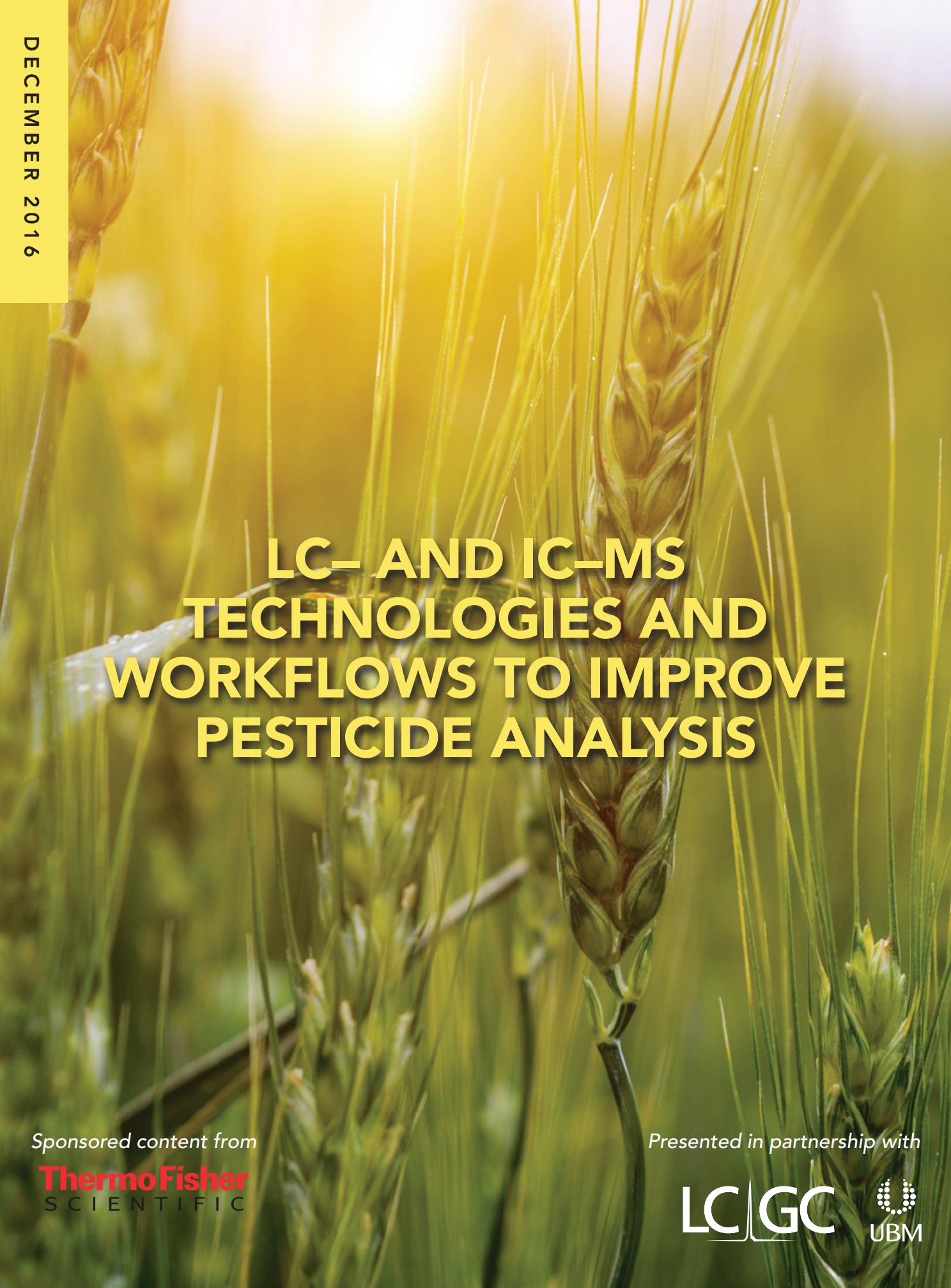
DECEMBER 2016



LC– AND IC–MS TECHNOLOGIES AND WORKFLOWS TO IMPROVE PESTICIDE ANALYSIS

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INTRODUCTION

There is increased demand for protection from chemical residues, such as pesticides, in food. This demand is putting increased pressure on pesticide residue laboratories, who often have to handle high sample volumes—as high as 50 samples per day—and provide results within one or two days of receipt and in compliance with strict quality control procedures. For these reasons, these laboratories must continue to implement advanced methods that make their analyses more effective and more efficient. This new e-book presents articles provided by our sponsor, Thermo Fisher Scientific, that share information designed to help analysts improve their methods and workflows for pesticide residue testing.

In the first article, Professor Amadeo Fernández-Alba, Ph.D., and doctoral student Łukasz Rajski of the University of Almería discuss coupling liquid chromatography to high-resolution accurate mass (HRAM) mass spectrometry, instead of to triple-quadrupole mass spectrometry, as a solution to the problem of false positives and false negatives in pesticide residue testing. This approach can involve three different workflows for pesticide analysis: data-dependent MS/MS, all-ion fragmentation, and variable data-independent acquisition.* They present data from a recent study evaluating and comparing these approaches for the analysis of pesticides in various matrices, including complex matrices like oranges, leeks, and onions.

In the second article, Stuart Adams of Fera Science Ltd. discusses the use of ion chromatography coupled with tandem mass spectrometry for the analysis of polar pesticides such as glyphosate. He presents studies using this approach for the analysis of 13 anionic pesticides in two commodity groups. The method provided excellent sensitivity and selectivity, with decreased system downtime and lower analysis costs compared to previous methods, while increasing sample throughput.

We close the e-book with an article from Ed George and Debadeep Bhattacharyya of Thermo Scientific. They describe the development and implementation of complete workflow solutions based on LC–triple quadrupole MS/MS and LC–HRAM MS/MS. These workflow solutions are designed to work as ready-to-implement methods and enable productivity and efficiency enhancements for startup laboratories as well as established laboratories that are adding new analytical capabilities to address evolving customer or industry demands.

We hope you enjoy this new e-book, and find it helpful in your laboratory's analysis of pesticide residues.

*vDIA is not available in the US

TOC
Table of contents

LC– AND IC–MS TECHNOLOGIES AND WORKFLOWS TO IMPROVE PESTICIDE ANALYSIS

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UTILIZING THE POWER OF LC-ORBITRAP MS TECHNOLOGY FOR THE MULTI-RESIDUE ANALYSIS OF PESTICIDES

By Amadeo Fernández-Alba and Łukasz Rajski

Introduction

The workload of pesticide residue laboratories can easily reach 50 samples per day, yet they have to provide results within 1–2 days of receipt and in compliance with strict quality control procedures. For that reason, and in order to provide accurate identification and quantification, these laboratories need instrumentation and software that are reliable and fully-automated. The technique of choice for most pesticide laboratories is liquid chromatography coupled with triple quadrupole mass spectrometry (LC–MS/MS). Using LC–MS/MS, the detection and identification of pesticide residues are based on a combination of the chromatographic retention time and ratios of multiple reaction monitoring (MRM) transitions in the sample compared to a known standard.

However, during the analysis of real samples, matrix co-extractives can cause issues with the correct identification of the pesticide. There are over 250 different plant matrices, each releasing thousands of co-extractives during extraction with solvent. It is possible that one of these co-extractives will co-elute with a pesticide of interest and both will produce the same MS/MS transition. When that happens, the identification will often fail because the ion ratio obtained from analysis of the sample extract will be different than the ion ratio of the corresponding standard. This is then classed as a false negative result. If the ratio of the transitions derived from the co-extractive corresponds to a pesticide standard (and there is no pesticide residue in the sample), then this is classed as a false positive result. **Figure 1** shows the example of the LC–MS/MS analysis of azinphos methyl in onion and the potential of matrix co-extractives from different solvent extracts to interfere with the ion ratios.

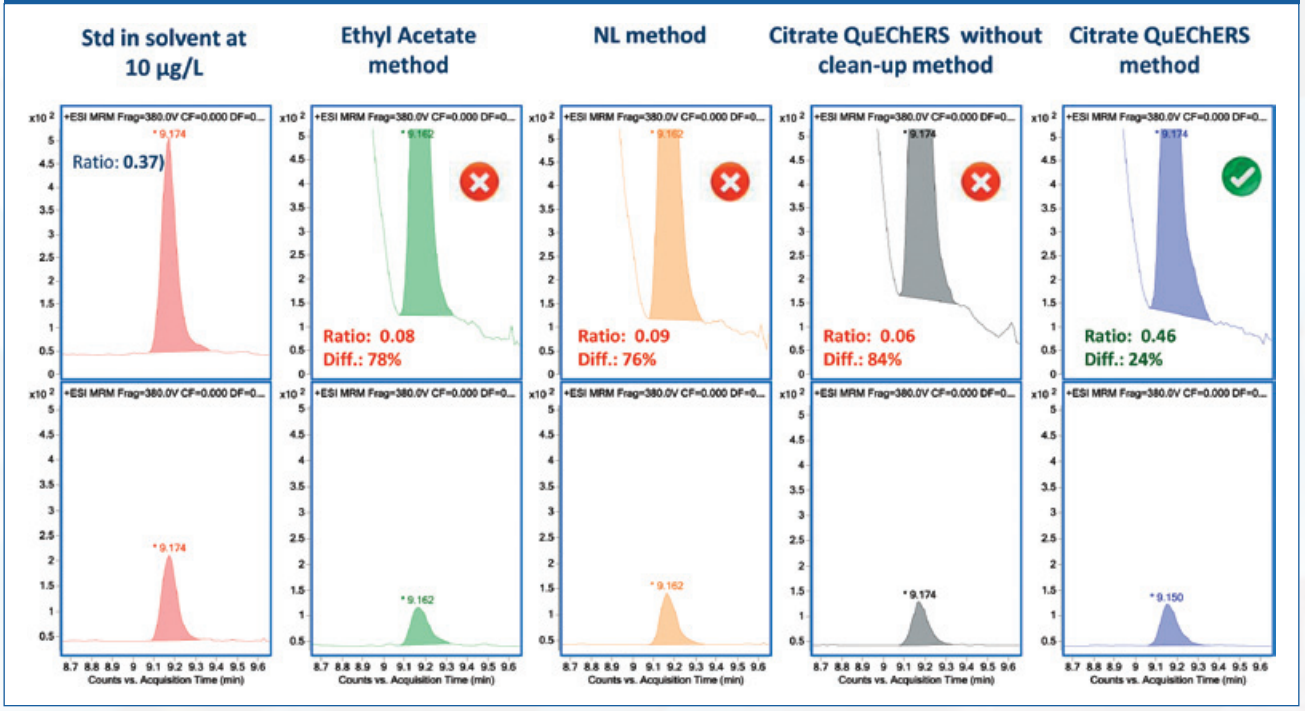
Rastko Belic / EyeEm/Getty Images

High Resolution Accurate Mass (HRAM) Mass Spectrometry

Every year, our laboratory coordinates round-robin proficiency testing (European Proficiency Test in Fruits and Vegetables [EUPT-FV]) using test samples containing both incurred residues and spiked residues. We prepare and distribute the samples to the participant laboratories. In the EU the scheme is compulsory for the official control laboratories (those laboratories submitting results for official control samples). The results of analysis of these proficiency test samples often contain a number of false positive and false negative results, not only from the presence of matrix co-extractives, but also because of the presence of co-eluting pesticides. So, the question is: how can these kinds of problems be avoided?

One approach is to use high resolution accurate mass (HRAM) mass spectrometry, instead of triple quadrupole mass spectrometry using nominal mass transitions. The main benefit of using the HRAM approach is that we obtain much more selectivity, dependent on the resolution. If we consider the example of the analysis of thiabendazole, or an ion with the same mass, then using a resolution of 25,000, we can obtain 12 molecular formulas for that ion; thiabendazole, and 11 other potential false positive ions. However, if we analyzed the same sample with

Figure 1: Potential for false positives of azinphos methyl (10 µg/kg) in onion by LC–MS/MS; based on comparison of ion transition ratios with a standard.



a resolution of 70,000, we obtain five molecular formulas only. This fact is shown in **Figure 2**, which is a plot of the number of possible molecular formulas against absolute mass error relative to thiabendazole.

This is further demonstrated by the example of the determination of linuron in coriander for which the nominal mass MS/MS ion ratio in the sample is very different from the ratio obtained in the solvent standard, as shown in **Figure 3**. Fortunately, using a mass resolution of 70,000, the linuron ion and the interfering ion from the matrix are very easy to separate, as shown in **Figure 4**. So, what can we do to avoid false positive results? Of course, we can work with higher resolution, but unfortunately we don't have instruments with infinite resolving power, and therefore, we need fragment ions for unambiguous identification of the analyte compounds. But the problem of working in full scan only, using typical ionization conditions, is the fact that we obtain fragment ions only for a small number of the pesticides. We can change the parameters of the electrospray ionization source in order to obtain fragments for a higher number of pesticides, but then we lose the sensitivity for the molecular ions. A better approach is to work simultaneously in both MS and MS/MS modes. We find that the MS data are best for detection and quantitation, while MS/MS (MS2) data work better for the identification of the pesticides.

Figure 2: Plot of the number of possible molecular formulas against absolute mass error relative to thiabendazole.

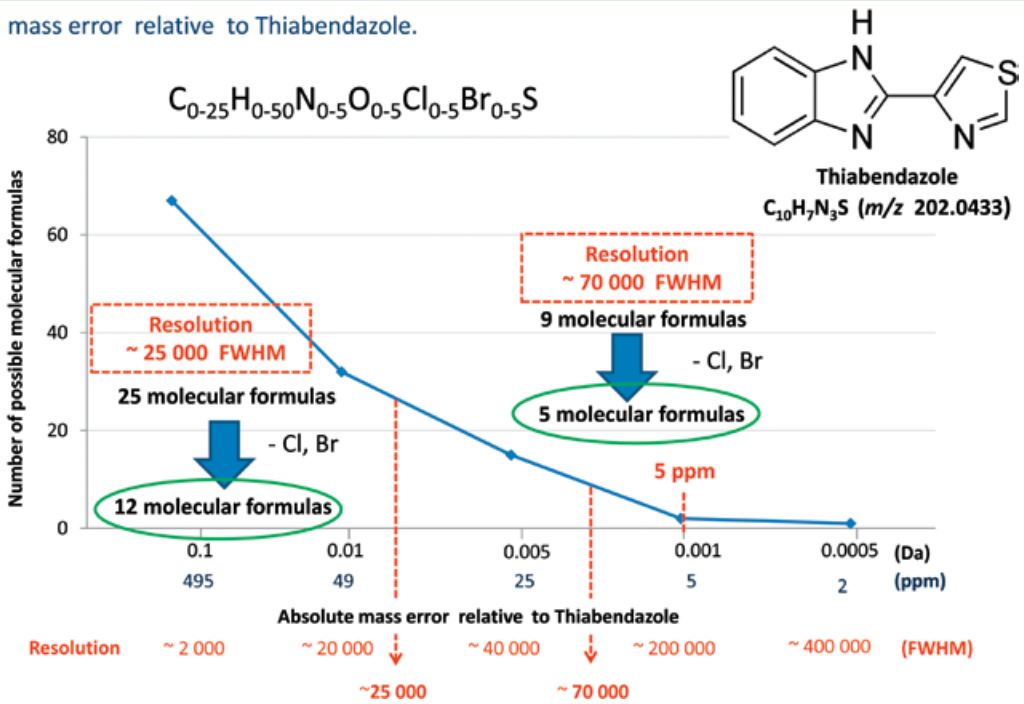


Figure 3: An example of a false negative result for linuron in an EUPT sample (coriander) as demonstrated by the ratio of the ion transition in the sample compared to the standard.

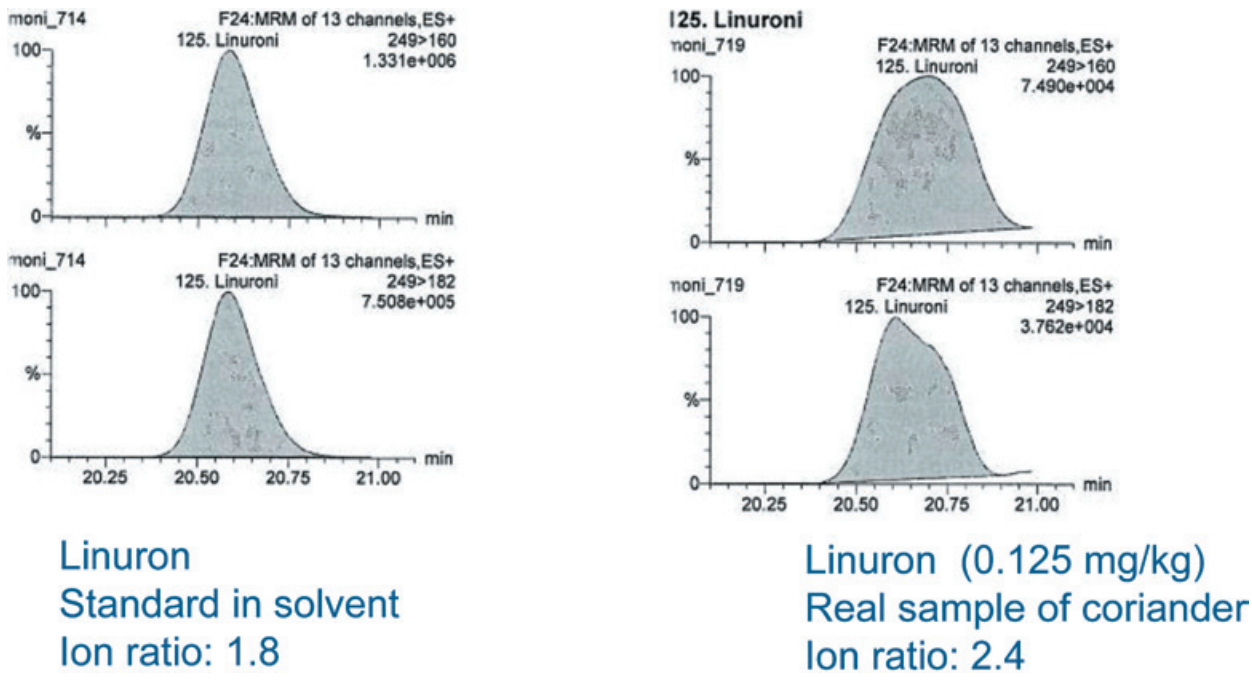
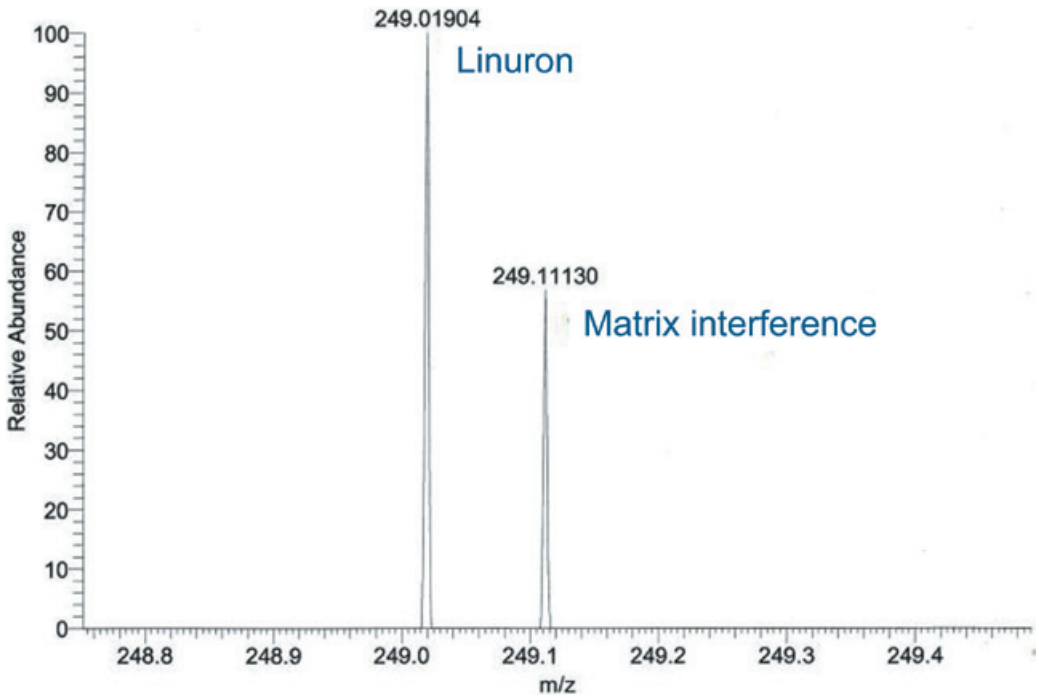


Figure 4: EUPT sample (coriander) analyzed by high resolution MS technology, showing unambiguous identification of the linuron in the presence of the matrix.



Q Exactive Focus hybrid quadrupole-Orbitrap Mass Spectrometer

When we are analyzing pesticides, we have to fulfill two criteria for detection. The first criterion is retention time and the second one is mass error. It is our experience that for positive identification, the mass error has to be lower than 5 ppm. Fortunately, using the Thermo Scientific™ Q Exactive™ Focus™ hybrid quadrupole-Orbitrap mass spectrometer instrument in full scan MS mode, we obtain mass errors of below 2 ppm, not only in samples like tomato and apple, but also in more complex matrices such as orange with a high number of co-extracted compounds as shown in **Figure 5**.

So why are fragments so important for identification? This point is emphasized by **Figures 6 and 7**. **Figure 6** shows three extracted ion chromatograms of the fungicide metalaxyl-M; one for a sample of green pepper spiked with metalaxyl-M at 10 µg/kg (upper trace) and two for different grapefruit samples that were not spiked. In all three cases, the ion chromatograms obtained using full scan acquisition at a resolution of 70,000 show peaks with the same *m/z* at the expected retention time as metalaxyl-M in the standard. An evaluation of the MS2 data in **Figure 7** shows four fragments characteristic of metalaxyl-M in the library spectrum and in the experimental MS/MS spectrum for the spiked pepper sample, but not for the grapefruit

Figure 5: Mass errors in full scan MS mode are below 2 ppm, even for orange, which is considered a difficult sample matrix containing a high number of co-extracted compounds.

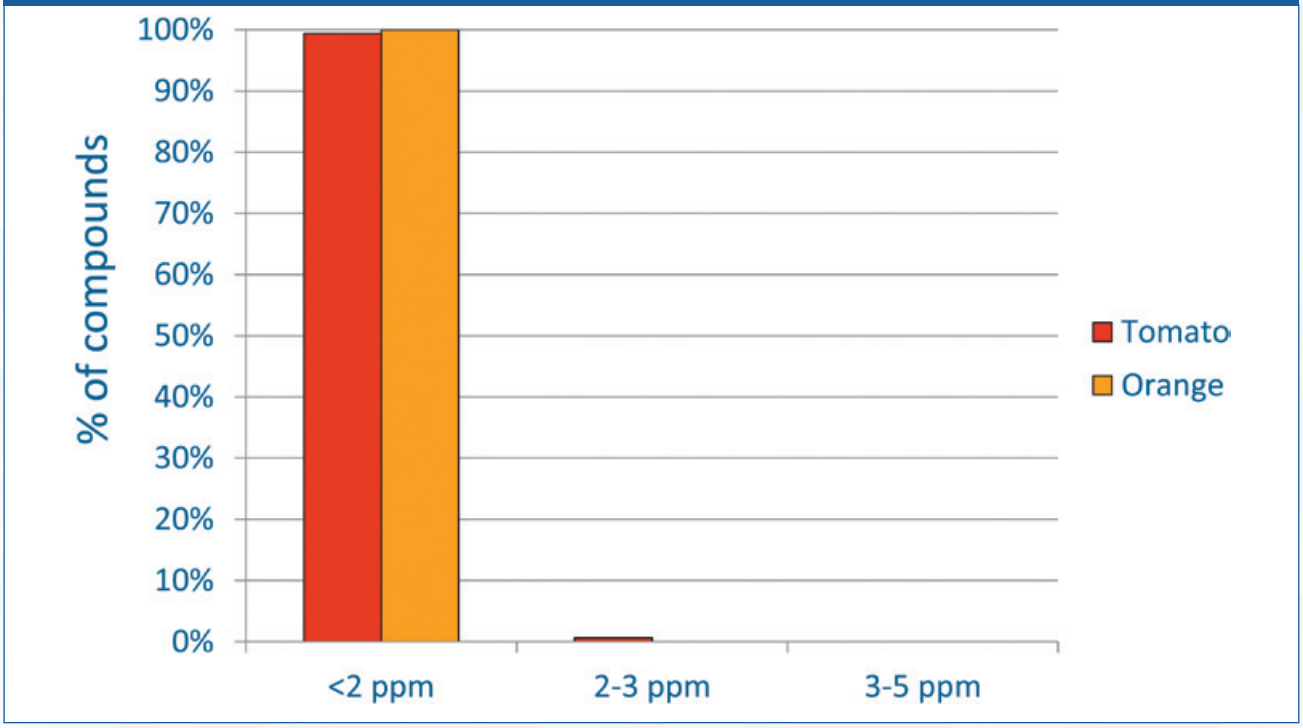


Figure 6: Determination of the fungacide, Metalaxyl-M in pepper and grapefruit in full mass scan mode.

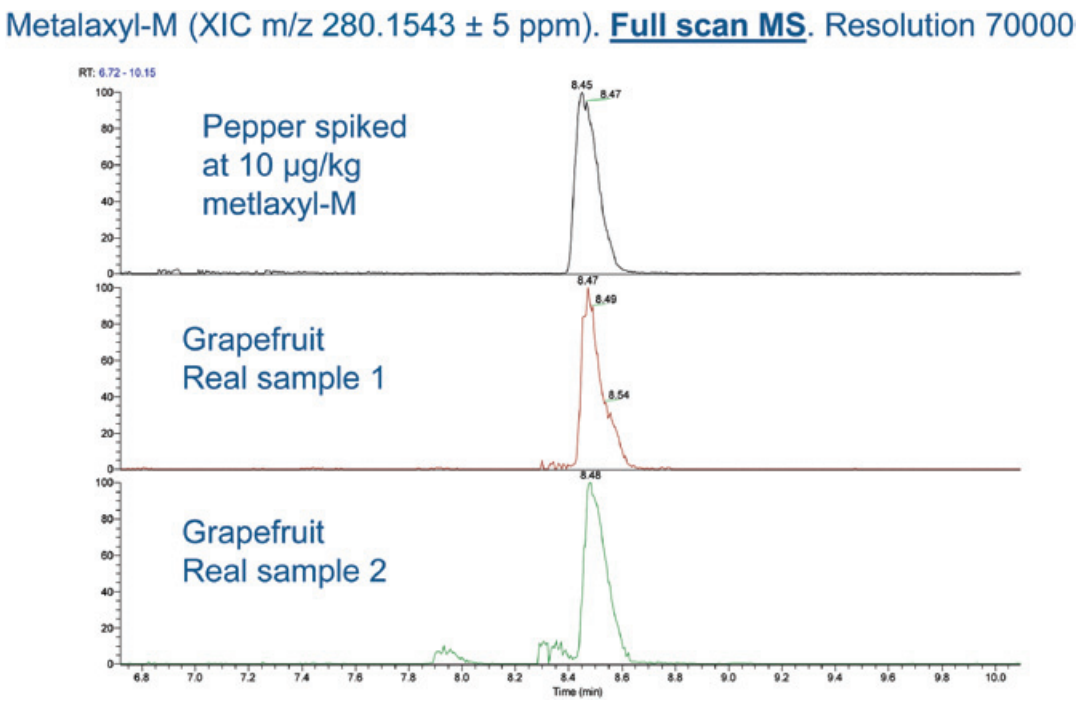
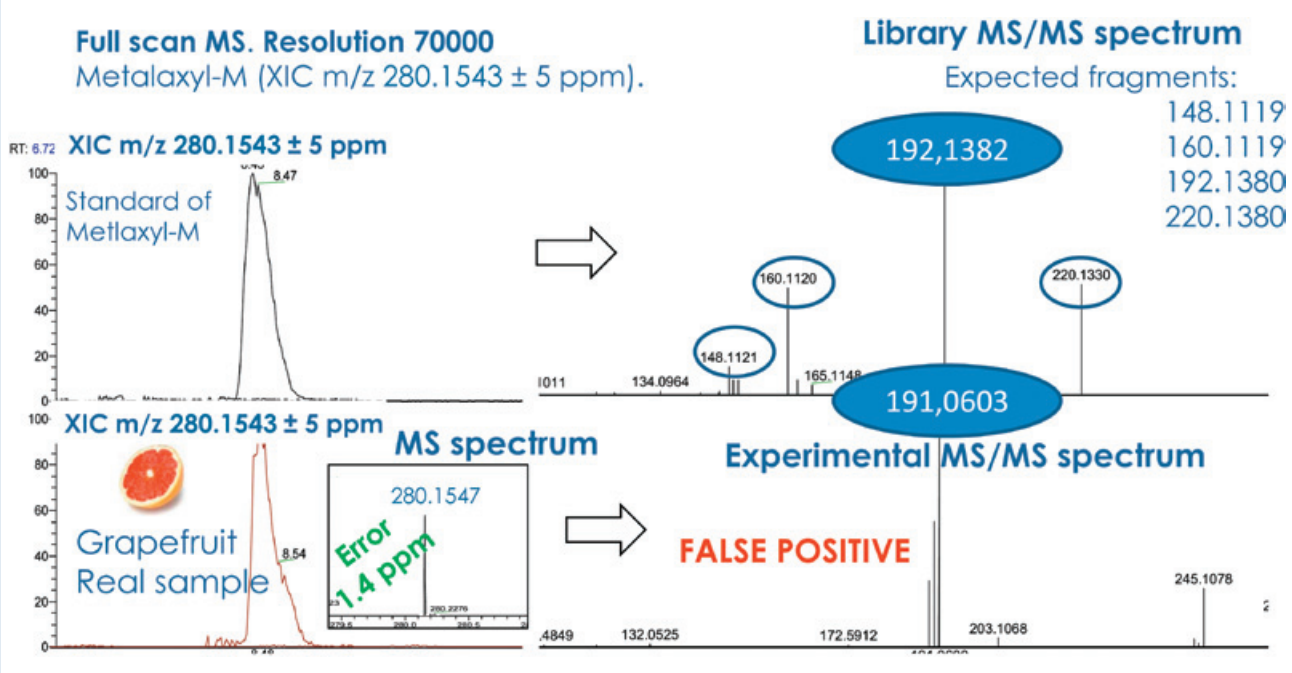


Figure 7: Fragments of Metalaxyl-M confirm its presence in the standard, but no fragments in the grapefruit sample show the full mass scan gave a false positive for the compound.



samples. This mismatch demonstrates that the ion detected in grapefruit using full scan at 70,000 RP was not metalaxyl-M, but some other compound, equating to a false positive response in full scan. We also measured the stability of ion ratios in dd-MS2 mode, by comparing the variations between two different matrices (10 µg/kg metalaxyl-M in tomato and orange) and two different concentration levels (10 µg/kg and 100 µg/kg metalaxyl-M diluted 1:5 in tomato extract). In all cases, we obtained very stable ion ratios with variations all <30%.

Choice of Workflows

So let's take a closer look at three selected workflow approaches using the Q Exactive Focus Orbitrap LC-MS/MS system and evaluate the suitability of each one for the analysis of pesticide residues.

- Data Dependent MS/MS (dd-MS2)
- All Ion Fragmentation (AIF)
- Variable Data Independent Acquisition (vDIA)

Data dependent MS/MS (dd-MS2) is a targeted-triggered MS2 workflow, in which the user has to submit an inclusion list containing the mass of the molecular ion(s) and retention time for each of the target pesticides. Using this approach, the mass spectrometer is acquiring data in full scan mode most of the time. However, when a compound from the inclusion list is detected, a single scan is then subjected to dd-MS2. A quadrupole mass filter selects the precursor ion, which is fragmented in a collision cell, and the fragments (product ions) are then analyzed in the Orbitrap analyzer. We obtain one MS2 spectrum for each chromatographic peak, which can then be used for identification purposes.

All ion fragmentation (AIF) is where the workflow is non-targeted. In this case, each full scan is followed by an MS2 scan. During the MS2 scan, the quadrupole is open so there is no filtering of the ions and therefore we fragment all of the precursor ions that we observe in full scan. For example, if we work in a full scan in the range of 100 to 1,000 Daltons, then ions in the same *m/z* range are passed to the collision cell, fragmented, and the fragment ions analyzed in the Orbitrap analyzer. Using this approach, we obtain fragment information for all the compounds present in the sample, but the fragment spectra are more complex compared to dd-MS2 or variable data independent acquisition (vDIA).

Variable data independent acquisition (vDIA)† is a variation of the AIF technique wherein the fragmentation scan is formed by a number of consecutive MS2 events, each with a predetermined and fixed mass range. In other words, the fragmentation across the full mass range of interest is divided into smaller mass segments. For example, the 100 to 1,000 Daltons range is covered by several fragmentation events; 100 to 200, another from 200 to 300 etc. Fragments in each selected mass range are analyzed separately so we gain selectivity because we can reduce

†vDIA is not available in the US

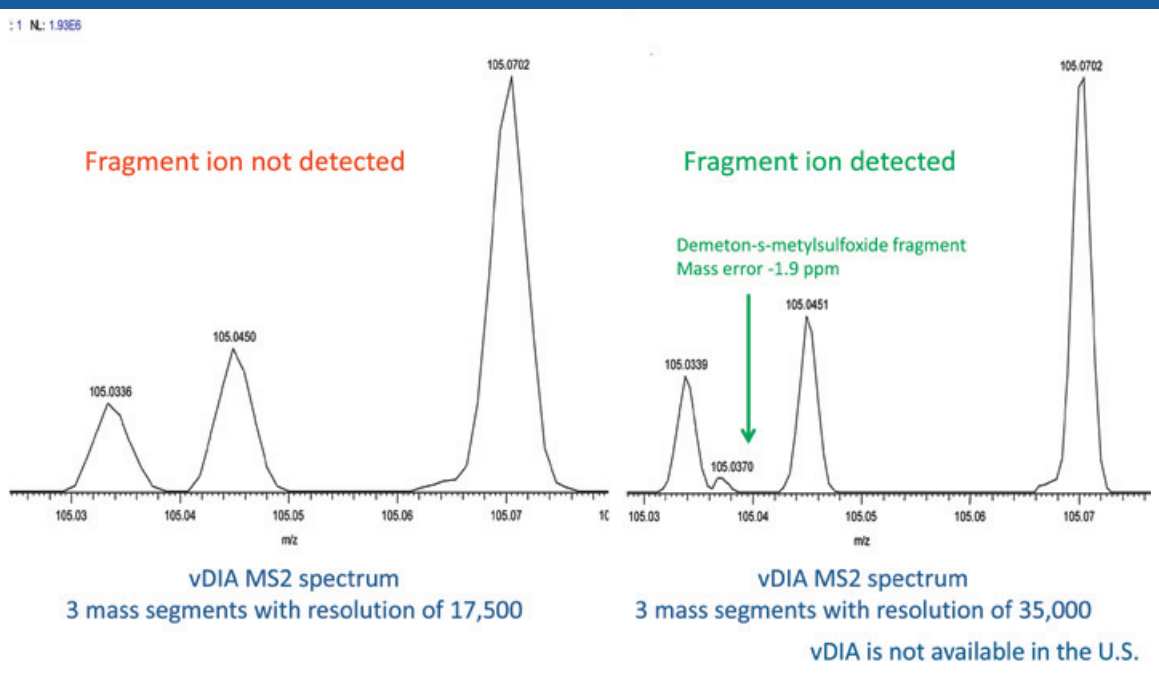
the number of ions observed in AIF. The vDIA technique is not dependent on the detection of a peak, but is a pre-programmed event. Also, it is variable because the number of segments and the range of each segment can be varied within certain limits.

Evaluation of Workflows with Real Samples

For this evaluation, we selected 11 representative matrices of different kinds of fruit and vegetables. Some of them were very straightforward such as tomato, apple, and cucumber, but we also selected very complex matrices like orange, leek, and onion. We spiked the fruit and vegetable extracts with 166 pesticides at two concentrations—100 and 10 µg/kg. We obtained almost 2,000 results at each spiking concentration, for each of the three workflows. For all of the workflows, we were able to identify practically 100% of the compounds at 100 µg/kg, and at the level of 10 µg/kg, over 95% were identified. The compounds that were the most problematic to identify were at low concentrations in complex matrices, particularly orange and leek, which have large numbers of co-extractive compounds.

Working at high resolution is not only important in full scan, but also in MS2 mode to gain improved selectivity. This is seen in **Figure 8**, which shows demeton-s-methyl sulfoxide in orange

Figure 8: Mass spectrum of 0.01 mg/kg demeton-s-methylsulfoxide in orange by vDIA, showing the fragmented ion can only be separated and positively identified at the higher mass resolution of 35,000.

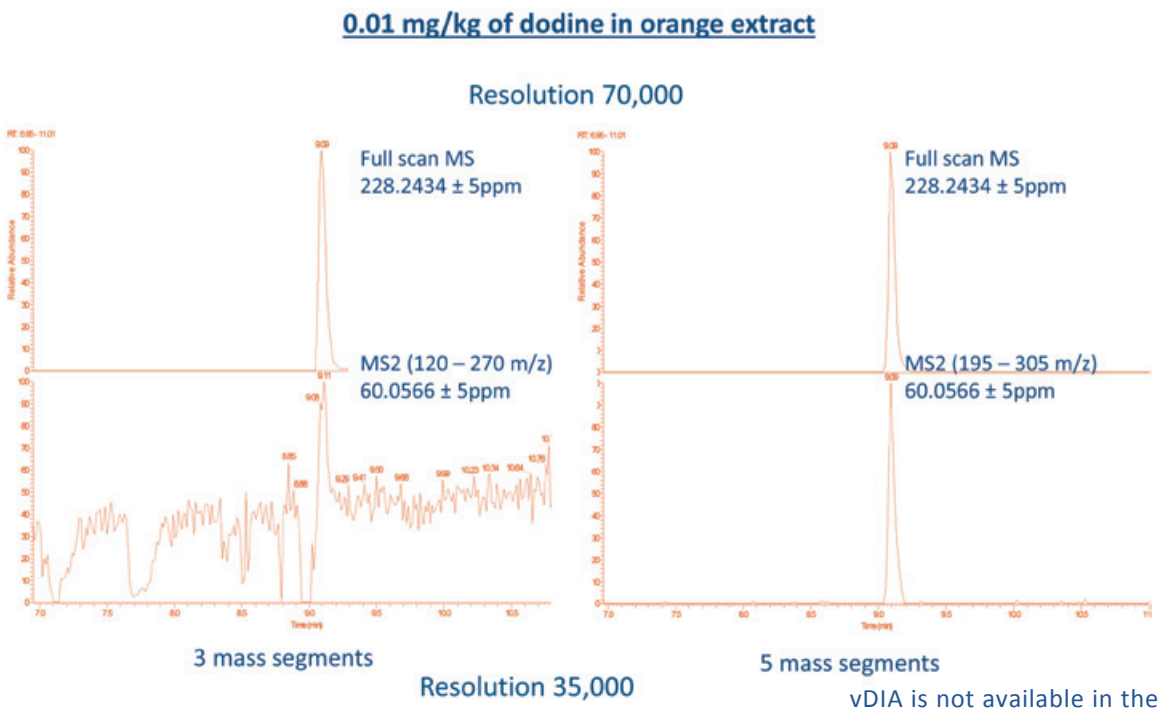


extract at a level of 10 μg/kg. On the left side, we have a mass spectrum obtained with a resolution of 17,500, and on the right side with a resolution of 35,000. At lower resolution, we were not able to identify the pesticide, whereas at higher resolution, we were able to separate a fragment ion of demeton-s-methyl sulfoxide from the matrix ion.

Using vDIA, we can also change the selectivity of the method by changing the number of mass segments. **Figure 9** shows the example of 10 μg/kg of dodine in an extract of orange. The two upper chromatograms are the extracted ion chromatograms from full scan mode using a resolution of 70,000 and in both cases dodine was detected. The two vDIA chromatograms were acquired using three and five segments, respectively, at a resolution of 35,000. We can see that in the case of the three mass segment vDIA acquisition, interferences and high background noise were observed, while for the five mass segment vDIA acquisition, a very clean peak without any interference was obtained. The reason we have such different results is that in the extract of the orange, the sample contained co-extractives with mass peaks between 120 and 195 Daltons, which produced fragment ions with the same mass as dodine.

In another example (propargite in leek)

Figure 9: In the identification of dodine in orange, the extracted compounds can cause poor identification, because of interfering peaks producing a fragment ion with the same mass as dodine.



vDIA is not available in the U.S.

shown in **Figure 10**, we are comparing AIF with vDIA, which is seen in the upper extracted ion chromatogram. This figure clearly shows that vDIA with a resolution of 35,000 can provide much better selectivity than AIF at a resolution of 70,000.

In all three MS2 modes of operation, we obtained fragments for practically all of the compounds, with a mass error below 2 ppm for more than 70% of the cases, and in the order of 5 ppm for the rest. It's important to point out that in all MS2 modes, we observe slightly higher errors compared to full scan. This is to be expected since fragments are smaller (*m/z* <100) than precursor ions, thus the relative error (expressed in ppm) is higher compared to the larger ions. Even in an orange matrix, over 70% of fragment ions had errors below 2 ppm (see **Figure 11**).

Detection Capability and Linearity

It's important to point out that the Q Exactive Focus is a very sensitive instrument. In this study, we were able to detect practically all of the pesticides at a level of 10 µg/kg for the majority of sample types. In addition to the excellent detection capability, the linear dynamic range of the Orbitrap analyzer is also very good because the number of ions entering into the Orbitrap analyzer is controlled by Automatic Gain Control (AGC); thus it is impossible to overfill/saturate the detector. This

Figure 10: Comparison of AIF with vDIA for a pesticide residue in leek extract.

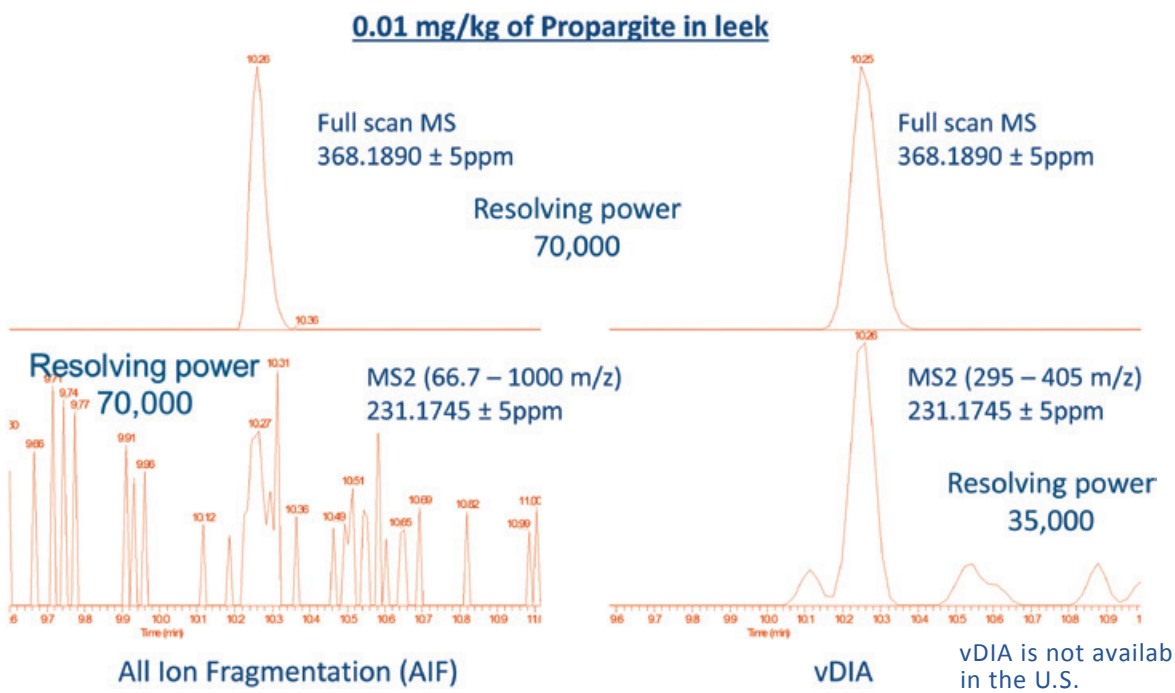


Figure 11: Mass errors in MS2 mode are below 2 ppm for 70% of compounds extracted using three different workflow techniques.

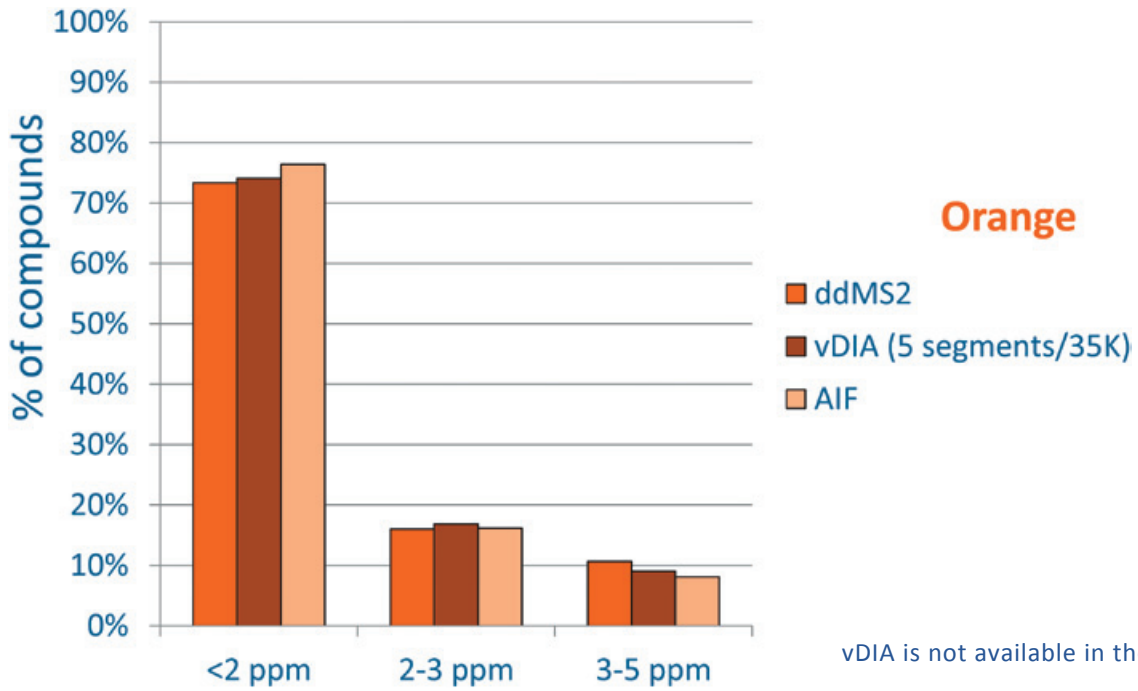


Figure 12: Linearity for three pesticides in spinach by dd-MS2.

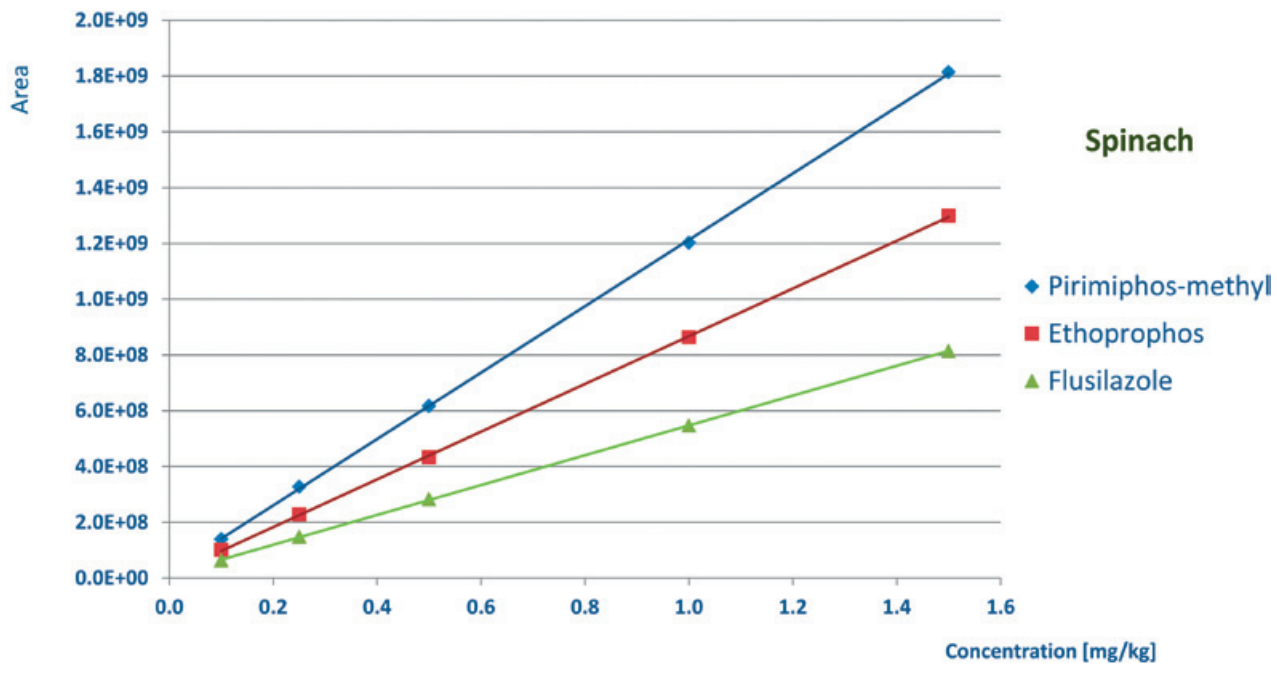
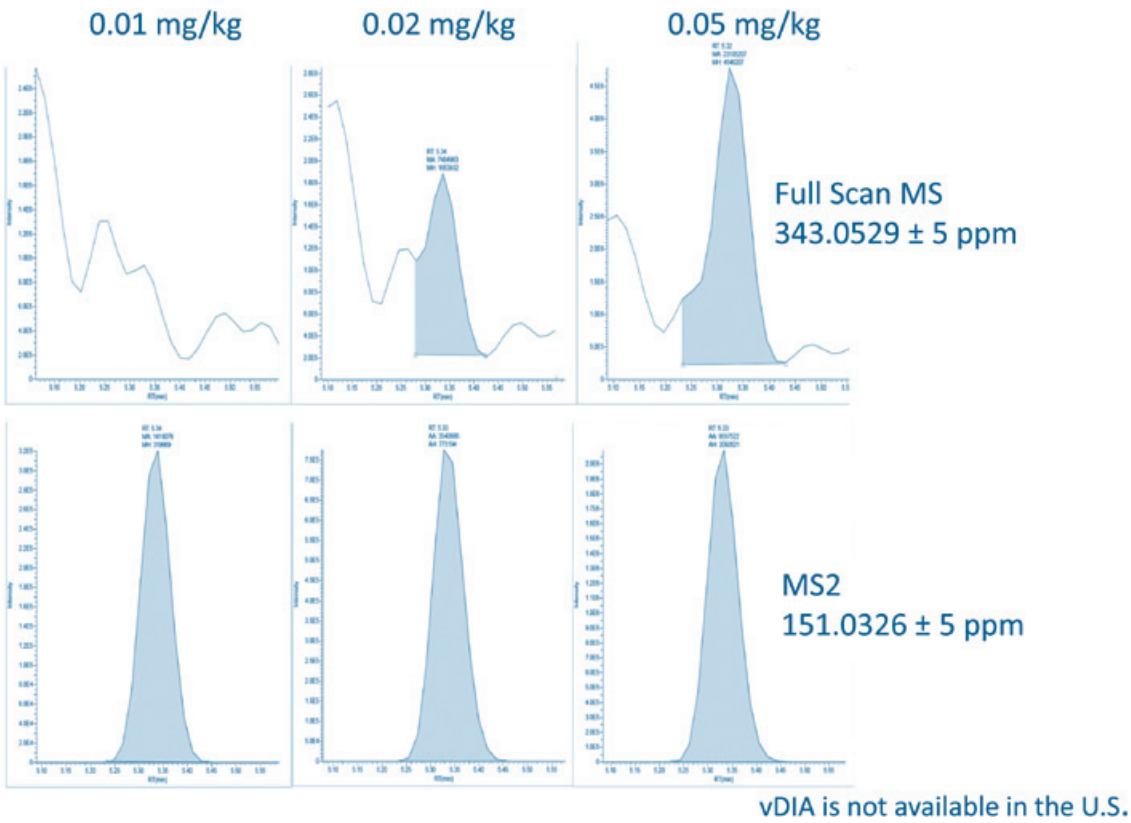


Figure 13: Quantitation of thiophanate methyl in onion (vDIA, 5 segments, 35,000 resolution), showing the impact of interferences on the analyte peak.

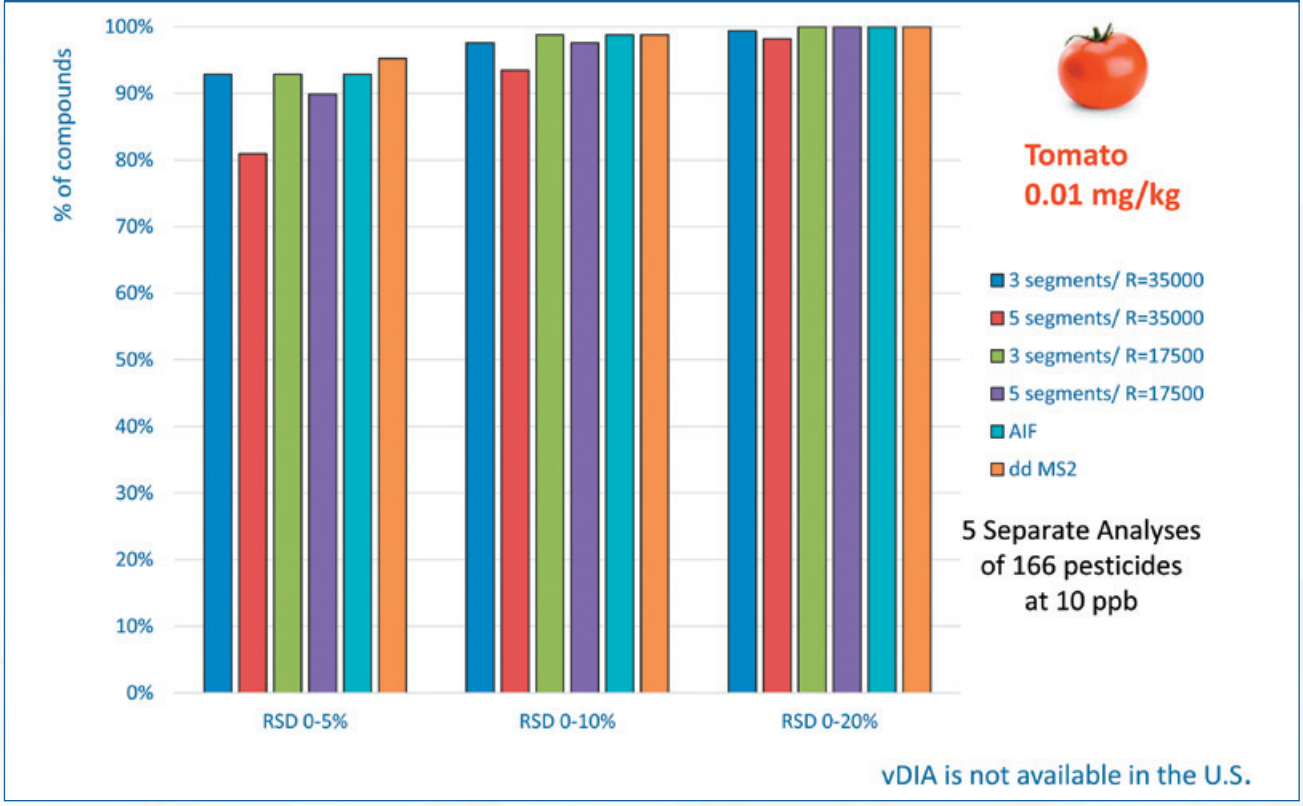


is demonstrated by **Figure 12**, which shows that linearity up to 1.6 ppm for three pesticides in a spinach sample using dd-MS2 can be achieved. Both the vDIA and AIF approaches showed similar linearity. It's also important to emphasize that the detector response of some other designs of high resolution instruments is not linear at higher concentration due to saturation of the detector.

Handling Interferences

The impact of interferences on the identification and quantitation is demonstrated by the example of thiophanate methyl in an onion extract as shown in **Figure 13**. Onion is a very complex matrix with a very large number of natural components. On the three upper full scan ion chromatograms, acquired with 70,000 resolving power, we see many co-extracted compounds that generate potential interferences. The level of interferences is so high that the peak for thiophanate methyl at 10 µg/kg is completely overlapped by the interference. At the level of 20 µg/kg, we start to see the peak for thiophanate methyl, but it's very difficult to quantify. Quantitation becomes more

Figure 14: Comparison of measurement repeatability between different workflows.



realistic at the level of 50 μg/kg but we still have some interference either side of the analyte peak. As mentioned previously, in dd-MS2 we obtain only one MS2 scan per chromatographic peak, and as a result it can be used only for identification purposes. However, in the case of vDIA or AIF, it is possible to extract peaks from MS2 data, so they can also be used for quantitation. This is shown in the lower scans in **Figure 13**, where we see peaks free from the interferences because the compounds present in the onion extract do not produce the same fragments as thiophanate methyl.

One of the inherent problems in LC–MS is matrix effects. In our laboratory, we usually dilute samples five-fold to reduce matrix effects because when we do this, 95% of compounds in tomato and apple extracts are free from interferences. In the case of orange, approximately 80% of compounds are free from matrix effects, while in the onion extract, which is a more complex matrix, the number is about 50%.

Repeatability

Another very important parameter of quantitative analysis is peak area repeatability of the molecular ion (not the fragment ions). In general we want to obtain precision below 20%. The histogram shown in **Figure 14** illustrates

Table 1: Analysis of EUPT-FV-15 potato (2013) reference material using the three different workflows described in this study.

Pesticide	Asigned value (mg/kg)	Obtained value (Difference)		
		AIF	dd-MS2	vDIA
Acephate	0,083 (±50%)	0,060 (-28%)	0,062 (-25%)	0,063 (-24%)
Azoxystrobin	0,203 (±50%)	0,193 (-5%)	0,195 (-4%)	0,199 (-2%)
Diazinon	0,195 (±50%)	0,152 (-22%)	0,154 (-21%)	0,158 (-19%)
Fosthiazate	0,08 (±50%)	0,069 (-14%)	0,070 (-13%)	0,070 (-13%)
Iprovalicarb	0,09 (±50%)	0,073 (-19%)	0,075 (-17%)	0,073 (-19%)
Linuron	0,098 (±50%)	0,088 (-10%)	0,089 (-9%)	0,087 (-11%)
Methiocarb	0,136 (±50%)	0,129 (-5%)	0,131 (-4%)	0,129 (-5%)
Pencycuron	0,269 (±50%)	0,264 (-2%)	0,266 (-1%)	0,258 (-4%)
Prochloraz	0,058 (±50%)	0,029 (-50%)	0,034 (-41%)	0,035 (-40%)
Spirodiclofen	0,444 (±50%)	0,280 (-37%)	0,284 (-36%)	0,284 (-36%)
Thiabendazole	1,71 (±50%)	1,83 (7%)	1,81 (6%)	1,88 (10%)
Thiacloprid	0,338 (±50%)	0,331 (-2%)	0,324 (-4%)	0,324 (-4%)

vDIA is not available in the U.S.

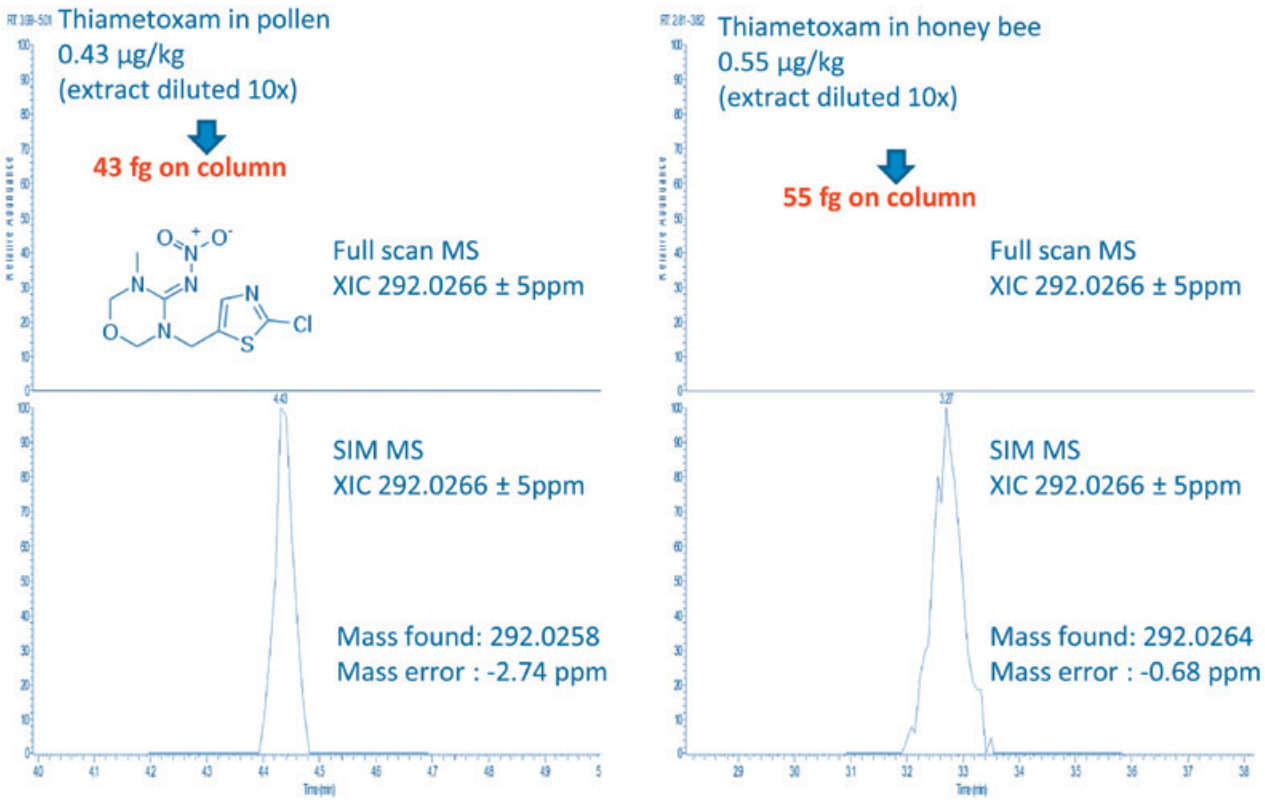
the results obtained for a tomato extract spiked with 166 different pesticides at 10 µg/kg, and analyzed using dd-MS2, AIF, and vDIA using four different settings. Almost 100% of the pesticides are below 20% RSD. However, if we look at how many of them are below 5%, we see differences between the workflows.

In this example, we have the best results for dd-MS2 because it has the shortest cycle time. In dd-MS2 with the Q Exactive Focus instrument, almost all the available dwell time is spent acquiring data in full scan. So, working with 70,000 resolution, we have more than three scans per second, which translates to more than 20 points per chromatographic peak. By contrast, vDIA, has the longest cycle time, requiring around one second for five MS2 segments, approximately 3x longer than dd-MS2.

Reference Materials

Finally, an evaluation of EUPT materials of potato, pepper and broccoli was carried out using the three different Q Exactive Focus workflows: full scan with AIF, dd-MS2, and vDIA. **Table 1** shows the data for the EUPT-FV-15 potato reference sample. The results obtained for every one of the test materials using all of the workflows were in good agreement with the assigned values.

Figure 15: Femtogram levels of Thiametoxam can be detected in SIM mode, but not in full scan MS mode.



Other Application Areas

Other application areas of the Q Exactive Focus worth mentioning are based on retrospective analysis. This becomes important when we are working with workflows such as AIF or vDIA. At a later date, and perhaps in response to new emerging information, we can return to the original raw data files and interrogate the acquired spectra by comparing raw data files with information contained in large databases to possibly detect new compounds of interest. We cannot only detect compounds, but we can also identify those detected compounds using their fragmentation products, because we previously obtained fragments from all compounds present in the sample.

Another very interesting application is operation of the Q Exactive Focus instrument in selected ion monitoring (SIM mode) for the analysis of analytes at very low concentrations. In our experience, SIM mode is 5–10 times more sensitive than full mass scan mode, as demonstrated by the detection of the thiametoxam residue in pollen and in honeybees in **Figure 15.** No residues were detected in full scan mode, but when the samples were reanalyzed in SIM mode we were able to detect

thiametoxam at around 50 femtogram on the column.

Conclusions

To summarize our investigation, we can say that the Q Exactive Focus Orbitap system operated in full scan with 70,000 resolution and dd-MS2 detected over 99% of pesticides with a mass error lower than 2 ppm. Also, by using this approach, all of the fragments were detected with mass errors below 5 ppm. All of the workflows (full scan-ddMS2, -vDIA, and -AIF) investigated showed very good quantitation capabilities for the vast majority of analytes down to 10 μg/kg with good linearity and peak area repeatability.

However, based on our studies, the best technique for quantitation was full scan-dd-MS2 (quantification in full scan) because this workflow has the shortest cycle time. On the other hand, AIF and vDIA offer additional quantification modes, which could potentially be very helpful in the case of very complex matrices. Based on concentration values obtained in analyzing standard reference samples, we can conclude that all evaluated workflows gave very similar and consistent results. For more information about this technology and a more exhaustive set of data for the determination of pesticides in various samples, please refer to the following publications.

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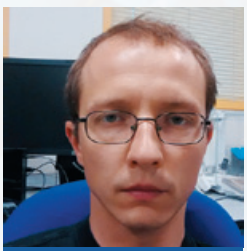
Prof. Amadeo Fernández-Alba, Ph.D., European Reference Laboratory for Pesticide Residues in Fruits and Vegetables, Analytical Chemistry Dept., University of Almeria, Spain



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ION CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY: A PERFECT MARRIAGE FOR POLAR PESTICIDES?

By Stuart Adams

Introduction

Polar ionic pesticides such as glyphosate, glufosinate, and chlorate are not usually amenable to common multi-residue methods so are typically extracted using the Quick Polar Pesticides Extraction (QuPPe) method.[1] To obtain sufficient chromatographic retention and acceptable peak shapes for all of the polar pesticides listed in the method, it is necessary to analyze an individual extract multiple times using different chromatographic columns and conditions. This increases the total sample analysis times and the overall cost. By contrast, the multi-residue capability of suppressed ion chromatography coupled to tandem quadrupole mass spectrometry can provide significant time and cost savings for these types of analyses. This manuscript will describe the challenges and successes in the development, validation, and implementation of this approach for routine analysis.

IC-MS/MS Analysis of Polar Ionic Pesticides

The Quick Polar Pesticide Extraction (QuPPe) method is often used for the analysis of polar ionic pesticides such as glyphosate, ethephon, chlorate, perchlorate, glufosinate, fosetyl aluminum, relevant metabolites, and the like. Glyphosate is of particular interest due to a number of factors, including the differences between the regulations in Europe and the US. The maximum residue limits (MRLs) in Europe, set by the European Commission, are lower than tolerance values set by the Environmental Protection Agency (EPA) in the US. Also, in 2015, the International Agency for Research on Cancer (IARC), which informs the World Health Organization (WHO) on cancer risk factors, classified glyphosate as a "probable carcinogen." As a result of the ongoing controversy, glyphosate continues to be featured in the news. In 2016, residues of glyphosate had been found in beer and many popular breakfast foods.

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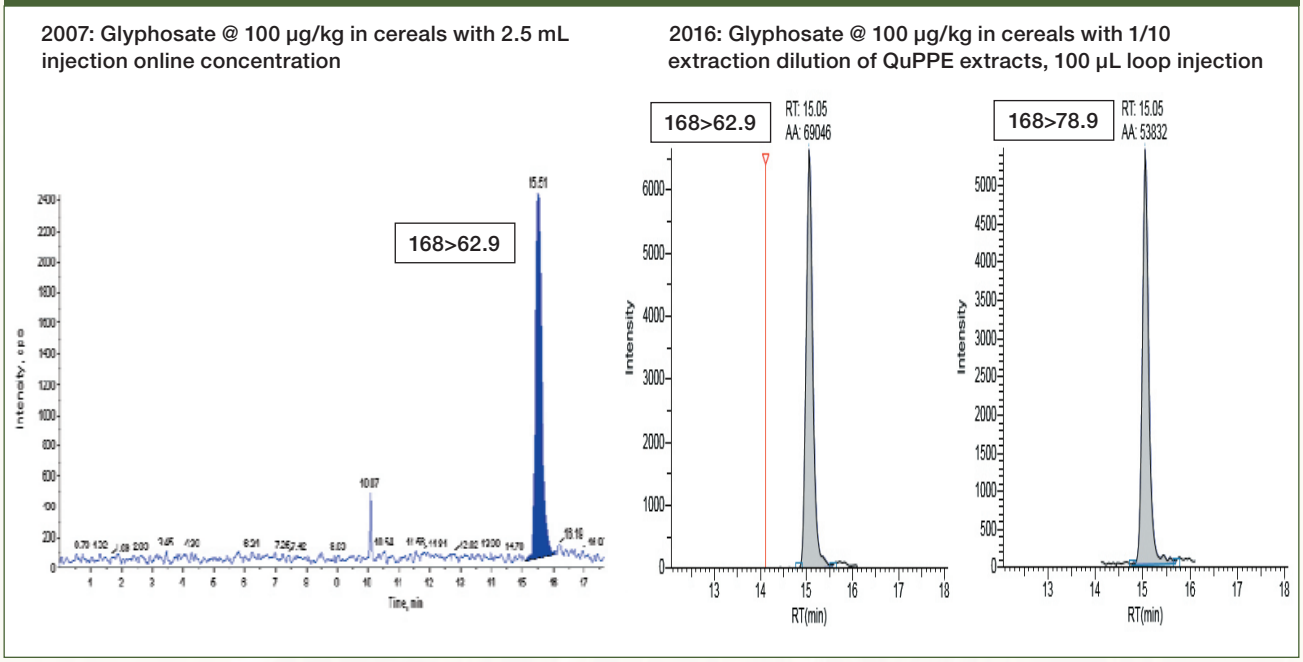
Figure 1: IC-MS/MS Then and Now—Glyphosate



Historically, glyphosate analysis required time-consuming derivatization prior to detection and quantification. More recently, ion chromatography has been used for the direct analysis of glyphosate and its metabolites without the need for derivatization. One advantage of using ion chromatography-tandem mass spectrometry (IC-MS/MS) is the capability to use in-line concentration cartridges, which can help with the removal of matrix components as well as concentrating the ionic analytes of interest.

In 2007, this approach was employed at Fera Science Ltd. for the analysis of glyphosate residues in sugars, which are used extensively in the manufacture of food products. The challenging target concentrations were 1 µg/kg for glyphosate and 5 µg/kg for glufosinate. At that time, a Dionex ICS 3000 (ion chromatography system) was coupled to a mass spectrometer with relatively low sensitivity by today's standards. To compensate for low MS sensitivity, large volume injections of several milliliters of sample were required. Although this approach enabled the target concentrations of 1 µg/kg and 5 µg/kg to be achieved, such high volumes of complex sample extracts resulted in relatively rapid contamination of the analytical column, the MS-system (after 100 Injections), and longer term contamination of the post column suppressor (i.e., loss of peak shape and response after 4,000 injections).

Fera is currently evaluating the performance of the latest IC-MS/MS system; the Thermo Scientific™ Dionex™

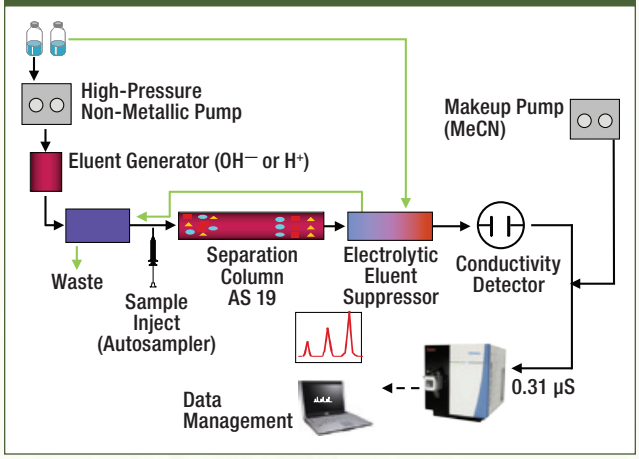
ICS-5000 HPIC™ system coupled to the Thermo Scientific™ TSQ™ Quantiva™ Triple Quadrupole MS. One of the advantages of using the Quantiva is a decrease in instrument downtime due to the ability to change the ion transfer tube while the system is still under vacuum. The Thermo Scientific™ TraceFinder™ 3.2 software makes the system significantly more user friendly as it eliminates the need for two previously required control software packages.Only one sequence, not two, is needed so that the risk of errors is reduced. Also, Fera is evaluating the new 4-µm particle size IC columns, which have the potential to provide better peak shapes. In combination with the high-sensitivity Quantiva MS (which has been optimized for low mass), the overall increase in sensitivity allows the use of smaller injection volumes (5–100 µL).

Where Are We Now?

Figure 1 shows that the new system with only a 100 µL injection (equivalent to 10 µL extract) can provide a better response compared to the system using 2,500 µL in 2007.

The new ion chromatography system configuration is shown in **Figure 2.** Compared to a standard LC–MS/MS configuration, there are some subtle and unique differences including a high-pressure non-metallic pump, an eluent generator, and an electrolytic eluent suppressor. The eluent generator only requires the addition of water to produce the mobile phase *in situ*

Figure 2: IC-MS/MS Configuration



resulting in very reproducible eluent gradients and retention times. The suppressor, which electrolyzes the KOH mobile phase to water at the exit of the column, and before the detector, is critical to enable the coupling to a mass spectrometer. It is important to monitor the back pressure on the suppressor at the start of each run, and to maintain the pressure at 100–150 psi to avoid damaging it. Both the MS system and the acetonitrile makeup flow will add system backpressure. In the unlikely event the suppressor fails, the conductivity signal will increase, and a feedback loop will shut down the system automatically to ensure that the MS isn't exposed to hydroxide eluent. The post suppressor addition of acetonitrile, to assist desolvation of water in the mass spectrometer, provides an average increase in signal response up to a four-fold without any distortion of peak shapes. Details of the chromatographic conditions used are

Figure 3: Multi Residue Separation Program

Column: AS19 250 × 2mm, 4 µm particle size
- Column flow: 0.35 mL/min
- Column temp: 40°C

Suppressor: AERS 500 2mm (used in external water mode)
- External water flow: 0.5 mL/min
- Suppressor current: 52 mA

Eluent: KOH gradient with EGC 500

Injection volume: 100 µL

Post column MeCN flow (make-up): 0.2 mL/min

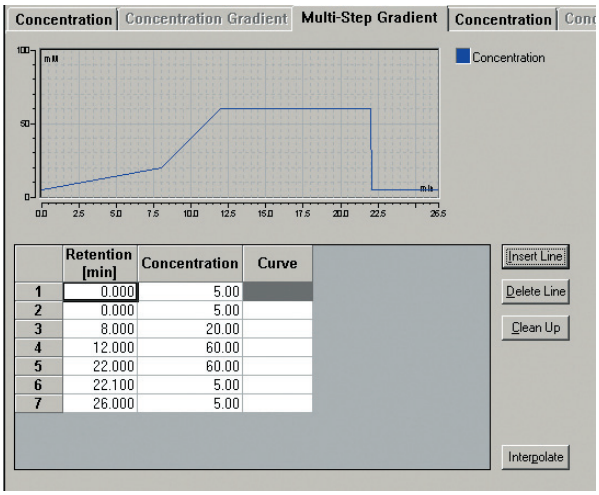
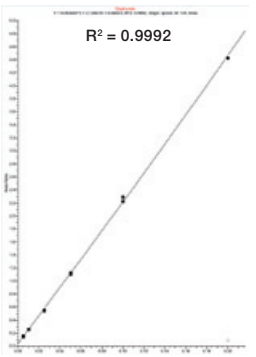
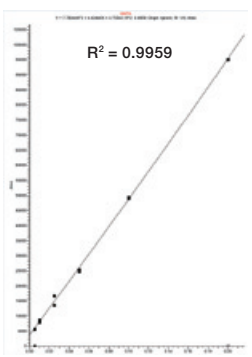


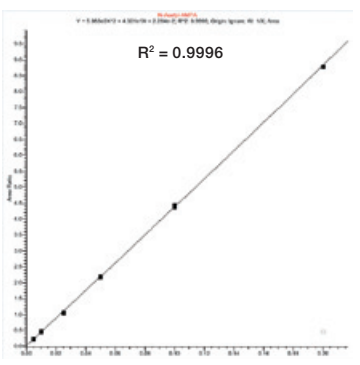
Figure 4: Glyphosate—Cereals (Flour)

Summary of validation results

Compound	Concn (µg/kg)	Mean % Recovery (n=5)	Mean % RSD
Glyphosate (IS)	10	112	15
	50	108	12
	100	111	7
AMPA (no IS)	10	92	22
	50	98	13
	100	97	3
N-Aectyl-AMPA (no IS)	10	85	7
	50	82	10
	100	86	2







shown in **Figure 3.** Since the presence of formic acid in the QuPPe extracts can affect the peak shape, the extracts were diluted 10-fold before injection of 100 µL. This dilution provided better peak shapes compared to direct injection of 10 µL of crude non-diluted extract. So, what is the QuPPe method?

The QuPPe Method

The QuPPe method was developed to consolidate several single residue methods into one generic extraction method. Version 1 was published in January 2009 and the current version (V9) was released in March 2016.

The method continues to evolve to include new compounds of interest and different liquid chromatography conditions to cover the analysis of some of the most challenging polar pesticides.

To perform the extraction, internal standards, if available, are added to the sample (10 g). Acidified methanol (10 mL) is added, and then the extract is shaken using a mechanical shaker for 20 minutes. The extract is centrifuged, filtered into a plastic LC–MS vial, and analyzed using the appropriate method conditions. This generic extraction approach is not without its challenges. There is no liquid–liquid partitioning stage so a large number of co-extractives can end up in the final extract. Also, there is no single clean-up

method applicable due to the diversity of chemical properties of the analytes. This can lead to co-eluting interferences and make it more challenging to meet the method performance criteria specified in the SANTE/11945/2015 document[2].

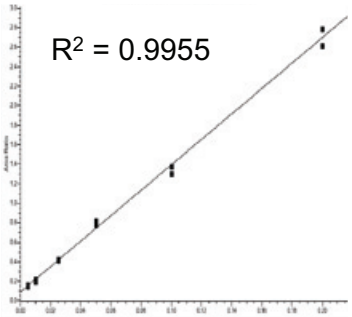
Method Validation (QuPPe-IC-MS/MS)

The QuPPe-IC-MS/MS method developed was validated for cereal and grape matrices, according to the SANTE validation criteria. These criteria include the recovery, which must be 70–120%; the associated %RSD, which should be ≤20%; a sensitivity/linearity check (residuals <±20%); and retention time (at least two-times the void volume of the column and within ±0.1 minute of the retention time of a calibration standard). If available, isotopically labelled internal standards were used during validation experiments.

The validation results for the analysis of glyphosate and two of its transformation products, AMPA and N-acetyl-AMPA, in cereals (flour) are presented in **Figure 4.** At 10, 50, and 100 µg/kg (ppb), the results for % recovery (in the range 82–112), %RSD (2–22), linearity (R2 values >0.99), and retention time stability (<0.1 min) were all, apart from one exception, within acceptable values for all analyte-concentration combinations. The one exception was the %RSD for AMPA at 10 µg/kg (not internally standardized), at 22%, fractionally outside the limit of 20%. The IC-MS/MS method is multi-residue and excellent recovery and precision data, compliant with SANTE criteria, were achieved for glufosinate, 3-MPPA, N-acetyl glufosinate, perchlorate, chlorate, and ethephon. Excellent data were obtained for Fosetyl-AL and phosphonic acid at 200, 1,000, and 2,000 µg/kg (ppb). These compounds were spiked at higher concentrations to reflect their higher EU MRLs. Clopyralid displayed poor sensitivity at 10 ppb, but provided good data at 50 and 100 ppb. Meanwhile, cyanuric acid gave insufficient response at 10 ppb and slightly variable results at 50 ppb.

Figure 5: Analysis of Ethephon in Grapes

Compound	Concn (µg/kg)	Mean Recovery (n=5)	Mean % RSD
Ethephon (IS)	10	114	17
	50	95	14
	100	109	10



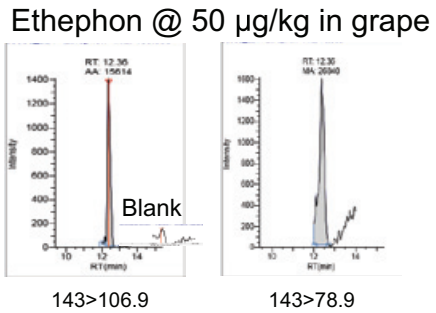


Figure 6: Glyphosate in Beer—No Extraction Required!

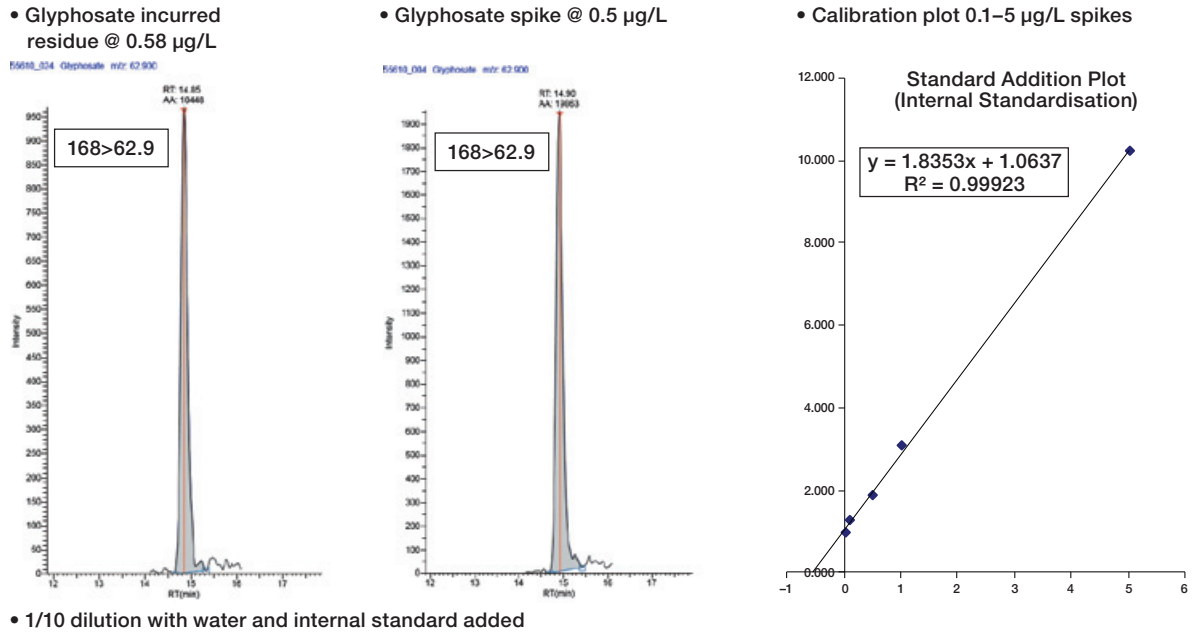
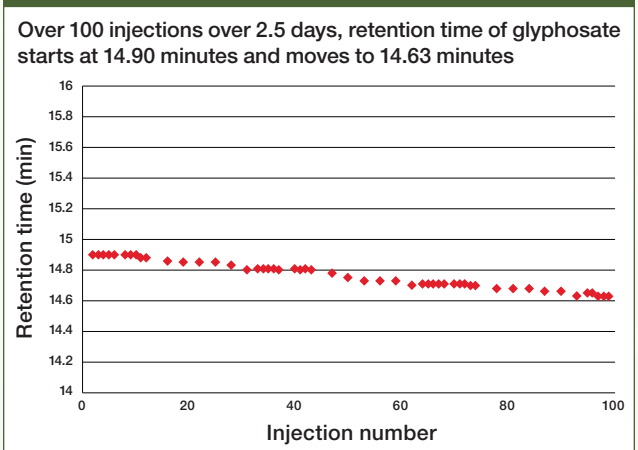


Figure 7: Glyphosate in Beer—Retention Time Stability



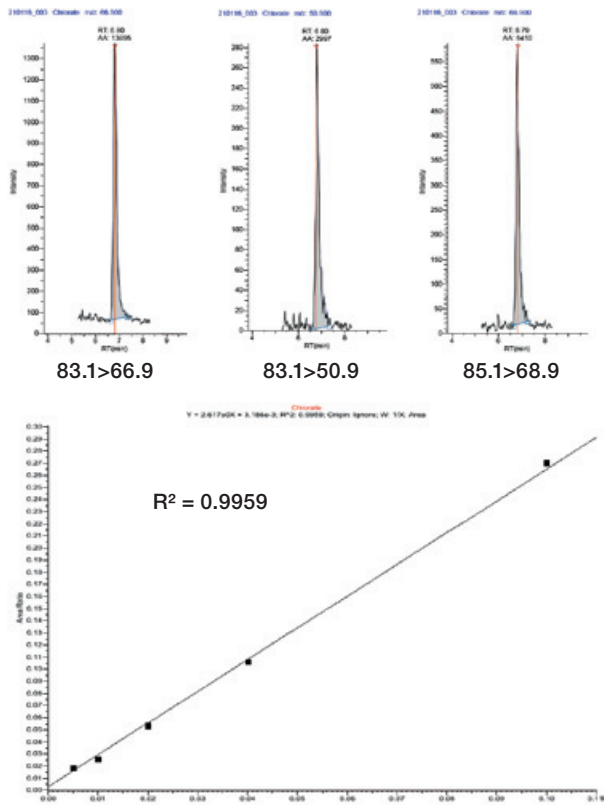
The results for grapes were even more impressive. All of the compounds, at the same concentrations as in cereals, were compliant with the SANTE validation criteria, with the exception of cyanuric acid, which gave poor response at 10 ppb. The validation results for the analysis of ethephon at 10–100 ug/kg in grape matrix are summarized in **Figure 5.**

Other Application Examples

Another application example, the analysis of glyphosate in beer is illustrated in **Figure 6.** Sample preparation in this case involved degassing the sample, then diluting 1/10 in water (no extraction required), followed by the addition of an internal standard. Excellent stability of retention time was obtained as seen in **Figure 7.** The retention time of glyphosate drifted by only about 0.3 minutes for 100 injections spread out over 2.5 days.

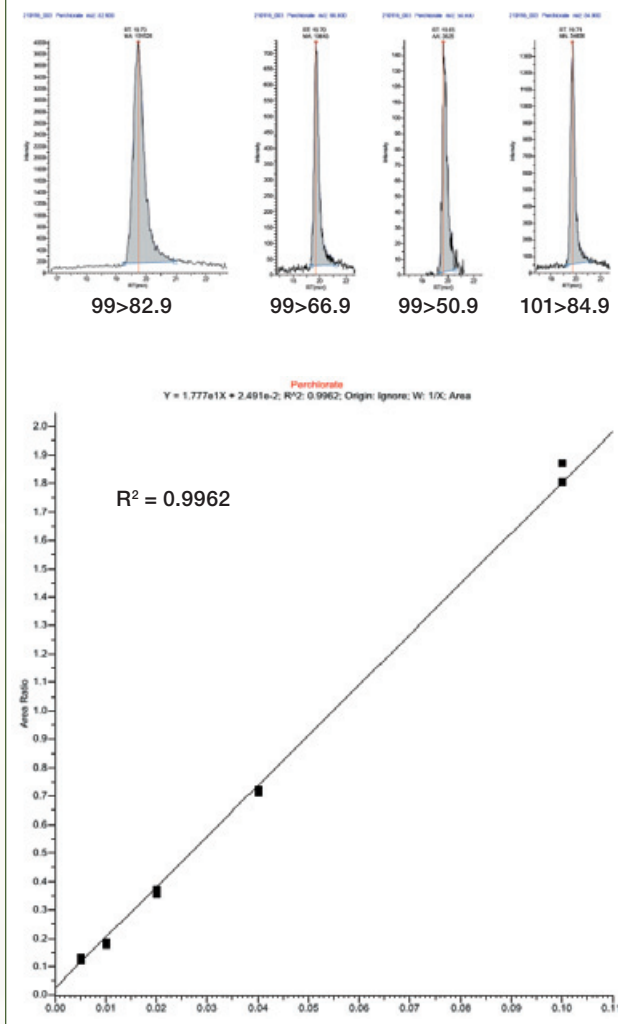
At the start of this decade, chlorate was identified as a potential problem and its presence in food was banned in the EU in 2010. It was determined that the presence

Figure 8: Chlorate in Dairy Products



Chlorate in dairy products: calibration 5–100 µg/kg

Figure 9: Perchlorate in Dairy Products



Perchlorate in dairy products: calibration 5 – 100 µg/kg

of chlorate in food most likely occurred as a result of a disinfection by-product in the water used in food preparation facilities, and in disinfection products used to clean equipment and work surfaces. The current default MRL of 10 µg/kg is applied. Because of ongoing concerns the European Food Safety Authority called for more data on the presence of chlorate residues in foods. In response, the IC-MS/MS approach has been used to successfully conduct several chlorate surveys in the UK. Using IC-MS/MS, chlorate can be detected at 5 µg/kg in dairy products, as shown in **Figure 8,** with good linearity over a calibration range of 5 to 100 µg/kg. Equally good results were obtained for the analysis of perchlorate in dairy products, as illustrated in **Figure 9.**

Conclusion

The IC-MS method developed was successfully validated for 13 anionic pesticides in two commodity groups,

displaying excellent sensitivity and selectivity. Simplified sample preparation, smaller particle size columns, a more sensitive MS instrument, and unified control software have made systems easier to use. The high system sensitivity and lower injection volumes have resulted in decreased system downtime (due to lesser contamination) and decreased analysis costs while improving sample throughput. In the future, it is easy to see this technology being extended to the analysis of other applications such as halo acetic acids, metals speciation, organic acids, carbohydrates, and other types of cations and amines. IC-MS is an essential tool for modern food and environmental analytical laboratories; and it is a very good, if not yet a perfect, marriage.

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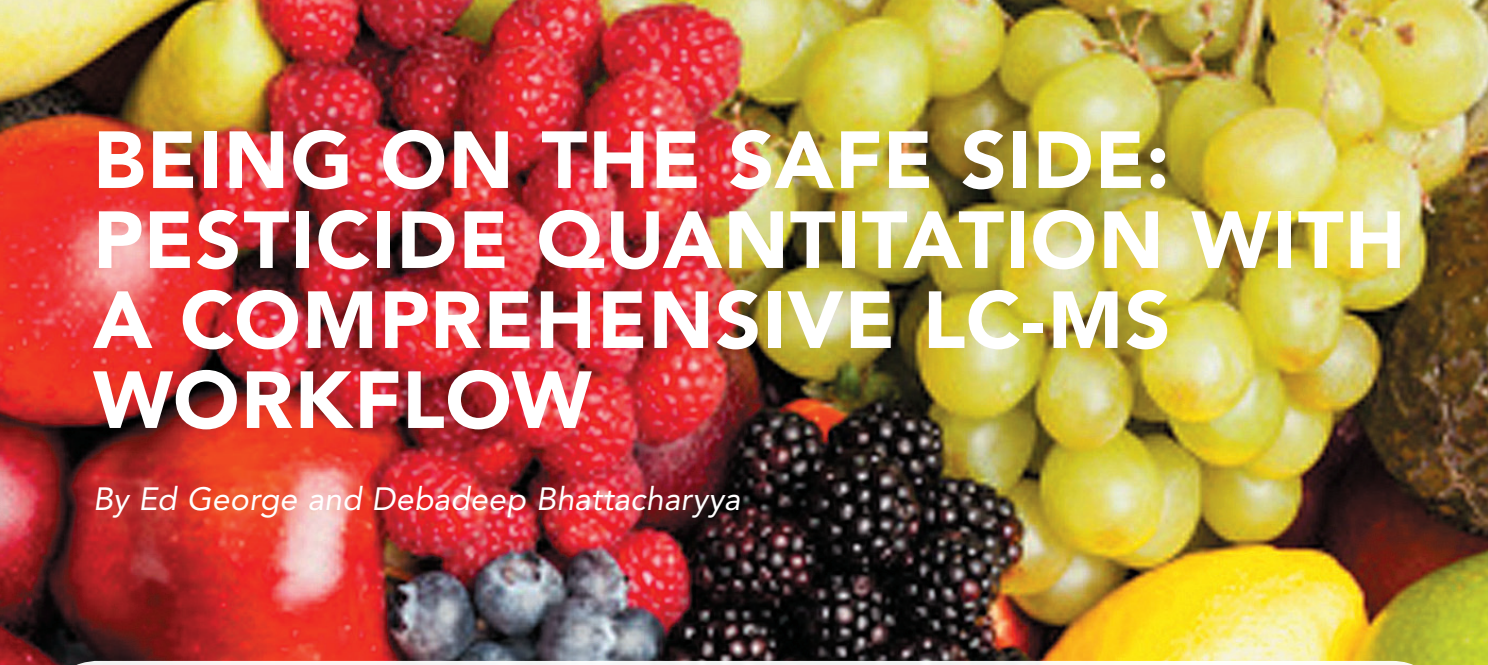
BEING ON THE SAFE SIDE: PESTICIDE QUANTITATION WITH A COMPREHENSIVE LC-MS WORKFLOW

By Ed George and Debadeep Bhattacharyya



Introduction

The screening and routine quantitation of pesticide residues in food products is one of the most important and demanding applications in food safety. Despite the recent technological advancements in LC-MS, it is still challenging to quantify hundreds of LC-amenable pesticides with a robust and sensitive workflow solution. This manuscript describes the development and implementation of complete workflow solutions based on LC-triple quadrupole MS/MS and LC-high-resolution accurate mass (HRAM) MS/MS. These ready-to-go solutions have been validated in three matrices across four different laboratories. Customized software is used for data acquisition and processing; this combination allows users to rapidly implement these analytical methods and enhance productivity.

The World of Food Safety and Testing

Food safety is a growing market and there is an increasing public demand for protection from chemical residues and contaminants that can be present in food and food products originating in different parts of the world. Changing agricultural practices, climatic conditions, and socioeconomic factors all contribute to food safety concerns. As a result, food products have to be thoroughly tested and evaluated according to the prevailing legislative and regulatory requirements before they reach the market and before they are ultimately consumed. At present, there are many different agricultural practices including the use of chemicals to control pests and improve crop yields. In fact, there are more than one thousand different active substances that are used as pesticides. Occasionally, pesticides are not applied in accordance with the intended purpose, for example, the use of a pesticide on a crop for which its use has not been approved. Whether accidental or intended, such misuse of pesticides can erode toxicological safety margins. This in turn drives the requirement for a holistic solution or workflow that enables detection, identification, and quantification of hundreds of different pesticides in hundreds of sample types, from simple

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matrices such as fruits to more complex samples such as tea and spices.

The solution has to be based on proven, robust methods that are quick, easy to implement, and cost effective. The Thermo Scientific™ Pesticide Explorer Collection is an extensive repository of LC-MS/MS methods that provide cost-effective, robust, sensitive, and selective solutions that are compliant with regulations and guidelines on method performance, and provide complete confidence in the results obtained.

The Thermo Scientific Pesticide Explorer Collection

The Thermo Scientific™ Pesticide Explorer Collection provides comprehensive start-to-finish workflow solutions for the analysis of pesticides in food matrices. Each complete workflow includes sample preparation, LC-MS method details, and data processing parameters. Each workflow is compliant with regulatory or guideline criteria recommended by the U.S. FDA, USDA, EU SANTE, and the Chinese GB standard of testing. The Pesticide Explorer Collection comprises two different mass spectrometer platforms: triple quadrupole and high-resolution accurate mass (HRAM). Depending on the study requirement, the user can choose the MS platform that is most optimal; whether performing routine, targeted quantitation using the Thermo Scientific™ TSQ Endura™ Triple Quadrupole Mass Spectrometer or the Thermo Scientific™ TSQ Quantiva™ Triple Quadrupole Mass Spectrometer, or simultaneous targeted and non-targeted analysis using an HRAM instrument (e.g. the Thermo Scientific™ Q Exactive™ Focus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer). Regardless of the LC-MS platform chosen, the Pesticide Explorer Collection offers tested methods, robust LC, and the requisite software to control the system and process the data.

In addition, the Collection offers a pesticide accessories kit that includes; columns, tubing, a QuEChERS sample preparation kit, QC standards and multi-pesticides standard options (a 276 pesticide mix or 440 pesticide mix) depending on analysis requirements.

The choice of the most optimal LC-MS system is dependent on the goals of the analysis. For routine, targeted analysis of a predefined list of pesticides, a triple quadrupole MS would be the most suitable option. However, if in addition to targeted analysis, there is an interest in screening for unexpected residues, an HRAM instrument like the Q-Exactive Focus system is a more appropriate option. The considerations to be made in choosing the right platform are summarized in **Figure 1.**

The Analytical Challenges of Pesticide Residue Analysis

There are many analytical challenges encountered during pesticide residue analysis. Apart from the large number of pesticides and variety of different matrices, losses of pesticides due to degradation or

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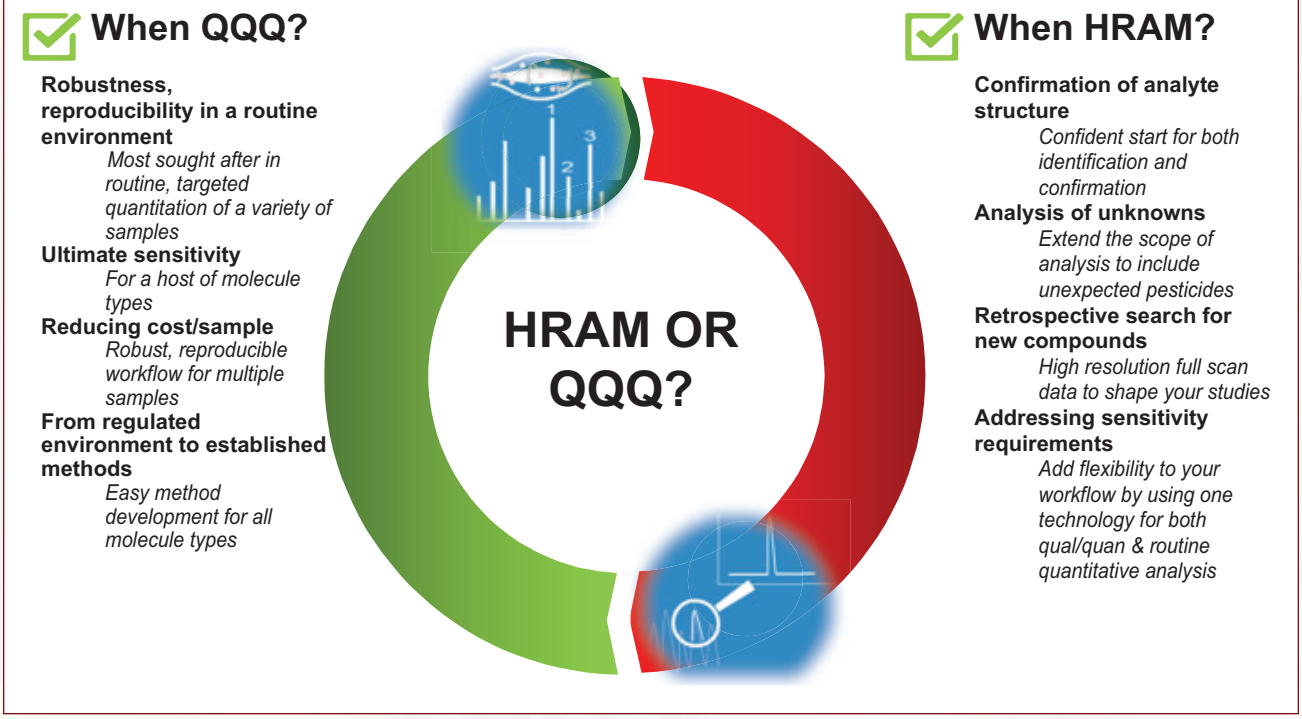
Figure 1: Femtogram levels of Thiametoxam can be detected in SIM mode, but not in full scan MS mode.



interaction of the pesticides with the matrix; quality assurance requirements [limits of detection/limits of quantitation (LOD/LOQ), reproducibility, recovery, identification and confirmation] can also pose some serious challenges. Testing laboratories are expected to analyze pesticides at low (~10 ppb) levels in many different sample types within very short turnaround times. The results are typically required within 48 hours, since perishable foods like fresh vegetables and fruits are unsuitable for storage over an extended period.

Getting Started with Sample Preparation: The sample preparation process (homogenization and extraction) is the most under-rated analytical challenge in the pesticide residue analysis workflow. The variation in matrix constituents and diversity of the chemical properties of the pesticides contribute significantly to the complexities of this process. Pesticide losses during the grinding or homogenization of the sample are sometimes observed, and some pesticides are very sensitive to the temperature used during sample preparation, as discussed elsewhere.[1]

Sample extraction has traditionally been performed by homogenization or blending with solvents such as acetone, acetonitrile, ethyl acetate, and/or methanol. Additional clean-up is usually performed with liquid–liquid extraction, gel permeation chromatography, and solid-phase extraction. Detection and quantitation have evolved from simple GC and LC with

non-selective detectors to GC-MS and LC-MS triple quadrupole, and more recently, HRAM technology.

Nowadays, food safety laboratories typically use the Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) approach. The most commonly used QuEChERS versions[2-4] are; easy to use, fast, require minimal bench space, require minimal laboratory equipment, environmentally friendly (require low amounts/volumes of reagents and solvents), and cover a broad range of analyses at acceptable cost. All of these QuEChERS versions are suitable for the analysis of dry, or high moisture content, samples and can be used prior to either LC-MS or GC-MS detection. Since the dispersive Solid Phase Extraction (dSPE) clean-up step only removes limited amounts of matrix co-extractives, it is often omitted prior to analysis by LC-MS. Non-cleaned QuEChERS extracts are relatively "dirty" and are very likely to contaminate the analytical instruments. Therefore, regular maintenance of instruments is highly recommended. More information on sample preparation, extraction and clean-

Figure 2: QuEChERS-LC-MS method outline.

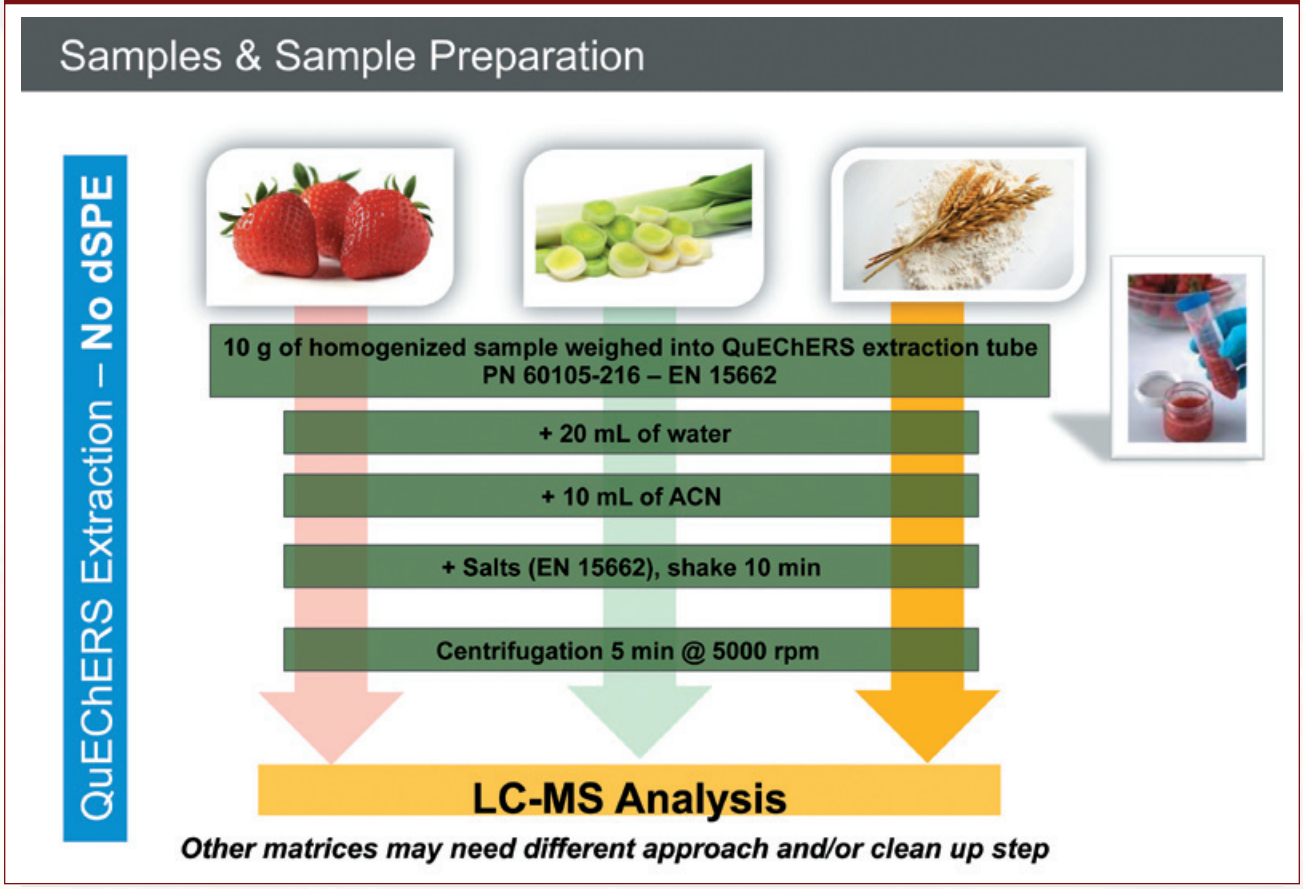
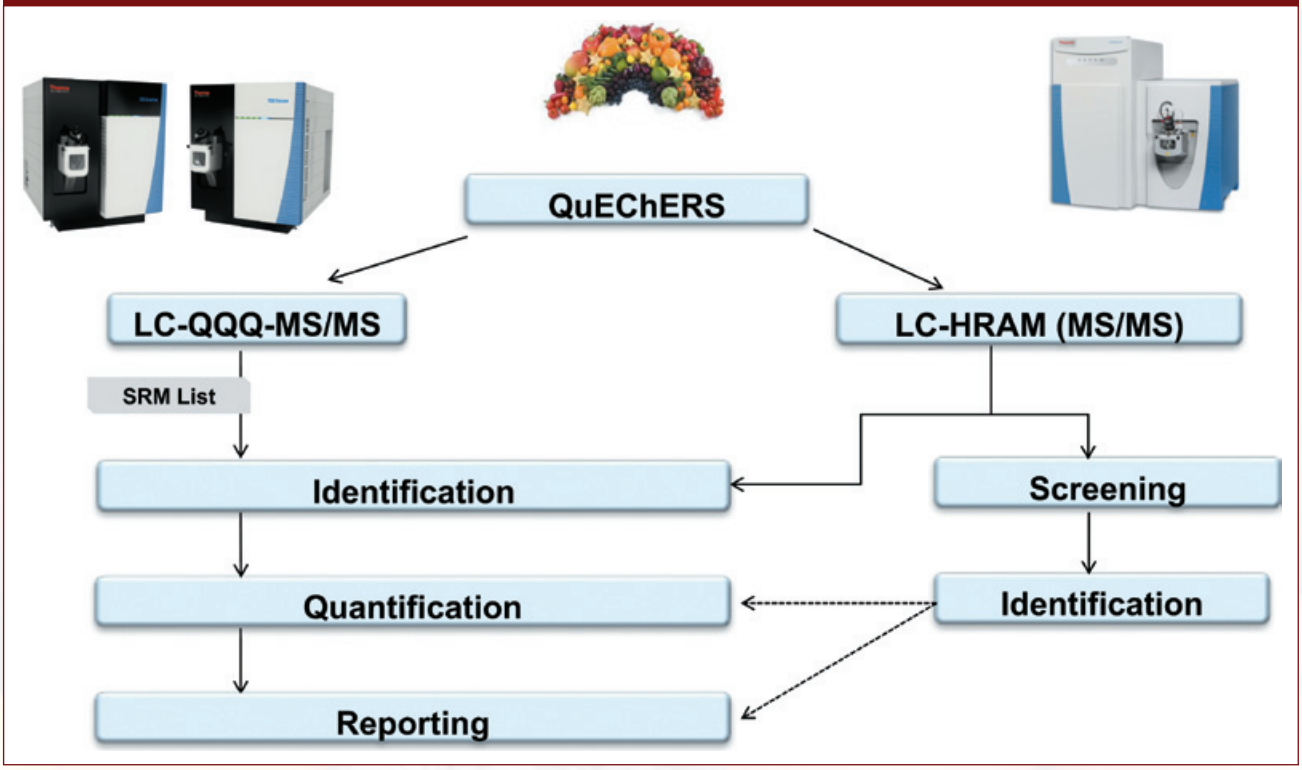


Figure 3: Selection of appropriate MS workflow.



up can be found in a white paper titled "Analytical Challenges for Pesticide Residue Analysis in Food: Sample Preparation, Processing, Extraction and Cleanup".[1]

Optimizing Sample Extractions

There are a few tips and tricks that can be used to improve QuEChERS extractions. **Figure 2** outlines the citrate buffered QuEChERS extraction approach, as used in the Pesticide Explorer Collection method, for three different representative matrices: strawberry (high water, simple matrix), leek (high water, complex matrix), and cereal flour (low water, complex matrix).

Different matrices can present different challenges to the LC-MS system, hence, in terms of matrix capacity, the performance of the Pesticide Explorer Collection methods were evaluated for these three specific and diverse matrices.

For dry commodities, the addition of water is recommended to desorb incurred pesticides residues from the matrix and to enable adequate partitioning of the pesticides into the organic solvent layer and the polar co-extractives into the aqueous layer. The methods described in the Pesticide Explorer Collection are applicable to the vast majority of food samples. However, samples containing high amounts of oils (e.g., avocado), fats (e.g., products of animal origin), tea, and spices will require modification of the methods, such as

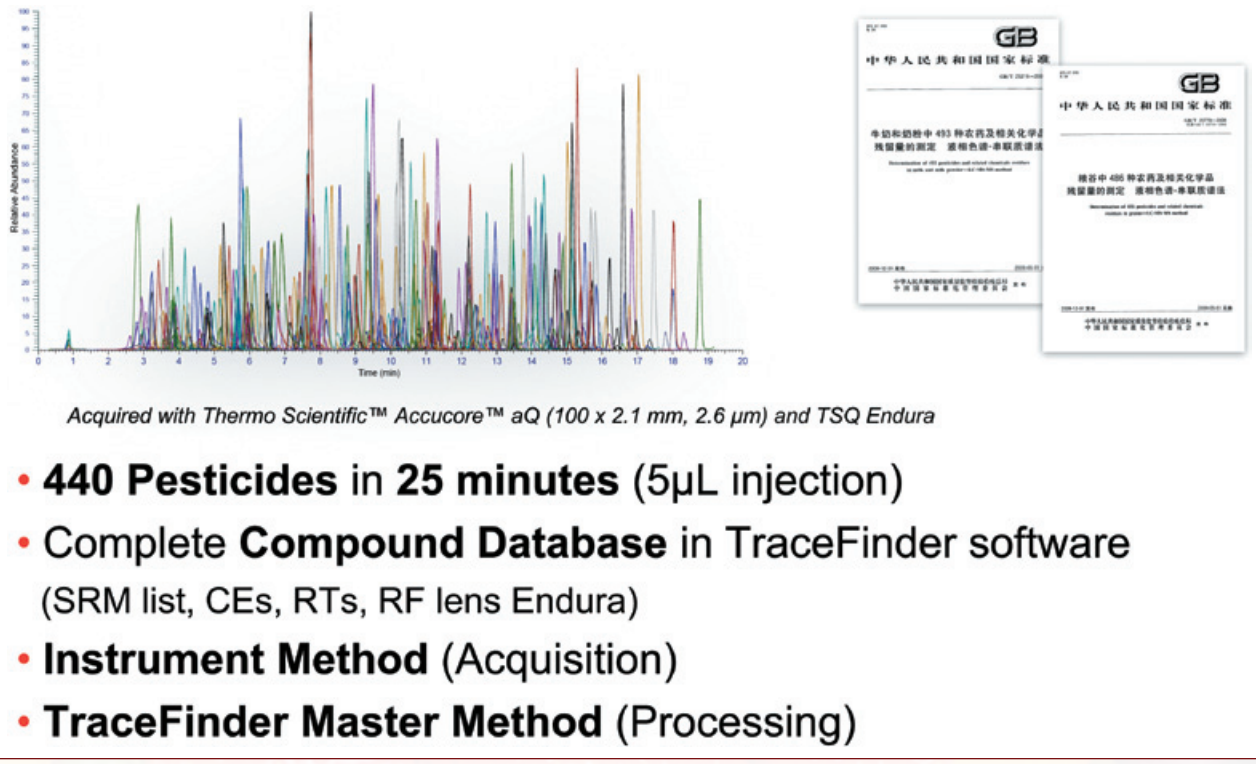
inclusion of clean-up steps. Regardless of any modification, extraction and clean-up methods, the LC-MS acquisition method for detection, identification and quantification of the pesticides in the final extract is still applicable.

The QuEChERS extraction approach can be used in a triple quadrupole instrument workflow for routine quantitation with SRM lists, or an HRAM workflow that includes quantitation, and identification for targeted compounds as well as screening for unknown compounds (see **Figure 3**).

The Pesticide Explorer Collections Methods

In order to ensure increased instrument uptime and enhanced productivity, it is critical for every analytical laboratory to initiate the preferred workflow and deliver results faster. The Pesticide Explorer Collection comprises all necessary components (platform solutions, software, and methods) that enable every analytical lab to achieve their goals without any delay. Two methods are available for both the TSQ Endura mass spectrometer and the TSQ Quantiva mass spectrometer; a 15-minute high-throughput quantification method for 276 compounds, and a 25-minute method for quantifying 440 pesticides; both with polarity switching. A total ion chromatogram acquired using the TSQ Endura is shown in **Figure 4**.

Figure 4: TIC for the 440 pesticides method acquired using the TSQ Endura Mass Spectrometer.



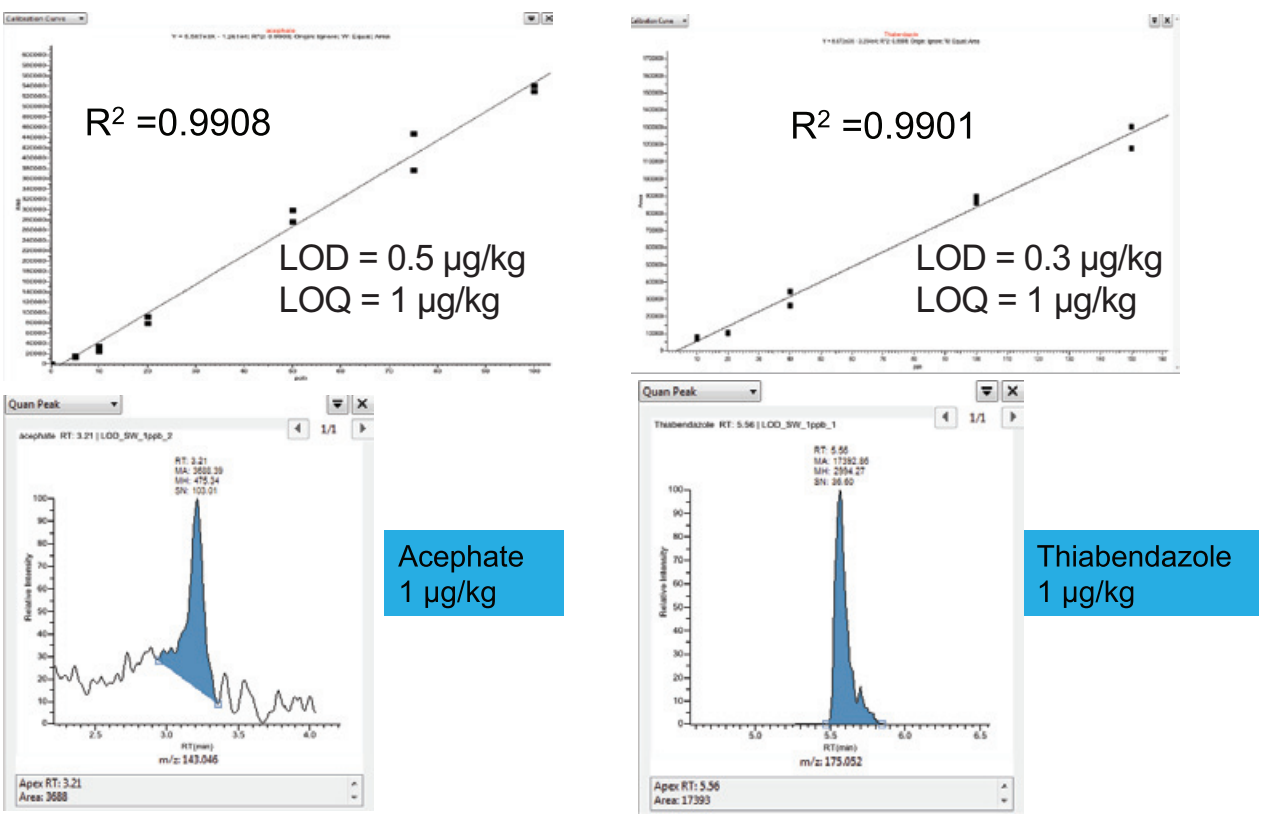
The TSQ Endura mass spectrometer offers sufficient sensitivity to meet all regulatory requirements, while ultimate high sensitivity for more challenging applications can be achieved using the TSQ Quantiva mass spectrometer. Pesticide Explorer methods each include; the analytical column and chromatographic conditions, the instrument method (acquisition parameters, RF lens settings, MS/MS transitions, collision energies, retention times etc.), and a Thermo Scientific™ TraceFinder™ software Master method for data-processing in conjunction with a comprehensive compound database.

TraceFinder Software is used for both HRAM and triple quadrupole MS instruments, hence, the methods can be easily transferred between these two MS platforms, and can be downloaded.

Method Validation

To ensure the robustness, reproducibility, and reliability of a method, it is critical to test and validate it. Typical parameters evaluated during such a validation protocol

Figure 5: LOQ data obtained for the high-throughput analysis of acephate and thiabendazole in strawberry matrix using TSQ Endura mass spectrometer.



include injection precision, accuracy (using reference materials), LOD, LOQ, specificity, and linearity.

Due to their polar nature, the analysis of acephate and thiabendazole can be challenging. The LOD for acephate and thiabendazole were 0.5 µg/kg and 0.3 µg/kg, respectively, with LOQs of 1 µg/kg, as shown in **Figure 5**. The peak shapes for both of these early eluting analytes were acceptable. Recovery and reproducibility of the method were demonstrated at both 10 and 100 µg/kg levels for a representative group of compounds in strawberry, leek, and flour matrices. Excellent RSDs typically <15% and percent recoveries in the range of 80% to 120% for the majority of pesticides were obtained.

Accuracy of the analysis can be established relative to reference materials with assigned values. Three such test samples (melon, lettuce, and wheat flour) with assigned values for several different pesticides were obtained from the Food Analysis Performance Assessment Scheme (FAPAS) in the UK. Once extracted and analyzed, results were compared with the accepted concentration range.

The measured values obtained for 12 pesticide-commodity combinations using the Pesticide Explorer Collection methods were all within the acceptable range

Figure 6: Results from analysis of FAPAS samples using the TSQ Endura Mass Spectrometer.

- Analysis of external certified reference material – FAPAS (3 different matrices)
- Found values in reported acceptance range

Analyte	FAPAS No.	FAPAS Matrix	Assigned value (µg/kg)	Acceptance range (µg/kg)	Measured value (µg/kg)	RSD (%)
Carbaryl	T19142	Melon Puree	89.0	49.9-128.2	91.1	1.1
Diniconazole			52.3	29.3-75.3	59.7	9.0
Zoxamide			91.7	51.4-132.1	108.4	3.0
Pencycuron	T19140	Lettuce Puree	73.2	41.0-105.4	45.9	6.0
Thiamethoxam			48.8	27.3-70.3	36.3	9.1
Azoxystrobin	19110	Lettuce Puree	188.0	110-265	132.5	15.4
Dimethomorph (sum of isomers)			181.0	106-256	160.1	11.9
Propyzamide			197.0	116-277	195.1	16.5
Azoxystrobin	T0983	Wheat Flour	383.0	241-524	361.2	1.7
Fenhexamid			110.0	61-158	125.4	10.4
Imazalil			161.0	93-229	157.2	8.2
			88.0	49.3-126.7	67.6	7.3

demonstrating excellent accuracy of the method. The results are summarized in **Figure 6**.

Method linearity was demonstrated using matrix standards from 0 to 100, or 0 to 200 µg/kg (equivalent in the sample), see **Figure 7**.

Why Work with High Resolution Accurate Mass (HRAM) Spectrometry?

The general approach of the Pesticide Explorer Collection workflow for HRAM is similar to that of the triple quadrupole MS; a QuEChERS extraction followed by analysis of the extracts using HRAM in full-scan with or without fragmentation. However, the HRAM workflow offers some significant advantages over the triple quadrupole workflows in its capability for simultaneous targeted detection, identification, and quantification, as well as the ability to perform non-targeted screening of additional pesticides that are not expected to be present in the sample. For targeted analysis of pesticides, the results obtained using triple quadrupole and HRAM are in good agreement with a broadly similar response for the majority of the pesticides studied, as shown in **Figure 8**. These results offer excellent encouragement for analysts considering or working with HRAM for the first time.

However, the high resolving power (70,000 at *m/z* 200) and excellent mass

Figure 7: Example calibration curves.

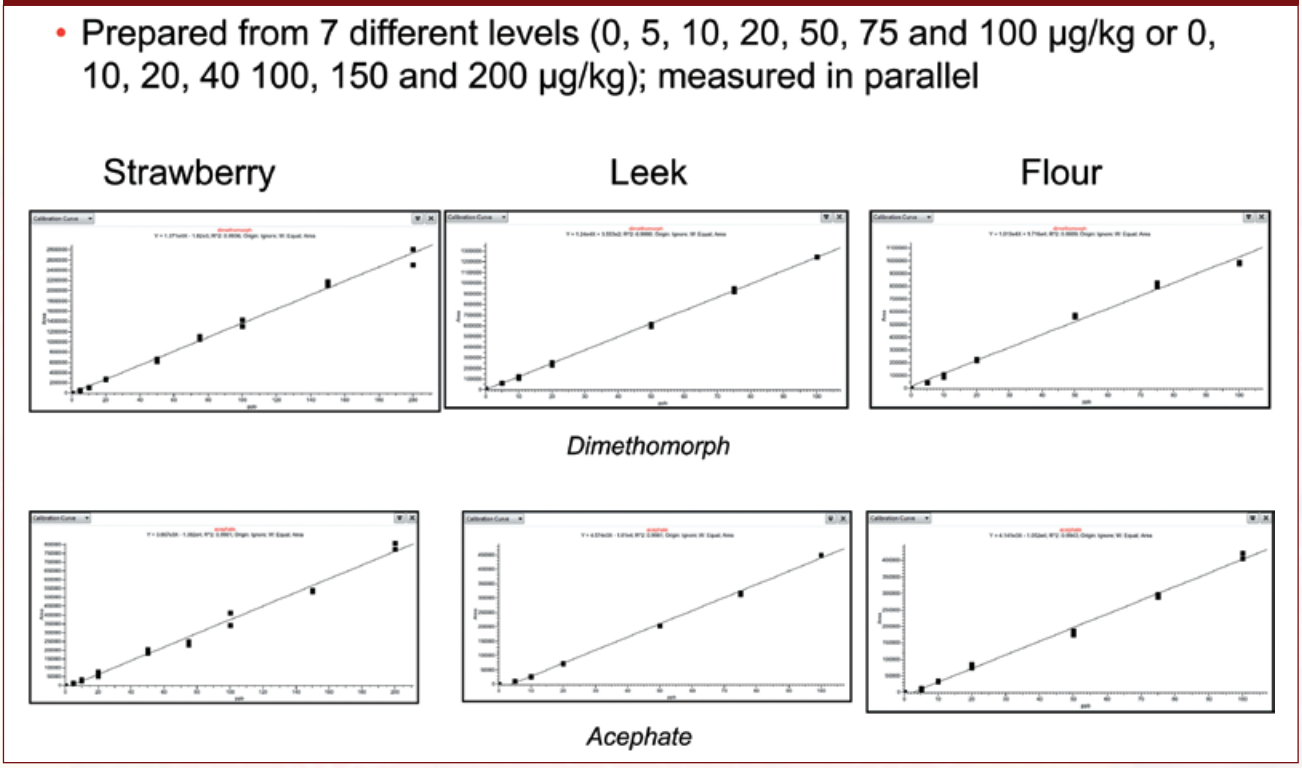
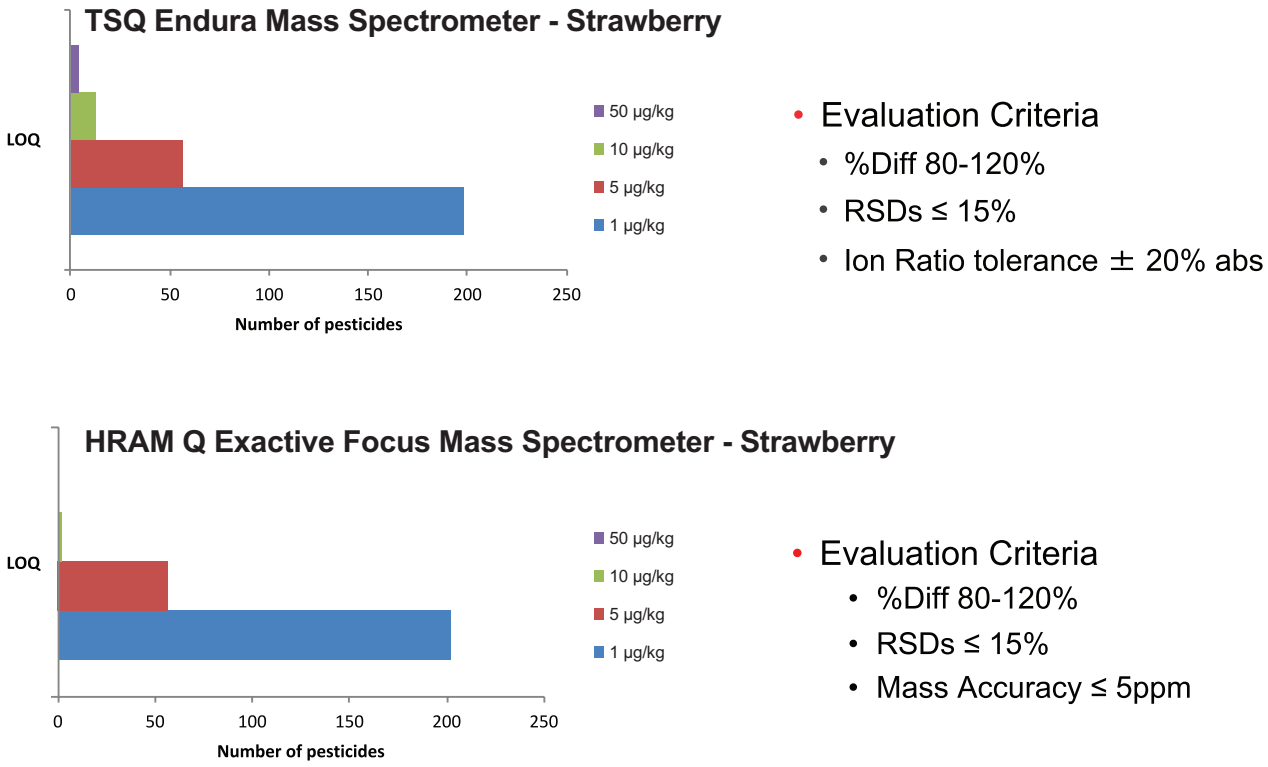


Figure 8: Quantitation performance by HRAM and triple quadrupole.



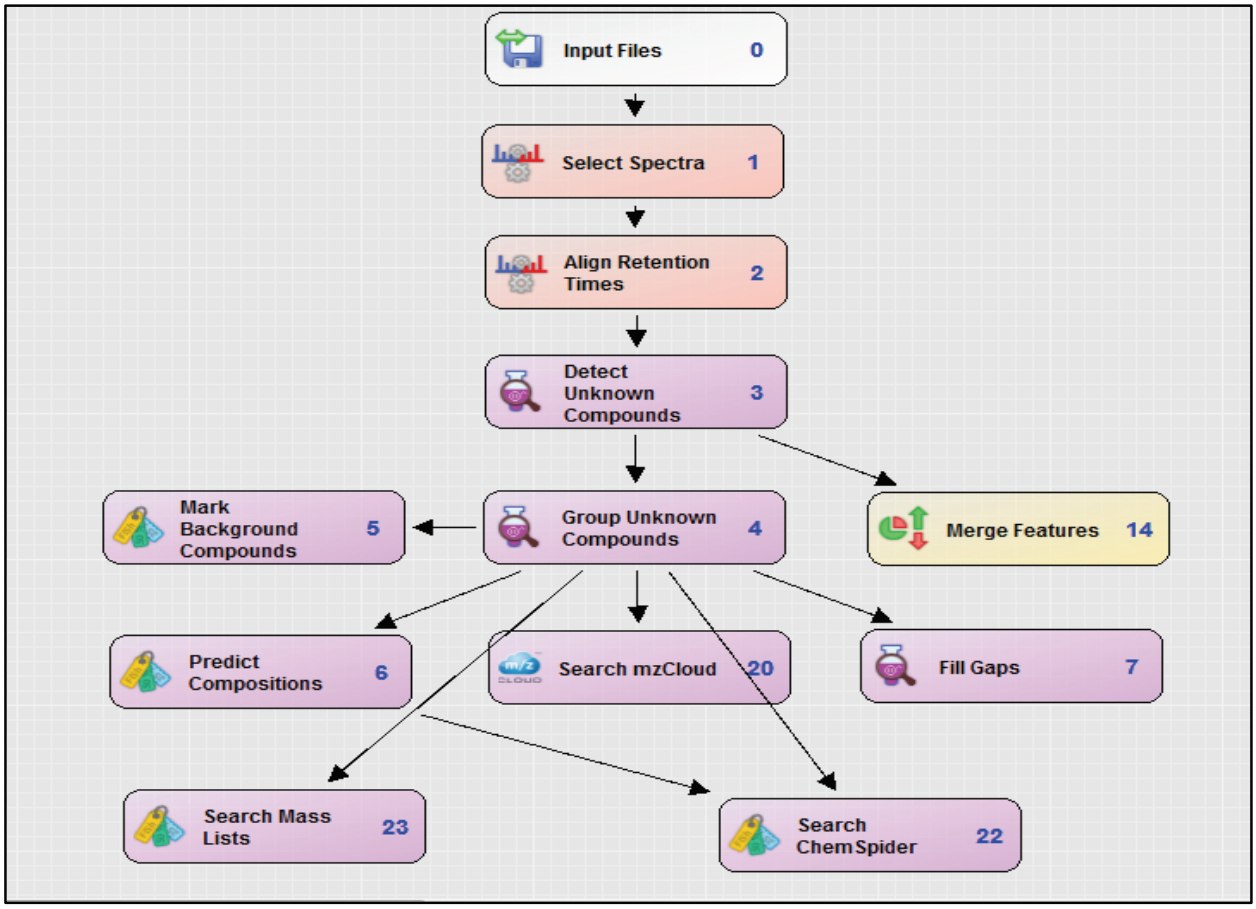
accuracy (typically 1–2 ppm for molecular ions in full scan and for fragment ions in MS/MS modes) available with HRAM instruments provide greater confidence in quantification and identification, especially for complex matrices. A mass extraction window, typically 5 ppm or less, can help to eliminate matrix interferences minimizing the possibility of false negative and false positive findings. Isotope pattern matching also provides additional information to help identify a compound. HRAM library searches against compound databases or spectral libraries (including fragment information with retention times) are extremely beneficial. They provide high confidence in compound identification and can help reduce method cost and development time by not having to purchase multiple standards or in performing manual method set up. Thermo Scientific™ TraceFinder software offers multiple MS/MS libraries that can be selected for use in identification of pesticides and also for confirmation of results.

Database Software and Workflows

Thermo Scientific™ Compound Discoverer™ software has ready-to-go workflows to help analytical laboratories identify and screen for unknown

Figure 9: Unknown screening using Compound Discoverer Software.

Compound Discoverer software has ready-to-go workflows to help laboratories identify unknown compounds and organize statistically significant findings for easy data review. Databases such as mzCloud and ChemSpider are readily available, along with local databases.



compounds and organize statistically significant findings for easy data review. In addition, databases such as mzCloud and ChemSpider (Raleigh, NC) are readily available, along with local databases.

Compound database or spectral library? An HRAM compound database is different than a spectral library. A spectral library is a collection of spectra obtained under different conditions for a variety of compounds, whereas, a database provides a lot more information including the metadata, information about all adducts, retention times, and fragments. mzCloud (https://www.mzcloud.org) is an extensively curated

database of high-resolution tandem mass spectra that are arranged into spectral trees. MS/MS and multi-stage MS^n spectra were acquired at various collision energies, precursor *m/z*, and isolation widths, using collision-induced dissociation (CID) and higher-energy collisional dissociation (HCD). Each raw mass spectrum has been filtered and recalibrated giving rise to additional filtered and recalibrated spectral trees that are fully searchable. Besides the experimental and processed data, each database record contains the compound name with synonyms, the chemical structure, computationally and manually annotated fragments (peaks), identified adducts and multiply charged ions, molecular formulas, predicted precursor structures, detailed experimental information, peak accuracies, mass resolution, InChi, InChiKey, and other identifiers. mzCloud is a fully searchable database that allows spectra searches, tree searches, structure and substructure searches, monoisotopic mass searches, peak (*m/z*) searches, precursor searches, and name searches.

Using the workflow outlined in **Figure 9**, an acquired dataset can be processed to separate compounds of interest from other matrix components.

Conclusion

The Pesticide Explorer Collection is a comprehensive, ready-to-implement solution that provides start-to-finish workflows tailored to assist every food monitoring and testing laboratory. The Collection provides compelling productivity and efficiency enhancements for both startup laboratories and laboratories adding new analytical capabilities to address evolving customer and industry demands. Regardless of staff or laboratory expertise and complexity of the matrices, the Pesticide Explorer Collection enables every user to achieve robust, sensitive, reliable, unambiguous, high-quality LC-MS/MS results. Further information is available on the Pesticide Explorer Collection bundle[5], pesticide analysis generally[6], triple quadrupole[7] and HRAM mass spectrometry[8], and software.[9]

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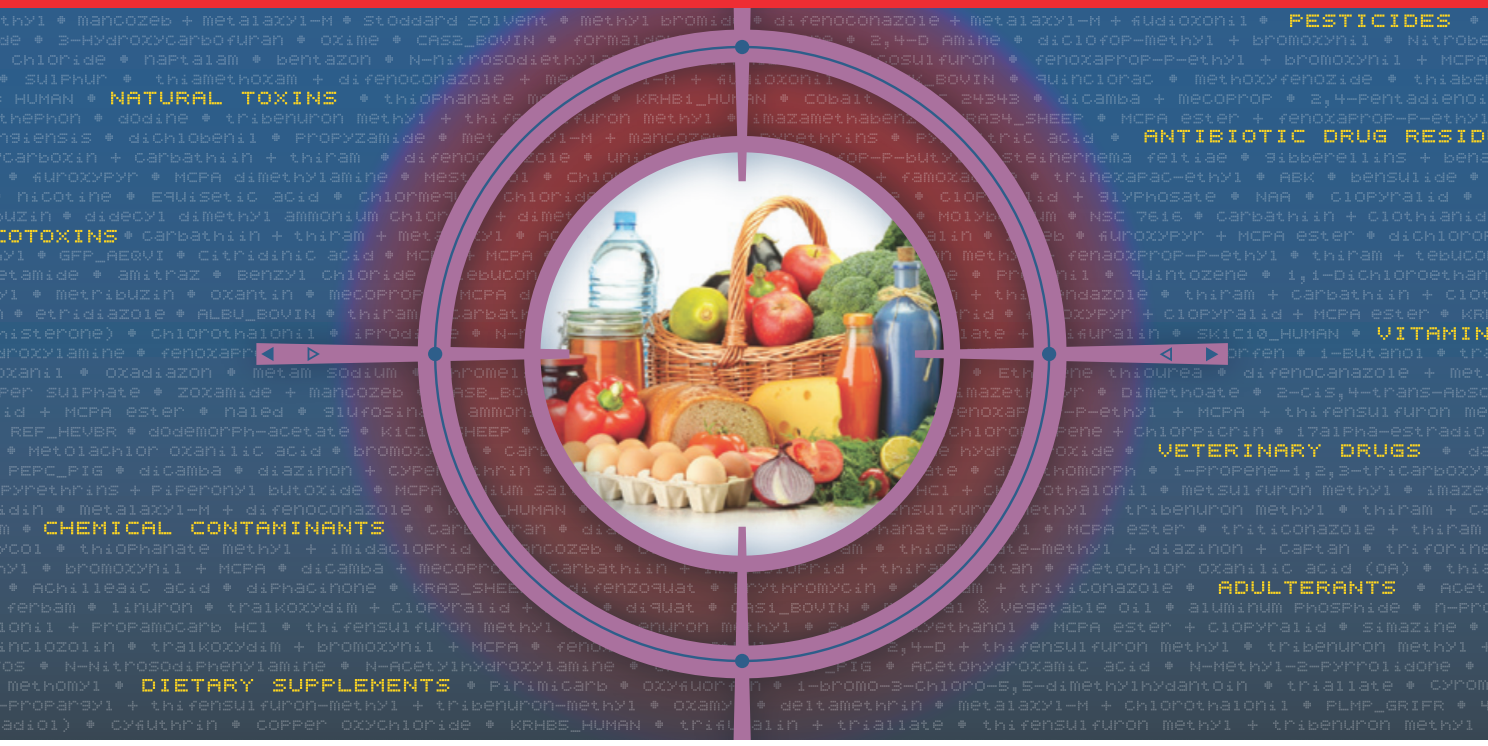

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