Application Note

Food Testing & Agriculture

Enhanced Food Safety Testing

A pesticide screening methodology using the Agilent 6546 LC/Q-TOF and MassHunter Quantitative Analysis Software 10.0 LC/Q-TOF Screener Tool

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Abstract

This Application Note describes a methodology for screening hundreds of pesticides in a variety of complex food matrices. Screening is done using an Agilent 6546 quadrupole time-of-flight LC/MS system with the LC/Q-TOF Screener tool of Agilent MassHunter Quantitative Analysis software. This workflow solution can confidently perform target quantitation and suspect screening, achieving excellent reproducibility, mass accuracy, and linearity. Combining of target quantitation and suspect screening workflows reduces the complexity of data analysis and the burden of data review.

Introduction

Pesticide testing laboratories routinely need to analyze samples for large lists of analytes with high confidence and high throughput. Conventionally, a targeted acquisition approach, using a triple quadrupole mass spectrometer, is used to quantitate a list of target analytes, the scope of which is usually determined by regional government plans, often set annually. Quantitative analysis typically requires routine calibration with an analytical-grade standard for each analyte. However, government plans are causing significant growth in the number of pesticides to monitor. Thus, the cost associated with standards and their preparation (usually in multiple matrices) sometimes becomes a limiting factor.

There is an increasing demand for alternate cost-effective methodology to allow efficient expansion of the testing capabilities beyond the target compound list of routine quantitation. Some compounds occur frequently enough in samples, that quantitating during the initial screening is cost-effective, and a final result with one injection is possible. Agilent refers to these as priority targets. However, for other compounds, it is practical to consider not building a calibration curve but rather quantitate after screening, depending on what is found initially in the sample. Agilent refers to compounds dealt with in this manner as suspects. For these compounds, this suspect screening approach can deliver increased productivity in the lab, due to a lower burden for routine calibration. This depends on two things:

- First, that the approach taken can reliably find these suspects, if they are present at a defined level
- Second, that the approach taken does not deliver too many incorrect results that later are rejected, during the follow up quantitation or confirmation step. If this happens, the productivity advantages mentioned will be nullified.

High-resolution quadrupole time of flight (Q-TOF) mass spectrometry is an attractive alternative to triple quadrupole systems because the Q-TOF platform has the capability of measuring accurate mass and isotope pattern for both molecular ions and fragments, enabling high confidence in compound identification. Q-TOF systems are also able to collect data at a fast rate, making it possible to collect quality data from both molecular ions and fragments while still maintaining sufficient data points across a chromatographic peak. This is key for those compounds that merit simultaneous quantitation but also for suspects, which will be more easily identified if a true representation of a chromatographic peak can be generated.

With recent technological breakthroughs, the quantitation suitability of Q-TOF systems has significantly improved owing to enhanced sensitivity, selectivity, and dynamic range. Q-TOF is growing into a fit-for-purpose platform for laboratories that are interested in simultaneously performing quantitation for priority targets and suspect screening for a broader list of pesticides.

A straightforward and ease-of-use data analysis workflow is important to achieve high-throughput and wide-scope Q-TOF screening in this manner. For pesticide testing laboratories, it is highly desired that a screening workflow software tool can efficiently turn the information-rich Q-TOF data into meaningful reports for suspects, and for priority targets that also have a quantitative result.

Regarding the latter, it is important that software reports data quality parameters required by guidelines from any relevant regional government, for example, SANTE/11813/2017[1]. Such a tool also needs to accommodate flexibility for method development and validation, since labs may often need to experiment and review which fragment ions are the best qualifiers for a given food matrix.

This Application Note introduces the 6546 LC/Q-TOF MS and the Agilent MassHunter Quantitative Analysis software LC/Q-TOF Screener—a streamlined data analysis workflow for priority target quantitation and suspect screening achieved simultaneously, both from the standpoint of data acquisition and data review. Information from the Agilent pesticide accurate mass spectral library was used to expedite method development.

Shown below is a proof of a principal study. To establish the scope of target quantitation for commonly found pesticides, a large set of pesticides were spiked into extracts of four representative food commodities for matrix-matched calibration. Less commonly found pesticides were treated as suspects, based on identification criteria of SANTE/11813/2017 guidelines. The high confidence of the results generated, and their excellent quantitative linearity were made possible by the improved low mass resolution (>30,000 for *m/z* 118), large spectrum dynamic range (five orders of magnitude), and excellent isotope fidelity of the 6546 LC/Q-TOF. Combined with hardware enhancements, laboratories may now reliably obtain both quantitation of priority targets and suspect screening results in one injection using this screening workflow enabled by MassHunter Quantitative Analysis software 10.0.

Experimental

Sample preparation, data acquisition, and analysis method setup

Four different food matrices: black tea, broccoli, avocado, and strawberry were prepared using the QuEChERS (EN) protocol. Figure 1 shows an example of the strawberry protocol. Ten grams of homogenized food fruit/vegetable samples and 2 g of dry black tea were weighed in a 50 mL conical tube, and extracted following the buffered EN 15662 method with an Agilent QuEChERS extraction kit (p/n 5982-5650CH). The black tea samples were wetted with 8 mL ultrapure water for two hours prior to extraction. Raw extracts were then cleaned up by corresponding dispersive SPE kits; avocado by Bond Elut EMR—Lipid (p/n 5982-1010), black tea by the dSPE kit for highly pigmented fruits and vegetables (p/n 5982-5356), broccoli by the kit for pigmented fruits and vegetables (p/n 5982-5256), and strawberry by the kit for general fruits and vegetables (p/n 5982-5056). For some of the matrices, additional food samples, either organic or conventional, were also prepared.

A pesticide standard mix was prepared from the Agilent LC/MS Pesticide Comprehensive Test Mix product (p/n 5190-0551). The pesticide mixture contained over 250 pesticides. Of these, only 195 were considered priority targets; since certain analytes in the standard mix did not ionize in positive mode, MS/MS spectra for the primary molecular ion was not in the PCDL. This mixture was diluted into five 20x stock solutions at different levels (100, 200, 400, 1,000, and 2,000 ng/mL). These were then diluted 20x into each matrix to prepare calibrators at 5, 10, 20, 50, and 100 ppb levels. To account for the black tea dilution during the extraction, these calibration stocks were diluted a further 5x into the matrix. In addition to the calibrators, a separate stock solution of 12 different compounds was prepared and spiked into the matrices at 10 and 100 ppb as fortified samples. Of these compounds, eight were a part of the priority target list and four were suspects. All would later be evaluated solely through the suspect screener software. Unknown strawberry samples were collected from different markets in the Delaware and California regions. These 16 samples were processed and analyzed as unknowns to determine what pesticides are present and quantify priority targets. Samples were held at 7 °C in silanized HPLC vials until injected.

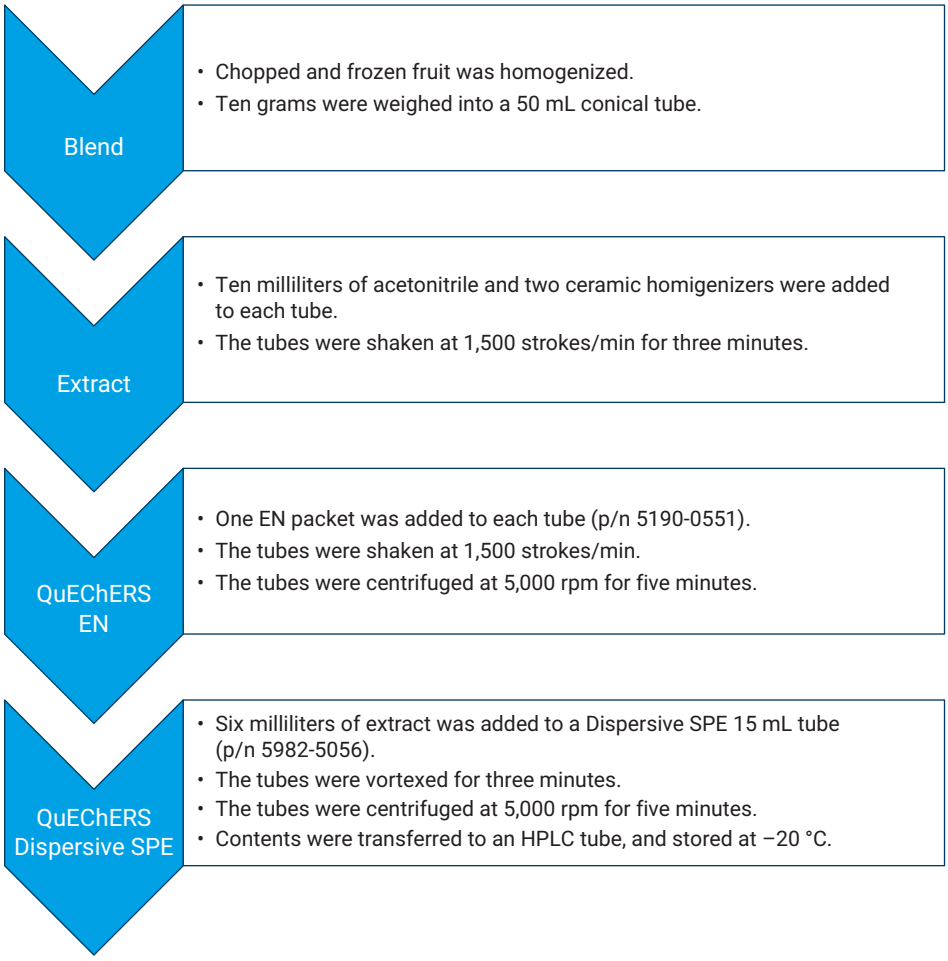

Figure 1. Example of the food sample preparation protocol for strawberry fruit.

An Agilent 1290 Infinity II Prime LC was coupled to a 6546 LC/Q-TOF. Table 1 shows the chromatographic details, and Table 2 describes the Q-TOF parameters[2]. All Ions MS/MS data are collected by acquiring a MS spectrum, followed by the acquisition of spectra where a defined collision energy (or energies) is applied. This nontargeted acquisition of molecular ions and fragmentation data is a desirable trait unique to Q-TOF acquisition, because it means that more compounds can be added to the data analysis without having to change acquisition method. This allows for greater flexibility to analyze data in a food lab, and is another key benefit of Q-TOF screening versus triple quadrupole screening.

Purine and HP-921 (hexakis (^{1}H, ^{1}H, ^{3}H-tetrafluoropropoxy) phosphazine)) were used as reference ions during the analysis. After an initial calibration of the system, the Q-TOF did not require maintenance or recalibration throughout the 10 days of data acquisition. The worklist consisted of matrix blanks followed by the calibration samples and fortified samples. If additional unknown samples were processed, they were acquired at the end. Calibration samples were acquired in triplicate, while duplicate injections were made for the fortified and unknown samples.

Following data acquisition, each sample was converted to the SureMass data format. SureMass data conversion searches the entire collection of profile spectra for features using only noise statistics and signal continuity; knowledge of sample chemistry is not required. Data analysis in SureMass format speeds up processing without compromising mass accuracy. Enabled in MassHunter Acquisition software 10.0, SureMass conversion happens automatically after each data file is acquired. Data conversion can also be performed prior to batch analysis using MassHunter Quantitative Analysis software 10.0.

In MassHunter Quantitative Analysis software 10.0, the quantitative method can be built easily from a personal compound database or library (PCDL). In general, the precursor ion is set as the quantifier, while selectively imported fragment ions, with a defined collision energy (CE), are the qualifiers. Once the compound and fragment masses from the PCDL are imported, various settings including calibration levels, mass extraction windows, coelution scores, and others can be customized, if desired, on an analyte-by-analyte basis. After optimization, this method included one reproducible fragment (qualifier) per molecular ion for priority targets, and four fragments for qualifiers for the suspects.

Table 1. Chromatographic conditions for the 1290 Infinity II LC.

Parameter	1290 Infinity II LC System
Analytical Column	Agilent ZORBAX Eclipse Plus C18 3.0 × 150 mm, 1.8 µm (p/n 959759-302)
Guard Column	ZORBAX Eclipse Plus C18, 2.1 mm, 1.8 µm, UHPLC guard column (p/n 821725-901)
Column Temperature	45 °C
Injection Volume	2 µL
Autosampler Temperature	7 °C
Needle Wash	10 seconds, standard (50:50 methanol:isopropanol)
Mobile Phase A	Water + 4.5 mM ammonium formate + 0.5 mM ammonium fluoride + 0.1 % formic acid
Mobile Phase B	Methanol + 4.5 mM ammonium formate + 0.5 mM ammonium fluoride + 0.1 % formic acid
Flow Rate	0.45 mL/min
Gradient	Time(min) %B 0.00 2 0.50 2 1.00 50 4.00 65 16.00 100 18.00 100 18.10 2 20.00 2
Post Time	4 minutes

Table 2. List of data acquisition parameters using the 6546 LC/Q-TOF.

Parameter	Value
Sheath Gas Temperature	400 °C
Sheath Gas Flow	12 psi
Gas Temperature	325 °C
Gas Flow	10 psi
Nebulizer	20 psi
Capillary Voltage	4,000 V
MS Tune	*m/z* 750, fragile
MS Mode	Positive
Acquisition	MS only with 0, 20, and 40 CE segments
MS Range	*m/z* 50 to 1,000
Reference Mass	121.0509 ($M+H^+$ for purine) 922.0098 ($M+H^+$ for HP-921)

Because there was no available standard for the suspects in this method, the retention time (RT) was estimated from previous work done with the same model column and LC. For full validation of suspect screening according to DG SANTE guidelines, it might be required to do more than this[1]. Figure 2 shows full details for the analysis method setup for this All Ions MS/MS pesticide method.

Results and discussion

Combined target quantitation and suspect screening with MassHunter Quantitative Analysis software 10.0

MassHunter Quantitative Analysis software 10.0 enables one method to analyze a batch of samples for both quantitation of priority targets and suspect screening. All compounds in the method can be reviewed in the software as a classic quantitative batch. The user navigates through compounds in the Compound Table or data files in the Batch Table, reviews chromatograms and spectra in the Compound Information pane, and checks the curve in the Calibration Curve pane. This analysis and review of hundreds of compounds for a batch of samples is inefficient and time-consuming.

The LC/Q-TOF Screener tool gives a green, orange, or red flag for every analyte in every sample depending on confidence of identification, and this makes reviewing data easy because the analytes can be filtered and reviewed by color (flag). In the screening workflow, a large RT shift, low signal-to-noise ratio (S/N), poor coelution score, isotopic fidelity, or mass accuracy is considered as outlier.

Compounds flagged as green show the highest confidence level because all set outliers are within bounds. The orange flags demand additional review because outliers exist for those compounds even though basic identification criteria are met. An example of this is when the RT of priority target compound is >0.2 minutes from the defined RT in the method. The red-flagged compounds are not detected because they fail to meet basic identification criteria. Outliers are adjustable and are set in the method editor during method analysis setup (Figure 2).

The Screener may be viewed in conjunction with the traditional quantitative analysis user interface (UI). This means that for compounds found by the Screener that also have a current calibration generated from the batch, the reviewer can instantly see a concentration level (plus calibration curve generated, and so on). The two views are linked for easy and fast navigation (Figure 3).

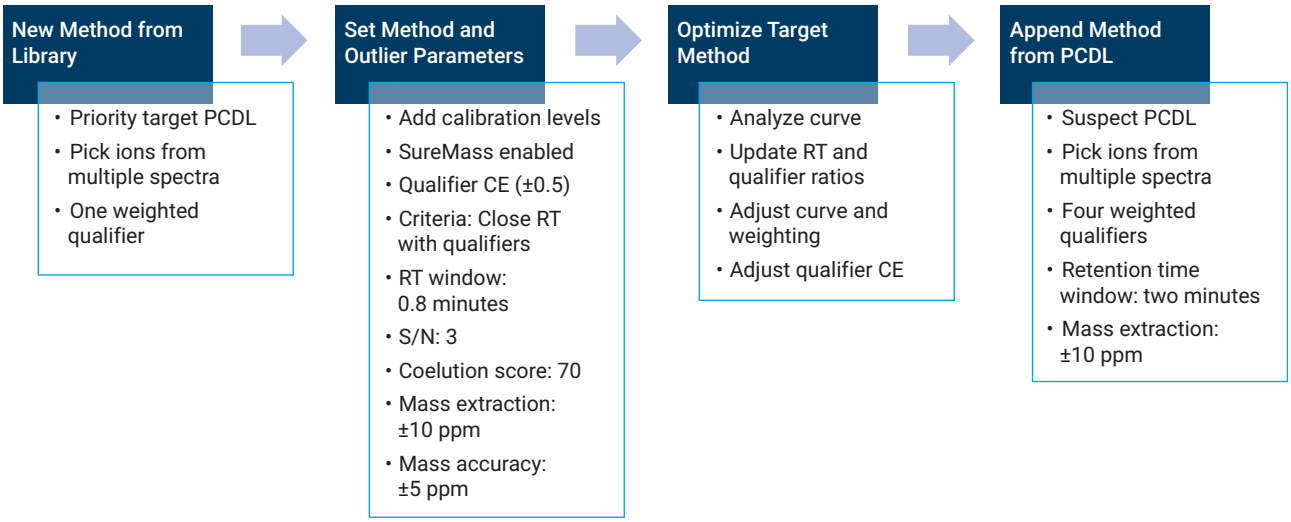

Figure 2. Analysis method development workflow from a PCDL and outlier parameters in MassHunter Quantitative Analysis software 10.0.

The Screener also displays an averaged full spectrum in the middle pane, a simplified spectrum only containing the ions defined by the method in the lower left pane, and an isotopic pattern match in the lower right pane (Figure 3) for users to inspect the data quality. These views are useful when a flag is orange, and the reviewer must decide what to do next.

The LC/Q-TOF Screener has been created to allow labs to develop Q-TOF-based broad screening methods that can be validated according to ISO 17025 and in accordance to the guidelines laid out by DG Sante[1]. This structure is common for controlled pesticide monitoring, and requires labs to define the scope of compounds they are analyzing (however large), and to show some form of initial validation that can reliably find each of those compounds down to a defined level.

However, it can also be used effectively by labs that wish to use it in a less controlled way, for example, when quickly screening for suspect compounds for which they do not have confirmed retention times or fragments. In this case, the isotopic pattern match in the lower right panel of Figure 3 can be particularly helpful in determining the confidence of identification. These types of workflows will inevitably lead to a higher false-positive rate, and are not so applicable to controlled testing of validated scope. But, they also have their place if labs have time to investigate what may be present beyond the validated compounds.

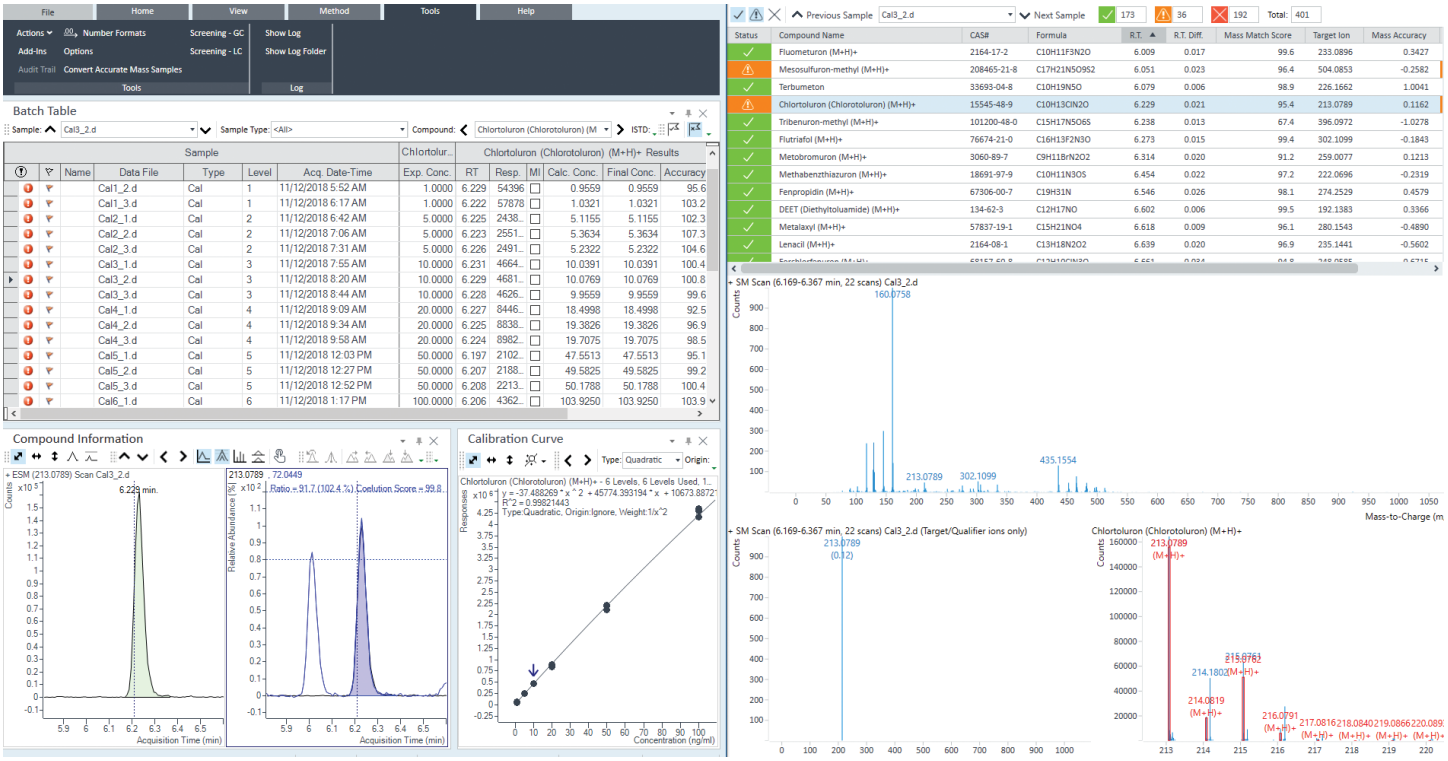

Figure 3. LC/Q-TOF Screener tool displayed next to the classic UI. Selected in the Screener results is a compound that has a calibration curve in the analysis. This analyte would be considered a priority target, and can be quantitated.

Pesticide screening method results

In this method, the concentration levels of the priority targets were fixed from 5 to 100 ppb. Table 3 summarizes their quantitative performance at the level of quantitation, 5 ppb. All 195 compounds were detected in all three injections at 5 ppb in strawberry, avocado, and broccoli matrices. Only 145 of the compounds were found at that concentration in the black tea matrix due to the further dilution. All of the detected analytes had relative standard deviations (RSD) of 20 % or lower, and most had an R^2 of 0.99 or greater. Figure 4 shows the quantitative result of an example analyte, paclobutrazol, at 5 ppb. The LC/Q-TOF Screener was designed to make the review of 195 priority targets more efficient and also search for an additional 182 suspect compounds in the same method. Therefore, the analysis contained a total of 377 pesticides.

Table 3. Pesticide results in the quantitative and suspect screening workflow. Results for black tea, strawberry, avocado, and broccoli matrices are at 5 ppb (n = 3).

	Strawberry	Avocado	Broccoli	Black Tea
Number of Targeted Compounds	195	195	195	145
Number of Targets with S/N >3 at 5 ppb	195	195	195	145
Number of Targets with RSD <20 % at 5 ppb	195	195	195	145
Number of Targets with R^2 >0.99	195	194	195	145
Number of Screened Compounds	182	182	182	182

In addition to the quantitative performance, high confidence in the identification is needed. With All Ions MS/MS data acquisition and SureMass data conversion, this can be achieved with the coelution of precursor and fragment ions, high mass accuracy, and isotopic fidelity. The coelution score is displayed in the qualifier pane (Figure 4). A high value here (>70) means that the qualifier ion overlaps well with the quantifier ion. This only happens if the fragment and precursor ion are detected at the same time, and this suggests that the fragment is made from the correct precursor structure.

The 6546 LC/Q-TOF shows high mass accuracy across the gradient, independent of analyte concentration. Figure 5 shows the sample chromatogram plotted against the mass accuracy of the analytes at their given RT. The blue dots are the mass accuracy of a 10 ppb sample, and the yellow crosses are from a 100 ppb sample. Nearly all the analytes are within ±2 ppm mass error (green bars). Those that are not fall within ±5 ppm (orange bars).

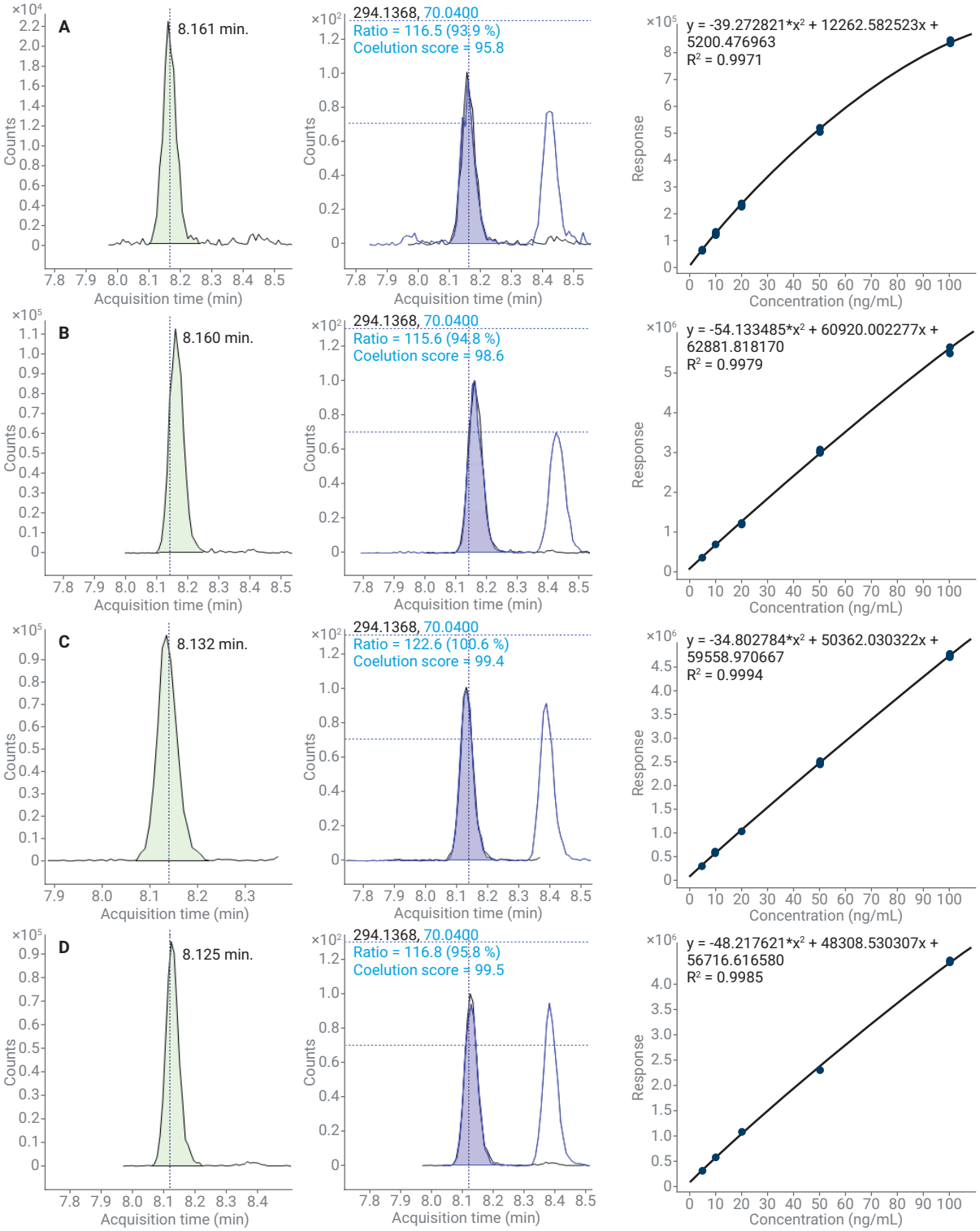

Figure 4. Quantifier signal, qualifier ratio, and calibration curve for paclobutrazol at 5 ppb in (A) black tea, (B) broccoli, (C) avocado, and (D) strawberry.

The fortified samples were analyzed with the LC/Q-TOF Screener tool with outliers set to align with SANTE guidelines. The analytes spiked in Table 4 are considered either priority targets or suspects. The results for each analyte, positive (green), for-review (orange), and not-detected (red) are reported for each matrix at two different levels. Most analytes were detected as positive. When an analyte was flagged for review, it was usually due to a low signal where mass match score needed review. These are flagged orange to promote the review process, but they pass the basic identification criteria in the SANTE guidelines.

In the unknown strawberry samples (n = 16) a range of pesticides were detected from less than 5 ppb to over 300 ppb. The number of found analytes in conventional produce was greater than organic produce, which follows use trends. The majority of the pesticides found were priority targets in this study. However, in a few cases, such as samples 4 and 15, the samples contained an analyte that is less commonly found (a suspect). In these cases, a conformational test may be desired.

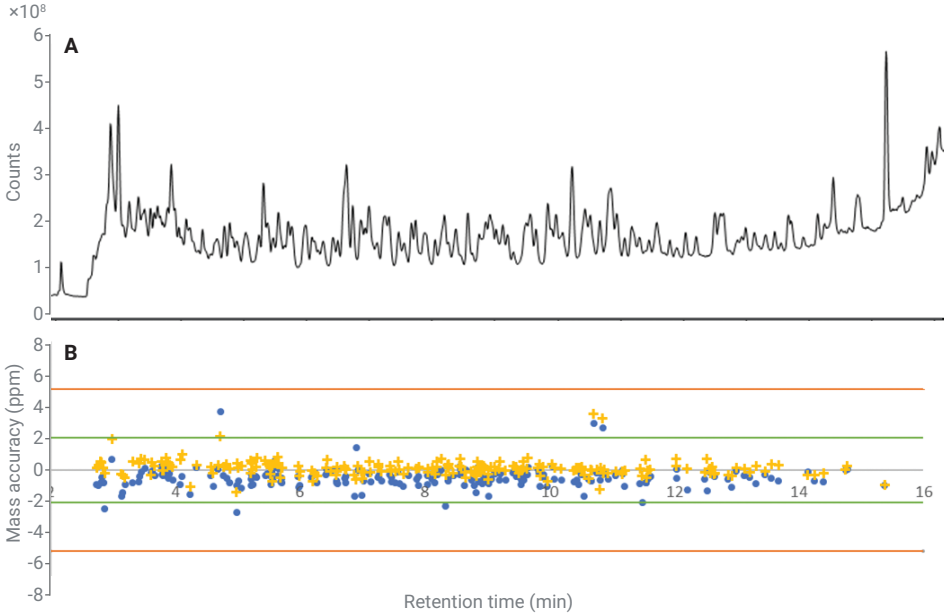

Figure 5. Total ion current (TIC) of a strawberry sample (A) and the mass accuracy of priority target ions in strawberry matrix at 10 ppb (blue circles) and 100 ppb (yellow crosses) plotted against their RT (B).

Table 4. LC/Q-TOF Screener results for the 10 and 100 ppb fortified samples.

	10 ng/mL				100 ng/mL			
Targets	**Black Tea**	**Broccoli**	**Avocado**	**Strawberry**	**Black Tea**	**Broccoli**	**Avocado**	**Strawberry**
Aminocarb	✗	✓	✓	✓	⚠	✓	✓	✓
Diazinon	✓	✓	✓	✓	✓	✓	✓	✓
Dimethoate	✓	✓	⚠	⚠	✓	✓	✓	✓
Imazalil	✓	✓	✓	✓	✓	✓	✓	✓
Malathion	✓	✓	✓	✓	✓	✓	✓	✓
Metazachlor	✗	✓	⚠	⚠	⚠	⚠	✓	✓
Pyraclostrobin	✓	✓	✓	✓	✓	✓	✓	✓
Thibendazole	✓	✓	✓	✓	✓	✓	✓	✓
Suspects								
Atrazine	✓	✓	✓	✓	✓	✓	✓	✓
Carbofuran	⚠	✓	✓	✓	✓	✓	✓	✓
Metosulam	⚠	✓	✓	✓	⚠	✓	✓	✓
Metoxuron	✓	✓	✓	✓	⚠	✓	✓	✓

Conclusions

The unified workflow solution combining the 6546 LC/Q-TOF and the MassHunter Quantitative Analysis software 10.0 Screening tool has been demonstrated to confidently perform target quantitation and suspect screening in various complex food samples. Excellent reproducibility, mass accuracy, and linearity were observed in the results. Integration of target quantitation and suspect screening in the same software with intuitive layout significantly reduces the complexity of data analysis and burden of data review.

References

1. Guidance document on analytical quality control and method validation procedures for pesticides residues and analysis in food and feed, SANTE/11813/2017, 21–22 November 2017. https://ec.europa.eu/food/sites/food/files/plant/docs/pesticides_mrl_guidelines_wrkdoc_2017-11813.pdf
2. Miladi, M.; *et al.* High-Throughput Pesticide Residue Analysis Using an Agilent Ultivo Triple Quadrupole LC/MS and the MassHunter Productivity App, *Agilent Technologies Application Note*, publication number 5994-0196EN, **2018**.

Table 5. LC/Q-TOF Screener results for 16 strawberry sample purchased in California and Delaware regions. Samples were both organic and conventional and purchased from different locations. Detected pesticides are reported and concentrations of the pesticide are given in parenthesis, when available.

Sample	Type	Pesticides Detected
Sample 1	Organic	Malaoxon (2.6 ppb), Carbaryl (29.4 ppb), Malathion (31.8 ppb), Methoxyfenozide (Intrepid) (4.7 ppb), Diazinon (Dimpylate) (2.5 ppb)
Sample 2	Organic	Spinosyn A (14.3 ppb), Spinosyn D (3.6 ppb)
Sample 3	Organic	None found
Sample 4	Organic	Prometryn
Sample 5	Organic	None found
Sample 6	Conventional	Flutriafol (12.0 ppb), Metalaxyl (28.6 ppb), Azoxystrobin, Pyrimethanil (218 ppb), Malathion (<5 ppb), Fenhexamid (5.6 ppb), Cyprodinil (26.6 ppb), Trifloxystrobin (6.2 ppb), DEET (Diethyltoluamide) (<5 ppb)
Sample 7	Conventional	Thiamethoxam (<5 ppb), Carbendazim (Azole)(163 ppb), Thiophanate-methyl, Chlorantraniliprole (5.6 ppb), Pyrimethanil (105 ppb), Boscalid (Nicobifen) (23.5 ppb), Methoxyfenozide (Intrepid) (6.8 ppb), Myclobutanil (<5 ppb), Bifenazate (D 2341), Tetraconazole (33.2 ppb), Fenhexamid, Cyprodinil (248 ppb), Pyraclostrobin (<5 ppb)
Sample 8	Organic	Boscalid (Nicobifen) (2.4 ppb)
Sample 9	Conventional	Flonicamid, Malaoxon (<5 ppb), Flutriafol (<5 ppb), Azoxystrobin (<5 ppb), Malathion (48.2 ppb), Myclobutanil (<5 ppb), Quinoxyfen (<5 ppb)
Sample 10	Conventional	Acetamiprid (<5 ppb), Metalaxyl (<5 ppb), Azoxystrobin (8.4 ppb), Myclobutanil, Bifenazate (D 2341) (<5 ppb), Fenhexamid (32.4 ppb), Cyprodinil (283 ppb), Pyraclostrobin (10.8 ppb), Trifloxystrobin (46.9 ppb), Etoxazole
Sample 11	Conventional	Thiamethoxam (13.1 ppb), Flonicamid (97 ppb), Carbendazim (Azole), Imidacloprid, Pyrimethanil (278 ppb), Methoxyfenozide (Intrepid) (14.8 ppb), Myclobutanil (<5 ppb), Bifenazate (D 2341), Tetraconazole (12.3 ppb), Cyprodinil (312 ppb), Trifloxystrobin (<5 ppb), Hexythiazox (22.6 ppb), Thiophanate-methyl
Sample 12	Conventional	Flonicamid (153 ppb), Acetamiprid (<5 ppb), Chlorantraniliprole (69 ppb), Methoxyfenozide (Intrepid) (<5 ppb), Cyprodinil (146 ppb), Trifloxystrobin (78 ppb), Quinoxyfen (34 ppb), Hexythiazox (<5 ppb)
Stample 13	Conventional	Flonicamid (64 ppb), Azoxystrobin, Boscalid (Nicobifen)(14.7 ppb), Cyprodinil (<5 ppb), Pyraclostrobin (19.4 ppb), Cyflufenamid
Sample 14	Conventional	Thiamethoxam (<5 ppb) , Flonicamid (29 ppb), Chlorantraniliprole (<5 ppb), Myclobutanil (<5 ppb), Cyprodinil (64 ppb), Trifloxystrobin (<5 ppb)
Sample 15	Conventional	Flonicamid (77 ppb) , Malaoxon (<5 ppb), Flutriafol (8.1 ppb), Azoxystrobin (<5 ppb), Malathion (47.5 ppb), Myclobutanil (5.9 ppb), Quinoxyfen, Cyflufenamid
Sample 16	Conventional	Imidacloprid (48.4 ppb), Metalaxyl (107 ppb), Chlorantraniliprole, Azoxystrobin (457 ppb), Boscalid (Nicobifen) (<5 ppb), Myclobutanil (<5 ppb), Tetraconazole (138 ppb), Quinoxyfen (21.4 ppb), Flonicamid (35.4 ppb)

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