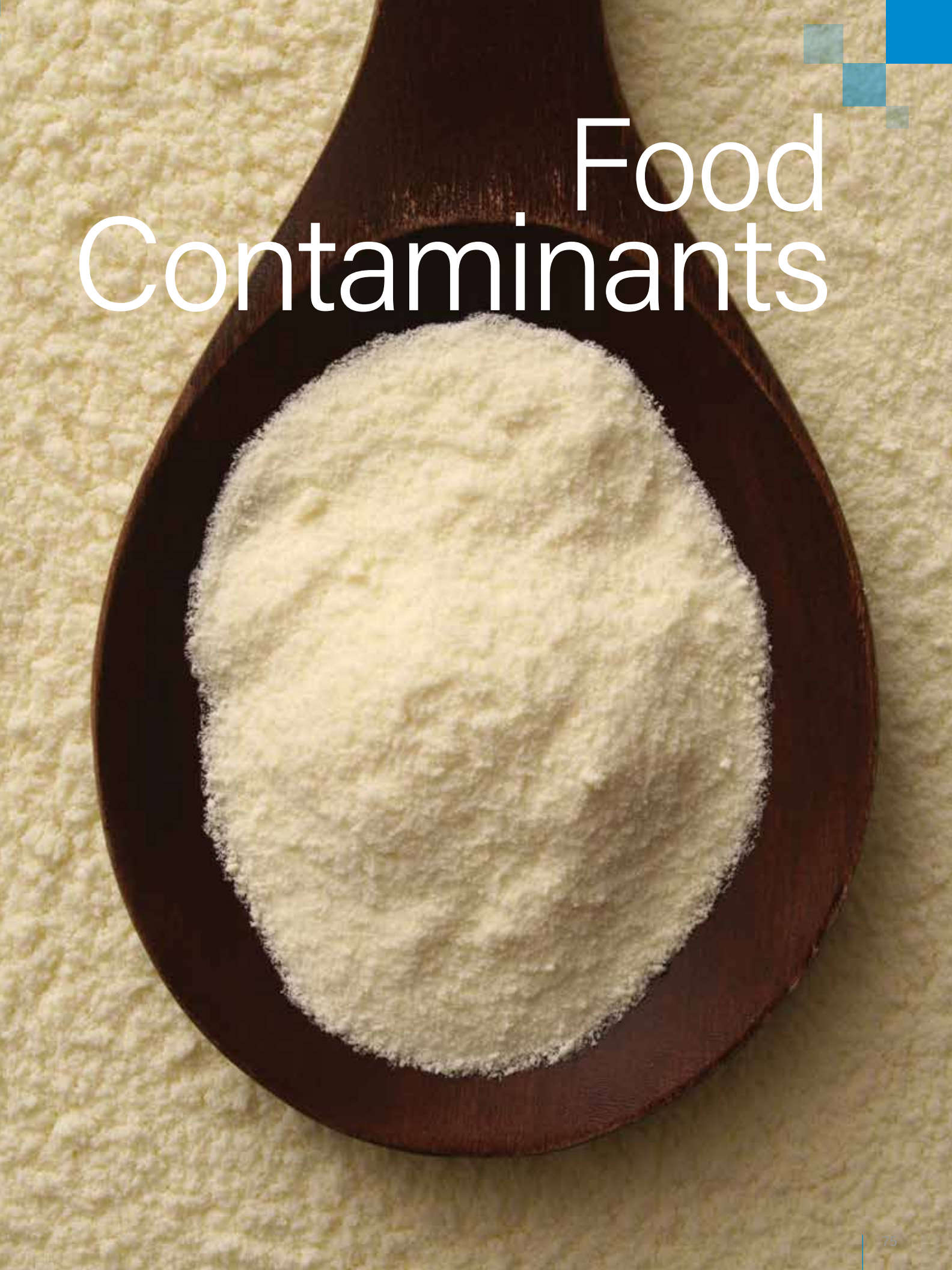

Food Contaminants

Food Contaminants

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Screening for Melamine, Cyanuric Acid, and Dicyandiamide in Powdered Milk and Infant Formula Using Mass Detection

Mark E. Benvenuti, Michael S. Young, Gareth E. Cleland, and Jennifer A. Burgess
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APPLICATION BENEFITS

- Separation of melamine, cyanuric acid, and dicyandiamide (DCD) in less than three minutes.
- Economical alternative to existing LC-MS/MS methods.
- Easily integrate mass detection into existing LC workflows.
- Quantitation of compounds with weak UV activity.
- Simple sample preparation procedure without the added cost of internal standards.
- Excellent recovery and repeatability.

WATERS SOLUTIONS

ACQUITY UPLC® H-Class System

ACQUITY QDa® Mass Detector

ACQUITY UPLC BEH Amide Column

Certified Sep-Pak® Silica Cartridges

MassLynx® Software

Empower® 3 Software

KEYWORDS

Melamine, cyanuric acid, dicyandiamide, DCD, mass detection, milk analysis, milk screening, protein adulteration

INTRODUCTION

Melamine and cyanuric acid (Figure 1) are low mass, nitrogen-rich compounds that have been linked to protein adulteration in various foodstuffs in the past.[1] While melamine and cyanuric acid are not individually toxic, in combination they can sometimes form an adduct compound through hydrogen bonding, melamine cyanurate, that produce sharp crystals which can cause internal organ failure and possible death.[2] A similar compound, dicyandiamide (DCD), which is used to minimize the environmental impact of grazing livestock was found in small amounts in dairy products in New Zealand.[3] Published limits on melamine in infant formula are 1 mg/kg, and 2.5 mg/kg in other foods and animal feed. These values are based on the TDI (tolerable daily intake) of melamine and its analogues of 0.64 mg/kg body weight (bw).[4] Recently a more stringent TDI for melamine and its analogs of 0.2 mg/kg body weight was established.[5] For DCD, the European Food Safety Agency has established a TDI of 1 mg/kg body weight.[6] As these compounds are quite polar, reverse-phase methods do not typically work well for these analytes. Current methods employ HILIC chemistry or ion pair mechanisms,[7] often with MS/MS detection.

In this application note we show a method using Waters® ACQUITY QDa Mass Detector coupled to the ACQUITY UPLC H-Class System for consistent and simple quantification of melamine, cyanuric acid, and dicyandiamide.

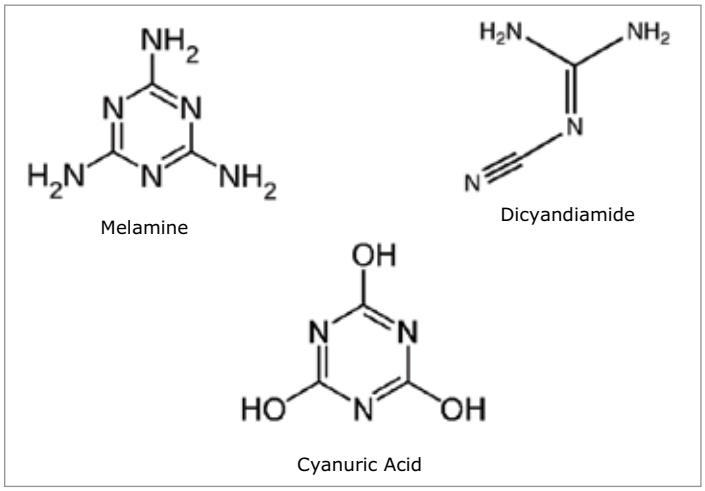

Figure 1. Structures of melamine, cyanuric acid, and dicyandiamide.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC H-Class
CDS data system:	Empower 3
Run time:	14.0 min
Column:	ACQUITY UPLC BEH Amide 1.7 µm, 2.1 x 150 mm
Column temp.:	35 °C
Mobile phase A:	50:50 water:acetonitrile, 10 mM ammonium formate, 0.125% formic acid
Mobile phase B:	10:90 water:acetonitrile, 10 mM ammonium formate, 0.125% formic acid
Flow rate:	0.6 mL/min
Injection volume:	5 µL

	Time (min)	Flow rate (mL/min)	%A	%B
1.	Initial	0.6	2	98
2.	3.0	0.6	2	98
3.	3.5	0.6	98	2
4.	4.0	0.6	98	2
5.	4.1	0.6	2	98
6.	14.0	0.6	2	98

Standard preparation

Individual 1000 mg/L standards of melamine, cyanuric acid, and dicyandiamide were prepared in water. From these, an intermediate mix of 2 mg/L melamine, 100 mg/L cyanuric acid, and 100 mg/L DCD was prepared in water. This standard was diluted 1:100 in 10:90 water:acetonitrile to produce a standard of 20 µg/L melamine, 1000 µg/L cyanuric acid, and 1000 µg/L DCD. Nine dilutions of this standard were made in 10:90 water:acetonitrile to produce calibration curves for the analytes with values listed in Table 1.

MS conditions

MS system:	ACQUITY QDa (Performance)
Ionization mode:	ESI+/-
Capillary voltage:	0.8 kV positive ion, 0.6 kV negative ion
Probe temp.:	Default (600 °C)
Source temp.:	Default (120 °C)

Melamine

SIR:	*m/z* 127.1, positive ion
Cone voltage:	15 V

Cyanuric acid

SIR:	*m/z* 128.0, negative ion
Cone voltage:	10 V

Dicyandiamide (DCD)

SIR:	*m/z* 85.1 positive ion
Cone voltage:	10 V
Acquisition rate:	5 Hz
Full scan acquisition:	*m/z* 50 to 300
Cone voltage:	15 V

Positive and negative ion, centroid

Standard	Melamine	Dicyandiamide	Cyanuric acid
1	20.0	1000.0	1000.0
2	10.0	500.0	500.0
3	5.0	250.0	250.0
4	4.0	200.0	200.0
5	2.0	100.0	100.0
6	1.0	50.0	50.0
7	0.5	25.0	25.0
8	0.4	20.0	20.0
9	0.2	10.0	10.0

Table 1. Concentration of standards in µg/L used to create calibration curves.

Sample preparation

1 g of powdered milk or infant formula was dissolved in 10 mL of 2% aqueous formic acid. For the liquid infant formula, 1 mL was added to 9 mL of 2% aqueous formic acid. 1 mL of this solution was added to 9 mL of acetonitrile and mixed well. The proteinaceous precipitate was allowed to settle for 20 minutes, then centrifuged for 20 minutes at rcf of 2233 g. 1 mL of the resulting supernatant was loaded onto a Certified Sep-Pak 6-cc Silica Cartridge (p/n 186004616), previously conditioned with 6 mL of 10:90 water:acetonitrile. The cartridge was eluted with 4 mL of 10:90 water:acetonitrile, and the resulting eluent injected. Five examples of powdered milk and infant formula (powder and liquid, dairy, and soy-based) samples were studied.

A spiking experiment was performed to determine recovery. One g (1 mL for liquid infant formula) was spiked with 1 mg/L of melamine, and 20 mg/L cyanuric acid, and dicyandiamide. This was done for the five examples mentioned above. Each sample was carried through the sample preparation protocol described above. Recovery values are listed in Table 2.

Matrix	Melamine	Cyanuric acid	DCD
Dry milk powder	85	105	98
Infant formula powder – dairy	103	123	105
Infant formula powder– soy	75.0	113	105
Infant formula liquid – dairy	99	119	112
Infant formula liquid – soy	91	115	97

Table 2. Percentage recovery for each of the analytes spiked into five different matrices. Spiking level for melamine was 1 mg/L. Spiking amount for cyanuric acid and DCD was 20 mg/L.

RESULTS AND DISCUSSION

METHOD DEVELOPMENT

For separating the three analytes in this application, HILIC is the ideal technique. Two different HILIC columns were investigated for this application, the BEH HILIC Column, and the BEH Amide Column. Figure 2 shows a comparison of the retention of the analytes on both columns, along with acenaphthene, which was used as a marker of no retention for both chemistries. As can be seen in Figure 2A, cyanuric acid and dicyandiamide showed little retention on the unbonded BEH particle deployed in the HILIC column. The tri-functional carbomoyl ligand of the ACQUITY UPLC BEH Amide Column provided vastly improved retention of the analytes of interest (Figure 2B), and was therefore selected as the better column. As shown in Figure 2B, excellent separation was achieved between the three analytes. Melamine is the most highly retentive of the three and eluted within three minutes.

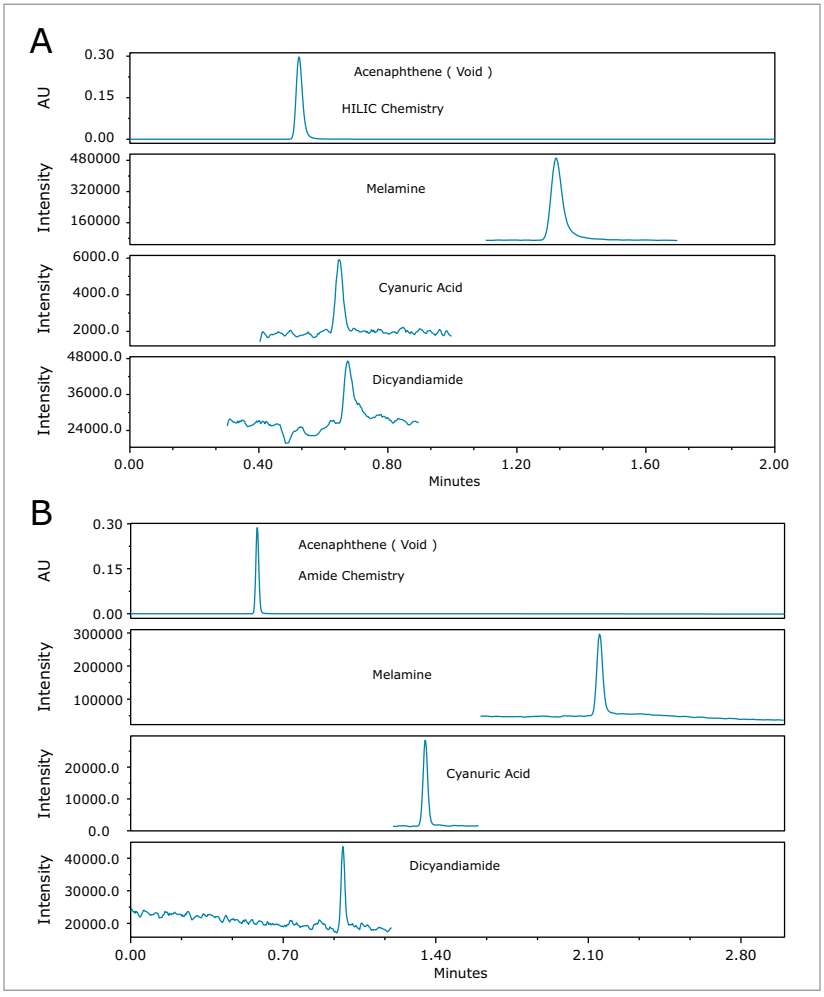

Figure 2A. SIR chromatograms of melamine, cyanuric acid, and dicyandiamide using the ACQUITY UPLC BEH HILIC Column. The first chromatogram shows the UV chromatogram at 280 nm for acenaphthene, which does not retain on this column, and therefore indicates the void volume of the column. Figure 2B. SIR chromatograms of melamine, cyanuric acid, and dicyandiamide using the ACQUITY UPLC BEH Amide Column. The first chromatogram shows the UV chromatogram at 280 nm for acenaphthene, which does not retain on this column and therefore indicates the void volume of the column.

Figure 3 shows an overlay of the UV max plot* of standard 1 with the SIR channels of each analyte (20 µg/L melamine, 1000 µg/L each, of cyanuric acid and dicyandiamide). In Figure 3, only dicyandiamide is weakly visible in the UV trace, while all three analytes showed a strong signal in their respective SIR channels, demonstrating the added sensitivity of using mass detection for these analytes.

To assess the chromatographic method with example sample matrices, five different samples, (nonfat dry milk, dairy-based powdered infant formula, soy-based powdered infant formula, dairy-based liquid infant formula, and soy-based liquid infant formula) were purchased from a local store. After analyzing these samples, it became apparent that an unknown compound within the samples eluted at a similar retention time to melamine. This compound resulted in a depression in the SIR chromatogram shortly following the elution of melamine. Full-scan MS data, acquired, along with the SIR chromatograms enabled further investigation of the cause. The compound was shown to have *m/z* 104.1 (data not shown), and was found to be present in all matrices that were tested. In order to avoid any suppression of the melamine response, a pass through cleanup using Certified Sep-Pak Silica Cartridges was deployed. The effectiveness of this method is illustrated in Figure 4, where a spiked infant formula is compared with and without the Sep-Pak Cartridge cleanup.

In Figure 4A, the SIR trace of melamine in the spiked infant formula with no cleanup is shown. The depression in the baseline following the elution of melamine suggests significant suppression of the signal, as previously mentioned. The extracted ion chromatogram of *m/z* 104.1 in Figure 4B shows the corresponding peak causing the suppression. The intensity of the chromatogram in Figure 4B also indicates that this compound is present at much higher levels than the analytes of interest.

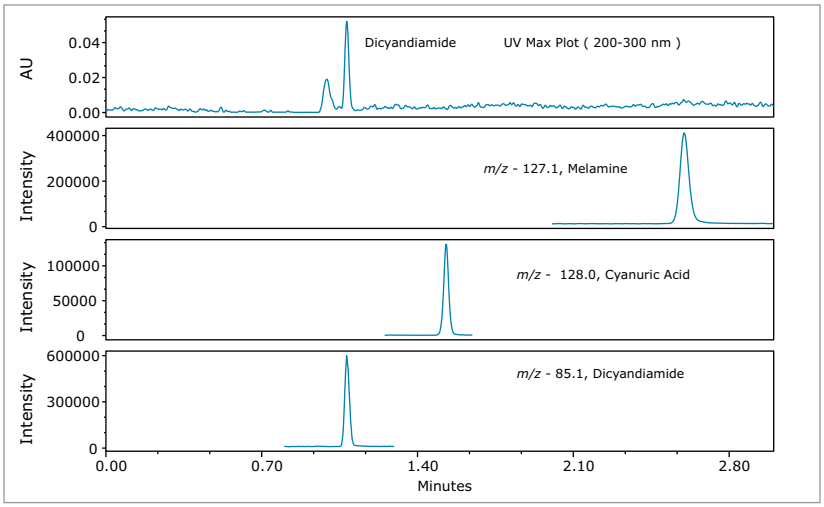

Figure 3. Overlay of UV Max Plot with SIR channels for melamine, cyanuric acid, and dicyandiamide for standard 1.*

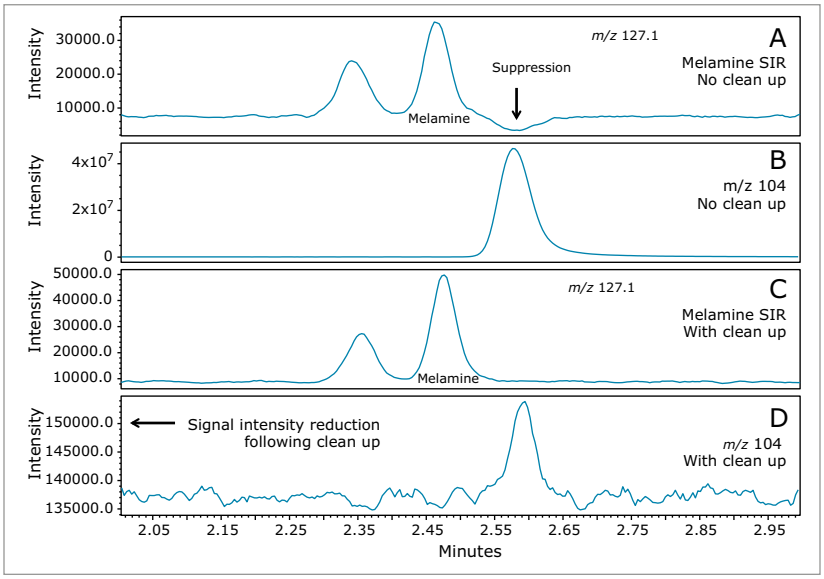

Figure 4. Comparison of melamine spiked infant formula with and without Certified Sep-Pak Silica Cartridge clean up.

As shown in Figure 4C, following the cleanup the baseline of the melamine SIR chromatogram is no longer affected. Figure 4D shows the extracted ion chromatogram of *m/z* 104.1 after cleanup.

The response of *m/z* 104.1 is approximately 250 lower than the sample without cleanup. This method development investigation and improvement was made possible by the use of simultaneous full-scan acquisition with the selected SIR traces of the analytes of interest.

**UV max plot is a 2D chromatogram plot derived from the 3D PDA data in which each data point is plotted at its maximum absorbance.*

SIR analysis delivers high sensitivity quantification at the lowest concentrations needed to screen for the analytes of interest. The full-scan MS data provided valuable information for method development and changes in the matrix background.

SAMPLE ANALYSIS

Calibration plots for the analytes are shown in Figures 5A–C. The calibration range was selected in order to use the linear portion of the calibration curve, which was a different range for the three compounds, as shown in Figure 5. The regression was <0.996 for all analytes with residuals <20%.

The comparison of spiked and unspiked nonfat milk powder, liquid dairy based infant formula, and powdered soy-based infant formula are shown in Figure 6. Melamine was spiked at 1 mg/L and is shown in Figure 6A. Cyanuric acid (Figure 6B), and DCD (Figure 6C) were spiked at 20 mg/L (within the calibration range shown in Figure 5). A low level peak prior to the melamine peak (retention time 2.5 mins, Figure 6A) was apparent in the dairy infant formula samples, but did not interfere with the integration of the melamine peak.

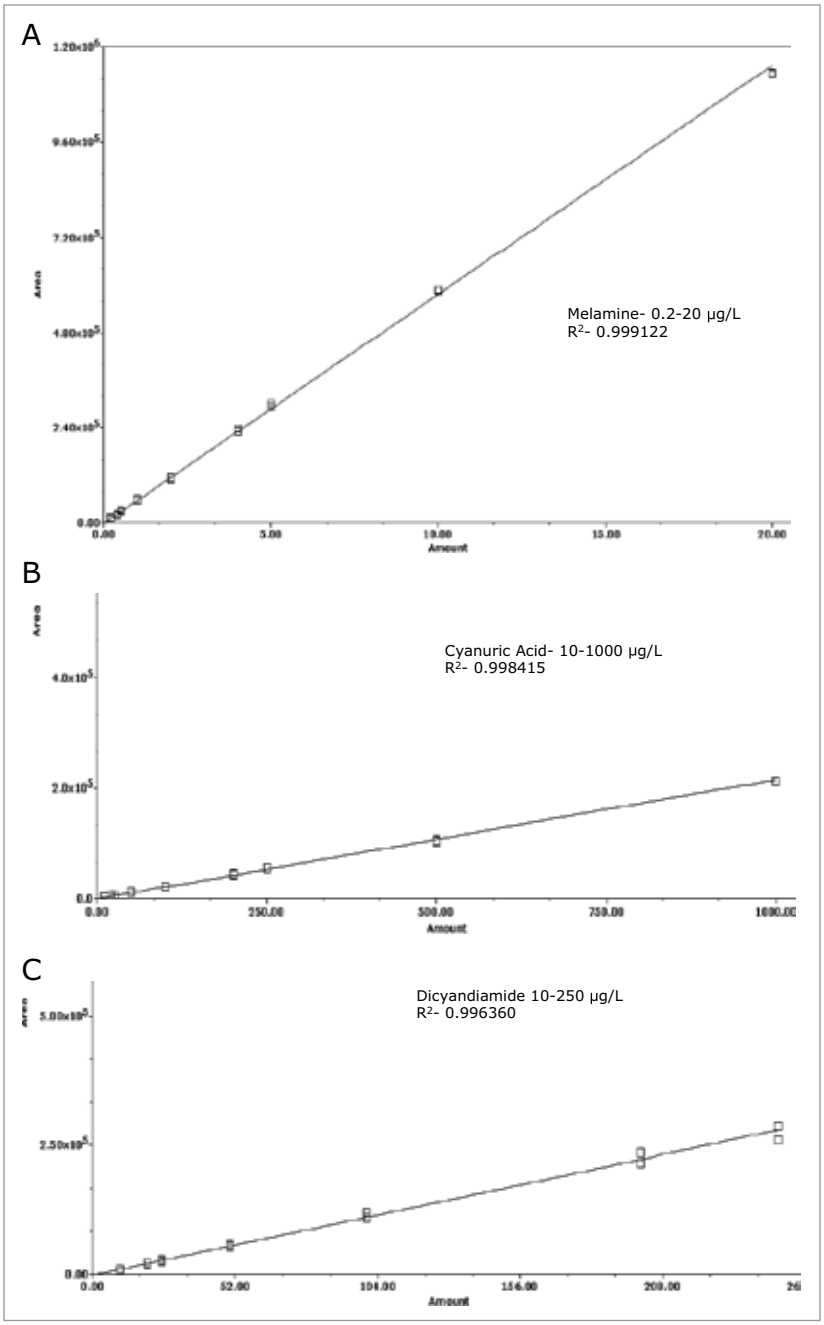

Figure 5. Calibration curves for A. melamine, B. cyanuric acid, and C. dicyandiamide.

To assess the method recovery, spiked amounts were compared to the amount quantified using the calibration curves in Figure 5. The resulting recoveries are listed in Table 2 on page 3 for the five different matrices that were spiked at the levels previously mentioned. Recoveries ranged from 75% to 123% for all analytes. Repeatability was assessed for 7 injections of a spiked liquid soybased infant formula and the percentage RSD for both retention time and amount are shown in Table 3 on page 3 for each of the analytes.

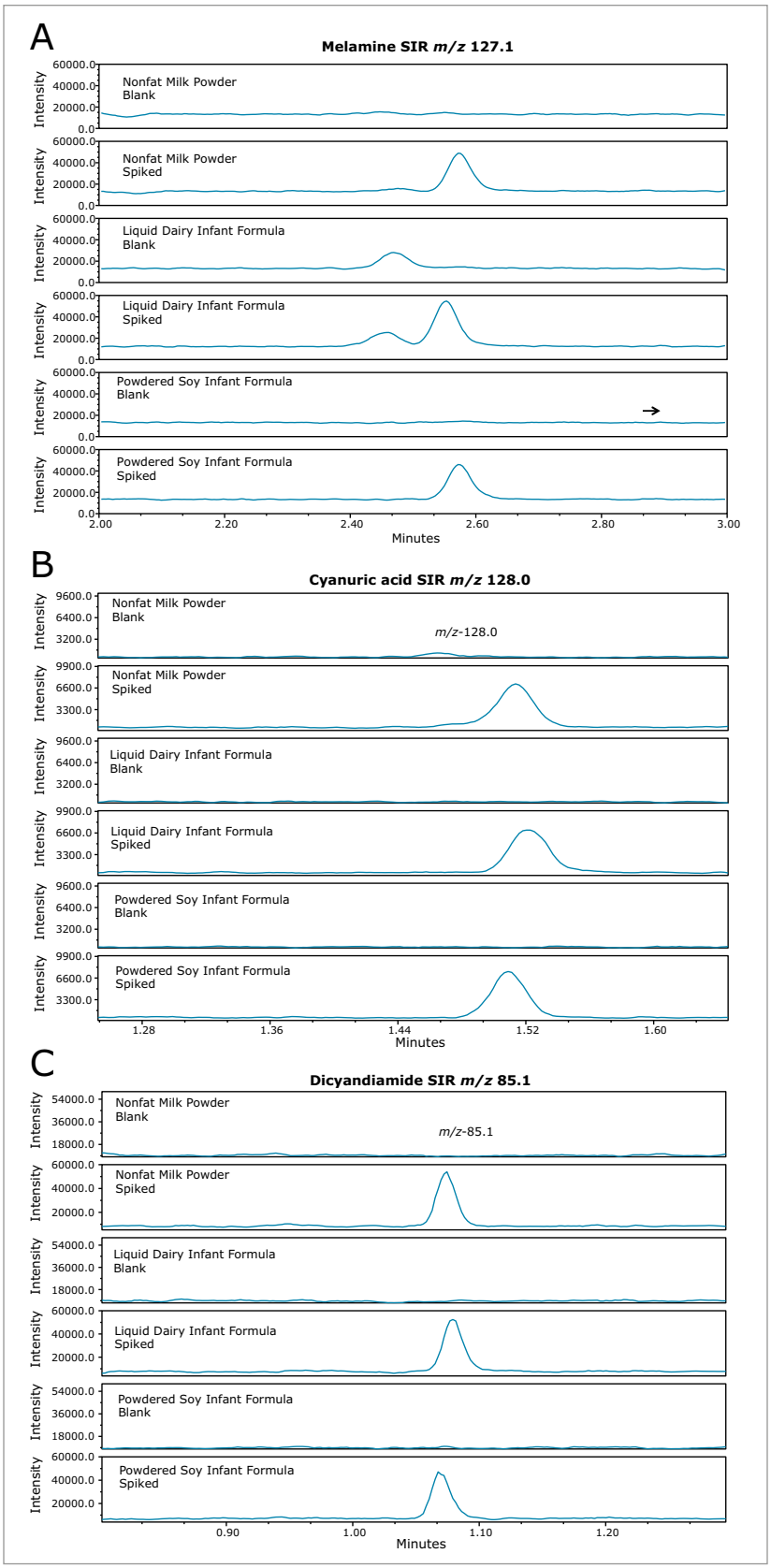

Figure 6. Comparison of SIRs in nonfat milk powder, liquid dairy infant formula and powdered soy-based infant formula blanks and spikes (1 mg/L melamine, 20 mg/L cyanuric acid, 20 mg/L dicyandiamide). (A) SIR chromatogram at m/z 127.1 (melamine). (B) SIR at m/z 128.0 (cyanuric acid), (C) SIR at m/z 85.1 (dicyandiamide).

CONCLUSIONS

A rapid screening method for melamine, cyanuric acid, and dicyandiamide in infant formula has been developed. Recoveries for the three analytes studied were in the range of 75% to 123% for the five spiked matrices studied. The use of the ACQUITY UPLC H-Class System with the ACQUITY QDa Detector and BEH Amide Column Chemistry provided:

- Retention of these difficult, highly polar analytes.
- Rapid baseline separation of these analytes in under three minutes.
- A simple pass through cleanup.
- Selectivity and sensitivity of mass detection, without the requirement of extensive mass spectrometry training.

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Analysis of 2- and 4-Methylimidazole in Beverages Using Alliance HPLC with ACQUITY QDa Mass Detection

Mark E. Benvenuti and Jennifer A. Burgess
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Analysis of 2- and 4-methylimidazole using HPLC-MS in less than 8.5 minutes.
- Sensitive and quantitative analysis without the need for SPE.
- High performance CORTECS® HPLC HILIC Columns effectively retain and separate these compounds.

WATERS SOLUTIONS

Alliance® HPLC System

ACQUITY® QDa® Detector

CORTECS Columns

KEYWORDS

Methylimidazoles, mass detection, 2-MEI, 4-MEI, imidazole, HILIC Column, caramel, soft drink, whiskey, food coloring, caramel color

INTRODUCTION

Caramel colorings are complex mixtures that are added to a variety of foods and beverages such as sauces, candies, desserts, soft drinks, beers, and liquors. The coloring is produced by heating carbohydrates in the presence of ammonia or other reagents. A byproduct of this reaction is the compound 4-methylimidazole (4-MEI), that has been identified as a possible carcinogen in animal models, and has hence been listed as 'possibly carcinogenic to humans' by the IARC Monographs.[1] The European Commission (EC) has therefore limited the concentration of 4-MEI in caramel colors to 250 mg/kg.[2] In the US, the State of California has listed 4-MEI as a cancer-causing chemical on Proposition 65[3] and currently gives a "No Significant Risk Level (NSRL) of 29 µg per day."

The recommended method for the analysis of 4-MEI and its related compounds is LC-MS, since photo diode array (PDA) detection does not provide sufficient sensitivity for detection of analytes at the levels found in beverage samples.[4] The need to quickly obtain occurrence data for methylimidazoles in food has also driven the need for methods that do not require solid phase extraction (SPE) for sample cleanup or enrichment.[4] In this application note we show a rapid analysis for 4-MEI and its isomer, 2-MEI using HPLC separation on a Waters® CORTECS Column with mass detection.

During method development, it was noted that with an isocratic method, the system pressure increased following the analysis of soft drink samples. To ensure that all of the matrix components were removed from the column, a gradient method with a cleaning step was programmed, as shown above. Schlee et al. also commented on the requirement for ensuring the column was sufficiently flushed following the analysis of beverage samples.[4] This provided a robust method for repeated analysis of all the beverage types shown in this application note.

EXPERIMENTAL

LC conditions

LC system:	Alliance
Runtime:	20.5 min
Column:	CORTECS HILIC 90 Å, 2.7 µm, 2.1 x 100 mm
Mobile phase A:	10 mM Ammonium formate pH-4 with formic acid
Mobile phase B:	Acetonitrile (0.1% formic acid)
Injection volume:	5 µL

Gradient:

	Time	Flow	%A	%B
1.	Initial	0.4	7.5	92.5
2.	8.5	0.4	7.5	92.5
3.	9.0	0.4	60.0	40.0
4.	11.0	0.4	60.0	40.0
5.	11.5	0.4	7.5	92.5
6.	20.5	0.4	7.5	92.5

MS conditions

MS system:	ACQUITY QDa (Performance)
Ionization mode:	ESI+
Capillary voltage:	0.8 kV
Cone voltage:	15 V
Probe:	600 °C
Source:	120 °C
SIR imidazole:	69.1 *m/z*
SIR 2 and 4-MEI:	83.1 *m/z*
Acquisition:	2 Hz

Standard preparation

Stock 1000 mg/L aqueous solutions of 2-MEI, 4-MEI, and imidazole were individually prepared. From these a mixed 10 ppm 2-MEI and 4-MEI standard, along with a separate 10 ppm imidazole standard were formulated. The 10 ppm mixed standard was diluted to prepare 10 individual standards with concentrations listed in Table 1. Each standard was fortified with 100 ppb imidazole as an internal standard. 100 µL of each standard was mixed with 900 µL acetonitrile prior to injection.

Sample preparation

Three soft drink colas (two diet and one regular), along with an orange flavored soft drink, and two samples of whiskey were purchased in local stores. The soft drink samples were sonicated to remove carbonation. Each of the six samples was fortified with 100 ppb imidazole as an internal standard. Separate portions of each sample were fortified with 100 ppb 2-MEI and 4-MEI to determine recovery.

Table 1. Concentrations used for the calibration curves for 2- and 4-methylimidazole and the internal standard (imidazole).

Standard	2- and 4-MEI (ppb)	Imidazole (IS) (ppb)
1	2000	100
2	1000	100
3	750	100
4	500	100
5	250	100
6	100	100
7	50	100
8	25	100
9	10	100
10	5	100

RESULTS AND DISCUSSION

Figure 1 shows a chromatogram of 4- and 2-MEI at *m/z* 83.1 overlaid with the internal standard, imidazole at *m/z* 69.1. Excellent separation of the MEI isomers was achieved in less than 8.5 minutes. Figure 2 shows the calibration curve for 4-MEI. Linearity (R^2) was 0.999 with residuals of <20% over the range of 50 to 2000 ppb. A chromatogram of the six samples studied is shown in Figure 3. 4-MEI was found in all of the cola samples, while 2-MEI was absent. Neither compound was found in the orange flavored soft drink as would be expected. A trace amount of both 4- and 2-MEI was found in one of the whiskey samples. Table 2 lists the amounts of 4-MEI found in each sample along with the recovery data.

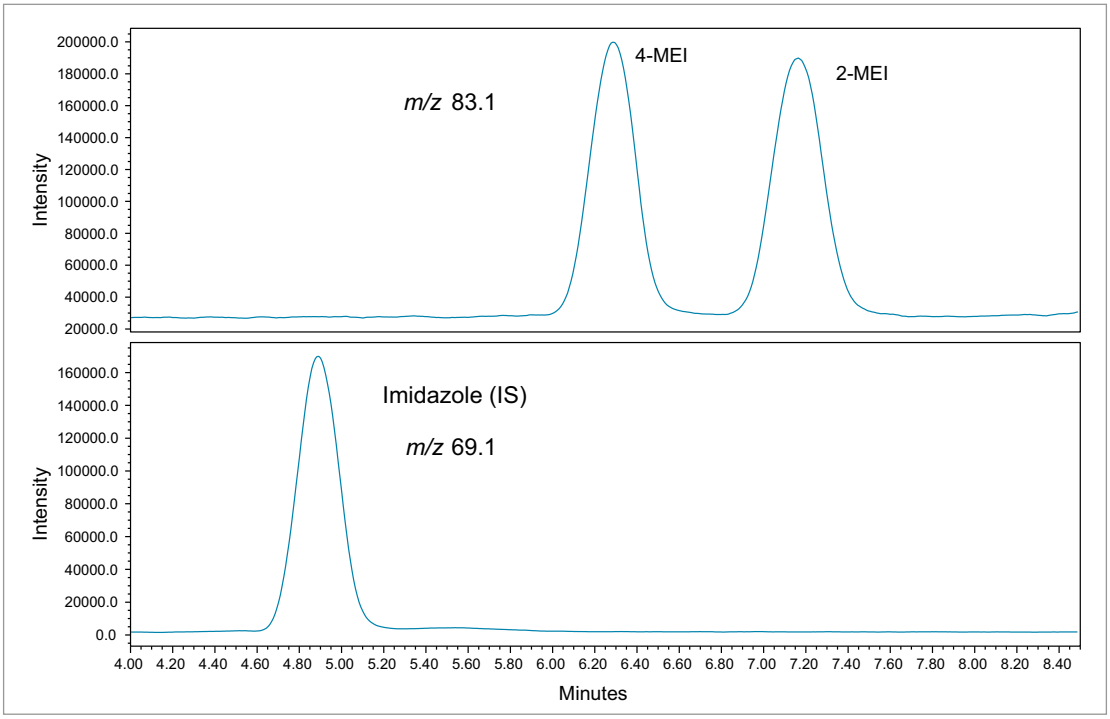

Figure 1. Chromatogram of Standard 6 (100 ppb).

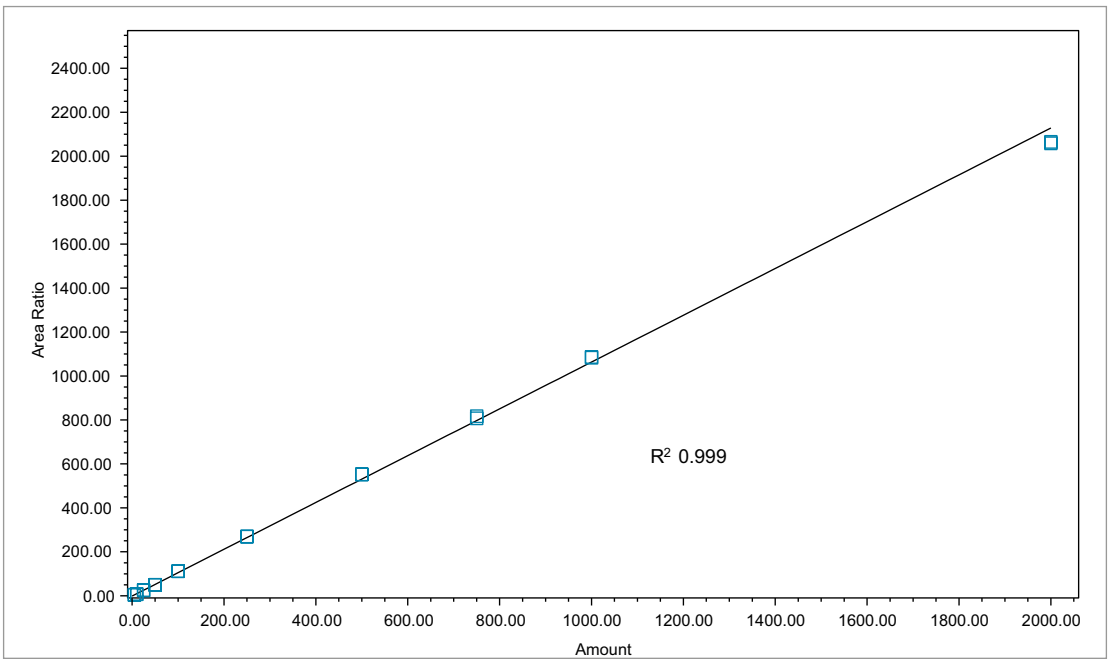

Figure 2. Calibration curve for 4-MEI.

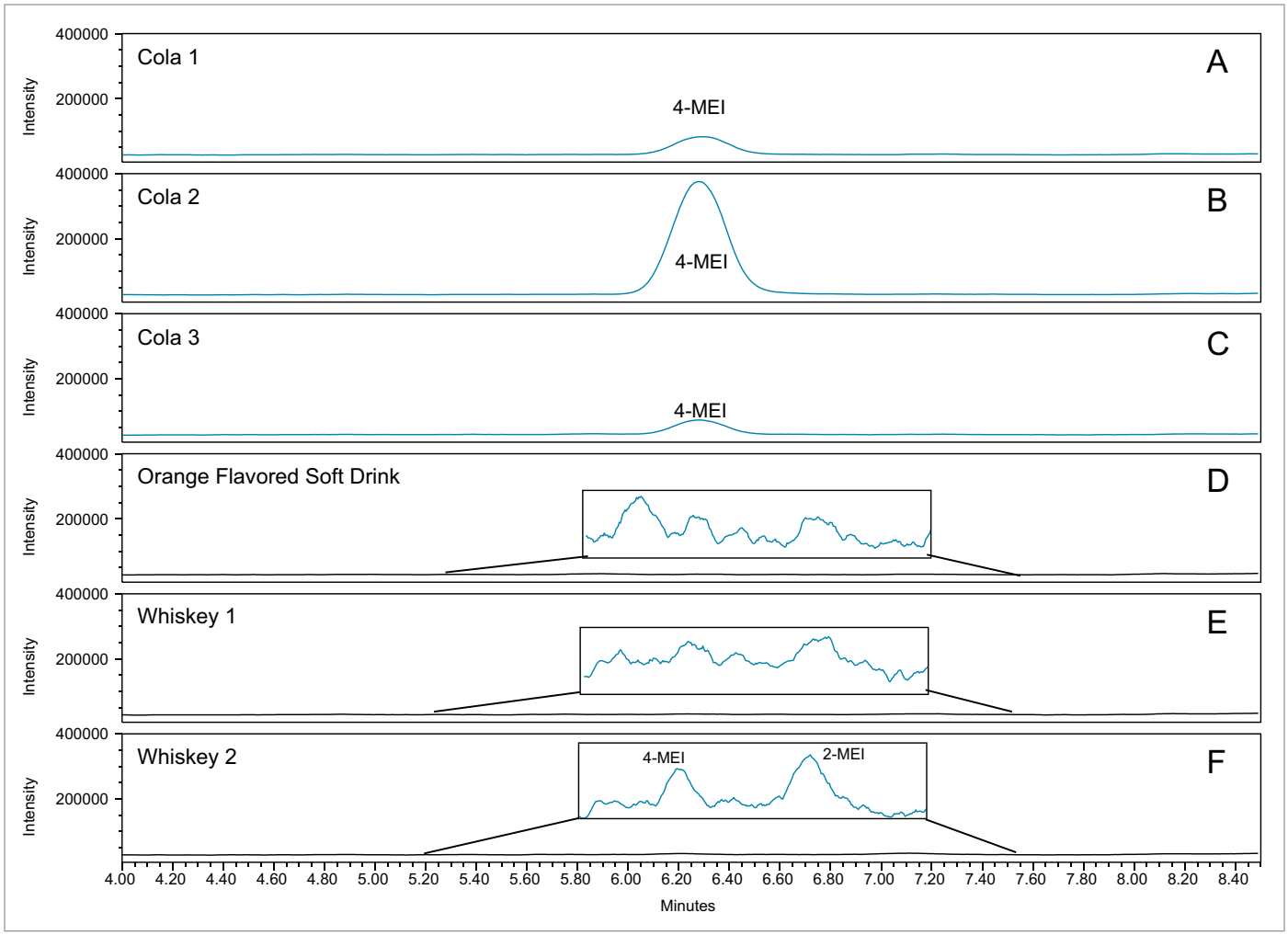

Figure 3. Overlay of SIR chromatograms of m/z 83.1 for the six samples. A–C: Cola samples 1–3. D: Orange-flavored soft drink. E–F: Whiskey samples 1 and 2. Vertical axes are displayed on the same scale; however, in order to clearly show the baseline and any detected peaks, a zoomed baseline region is shown for chromatograms D–F.

Table 2. Spike recovery data (ppb) for the samples studied.

Sample	4-MEI (ppb)	2-MEI (ppb)	4-MEI spike (ppb)	2-MEI spike (ppb)	%R 4-MEI (ppb)	%R 2-MEI (ppb)
Cola 1	14.37	ND	128.49	110.64	114.1	110.6
Cola 2	83.93	ND	194.78	109.01	110.8	109.0
Cola 3	14.93	ND	136.53	123.40	121.6	123.4
Orange SD	ND	ND	119.22	116.59	119.2	116.6
Whiskey 1	ND	ND	119.65	115.37	119.6	115.4
Whiskey 2	1.97	2.25	110.20	109.27	108.2	107.0

While one of the colas exceeded the California advisory guidelines for 4-MEI, all samples were well below the EU criteria of 80 mg/kg of body weight[2] with no observed adverse effect level. Recoveries ranged from 110% to 123% for 2- and 4-MEI for all samples. Table 3 lists the reproducibility data for retention time and amount for seven injections of the spiked Cola Sample 2. The %RSD was <0.55% for retention time for both analytes, demonstrating that the column was properly equilibrated between injections. The %RSD for the calculated amount for the seven injections of 4-MEI was 0.82% and 1.02% for 2-MEI.

Table 2. Spike recovery data (ppb) for the samples studied.

Analyte	RT (min)	%RSD	Concentration (ppb)	%RSD
4-MEI	6.28	0.44	194.78	0.82
2-MEI	7.16	0.52	109.01	1.02

CONCLUSIONS

A rapid and simple gradient method for the determination of 4-MEI has been developed. 4-MEI, but no 2-MEI, was found in all of the colas sampled. A trace of both 4-MEI and 2-MEI was found in one of the whiskey samples. The orange soft drink sample did not contain either analyte.

The use of the Alliance HPLC System in combination with the ACQUITY QDa Mass Detector and CORTECS HILIC Columns provided multiple benefits, including:

- Baseline resolution of 2-MEI and 4-MEI in under 8.5 minutes
- SIR mass analysis for UV transparent analytes
- Retention of highly polar analytes.

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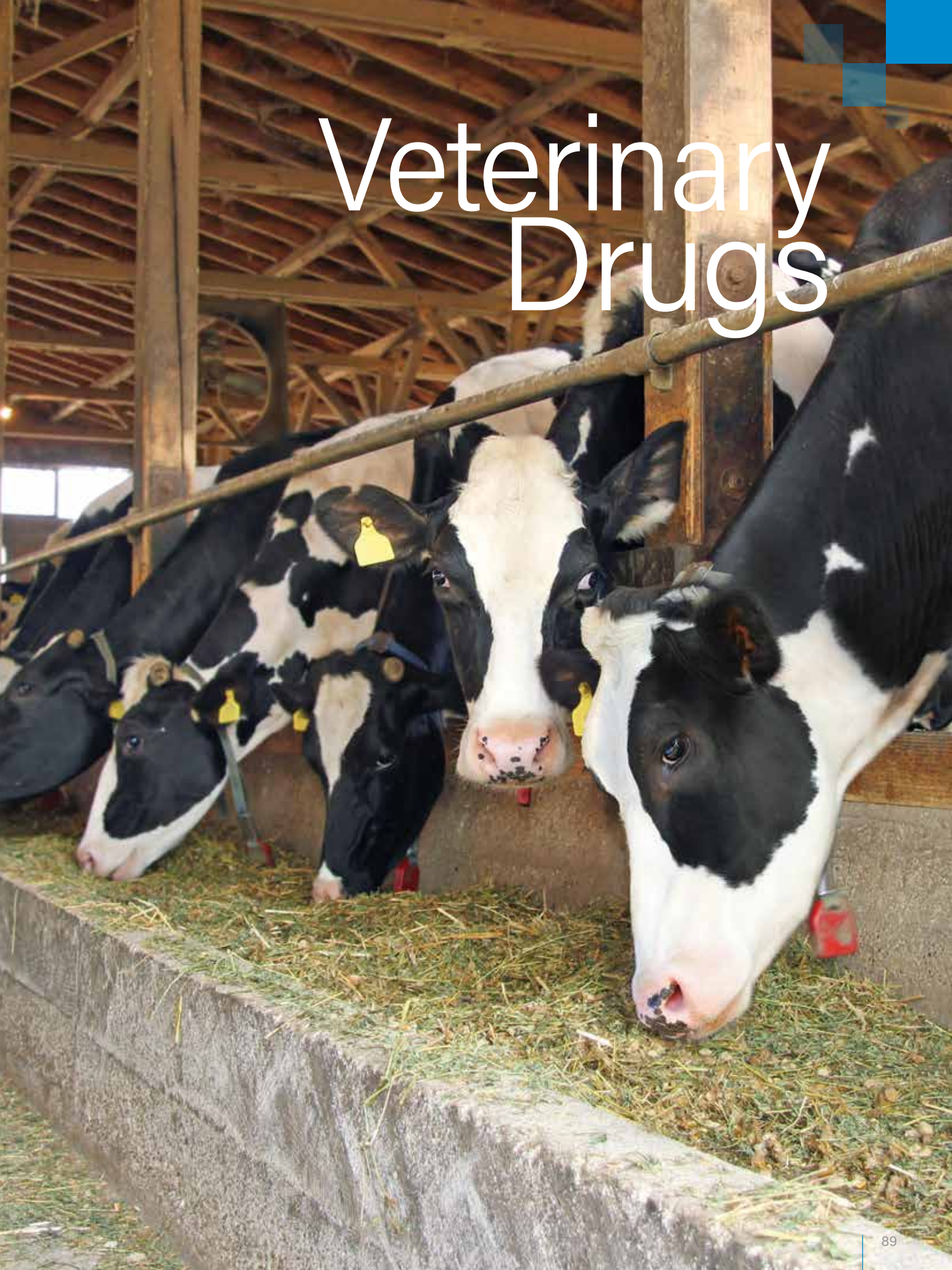
Veterinary Drugs



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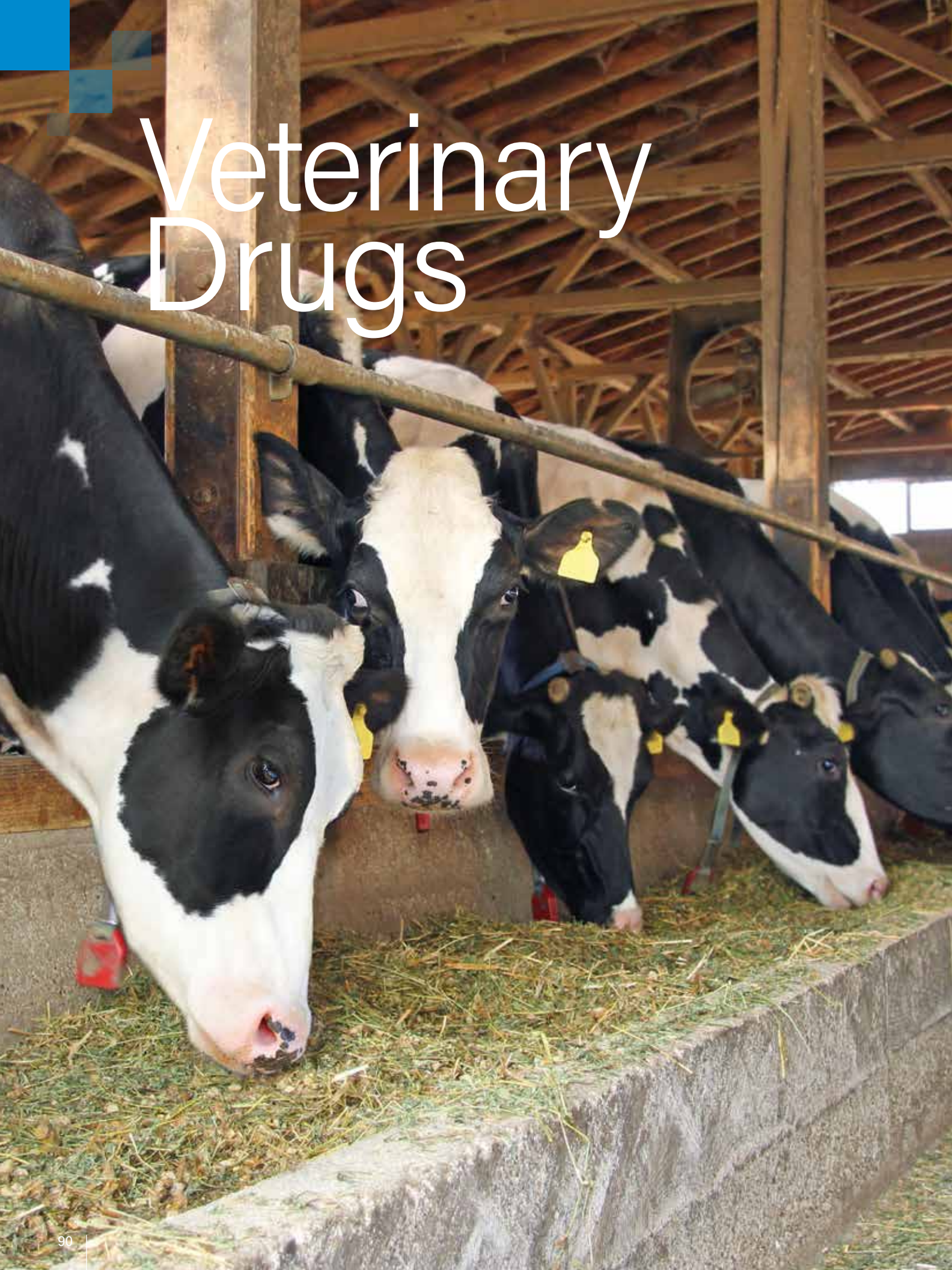

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Determination of Lasalocid and Tylosin at Therapeutically Relevant Levels in Animal Feed Using the ACQUITY UPLC H-Class System and ACQUITY QDa Mass Detector

Mohammed S. Ahmed,[1] Jeff Bieszki,[2] and Jason S. Kong[1]
[1] Ohio Department of Agriculture, Reynoldsburg, OH, USA
[2] Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Superb detection selectivity as compared to UV/Vis and fluorescence detection.
- Allows simpler sample preparation protocols for complex matrices.
- Reduced interference and noise from matrices for unambiguous identification and accurate quantification.
- Easy to use, fit-for-purpose LC-MS system for feed testing laboratories.

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ACQUITY UPLC® H-Class System

ACQUITY® QDa® Detector

Empower® 3 CDS Software

CORTECS® UPLC® C_{18}+ Columns

ACQUITY UPLC BEH C_{18} Columns

KEYWORDS

Veterinary drug, lasalocid, tylosin, feed

INTRODUCTION

Feed regulatory laboratories are tasked with protecting against consumer fraud by ensuring "truth in labeling." These laboratories utilize analytical methods distributed by AOAC INTERNATIONAL (Official Methods of Analysis)[1] and other publications to perform analytical testing, especially in regards to permitted veterinary drugs supplemented into animal feed. Current official HPLC methods use different types of detectors or attachments such as PDA, FLD, RI, or post-column derivitization for testing individual types of compounds. There is no UNIFIed multi-analyte approach for the determination of veterinary drugs. Traditional methods are also challenged by newer feed formulations which may result in more complex matrices and leads to chromatographic peaks that cannot be easily integrated or separated from interfering matrices.

While mass spectrometry (MS) has been adopted in a variety of regulatory work to solve the above problems, the cost of a high-end complete MS system can be prohibitive to be widely equipped in many feed regulatory laboratories. Typical feed testing does not necessarily require the level of sensitivity that is offered by high-end MS systems, especially for label claim verification of levels required for a therapeutic effect. Furthermore, the difficulty of training employees simply makes the transition to MS detection an all-around challenge.

The ACQUITY QDa is a high quality mass detector that has been specifically designed for integration with a liquid chromatography (LC) system. The ACQUITY QDa has been built on familiar LC detector design principles: it fits neatly into an existing LC stack; it is pre-optimized with zero adjustments required and no special expertise is required to tune or operate. It automatically performs mass calibration verification and advanced health check monitoring at power up. The ACQUITY QDa Detector is ready to operate around seven minutes after pressing the power button. It uses a pre-aligned electrospray source and has a mass range of 30 to 1250 *m/z* producing both Single Ion Recording (SIR) and full scan data at 10,000 AMU/sec. It is controlled by either MassLynx or Empower software. These design features have facilitated the adoption of MS detection in laboratories without the requirement of extensive training of employees.

The ACQUITY QDa Detector makes implementation of mass detection possible for any LC laboratory. The selectivity of mass detection allows for the unambiguous quantification of feed supplements at low levels, enabling simpler sample prep protocols from complex matrices.

In this application note, we demonstrate as a proof-of-concept a comparison of two veterinary drugs, lasalocid and tylosin, analyzed through a traditional HPLC-PDA and HPLC-FLD with Waters® ACQUITY UPLC System and the ACQUITY QDa Mass Detector. Figure 1 shows the molecular structures of lasalocid and tylosin.

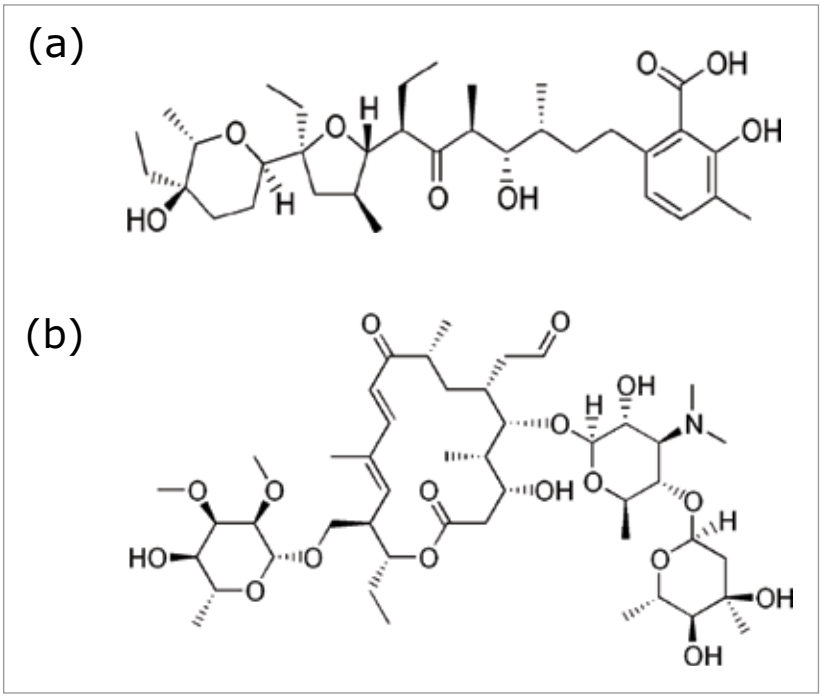

Figure 1. Molecular structures of (a) lasalocid (MW: 590.8 Dalton) and (b) tylosin (MW: 916.1 Dalton).

EXPERIMENTAL

Sample description

Lasalocid sodium was obtained from Alpharma Animal Health (Willow Island, WV). Tylosin standard was obtained from USP (Rockville, MD). The samples were from a variety of different feed base types (e.g. soy, corn, and non-grain based) and delivery forms (e.g. pellets, meal). Since there is no certified reference material available for these compounds in feed matrices, a sample that had previously tested within many different batches was used as a QC sample.

Preparation of standard solution

Stock solutions (600 µg/mL) of the individual antibiotics were prepared by measuring 30 mg of each analyte in 50-mL volumetric flasks and diluting to volume with the specified extraction solution (acidified methanol for lasalocid or phosphate buffer and methanol for tylosin. Intermediate standard solutions (60 µg/mL) were prepared by a ten-fold dilution of the stock solutions with the extraction solution. Working standard solutions were then prepared for each analyte by serial dilution. Lasalocid's curve was prepared from 1.5 to 7.6 µg/mL. Tylosin's curve was prepared from 3.1 to 30.7 µg/mL.

Lasalocid extraction

Extraction was adapted from AOAC OMA 2008.01.[2] 10 g of homogenized animal feed was combined with 100 mL acidified methanol (0.005% formic acid) in a 250-mL Erlenmeyer flask. The flask was sonicated for five minutes and shaken for 30 minutes in an orbital shaker. The solution was filtered through a 25-mm syringe filter discs with 0.45-µm nylon membrane filter and vialed for analysis.

Tylosin extraction

10 g of homogenized animal feed was combined with 100 mL of a 1:1 ratio of phosphate buffer (16.7 g K_2HPO_4, 0.5 g KH_2PO_4 diluted to 1 L with water), and methanol. Extraction procedure followed as above.

HPLC-FLD/PDA conditions

Sample vials were analyzed after extraction at the Consumer Protection Laboratory in Reynoldsburg, OH. Lasalocid and tylosin were analyzed using the Waters Alliance® 2695 Separations Module, equipped with a Waters 474 Scanning Fluoresence (FLR) detector, and a Waters 996 Photodiode Array (PDA) Detector, as described in Table 1.

UPLC-MS conditions

Samples were analyzed using a Waters ACQUITY UPLC System and ACQUITY QDa Mass Detector (Performance version). The experimental parameters are described in detail in Table 1.

Chromatograms from the UPLC-MS analysis were smoothed prior to integration using "mean" smoothing with five points.

The difference (or deviation) between the different methods for the same sample were calculated by taking the root of the square of the difference divided by the average of the two results for each sample.

	Lasalocid (HPLC-FLD)	Lasalocid (UPLC-MS)	Tylosin (HPLC-PDA)	Tylosin (UPLC-MS)
Run time (min)	10	2	20	2
Injection volume (µL)	20	2.0	20	0.2
Column	Phenomenex C_{18} (3 µm, 4.6 x 100 mm) at ambient	Waters CORTECS UPLC C_{18}+ (1.6 µm, 2.1 x 50 mm) at 40 °C	Symmetry® C_{18} (3 µm, 4.6 x 150 mm) at ambient	ACQUITY UPLC BEH C_{18} (1.7 µm, 2.1 x 50 mm) at 40 °C
Mobile phase	85:15 Acetonitrile: 125 mM Acetate buffer	A: 0.1% Formic acid in water B: 0.1% Formic acid in acetonitrile	A: 5 g Tetramethyl ammonium chloride in 1 L water, add 5 mL glacial acetic acid B: methanol	A: 0.1% Formic acid in water B: 0.1% Formic acid in methanol
Flow rate	1.0 mL/min	1.0 mL/min	1.0 mL/min	0.8 mL/min
Flow conditions	Isocratic	Gradient (25% A initial; 25% A 0.6 min; 5% A 0.9 min; 5% A 1.4 min; 25% A 1.5 min)	Gradient (60% A initial; 60% A 2 min; 40% A 14 min; 40% A 16 min; 60% A 18 min)	Gradient (55% A initial; 15% A 0.4 min; 15% A 0.7 min; 55% A 0.8 min)
Retention time (min) of analyte	6.7	0.81	13.5	0.78
Detection parameters	Excitation at 314 nm Emision at 418 nm	ESI-: *m/z* = 589.6 Cone 15 V	Abs at 286.4 nm	ESI +, *m/z* = 916.6 cone 15 V (tysolin) ESI+, *m/z* = 958.6 cone 50 V (TUA) ESI+, *m/z* = 1018.7 cone 15 V (TUA)

Table 1. Chromatographic conditions for the comparison study.

RESULTS AND DISCUSSION

Calibration curves (peak area versus concentration) were prepared for lasalocid and tylosin. The R^2 values for the lasalocid curve were 0.999984 for HPLC-FLD 0.999458 for UPLC-MS. The R^2 values for the tylosin curve were R^2 = 0.999996 for HPLC-PDA and 0.998943 for UPLC-MS.

Determination of analytes using UV or fluorescence detection requires chromatographic baseline separation. The high selectivity (unit mass detection resolution) in the detection of the analytes using the ACQUITY QDa allowed for the determination of lasalocid and tylosin without interference from the matrix.

Results of lasalocid and tylosin for QC and actual samples determined from the UPLC-MS system were compared with those from conventional HPLC systems (existing methods, see Figure 2). Table 2 shows the comparison results, as well as the differences between these two result sets. More importantly, the results for the QC sample was in close agreement for both methods with a deviation of <10% for both lasalocid and tylosin.

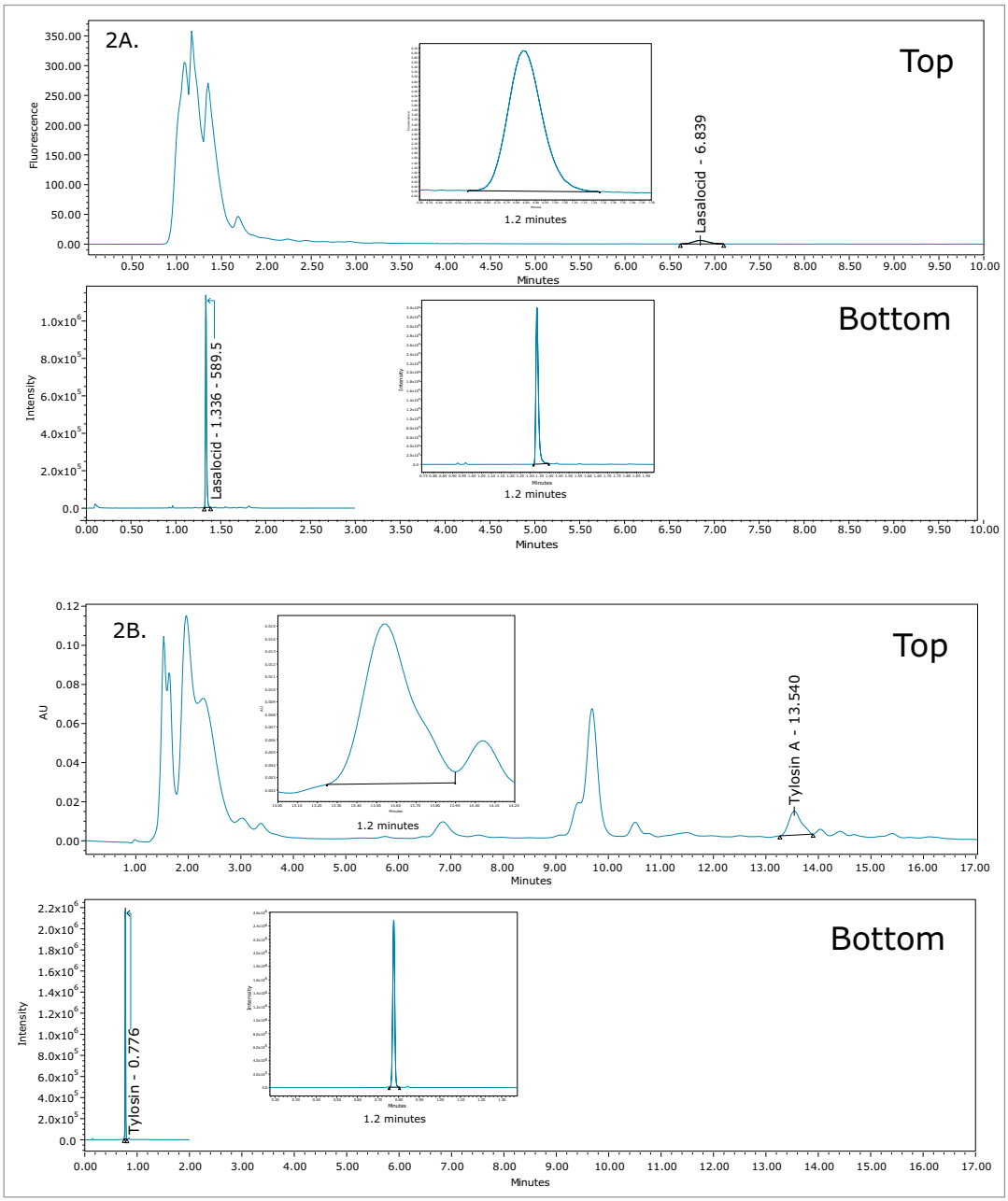

Figure 2A. Top: HPLC-FLD at Ex 314 nm and Em 418 nm; Bottom: a chromatogram depicting lasalocid from sample 1402984 with UPLC-MS SIR at m/z 589.6.

Figure 2B. Top: Chromatogram depicting tylosin from sample 1403969 with HPLC-PDA at UV 286.4 nm; Bottom: UPLC-MS SIR at m/z 916.6.

Given the variety of methods and complexity of feed samples, variations within proficiency test studies have resulted in widely acceptable variations recommended by the Association of American Feed Control Officials (AAFCO).[3] For all the different matrices tested, the lasalocid samples showed a deviation from 2.3% up to 27.5% for the two different methods employed. The tylosin samples carried a deviation from 0.1% up to 11.8%.

Given the challenging matrices included in the analysis, and the wide range of variation reported from PT studies, the good agreement for the majority of samples is very encouraging. For the two samples that showed a >20% deviation for lasalocid, further investigation is warranted.

Lasalocid results comparisons

Sample #	HPLC-FLD result (g/T)	UPLC-MS result (g/T)	Difference (g/T)	Deviation (%)
QC	68.1	64.05	-4.05	4.33%
1402968	131.4	127.24	-4.16	2.27%
1402978	132.1	140.57	8.47	4.39%
1402982	75.8	80.27	4.47	4.05%
1402984	29.3	37.06	7.76	16.54%
1403466	84.1	93.16	9.06	7.23%
1403706	5.01	7.42	2.41	27.46%
1403725	36.6	51.44	14.84	23.84%
1403726	91.1	111.41	20.31	14.18%
1403728	134.8	168.80	34.00	15.84%
1403735	304.8	375.00	70.20	14.60%
1403736	369.3	440.76	71.46	12.48%

Tylosin results comparisons

Sample #	HPLC-PDA result (g/T)	UPLC-MS result (g/T)	Difference (g/T)	Deviation (%)
QC-TYL-11	47.9	43.14	-4.74	7.37%
1403733	75.5	89.22	13.72	11.78%
1403741	85.6	93.25	7.68	6.07%
1403746	106.5	106.66	0.12	0.08%
1403923	111.1	116.49	5.35	3.32%
1403941	92.5	89.99	-2.55	1.98%
1403952	107.7	110.23	2.51	1.63%
1403957	95.3	102.45	7.20	5.15%
1403965	35.3	34.05	-1.25	2.54%
1403966	81.4	79.09	-2.35	2.07%
1403969	68.5	70.18	1.70	1.73%
1402993	70.6	74.98	4.38	4.25%

Table 2. Top, a comparison of results obtained from HPLC-FLD and UPLC-MS for lasalocid. Bottom, a comparison of results obtained from HPLC-PDA and UPLC-MS for tylosin. TUA was not included in this table.

The repeatability of lasalocid on the UPLC-MS system was tested by injecting sample number 1403736 10 times in sequence, as shown in Figure 3. The excellent repeatability from 0.2 µL injection volumes provide evidence of the ability of the ACQUITY QDa Detector to handle high volumes of feed matrices without the need for constant maintenance.

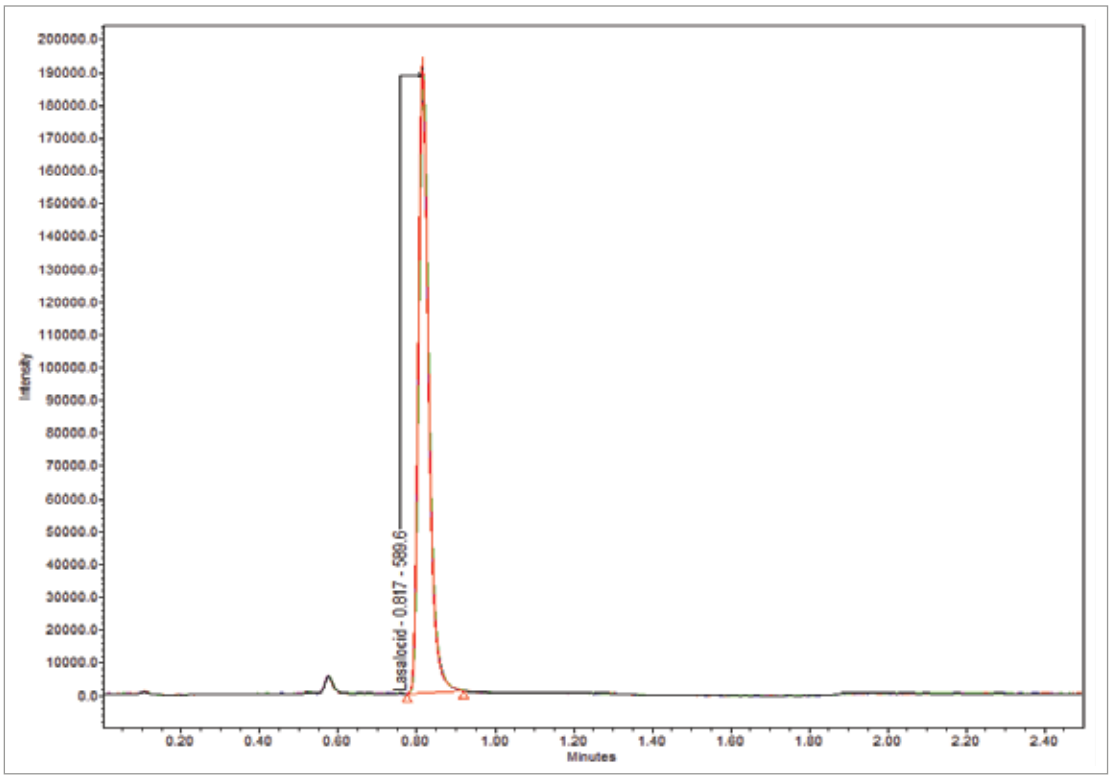

Figure 3. A chromatogram depicting 10 replicate injections of sample 1403736 on the UPLC-MS at 0.2 µL injection volumes. Retention time relative standard deviation (RSD) was 0.07% and area RSD was 1.2%. See Table 2 for concentrations.

TYLOSIN UREA ADDUCT

Feeds produced for ruminants often contain urea (*m/z* = 60.06), and are sometimes used as an inexpensive replacement for a part of the protein in feed. Tylosin is known to form an adduct with urea in feeds to create a tylosin urea adduct (TUA) which complicates conventional HPLC analyses of tylosin with the need to confirm an additional peak with an analyte with which there is no readily available standard.

TUA was identified using the UPLC-MS system at *m/z* = 958.6 (tylosin + 1 urea - H_2O) and *m/z* = 1018.7 (tylosin + 2 urea - H_2O). Figure 4 details the chromatogram of a tylosin-fortified feed sample where TUA has been clearly detected.

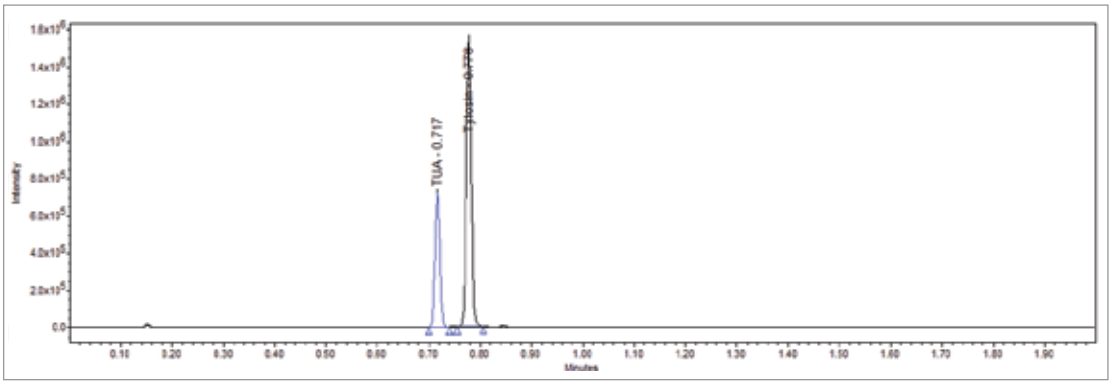

Figure 4. A chromatogram of sample 1403965 displaying m/z = 916.61 for Tylosin (black) and m/z = 1018.70 for TUA (tylosin + 2 urea – H_2O, blue) using UPLC-MS.

Using the same procedure outlined for previous conversion studies of TUA to tylosin A,[4] an estimate of approximately 30 g TUA per ton of feed in sample 1403965 was obtained. Figure 5 details the chromatogram of a sample that was not labeled to have fortified tylosin A, but which showed residual levels of TUA, which could not have been detected using conventional HPLC-PDA chromatography. The amount of TUA was estimated against a standard of unknown purity to be present at around the 100 ng/mL level. The ability to detect low levels of residual compounds is a significant advantage with a selective mass detector, and we foresee potential applications with complete tylosin determination in the future.

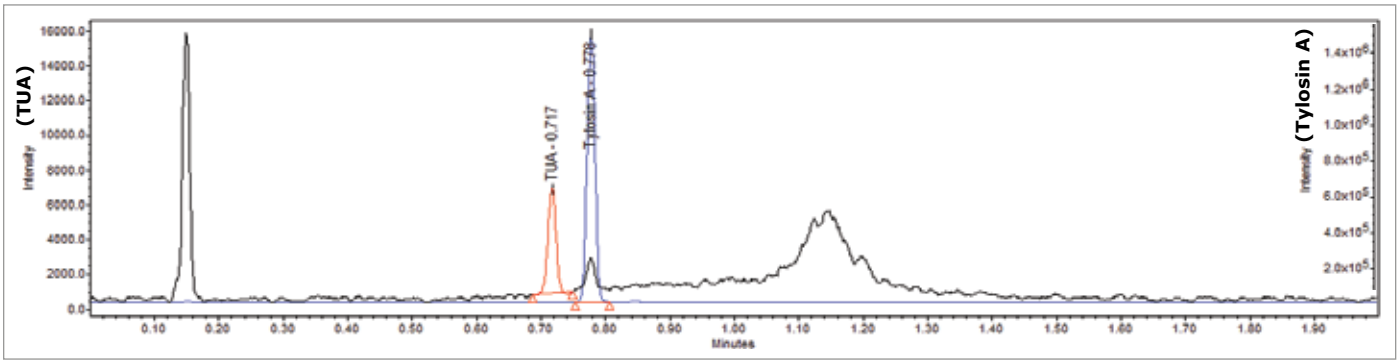

Figure 5. A SIR chromatogram overlay of sample QC-TYL-11 displaying m/z = 916.61 for tylosin (blue, scale on right) and m/z = 1018.70 for TUA (black with red peak, scale on left). The chromatograph displays a residual level of TUA that was not detected with HPLC-PDA.

CONCLUSIONS

The ACQUITY UPLC System with the ACQUITY QDa Mass Detector offers a more selective tool for determining lasalocid and tylosin in complex animal feed matrices and allows an accessible means to incorporate mass spectrometry into a feed regulatory laboratory, both in terms of cost and in ease of use.

The tylosin urea adduct (TUA) was identified at both residual and feed-level concentrations. The ACQUITY QDa Mass Detector provided confirmation of TUA through selective ion monitoring.

Further method development for the UPLC-MS is planned for consolidating extraction methodology, adding additional antibiotic analytes, and for incorporating single-injection, multi-analyte quantitation.

Acknowledgements

We thank Alpharma/Zoetis and USP for their provision of standards. We kindly acknowledge Nancy Thiex of South Dakota for TUA consultation over the years. We are grateful to David Wait of Waters Corporation for facilitating and coordinating the collaboration between the Ohio Department of Agriculture Consumer Protection Laboratory and Waters Corporation.

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Selective and Sensitive Screening of Chloramphenicol in Milk Using the ACQUITY UPLC I-Class System and the ACQUITY QDa Mass Detector

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[2] Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- A rapid screening method for chloramphenicol in milk.
- Quantitative screening of samples with detection below regulatory limits.
- Simple and sensitive detection of a regulated antibiotic.
- Easy implementation of mass detection within existing LC workflows.

WATERS SOLUTIONS

Oasis® HLB Column

ACQUITY UPLC® HSS C_{18} Column

ACQUITY UPLC I-Class System

ACQUITY® QDa® Mass Detector

KEYWORDS

Chloramphenicol, antibiotic, mass detection, milk, veterinary drug

INTRODUCTION

Chloramphenicol is an inexpensive broad spectrum antibiotic. In certain susceptible individuals, chloramphenicol has been associated with toxic effects. It is suspected to be both a carcinogen and genotoxic compound, and cases of fatal aplastic anemia (depression of bone marrow) are widely documented. Therefore, its use in food producing animals, insects, and aquaculture is prohibited in the EU, Canada, the U.S., along with a number of Asian and South American countries. Within the EU, Commission Decision 2003/181/EC requires that any method for the detection of chloramphenicol in food have a minimum required performance limit (MRPL) of 0.3 $\mu g.kg^{-1}$.

Dairy testing laboratories traditionally screen large volume samples using enzyme-linked immunosorbent assay (ELISA), where a quick turnaround time is essential given the short shelf life of the product. Although ELISA allows for sensitive and rapid screening of chloramphenicol, one drawback is the significant level of false positive results, where a false positive level as high as 16% has been reported in certain instances.[1]

Due to cross reaction of the immunosorbent with interfering matrix, chloramphenicol can often be misdetected in milk resulting in the unnecessary destruction of the product. Mass detection offers increased selectivity, which helps differentiate between chloramphenicol and contaminating matrix. Furthermore, mass detection coupled with liquid chromatography (LC) eliminates the need for time-consuming derivatization typically required for screening of chloramphenicol by gas chromatography. The ACQUITY QDa Mass Detector can easily be integrated into LC workflows, with the ease of use that does not require specialist mass spectrometry knowledge.

EXPERIMENTAL

UPLC conditions

UPLC system:	ACQUITY UPLC I-Class
Run time:	5 min
Column:	ACQUITY UPLC HSS C_{18} 1.8 µm, 2.1 x 100 mm
Mobile phase:	10 mM ammonium acetate in 45:55 methanol:water
Injection volume:	10 µL

MS conditions

MS system:	ACQUITY QDa
Ionization mode:	ESI-
SIR channel:	*m/z* 321.0
Cone voltage:	15 V
Probe temp.:	Default (600 °C)
Capillary voltage:	Default (0.8 kV)
Sampling rate:	Default (5 Hz)

Sample preparation

Semi-skimmed cow's milk (5 g) samples were weighed into 25-mL centrifuge tubes and fortified to allow for 0.3 µg.kg^{-1} in the sample. Fat removal and protein precipitation were completed by adding hexane (5 mL) and 70% acetonitrile solution (15 mL) to the sample. Samples were shaken then centrifuged for 10 min at 3,900 G (4 °C). The lower acetonitrile layer (5 mL) was removed and diluted to a total volume of 15 mL with 10 % methanol.

Oasis HLB 3 cc (60 mg) Cartridges were conditioned with methanol (2 mL) and water (4 mL). The diluted samples (15 mL) were loaded on to the cartridge, which was then washed with 5% methanol solution (6 mL). Chloramphenicol was eluted using 100% methanol (6 mL). This eluate was subsequently evaporated under nitrogen, reconstituted with 10% methanol solution (300 µL), and transferred for LC-MS analysis.

Preparation of standards

Blank milk samples (unfortified) were prepared through the sample method outlined. Once dried under nitrogen, the blank matrix samples were reconstituted using standard solutions of different concentrations to allow for a six-point calibration curve over the range equating to 0.075 to 1 µg.kg^{-1}.

RESULTS AND DISCUSSION

Prior to the analysis of the milk extracts, the optimum source conditions were investigated for chloramphenicol, where the pre-optimized source parameters of the ACQUITY QDa Detector provided for the most efficient and sensitive detection. Chloramphenicol was most sensitive in negative ionization mode with a voltage of 15 applied to the cone. Using the method described, a matrix-matched calibration curve allowed for accurate quantification of milk samples fortified at the EU MRPL. Excellent linearity (R^2>0.995) was observed for chloramphenicol over the selected working range of 0.075 to 1 µg.kg,$^{-1}$ as shown in Figure 1.

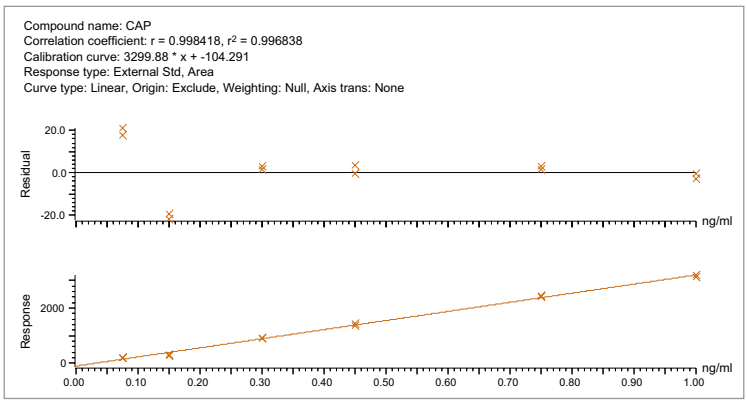

Figure 1. Matrix-matched calibration curve with duplicate injections, equating to range of 0.075 to 1 µg kg^{-1}.

In order to assess matrix effects, the matrix matched calibration curve was compared to a solvent calibration curve over the same range. Figure 2 shows an overlay of the matrix-matched and solvent calibration curves. From this overlay, matrix effects (ion enhancement) are evident. This can also be demonstrated statistically using the slope of each of the calibration curves ($\frac{\text{Slope sample}}{\text{Slope standard}}$ x 100), where matrix effects of 177% are calculated, confirming ion enhancement. Despite the matrix effects observed, the accurate quantitative screening of chloramphenicol in milk is readily achievable with the use of the matrix-matched calibration curve.

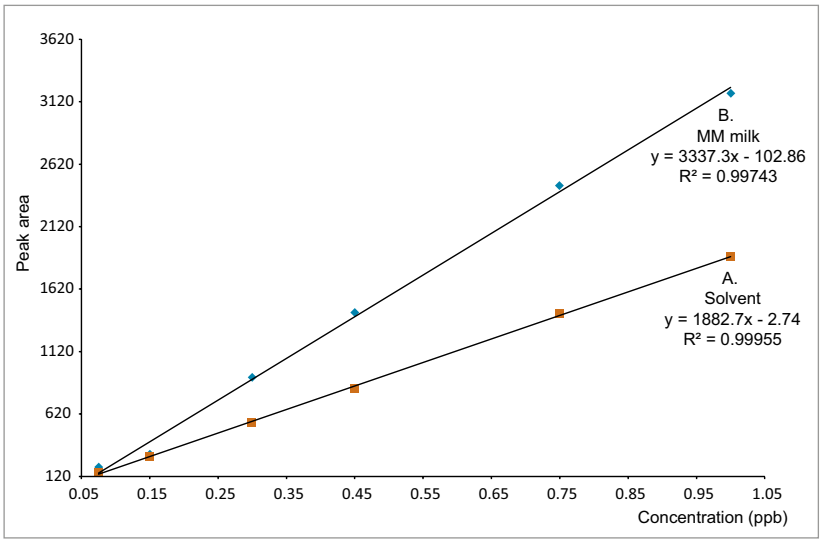

Figure 2. Overlay of calibration curves 2A. solvent (10% methanol), and 2B. matrix matched (in milk) equating to 0.075 to 1 µg.kg^{-1}.

Replicate blank samples of milk were analyzed, where no false positives were detected. An example chromatogram for an extracted blank is shown in Figure 3A, where only baseline noise was observed. Four replicate milk samples were fortified at the EU MRPL, extracted and analyzed in duplicate (n= 8), in the absence of expensive deuterated internal standard. The resultant samples were quantified against the matrix-matched calibration curve. Figure 3B shows the chloramphenicol peak observed for replicates at the fortification level (0.3 µg.kg^{-1}), where good peak-to-peak signal-to-noise ratio (S/N) of ≥50 was achieved.

As shown in Figure 3B, good repeatability was observed for the retention time. This is also interpreted statistically, along with recoveries, in Table 1. An average recovery of 72.7% was determined, which is acceptable in accordance with European Commission Decision 2002/657/EC. Excellent repeatability was obtained over eight replicates at the regulatory limit with relative standard deviation (RSD) of <4.6%. This demonstrates that the method is both robust and sensitive for the quantitative screening of chloramphenicol in milk at the regulatory limit.

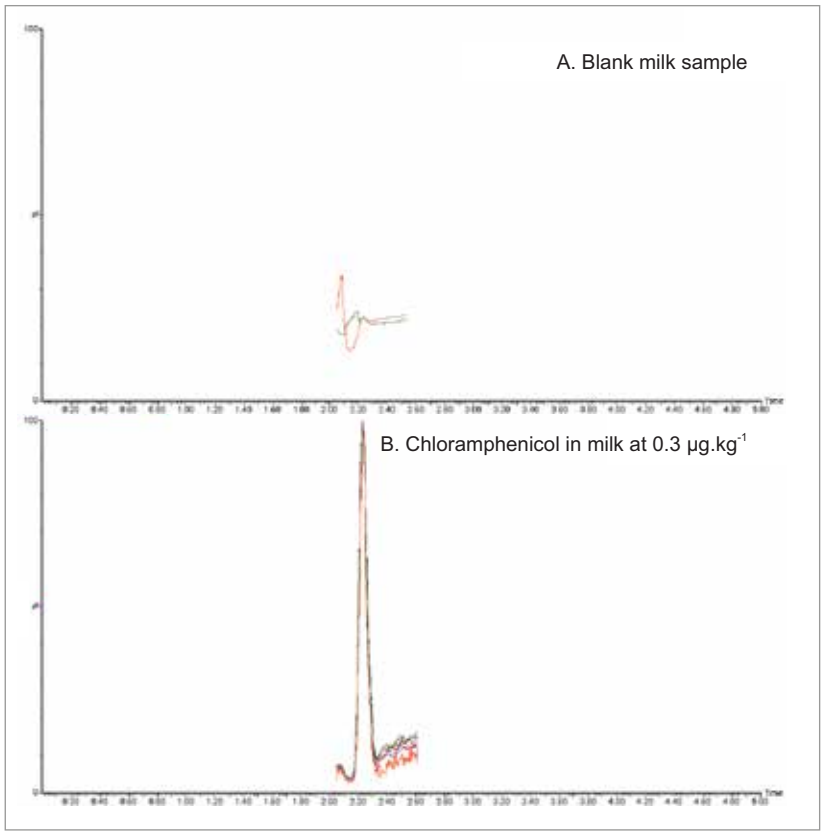

Figure 3. Selected ion recording (SIR) chromatogram of chloramphenicol (m/z 321) for: 3A. Blank milk extracts (n= 2); 3B. Milk fortified at MRPL (0.3 µg.kg^{-1}). Replicate injections (n= 8) are overlaid with S/N ≥50.

Table 1. Recoveries (n= 8) achieved from SPE method, showing good repeatability of sample preparation and instrument.

Replicate number	%Recovery	Retention time (min)
1	77.0	2.23
2	69.7	2.23
3	76.3	2.23
4	71.7	2.23
5	72.3	2.23
6	67.3	2.23
7	75.0	2.24
8	72.3	2.23
Average	**72.7**	**2.231**
Std dev	**3.3**	**0.004**
%RSD	**4.5**	**0.158**

CONCLUSIONS

An accurate and robust method has been developed for the reliable quantitative screening of chloramphenicol in milk. This method has proven to achieve levels of detection that can easily meet regulatory requirements, in the absence of an internal standard, while showing excellent repeatability at the EU MRPL of 0.3 µg.kg^{-1}. The additional discrimination that mass detection affords results in increased specificity, where no false positives were detected.

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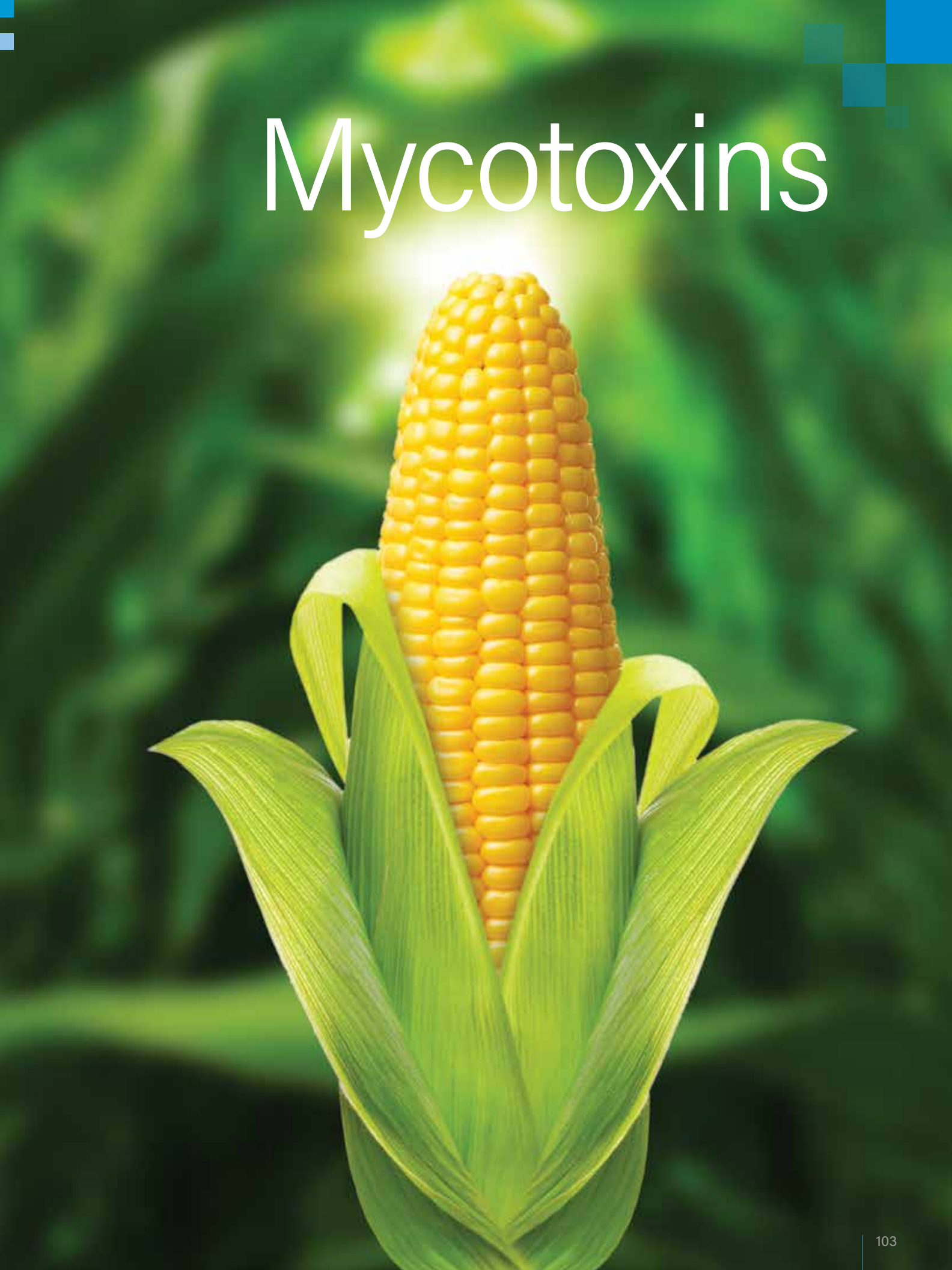
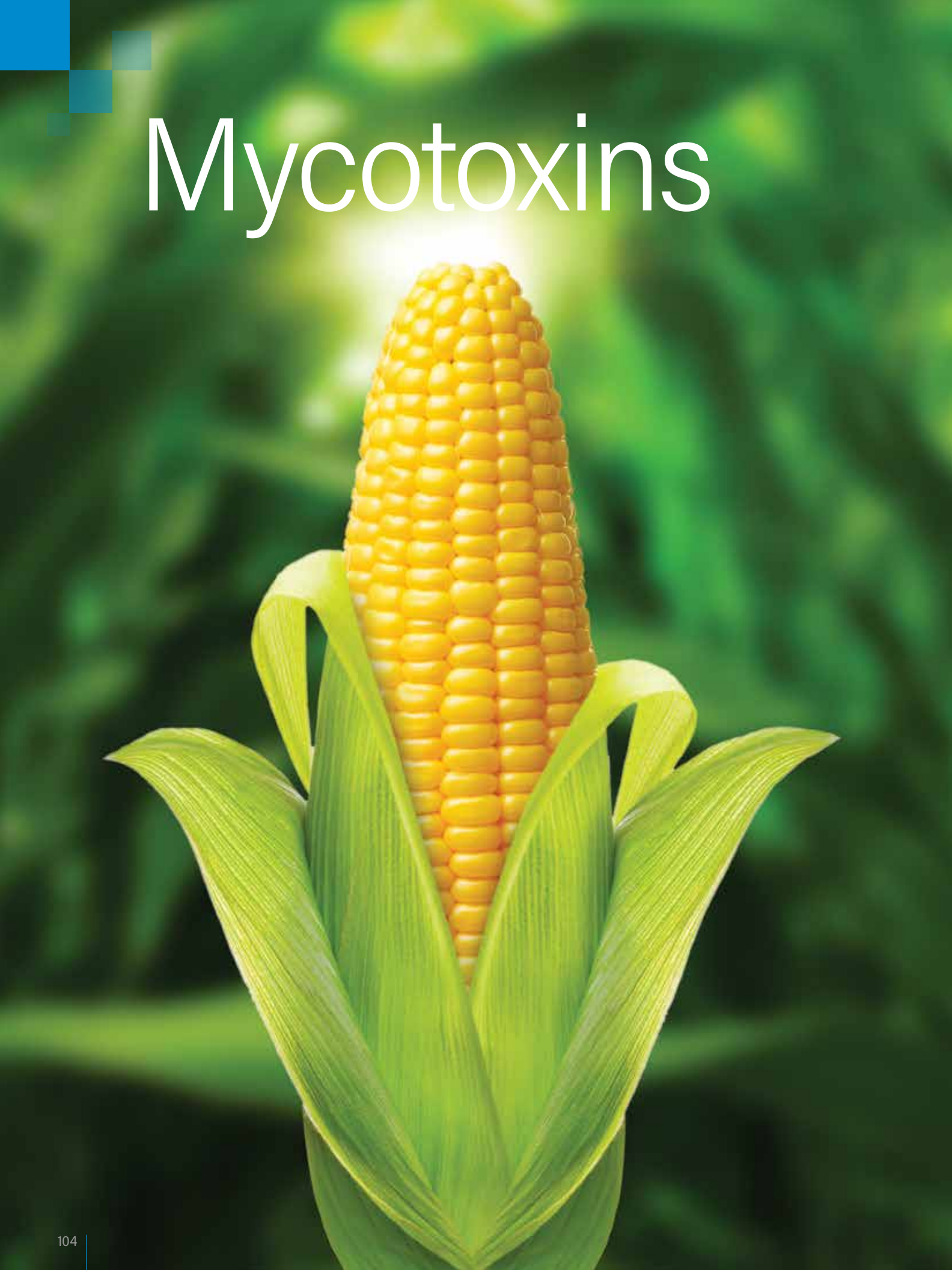
Mycotoxins



Mycotoxins



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Rapid Quantitative Analysis of 12 Mycotoxins in Processed Maize Using Myco6in1+ Immunoaffinity Clean-Up and the ACQUITY QDa Mass Detector

Dominic Roberts,[1] Sara Stead,[1] Veronica Lattanzio,[2] Biancamaria Ciasca,[2] Eimear McCall,[1] Jennifer Burgess,[3] and Stephen Powers[4]
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[3]Waters Corporation, Milford, MA, USA; [4]VICAM, Milford, MA, USA

APPLICATION BENEFITS

- Multiple mycotoxin detection in a single consolidated method.
- Sensitive detection of 12 mycotoxins at regulatory limits in complex cereal-based foods.

WATERS SOLUTIONS

CORTECS® UPLC® C_{18} Column

ACQUITY UPLC® I-Class System

ACQUITY® QDa® Mass Detector

VICAM® Myco6in1+® Immunoaffinity Column (IAC)

KEYWORDS

Mycotoxin, trichothocene, nivalenol, fumonisin, aflatoxin, ochratoxin, zearalenone, mass detection

INTRODUCTION

Mycotoxins are naturally occurring secondary metabolites produced by fungi. A variety of mycotoxins can contaminate a wide range of fruits, cereals and grains. Chemically stable and resistant to different forms of decomposition, many of these compounds are known to have carcinogenic, estrogenic, and immunotoxic effects *in vivo*. Therefore, extensive global regulations are enforced laying down sampling and testing methods, along with permitted limits for specific mycotoxins.

Within the European Union, maximum permitted levels for deoxynivalenol (DON), fumonisins (B1 and B2), aflatoxins (B1, B2, G1, and G2), ochratoxin A, and zearalenone are regulated in EU Commission Regulation 1881/2006 and 1126/2007. More recently, recommended levels for the sum of T2 and HT2 toxins have been provided in Recommendation 2013/165. Additionally, rules for sampling and performance of analytical methods are specified in Regulation 401/2006. Although currently not regulated, attention is paid in this study to the occurrence of nivalenol (NIV), another *Fusarium* toxin that frequently contaminates cereals in combination with DON.

Due to the variety of complex food commodities naturally contaminated by fungal species multi-mycotoxin analysis can prove both challenging and time consuming. Therefore, the availability of rapid and sensitive screening tools that are capable of achieving the regulatory levels for the relevant mycotoxins and commodities is essential. The development of methods for the determination of multiple mycotoxins is highly desirable, where LC-MS plays an important role in this field. However due to the differences in chemical and physical properties of the major mycotoxins, sample preparation can become the most challenging task.

Given the frequency of naturally contaminated food and feeds by various mycotoxins, the need exists for the simple and sensitive detection of regulated mycotoxins. This work aims to develop a simple and accurate method for the quantitative analysis of multiple mycotoxins in processed maize using an immuno-affinity cleanup method and mass detection.

EXPERIMENTAL

UPLC conditions

LC system:	ACQUITY UPLC I-Class
Run time:	12 min
Column:	CORTECS C_{18} 1.6 µm, 2.1 x 100 mm
Mobile phase A:	2 mM ammonium acetate with 0.1% formic acid in water
Mobile phase B:	2 mM ammonium acetate with 0.1% formic acid in methanol
Flow rate:	0.4 mL.min^{-1}
Injection volume:	10 µL

Gradient:

Time (min)	A (%)	B (%)
Initial	99.0	1.0
7.00	50.0	50.0
10.0	1.0	99.0
11.5	1.0	99.0
11.6	99.0	1.0
14.00	99.0	1.0

MS conditions

MS system:	ACQUITY QDa
Ionization mode:	ESI±
Desolvation temp.:	600 °C
Capillary voltage:	Default (0.8 kV)
Sampling rate:	Default (5 Hz)
SIR channels:	See Table 1

Sample preparation

Ten grams of ground sample were extracted by high speed blending with 40 mL of water followed by 60 mL of methanol. The extract was filtered and a 5-mL aliquot was reduced to approximately 2 mL under nitrogen. Phosphate buffer (5 mL) was added and the resulting solution was loaded onto the VICAM Myco6in1^{+} Immunoaffinity Column (IAC). The column was washed with 10 mL water and the toxins were eluted, first with methanol (3 mL) followed by water (2 mL). The eluate was subsequently evaporated to dryness, under a gentle flow of nitrogen, and reconstituted to 0.2 mL in 10:90 *v/v* methanol:mobile phase A.

Preparation of standards

Blank cereal food samples (unfortified) were prepared using the Myco6in1^{+} IACs. The resultant eluates were fortified with a mixed mycotoxin standard solution to allow for a 5-point calibration curve, where the midpoint range on all of the calibration curves were equal to the permitted level of each mycotoxin. The remaining points were equally distributed with two standards below and two standards above the permitted limit for each relevant mycotoxin. These standards were then evaporated to dryness and reconstituted in 10:90 *v/v* methanol:mobile phase A.

RESULTS AND DISCUSSION

The default source conditions of the Waters® ACQUITY QDa Mass Detector offered the optimum performance for all 12 mycotoxin analytes. The individual masses, cone voltages and electrospray ionization mode were identified and are shown in Table 1.

	Mycotoxin	Abbreviation	RT (min)	SIR (*m/z*)	Cone voltage (V)	Calibration range ($\mu g.kg^{-1}$)
1.	Nivalenol	[NIV-H_2O+H^+]	2.2	295.0	15	468.75 to 5625.00
2.	Deoxynivalenol	[DON+H^+]	2.9	297.0	10	468.75 to 5625.00
3.	Aflatoxin G2	[AFG2+H^+]	5.8	331.0	20	0.625 to 7.50
4.	Aflatoxin G1	[AFG1+H^+]	6.2	329.0	20	0.625 to 7.50
5.	Aflatoxin B2	[AFB2+H^+]	6.5	315.0	20	1.250 to 15.00
6.	Aflatoxin B1	[AFB1+H^+]	6.8	313.0	20	0.625 to 7.50
7.	HT2 toxin	[HT2+Na^+]	8.2	447.0	15	31.250 to 375.00
8.	Fumonisin B1	[FB1+H^+]	8.3	722.0	20	500.000 to 6000.00
9.	T2 toxin	[T-2+NH_4^+]	8.6	484.0	15	31.250 to 375.00
10.	Ochratoxin A	[OTA+H^+]	8.8	404.2	20	62.500 to 750.00
11.	Zearalenone (negative mode)	[ZEA-H]$^-$	8.8	317.0	20	1.875 to 22.50
12.	Fumonisin B2	[FB2+H^+]	9.0	706.0	20	125.000 to 1500.00

Table 1. The 12 mycotoxins with experimental parameters.

Good linearity was obtained for all 12 mycotoxins over the relevant calibration ranges. A minimum of 12 data points per chromatographic peak were obtained for each analyte. An example of the linearity is shown in Figure 1, where AFG1 and DON depict the difference in the regulatory limits and calibration ranges required. AFG1 runs over the range of 0.125 to 4.0 $\mu g.kg^{-1}$ due to the low regulatory limit assigned to aflatoxins; while the calibration required for DON is over the range of 94.0 to 1500 $\mu g.kg.^{-1}$ The linearity observed for all analytes over these varying calibration ranges shows the instrument's excellent robustness over the wide dynamic range in the presence of complex matrix.

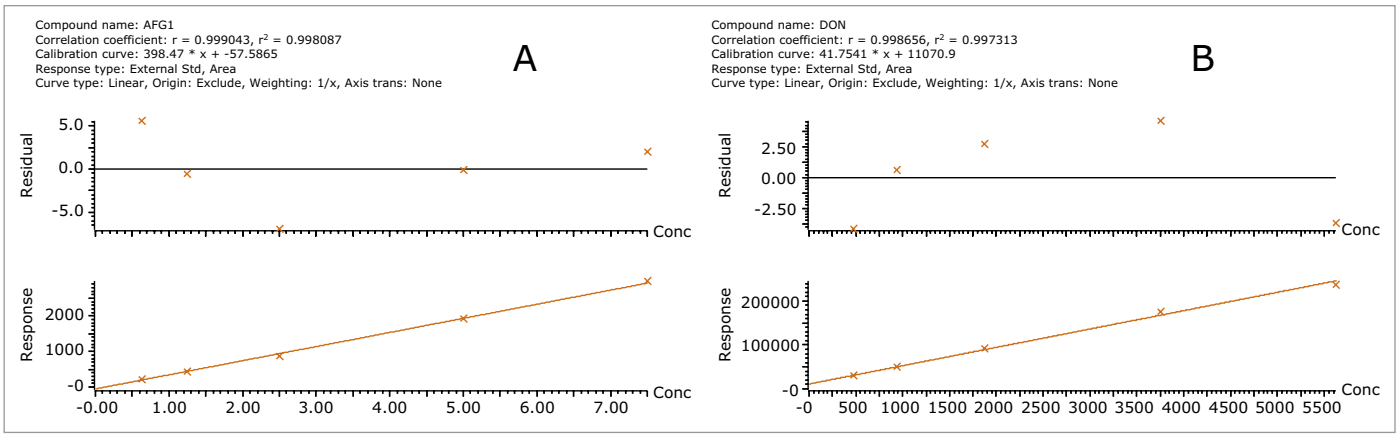
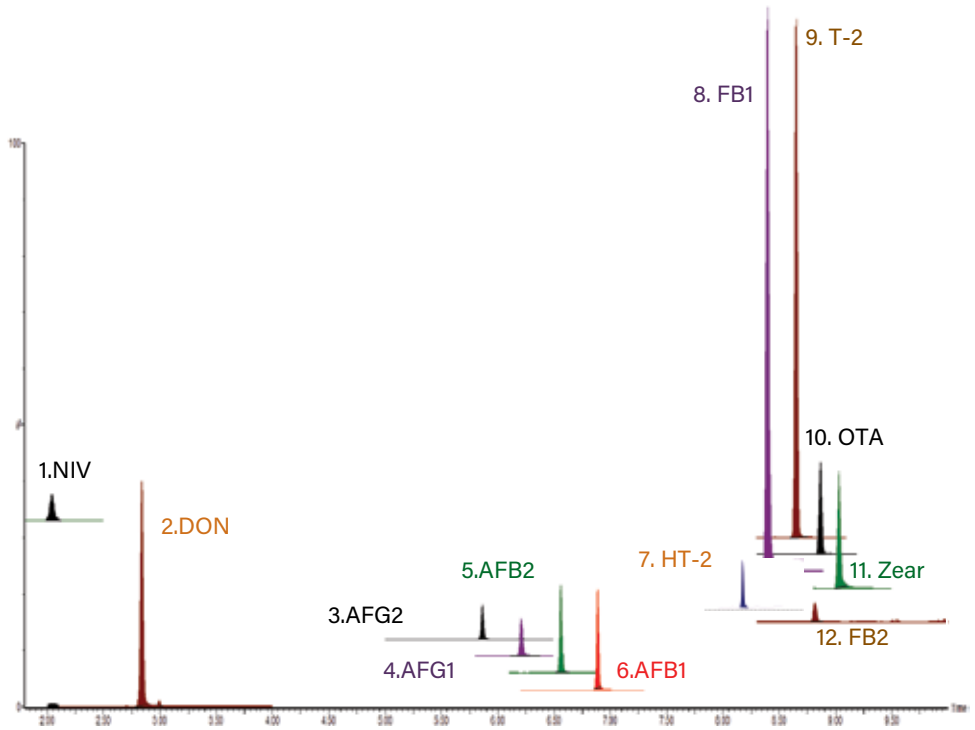


Figure 1. Examples of the matrix matched calibration curves for 1A. Aflatoxin G1 equating to the range of 0.125 to 4 $\mu g.kg.^{-1}$ and 1B. Deoxynivalenol equating to the range of 94.0 to 1500 $\mu g.kg.^{-1}$

An example of the chromatography achieved is shown in Figure 2, where the maize snack food was fortified to the regulatory limits. Satisfactory sensitivity was reported for each of the analytes, and excellent signal-to-noise (S/N) ratios were achieved. All four aflatoxins, plus the additional eight mycotoxins can readily be detected by LC coupled with mass detection.



Mycotoxin spiked concentration S/N (µg kg⁻¹)		
NIV	750	1188
DON	750	20955
AFG2	1	5149
AFG1	1	18272
AFB2	1	1967
AFB1	2	12766
HT2	50	430
FB1	800	2682
T-2	50	64641
OTA	3	993
Zear	100	6682
FB2	200	1967

Figure 2. Processed maize food sample fortified at the displayed concentrations (EU regulatory limits). Chromatographically resolved peaks (normalized) were detected with excellent S/N ratios at legally permitted levels.

CONCLUSIONS

A simple, sensitive, and cost-effective method has been developed for the quantitative analysis of 12 regulated mycotoxins in processed maize extract. Using a single sample preparation procedure, all analytes were extracted and readily detected on the ACQUITY QDa Mass Detector. No additional or time-consuming sample preparation was required, thus allowing for the rapid screening of multiple mycotoxins on a single detector.

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Selective Analysis of Patulin in Apple Juice Using the ACQUITY UPLC H-Class System with the ACQUITY QDa Mass Detector

Brian Tyler and Jennifer A. Burgess
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

- Ensures unambiguous detection of patulin at levels ten times lower than legislative requirements for apple juice and half the legislative requirements for baby food.
- The ACQUITY® QDa® Mass Detector is designed for integration with UPLC® and UHPLC systems to provide robust, reliable orthogonal detection.
- Selective detection using SIR mode is used for reliable and quantitative detection while RADAR™ Technology, which enables the simultaneous acquisition of full mass spectra, provides information on any background interference during method development and routine analysis.
- The ACQUITY QDa Mass Detector can easily be added to existing liquid chromatography workflows in order to vastly increase the selectivity that could previously be obtained with LC detectors.

WATERS SOLUTIONS

ACQUITY QDa Mass Detector

ACQUITY UPLC® H-Class System

MassLynx® Software

TargetLynx™ Application Manager

Oasis® HLB Sample Extraction Products

KEYWORDS

Patulin, mycotoxin, mass detection, food safety, apple juice , 5-hydroxymethylfurfural (HMF)

INTRODUCTION

Patulin is a mycotoxin produced by several Penicillium, Aspergillus, and Byssochlamys mold species that are commonly found on apples. Apples that have been damaged or bruised prior to processing are more susceptible to contamination by patulin producing molds.

Proper handling and storage of apples is important to prevent the growth of patulin producing molds. When patulin-contaminated apples are processed into juice high levels of patulin are possible. The thermal stability of patulin prevents its decomposition during pasteurization.

The effects of patulin on humans are not known but intestinal lesions and stomach hemorrhages have been observed in mice and rats. Owing to this toxicity, the U.S. FDA has set a maximum residue level (MRL) of 50 µg/kg of patulin in apple juice and apple juice concentrates.[1] Other countries including China, Japan, and the EU have also set the maximum contamination of patulin to 50 µg/kg in apple juice products. The EU also has lower limits for patulin in solid apple products (25 µg/kg), such as apple puree, and products designed for infants and young children (10 µg/kg).[2]

In order to protect both producers and consumers, accurate testing is required to prevent the contamination of apple juice with patulin. Patulin testing has typically employed LC separation with UV detection at 273 nm. Also absorbing at this wavelength, however, is 5-hydroxymethylfurfural (HMF), which is produced in the pasteurization of apple juice. The structures are shown in Figure 1.

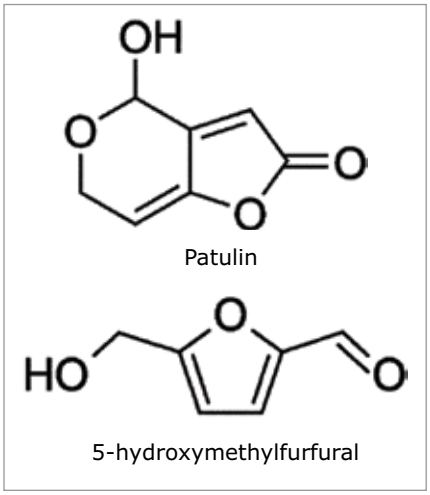

Figure 1. Structures of Patulin and HMF.

EXPERIMENTAL

UPLC conditions

UPLC system:	ACQUITY UPLC H-Class
Column:	ACQUITY UPLC BEH Shield RP18 1.7 µm, 2.1 x 100 mm
Column temp.:	40 °C
Injection volume:	10 µL
Flow rate:	0.60 mL/min
Mobile phase A:	Water + 0.1% NH_4OH
Mobile phase B:	Acetonitrile + 0.1% NH_4OH
Weak needle wash:	50:50 water:methanol
Strong needle wash:	50:50 water:methanol
Seal wash:	90:10 water:acetonitrile

Time (min)	Flow rate (mL/min)	%A	%B	Curve
Initial	0.60	99	1	6
1.80	0.60	99	1	6
2.30	0.60	10	90	6
2.80	0.60	10	90	6
2.81	0.60	99	1	6
5.00	0.60	99	1	6

Table 1. UPLC gradient method for analysis of patulin in apple juice.

MS conditions

MS system:	ACQUITY QDa (Performance)
Ionization mode:	ESI-
Capillary voltage:	0.8 kV
Desolvation temp.:	500 °C
Source temp.:	150 °C
Cone voltage:	2 V
Patulin SIR:	152.9
HMF SIR:	124.8
Sampling rate:	5 Hz

Solid phase extraction (SPE)[3]

Apple juice, which had been filtered and pasteurized prior to bottling, was purchased from a local supermarket. The juice was extracted using SPE.

Cartridge:	Oasis HLB 3 cc/60 mg
Condition:	3 mL methanol 3 mL water
Load:	2.5 mL sample
Wash 1:	3 mL 1% $NaHCO_3$ (1 g/100 mL)
Wash 2:	1 mL 0.1% acetic acid Dry under vacuum
Elute:	2 x 1.5 mL 10 ethyl acetate in methyl t-butyl ether (MTBE)
Reconstitute:	500 µL water

Overall the sample preparation resulted in a five-fold concentration. The calibration standards were prepared to take into account this concentration step (the range 5 to 200 µg/L in apple juice equates to an in-vial calibration range of 25 to 1000 µg/L).

HMF therefore has the potential to interfere in the analysis of patulin when using UV detection. Furthermore, UV methods struggle to detect down to the lowest levels required for products that are destined for consumption by infants and children. In order to improve the selectivity and reduce limits of quantification, mass detection is desirable in the analysis of patulin.[3] Deployment of mass spectrometry in some laboratories may require workflow, infrastructure, or resource modifications to operate and maintain the systems. ACQUITY QDa Detector offers laboratories the opportunity to capture the benefits of mass detection without the challenges associated with the adoption of mass spectrometers.

In this application note, we present an ACQUITY UPLC method using mass detection with the ACQUITY QDa Detector. This ACQUITY QDa can easily be added to existing liquid chromatography workflows in order to vastly increase the selectivity that could previously be obtained with LC detectors. It has been designed for chromatographic systems so it can be easily implemented into existing laboratory configurations.

RESULTS AND DISCUSSION

DETECTION AND QUANTIFICATION

Single ion recording (SIR) was used to monitor both patulin and 5-hydroxymethylfurfural (HMF), while simultaneously acquiring full-spectrum data using RADAR Technology. When analyzing patulin using UV detection, baseline resolution between the two compounds is required as they both absorb at 273 nm. When patulin is analyzed using mass detection, the requirement of baseline separation is removed as the two compounds have different masses (*m/z* 153 and *m/z* 125 for patulin and HMF, respectively). While separation from HMF is not required when mass detection is employed, the separation of HMF and patulin does allow for UV detection to be included in line with mass detection if desired (not shown here). However, the mass detection will have lower limits of detection.[3]

As shown in Figure 2, patulin was successfully detected in apple juice and mass detection with SIR provided high selectivity for this analysis. Patulin was detected down to spiked concentrations of 1 µg/L. The lowest spiked concentration that resulted in a signal-to-noise (S/N) ratio above 10 (using the peak-to-peak method) was 5 µg/L. This level is ten times lower than required by the EU and FDA regulations for apple juice and half of the strictest EU level for baby food.

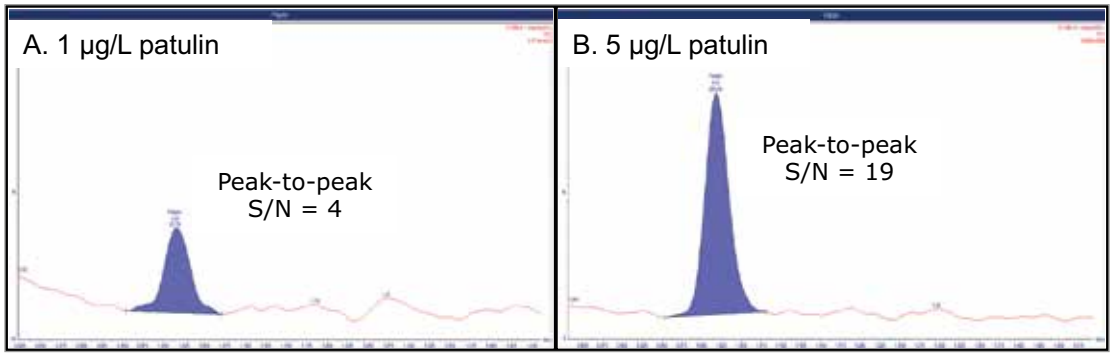

Figure 2. Single ion recording (SIR) chromatograms of patulin spiked into extracted apple juice equivalent to A. 1 µg/L and B. 5 µg/L. The signal-to-noise ratio (calculated using peak-to-peak) is annotated on each chromatogram.

Figure 3 shows the calibration curve obtained for patulin post-spiked into extracted apple juice to create a matrix-matched calibration curve from 5 to 200 µg/L. Excellent linearity was achieved in this range, as evidenced by the correlation coefficient (r^2) of 0.999.

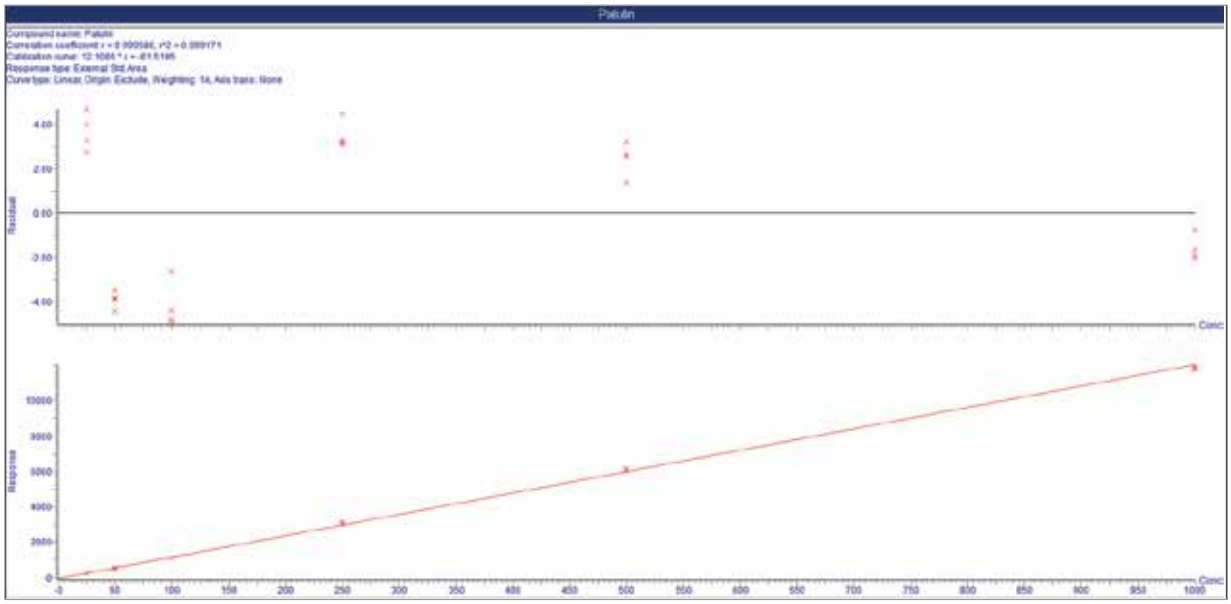

Figure 3. Calibration curve for patulin in extracted apple juice, equivalent to 5 to 200 µg/L.

MATRIX EFFECTS AND RADAR

The analysis of food and beverages is often complicated by matrix interferences that are co-extracted with the analytes of interest. It is therefore important to assess effects introduced by the matrix, and if necessary, adjust sample preparation protocols or chromatographic methods to ensure a robust method. Using the described sample preparation procedure, the response of patulin spiked into extracted apple juice was compared to the response of patulin spiked into water across the calibration range. This was achieved by comparing the slope of the calibration curve in matrix to the slope of the calibration curve in solvent standards (water). Figure 4 shows that the calibration curve of patulin in water was within the same range as the matrix matched calibration curve shown in Figure 3. The signal suppression observed from the matrix effect of apple juice was approximately 10%, providing confidence in the robustness of the method.

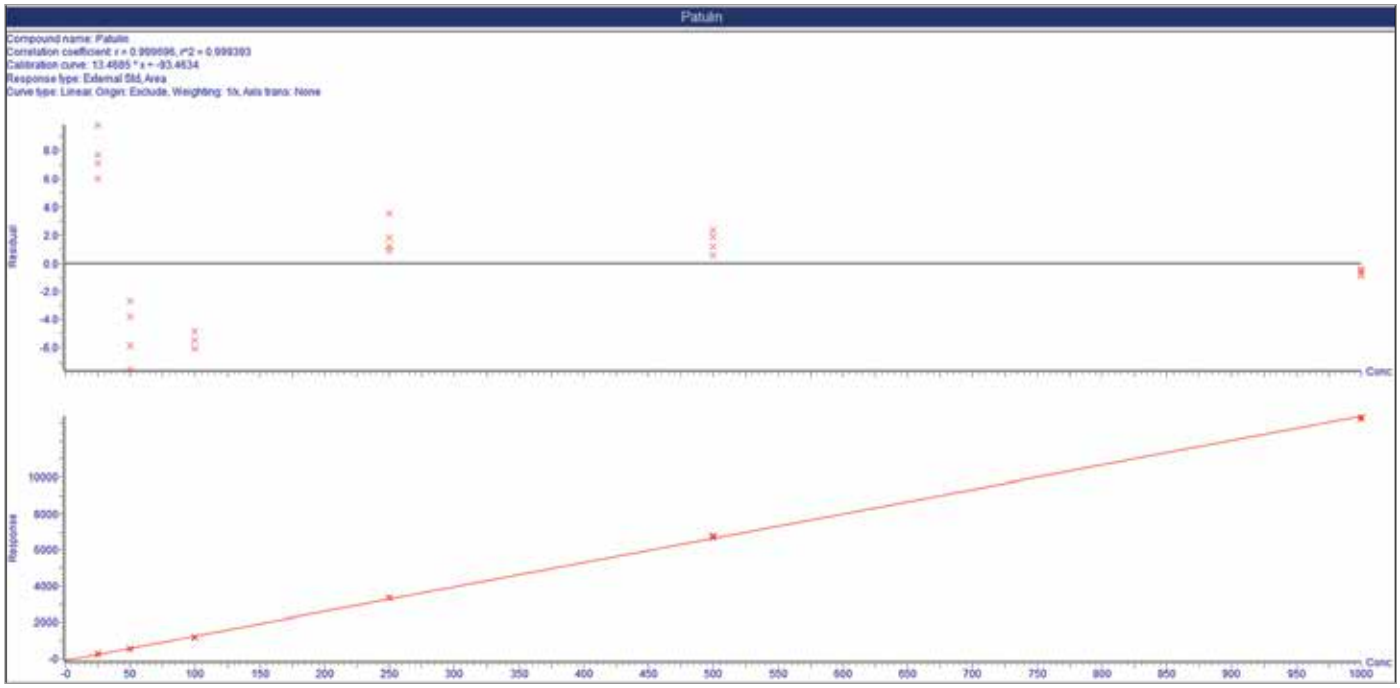

Figure 4. Calibration curve for patulin in water, equivalent to 5 to 200 µg/L.

The use of SIR with a mass detector vastly improves the selectivity of the method and reduces any impact from co-extracted components that could interfere in UV analysis. Another useful feature of mass detection is the ability to acquire full-spectrum data across the entire mass-to-charge (*m/z*) range of interest. Waters' RADAR Technology enables the simultaneous acquisition of SIR channels with full spectrum background data. This technology is especially useful to monitor high-level background matrix interference in food and beverage matrices. In the current work, full spectrum data was acquired in the same ionization mode used to analyze patulin (negative ion ESI). The resulting background base peak intensity chromatogram is shown in Figure 5, along with the simultaneously acquired SIR channels of patulin and HMF. As can be seen from these chromatograms, patulin elutes in a region of low matrix interference. This provides further confidence that the method is robust and minimal matrix effects are likely to occur for patulin in apple juice using this methodology.

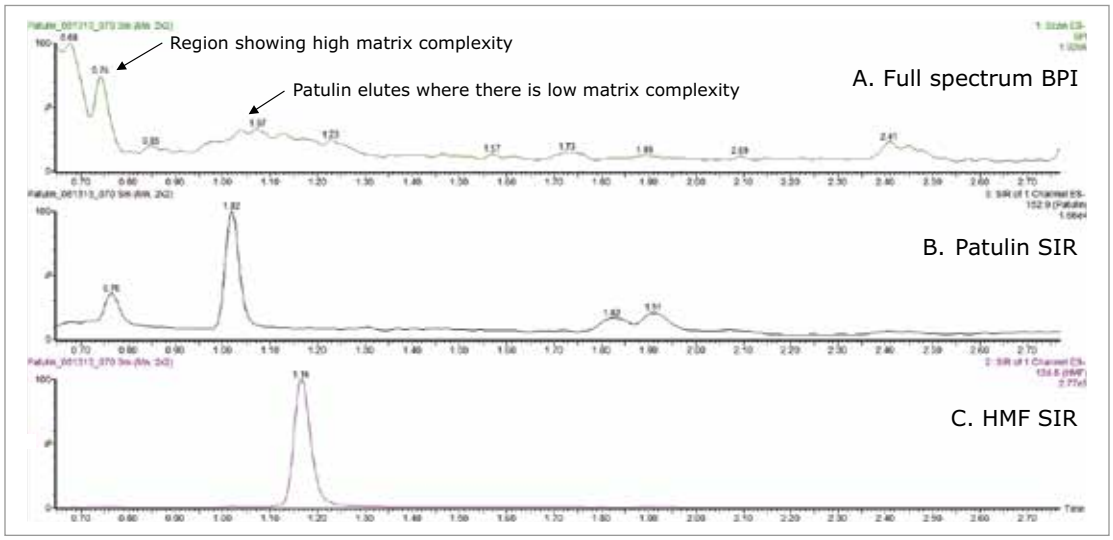

Figure 5. BPI chromatogram of the full spectrum base peak intensity (BPI) chromatogram (A) patulin SIR (B) at 50 µg/L and HMF SIR (C) in extracted apple juice. Full spectrum data was acquired simultaneously using RADAR Technology.

REPEATABILITY

In order to assess repeatability, a series of 50 replicate injections at 5 µg/L in apple juice were performed. The TrendPlot™ result, which is automatically plotted within the TargetLynx Application Manager, is shown in Figure 6. The repeatability was 9%, well within the 20% tolerance as specified in EU legislation 2002/657/EC.[4]

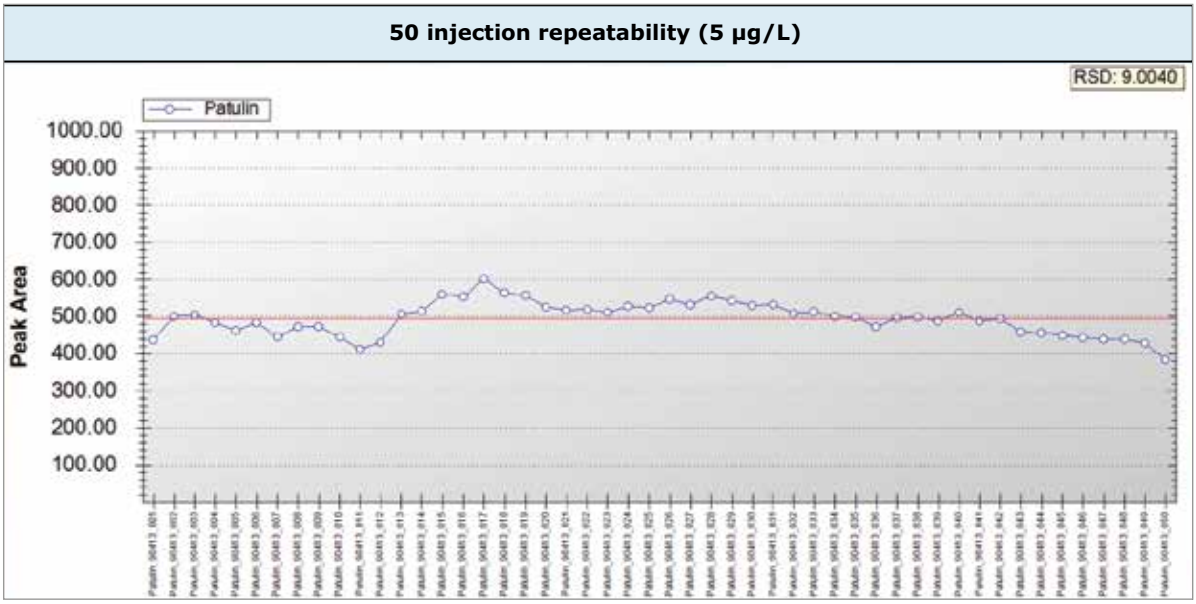

Figure 6. Peak area repeatability for 50 injections of patulin in extracted apple juice at 5 µg/L.

CONCLUSIONS

- The ACQUITY UPLC H-Class System with the ACQUITY QDa Mass Detector is capable of quantifying patulin in apple juice at ten times below regulatory requirements for juice and half the strictest EU regulation for baby food.
- The ability to simultaneously acquire highly selective SIR channels with full mass spectrum data using RADAR Technology provides a powerful tool to assess background interference. This is especially useful during the method development process and also throughout routine analysis when changes in matrix interference may impact results.
- The ACQUITY QDa Detector facilitates the implementation of mass detection in laboratories. The addition of mass detection enhances confidence in compound identification and increases detection selectivity in the analysis of food and beverages.

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2. Commission Regulation (EC) No. 1881/2006 Section 2.3.
3. J Morphet, Rapid Analysis of Patulin Contaminination in Apple Juice. Waters application note no. 720002410en, 2008.
4. Commission Directive 2002/657/EC, *Off. J. of the European Communities* No. L221/8.

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