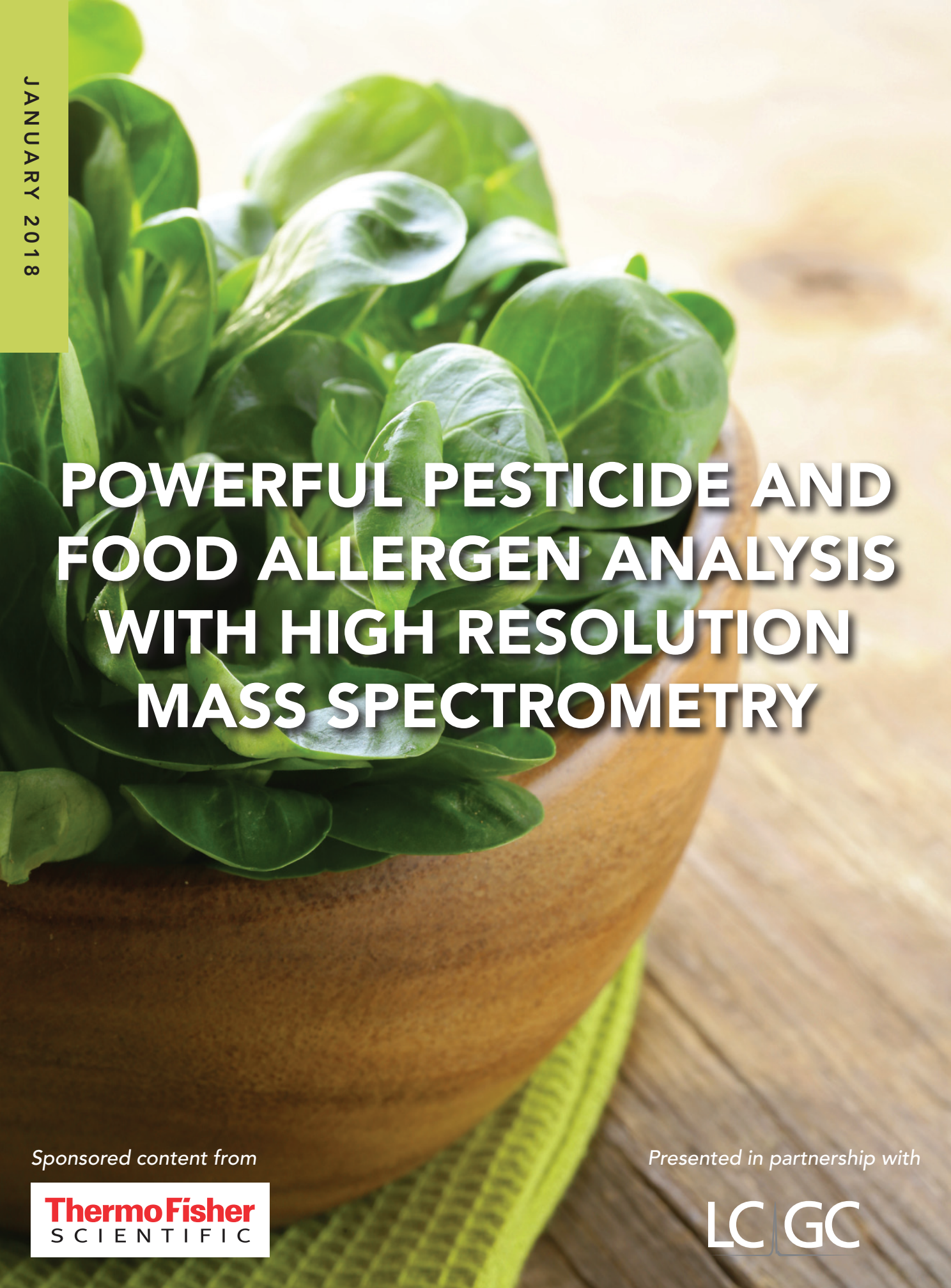
JANUARY 2018

POWERFUL PESTICIDE AND FOOD ALLERGEN ANALYSIS WITH HIGH RESOLUTION MASS SPECTROMETRY



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INTRODUCTION

From pesticides analysis to allergen testing, food sample analysis is a critical part of ensuring the safety of the food supply. Laboratories are increasingly faced with increased workload and demands for fast turnaround time of results. With these challenges, what are the best analytical tools for ensuring accurate and fast food testing?

In the new *LCGC* ebook, *Powerful Pesticide and Food Allergen Analysis with High Resolution Mass Spectrometry* (with sponsored content from Thermo Fisher Scientific), experts discuss the use of high-resolution accurate mass (HRAM) as an important way to improve food testing analysis.

First, Professor Amadeo Fernández-Alba, PhD, and Łukasz Rajski, from the University of Almería in Spain, evaluated the suitability of three workflow approaches for pesticide residues analysis using the Thermo Scientific™ Q Exactive™ Focus hybrid quadrupole-Orbitrap™ mass spectrometer instrument in full scan MS mode.

Next, learn why HRAM-MS instruments offer advantages over triple quadrupole and Q-ToF systems in terms of detecting and quantifying large numbers of peptides from allergenic foods.

Last, a group of regulatory experts offer some practical examples of how to correct for matrix effects to obtain reliable quantitative data using LC–MS and GC–MS. They suggest, "With newer instrumentation being developed and technologies that will address the root cause of the matrix effects, it is quite possible to take advantage by just diluting the sample to eliminate or minimize matrix effects."

These pieces demonstrate how HRAM methods will benefit analytical laboratories in numerous ways.

TOC
Table of contents

POWERFUL PESTICIDE AND FOOD ALLERGEN ANALYSIS WITH HIGH RESOLUTION MASS SPECTROMETRY

This ebook is sponsored by Thermo Fisher Scientific and presented in partnership with *LCGC*.

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

Amadeo Fernández-Alba and Łukasz Rajski

Introduction

Pesticide residue laboratories typically have a heavy workload, easily reaching 50 samples per day. At the same time, they often must provide results within 1–2 days of receipt and maintain compliance with strict quality control procedures. To address these challenges while providing accurate identification, 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, laboratories can detect and identify pesticide residues 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 sample analysis, matrix co-extractives can cause issues with the correct

identification of the pesticide. In addition, there are hundreds of pesticides in use making analysis much more complicated. 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 could 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 from the ion ratio of the corresponding standard. This is then classified as a false-negative result. If the ratio of the transitions derived from the co-extractive corresponds with a pesticide standard (and there is no pesticide residue in the sample), then this is classified as a false-positive result. **Figure 1** shows the example of 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.

High Resolution Accurate Mass (HRAM) Mass Spectrometry

Every year, the European Reference Laboratory (EURL) for Pesticide Residues in Fruits and Vegetables at the University of Almería, Spain, coordinates round-robin proficiency testing (European

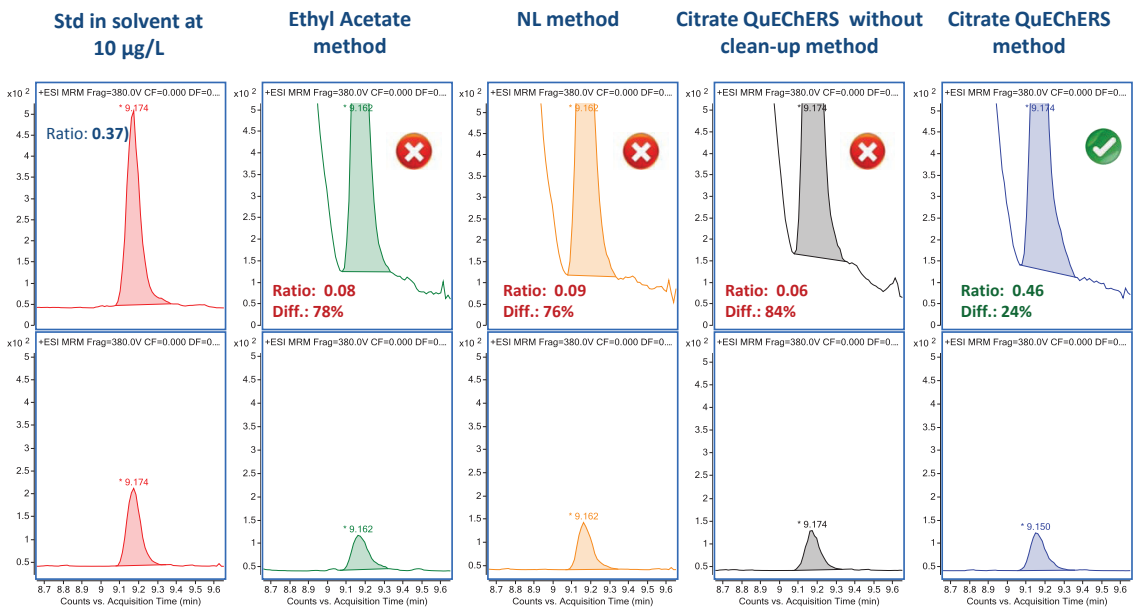


Figure 1: Potential for false positives of azinphos methyl (10 µg/kg) in onion by LC-MS/MS; based on comparison of ion transition ratios with a standard.

Proficiency Test in Fruits and Vegetables [EUPT-FV]) using test samples containing both incurred residues and spiked residues. EURL analysts prepare the samples and distribute them to participant laboratories. In the European Union, participation is compulsory for official control laboratories (i.e., laboratories that submit results for official control samples). Analysis results of proficiency test samples often contain false positive and false negative results because of the coelution of matrix co-extractives with the pesticides of interest. So, the question is: How can these 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 it offers much more selectivity, depending on the resolution. When EURL scientists analyzed thiabendazole using a Q-ToF instrument with a resolution of 25,000, for instance, they obtained 12 molecular formulas for that ion, plus 11 potential false-positive ions. However, when the team analyzed thiabendazole with a resolution of 70,000 with Thermo Scientific™ Orbitrap™ mass analyzer technology, they obtained just five

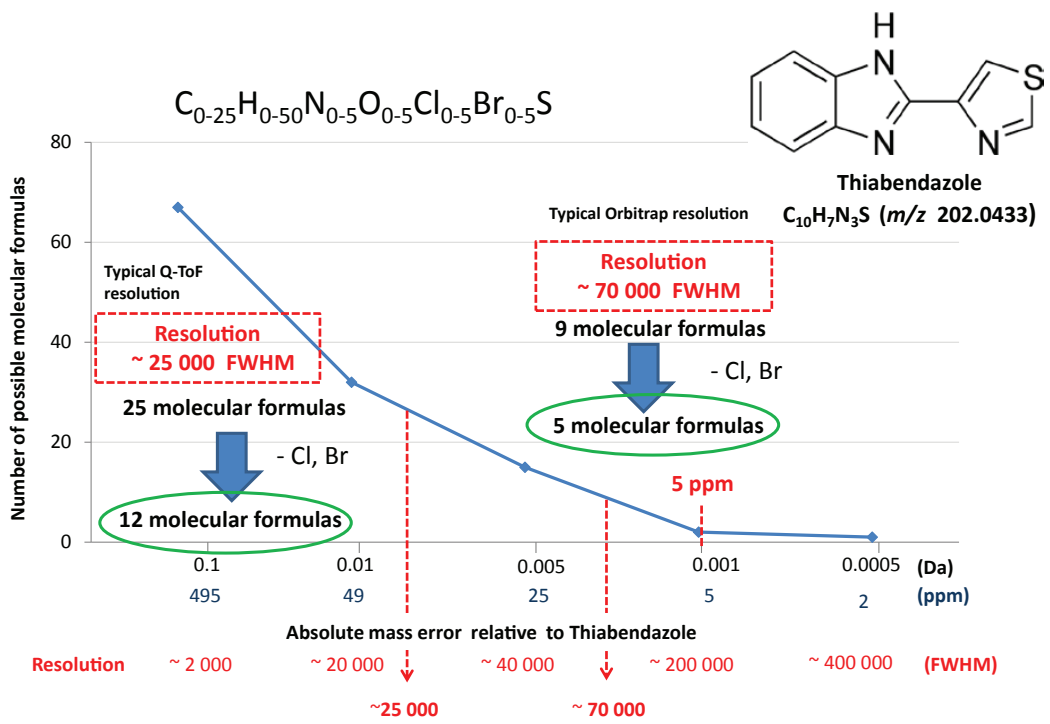


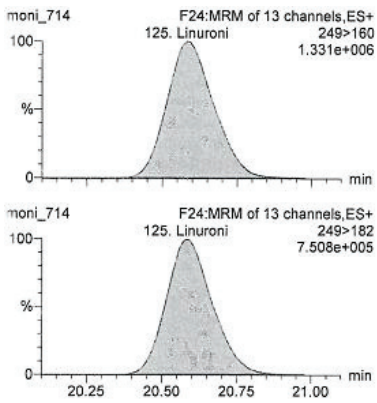
Figure 2: Plot of the number of possible molecular formulas against absolute mass error relative to thiabendazole.

molecular formulas. In this case, the Q-Tof instrument is less suited for these types of applications (see **Figure 2**).

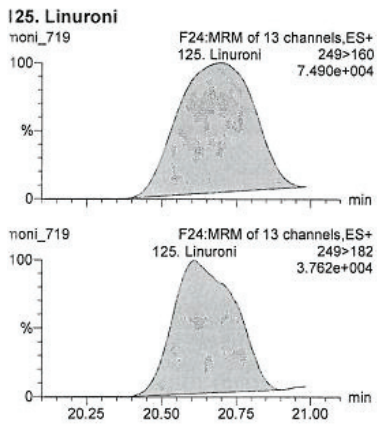
Another challenge was the determination of linuron in coriander for which the nominal mass MS/MS ion ratio in the sample is very different from the ratio obtained in the solvent standard (see **Figure 3**). Using a mass resolution of 70,000, however, the linuron ion and the interfering ion from the matrix are very easy to separate (see **Figure 4**).

How can false-positive results be avoided? One can work with higher resolution, but unfortunately, instruments do not have infinite resolving power, and therefore fragment ions are needed for unambiguous identification of the analyte compounds. But the challenge of working in full scan only, using typical ionization conditions, is that one can only obtain fragment ions for a small number of pesticides. Analysts can attempt to change the parameters of the electrospray ionization source to obtain fragments for a greater number of pesticides, but then one loses sensitivity for the molecular ions.

A better approach is to work simultaneously in both MS and MS/MS modes. EURL analysts find that MS data are best for detection and quantitation, while MS/MS (MS^2) data are better for the identification of the pesticides.



Linuron
Standard in solvent
Ion ratio: 1.8



Linuron (0.125 mg/kg)
Real sample of coriander
Ion ratio: 2.4

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

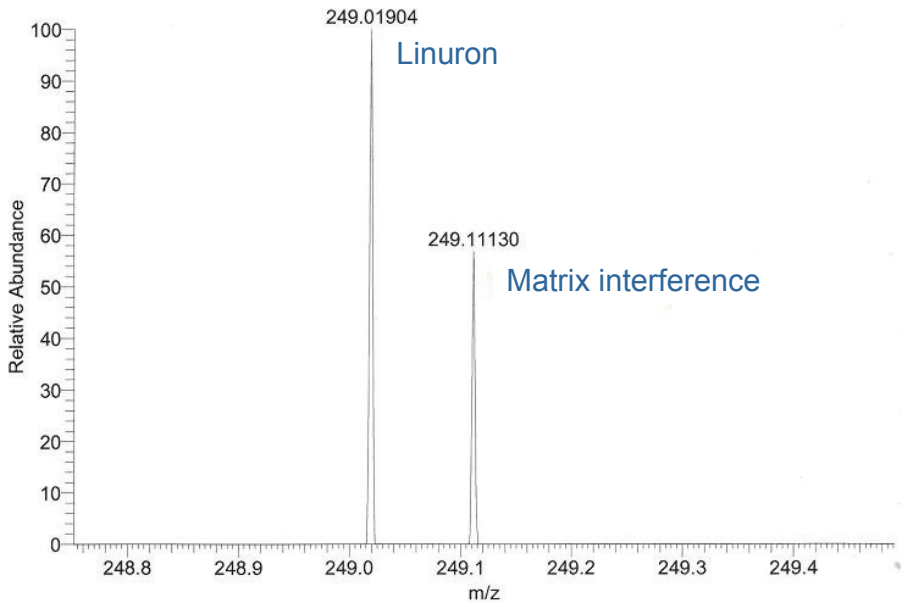


Figure 4: EU proficiency test sample (coriander) analyzed by high resolution MS technology, showing unambiguous identification of linuron in the presence of the matrix.

Q Exactive Focus hybrid quadrupole-Orbitrap Mass Spectrometer

When analyzing pesticides, two criteria must be met: retention time and mass error. For positive identification, the EURL recommends that the mass error must be lower than 5 ppm. Using the Q Exactive Focus mass spectrometer in full scan MS mode, one can obtain mass errors of below 2 ppm, not only in samples like tomato and apple, but also in more complex matrices with a high number of co-extracted compounds such as oranges (see **Figure 5**).

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 MS^2 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 samples. This mismatch demonstrates that the ion detected in grapefruit using full scan at 70,000 was not metalaxyl-M, but another compound, equating to a false-

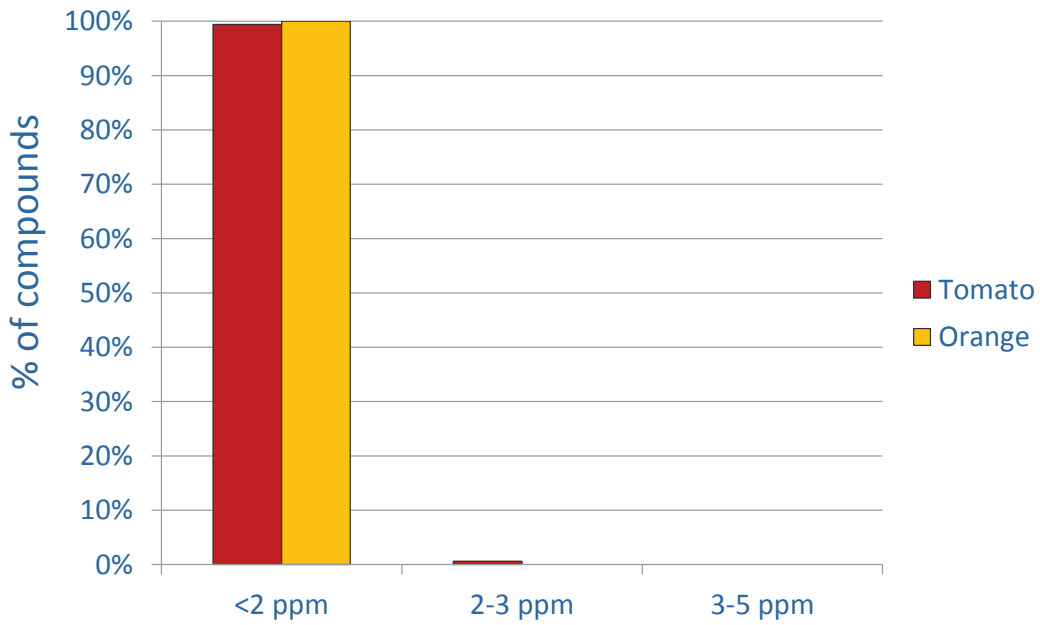


Figure 5: Mass errors in full scan MS mode are below 2 ppm, even for orange, which is considered a difficult sample matrix containing a high number of co-extracted compounds.

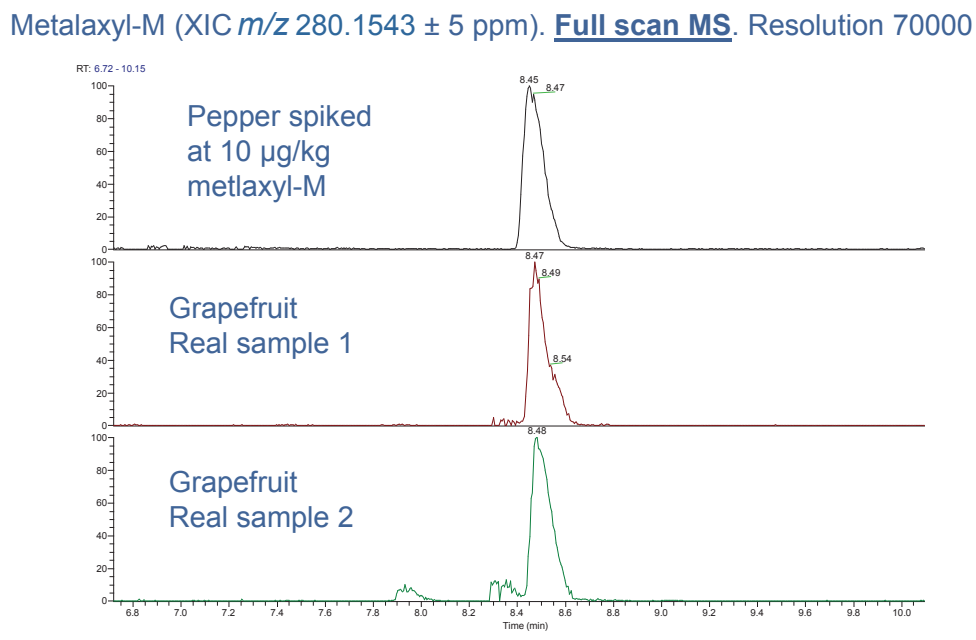


Figure 6: Determination of the fungacide Metalaxyl-M in pepper and grapefruit in full mass scan mode.

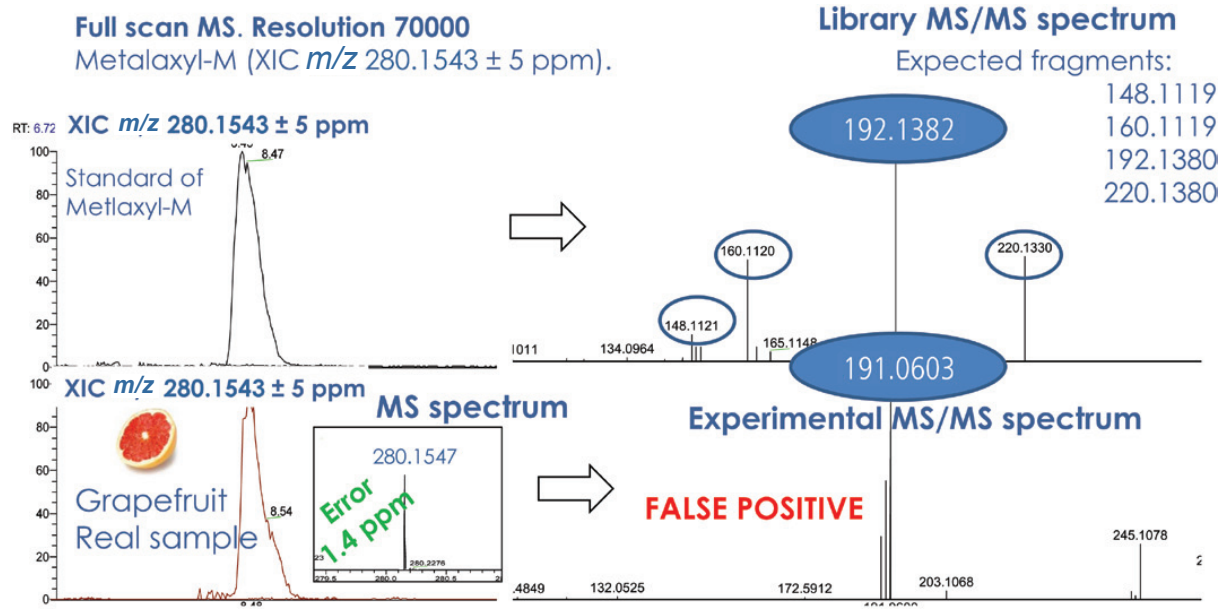


Figure 7: Fragments of Metalaxyl-M confirm its presence in the standard, but no fragments in the grapefruit sample show the full mass scan gave a false positive for the compound.

positive response in full scan. EURL analysts also measured the stability of ion ratios in data dependent MS^2 (dd-MS^2) 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, very stable ion ratios with variations all <30% were obtained.

Choice of Workflows

What follows is an evaluation of the suitability of three selected workflows using the Q Exactive Focus MS for the analysis of pesticide residues:

- Data dependent MS/MS (dd-MS^2)
- All ion fragmentation (AIF)
- Variable data independent acquisition (vDIA)†

Data dependent MS/MS (dd-MS^2) is a targeted, triggered MS^2 workflow in which the user must submit an inclusion list containing the mass of the molecular ion(s) and the retention time for each target pesticide. Using this approach, the mass spectrometer is acquiring data in full-scan mode most of the time. When a compound from the inclusion list is detected, however, a single scan is then subjected to dd-MS^2. A quadrupole mass filter selects the precursor ion, which is fragmented in a collision cell, and

†vDIA method is not available in the United States.

the fragments (product ions) are then analyzed in the Orbitrap mass analyzer. One MS^2 spectrum is obtained for each chromatographic peak, which can then be used for identification purposes.

All ion fragmentation (AIF) is used when the workflow is non-targeted. In this case, each full scan is followed by an MS^2 scan. During the MS^2 scan, the quadrupole is open, so there is no filtering of the ions. Therefore, all precursor ions observed in full scan are fragmented. For example, if one works in a full scan in the range of 100–1,000 Daltons, then ions in the same *m/z* range are passed to the higher energy collisional dissociation (HCD) collision cell and fragmented. The fragment ions are analyzed in the Orbitrap mass analyzer. Using this approach, one can obtain fragment information for all the compounds present in the sample, but the fragment spectra are more complex compared with dd-MS^2 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 several consecutive MS^2 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. Fragments in each selected mass range are analyzed separately, which gains selectivity because one can reduce 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. In addition, 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, EURL scientists selected 11 representative matrices of different kinds of fruit and vegetables. Some were very straightforward such as tomato, apple, and cucumber, while others were very complex matrices such as orange, leek, and onion. Fruit and vegetable extracts were spiked with 166 pesticides at two concentrations: 100 µg/kg and 10 µg/kg. Nearly 2,000 results were obtained at each spiking concentration, for each of the three workflows. For all workflows, researchers identified practically 100% of the compounds at 100 µg/kg; at 10 µg/kg, more than 95% of the compounds were identified. The compounds that were the most difficult to identify were those 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 mode, but also in MS^2 mode to gain improved selectivity. This is seen in **Figure 8**, which shows demeton-s-methyl sulfoxide in orange extract at a level of 10 µg/kg. On the left side, a mass spectrum was obtained with a resolution of 17,500, and the right-hand

†vDIA method is not available in the United States.

spectrum was obtained with a resolution of 35,000. At lower resolution, analysts could not identify the pesticide, whereas at higher resolution they could separate a fragment ion of demeton-s-methyl sulfoxide from the matrix ion.

Using vDIA†, analysts 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. In the case of the three mass-segment vDIA† acquisition, interferences and high background noise were observed. For the five mass-segment vDIA† acquisition, a very clean peak without any interference was obtained. Such different results were obtained because the extract of the orange 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), researchers compared AIF with vDIA†, which is seen in the upper extracted ion chromatogram in **Figure 10**. Figure 10 clearly shows that vDIA† with a resolution of 35,000 can provide much better selectivity than AIF at a resolution of 70,000.

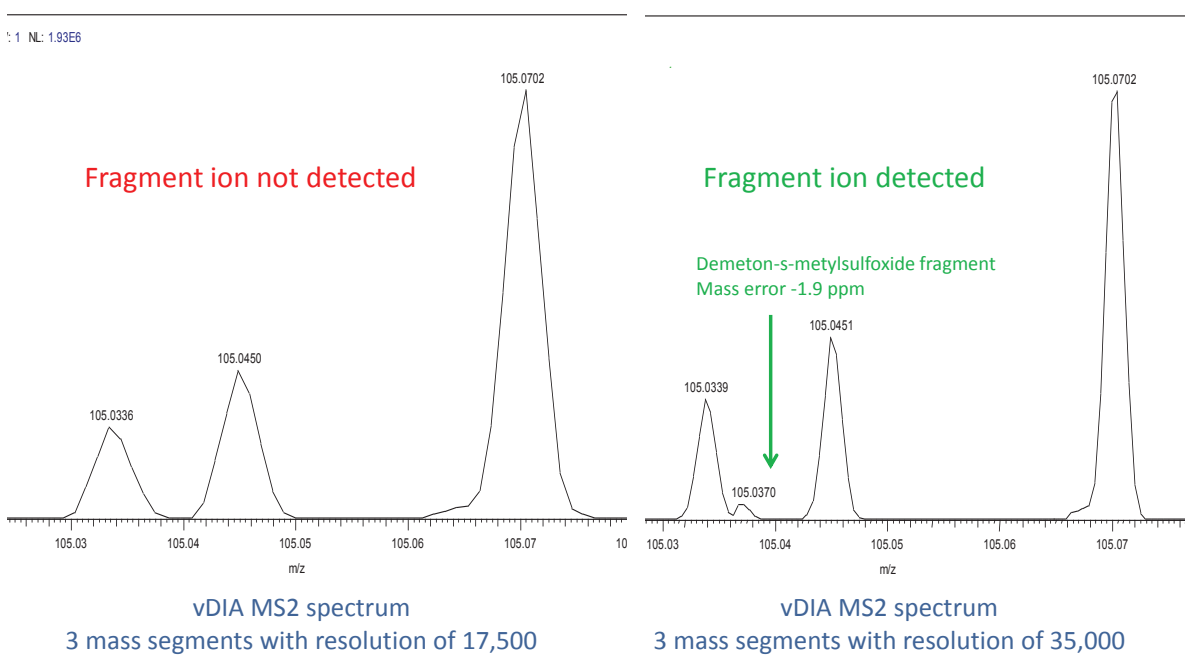


Figure 8: Mass spectrum of 0.01 mg/kg demeton-s-methylsulfoxide in orange by vDIA, showing the fragmented ion can only be separated and positively identified at the higher mass resolution of 35,000.

†vDIA method is not available in the United States.

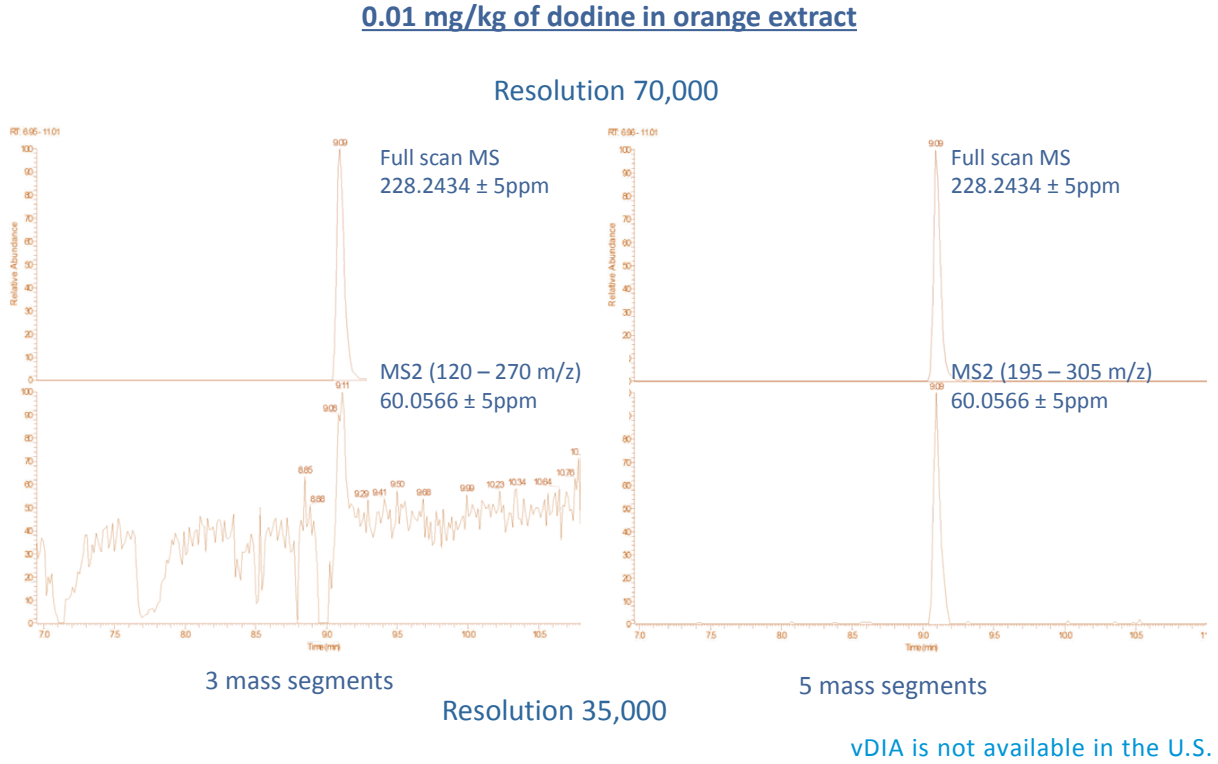


Figure 9: In the identification of dodine in orange, the extracted compounds can cause poor identification, because of interfering peaks producing a fragment ion with the same mass as dodine.

In all three MS^2 modes of operation, analysts obtained fragments for practically all 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. In all MS^2 modes, the EURL scientists observed slightly more errors than with 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 is important to point out that the Q Exactive Focus MS is a very sensitive instrument. In this study, researchers 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 mass analyzer is also very good because the number of ions entering into the Orbitrap mass analyzer is controlled by Automatic Gain Control (AGC); thus, it is impossible to overfill or saturate the detector. This is demonstrated in **Figure 12**, which shows

†vDIA method is not available in the United States.

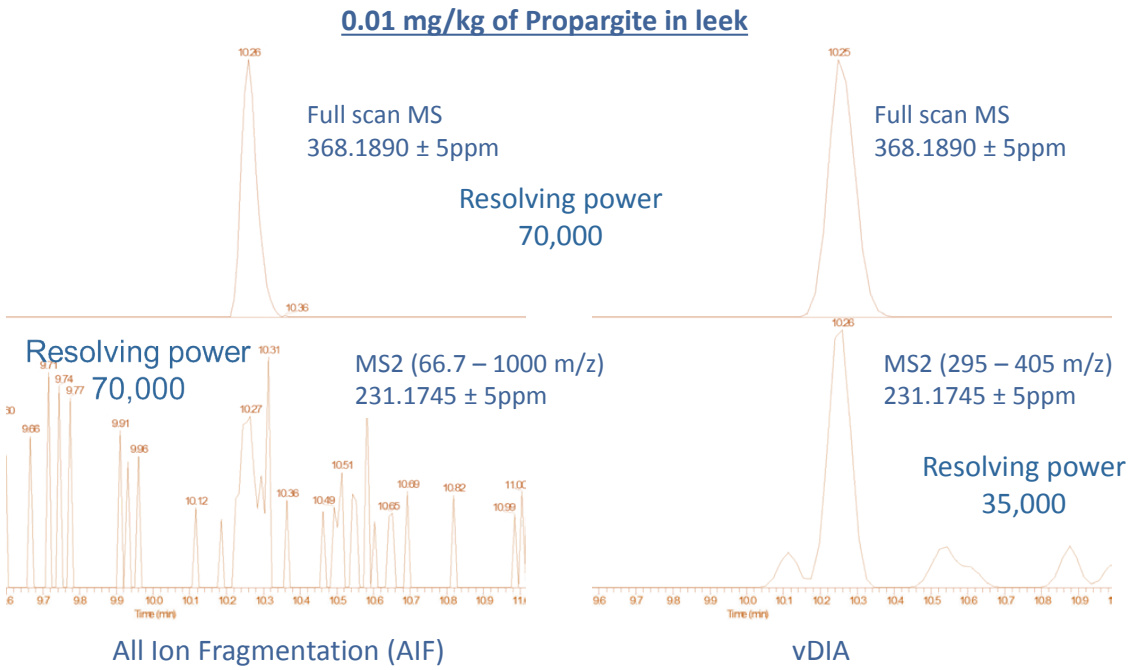


Figure 10: Comparison of AIF with vDIA for a pesticide residue in leek extract.

that linearity up to 1.6 ppm for three pesticides in a spinach sample using dd-MS^2 can be achieved. Both the vDIA† and AIF approaches showed similar linearity. It is 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 (see **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, one can see numerous 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, the peak for thiophanate methyl starts to be visible, but it is very difficult to quantify. Quantitation becomes more realistic at the level of 50 µg/kg, but some interference still occurs on either side of the analyte peak. As mentioned previously, in dd-MS^2, analysts obtained only one MS^2 scan per chromatographic

†vDIA method is not available in the United States.

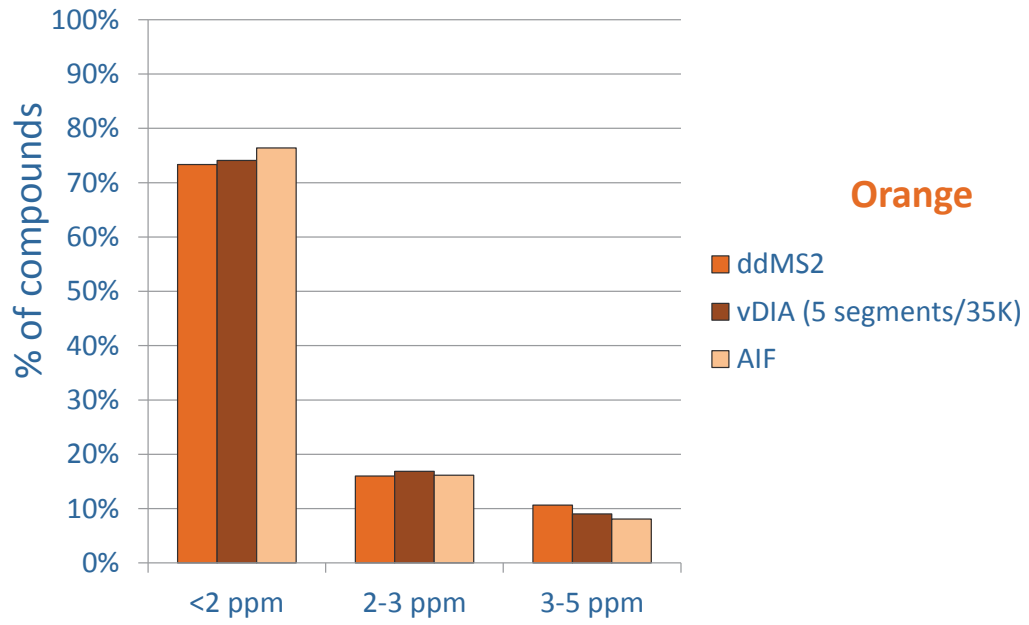


Figure 11: Mass errors in MS^2 mode are below 2 ppm for 70% of compounds extracted using three different workflow techniques.

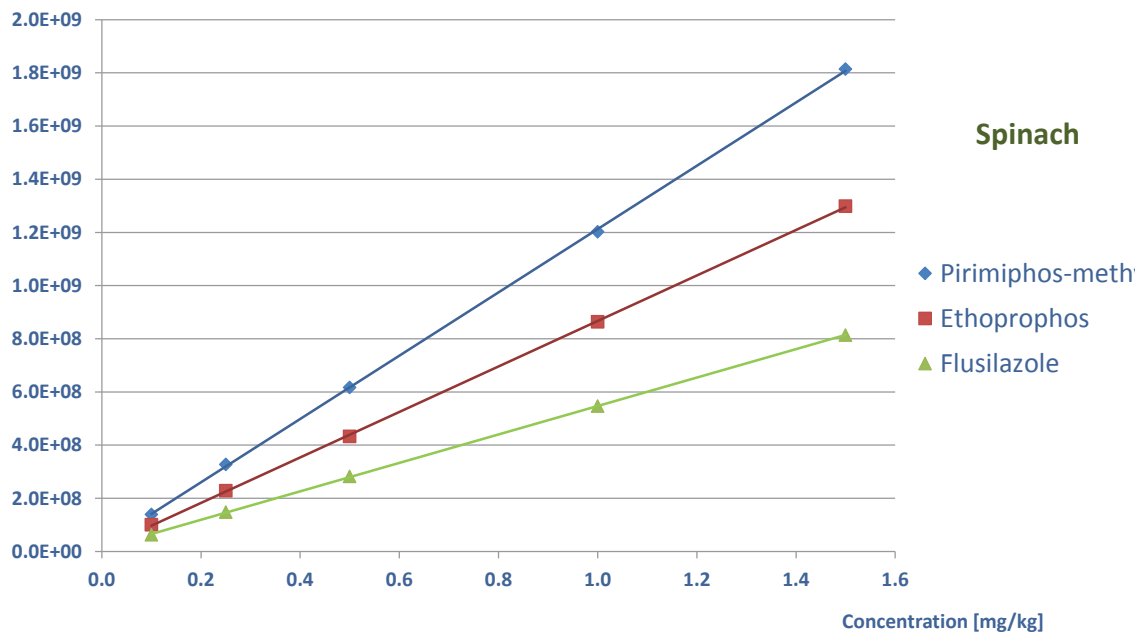


Figure 12: Linearity for three pesticides in spinach by dd-MS^2.

peak, and as a result, it can only be used for identification purposes. However, in the case of vDIA† or AIF, it is possible to extract peaks from MS^2 data so they can also be used for quantitation. This is shown in the lower scans in Figure 13, where peaks are 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. At the EURL, scientists usually dilute samples five-fold to reduce matrix effects. Using this technique, 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, one wants to obtain precision below 20%. The

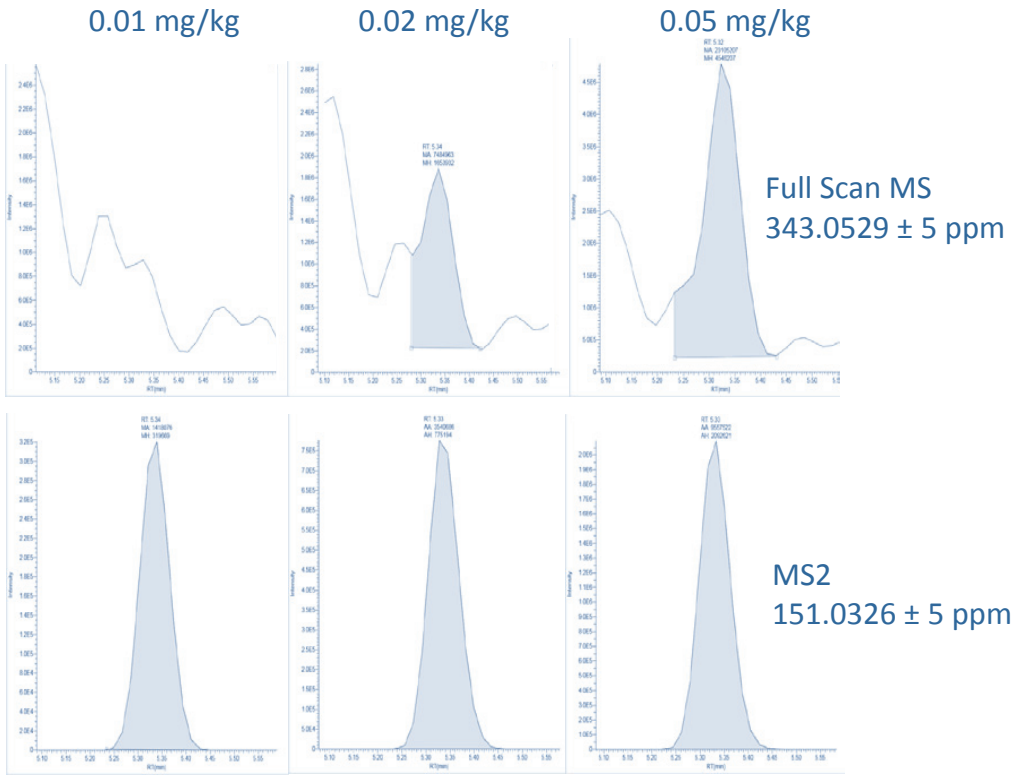


Figure 13: Quantitation of thiophanate methyl in onion (vDIA†, 5 segments, 35,000 resolution), showing the impact of interferences on the analyte peak.

†vDIA method is not available in the United States.

histogram shown in **Figure 14**, illustrates the results obtained for a tomato extract spiked with 166 different pesticides at 10 µg/kg, and analyzed using dd-MS^2, AIF, and vDIA† using four different settings. Almost 100% of the pesticides are below 20% RSD. However, given how many are below 5%, one can see differences between the workflows.

In this example, the best results were for dd-MS^2 because it had the shortest cycle time. In dd-MS^2 with the Q Exactive Focus MS instrument, almost all the available cycle time was spent acquiring data in full scan. Thus, working with 70,000 resolution, there are 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 approximately one second for five MS^2 segments, approximately three times longer than dd-MS^2.

Reference Materials

Finally, an evaluation of EU proficiency test materials of potato, pepper, and broccoli was carried out using the

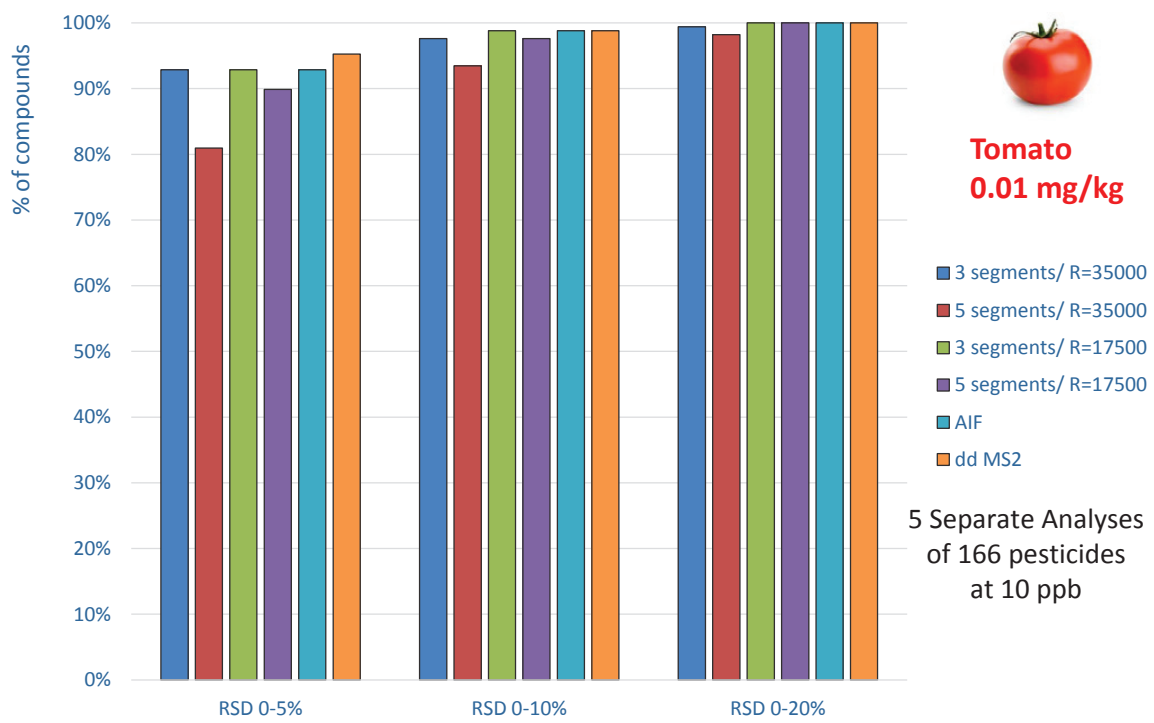


Figure 14: Comparison of measurement repeatability between different workflows.

†vDIA method is not available in the United States.

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%)	0.88 (-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%)

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

three different Q Exactive Focus MS workflows: Full scan with AIF, dd-MS^2, 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.

Other Application Areas

Other application areas of the Q Exactive Focus MS are based on retrospective analysis. This becomes important when working with workflows such as AIF or vDIA†. At a later date, and perhaps in response to emerging information, analysts can return to the original raw data files and further investigate the acquired spectra by comparing raw data files with information contained in large databases to possibly detect new compounds of interest. In addition to detecting compounds, analysts can also identify those detected compounds using their fragmentation products, because fragments were previously obtained from all compounds present in the sample. Another very interesting application is the operation of the Q Exactive Focus MS instrument in selected ion monitoring (SIM mode) for the analysis of analytes at very low concentrations. SIM mode is 5–10 times more sensitive than full mass scan mode, as demonstrated by the detection of the thiametoxam residues in pollen and in honeybees in **Figure 15**. No

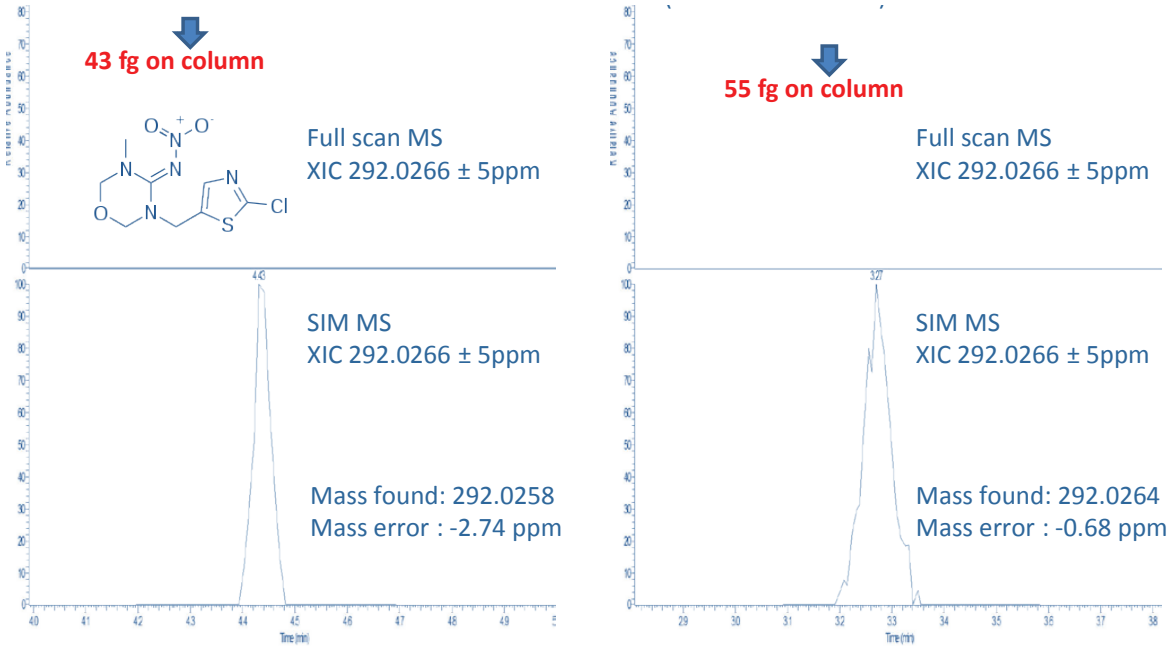


Figure 15: Femtogram levels of thiametoxam can be detected in SIM mode, but not in full scan MS mode.

residues were detected in full-scan mode, but when the samples were reanalyzed in SIM mode, scientists were able to detect thiametoxam at around 50 femtogram on the column.

Conclusions

In summary, one can say that the Q Exactive Focus MS operated in full scan with 70,000 resolution and dd-MS^2 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 workflows (full scan-ddMS^2, -vDIA^t, 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 these studies, the best technique for quantitation was full scan-dd-MS^2 (quantification in full scan) because this workflow has the shortest cycle time. On the other hand, AIF and vDIA^t 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, the researchers conclude that all evaluated workflows gave very similar and consistent results. For more information about this technology and a more comprehensive set of data for the determination of pesticides in various samples, please refer to the references.

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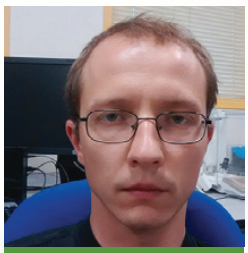

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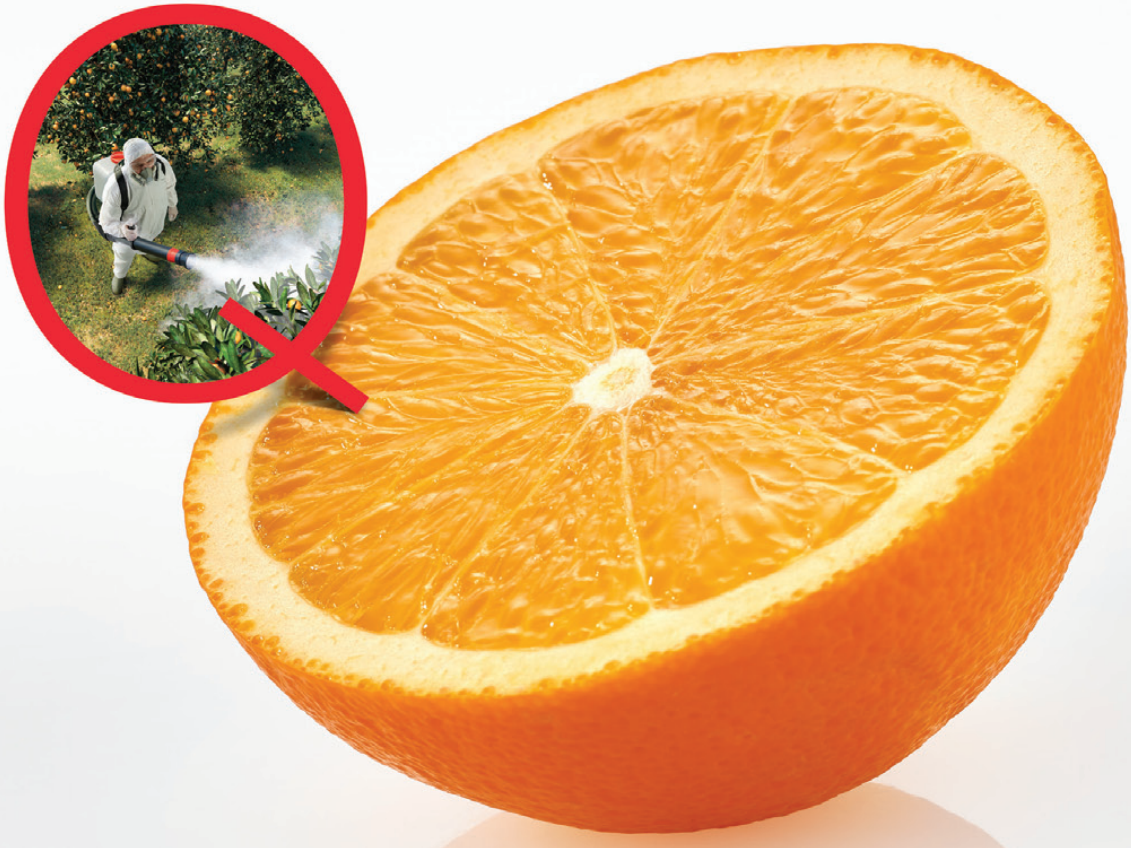

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FOOD ALLERGEN ANALYSIS: DEVELOPING METHODS TO HARNESS THE POWER OF HIGH RESOLUTION, ACCURATE MASS DETECTION

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Using the Thermo Scientific Q Exactive hybrid quadrupole-Orbitrap MS system for accurate food allergen detection and quantification.

Introduction

Food allergens are a critical food safety hazard that food manufacturers must manage appropriately. To validate allergen control plans and ensure regulatory compliance, robust detection and quantitation methods for food allergens in a variety of complex matrices are a necessity. Currently, mass spectrometry is the analytical strategy with the highest potential for use in confirmatory methods for food allergen detection and quantitation. Yet, few fully quantitative methods have been published for the detection of food allergens in complex food matrices. The complexity and diversity of food allergens themselves, the food matrices in which they need to be detected, and the types of food processing used in their production give rise to inherent challenges for the development of

widely applicable food allergen detection methods. This summary reviews the essentials of food allergen analysis and discusses novel strategies for method development to harness the power of HRAM-MS to untangle the complexities of food allergens.

Food Allergens Background

Approximately 90% of all food allergies are caused by just eight foods: milk, eggs, crustacean shellfish, fish, peanuts, soybean, tree nuts, and wheat. Identifying the presence of these allergens in ingredients and finished goods is an important issue that manufacturers cannot take lightly. The inadvertent presence of undeclared allergens in foods and beverages can cause serious health risks to food-allergic individuals. Food-induced IgE-mediated reactions can include gastrointestinal discomfort, skin reactions, and respiratory reactions such as rhinitis, throat swelling, or asthma. In severe cases, systemic symptoms can escalate to anaphylactic shock, which can be life-threatening.

The food components responsible for allergic reactions are almost always naturally occurring proteins that frequently are resistant to heat, proteolysis, and extremes in pH. Many times, food allergens are not a single causative protein; rather, several proteins may be responsible for allergic reactions and individual reactions to each protein can vary. Thus, an avoidance diet is often the only way to eliminate the possibility of exposure for food-allergic consumers. To help with this endeavor, the Food Allergen Labeling and Consumer Protection Act of 2004 requires the eight major food allergens to be clearly declared on the label of FDA-regulated products, which has dramatically improved the ability of food-allergic consumers to avoid allergens.

While the food industry is continually improving allergen management strategies, the number of food-allergen related recalls has increased over time, as illustrated in **Figure 1.** Since recalls represent a significant risk to the food industry (in terms of public safety, consumer confidence, and sales), better food allergen management strategies are needed, and detection methods can play an important role.

Allergen Detection Methods

The core role of allergen detection methods is to identify and quantify allergenic residues in foods to ensure products are safe and compliant with regulations. In addition, allergen detection methods are also used to support regulatory enforcement, validate food allergen management plans, provide data for industry risk assessments, and answer questions such as:

- How much of the allergenic food is present?
- Are manufacturers cleaning processing equipment well enough?

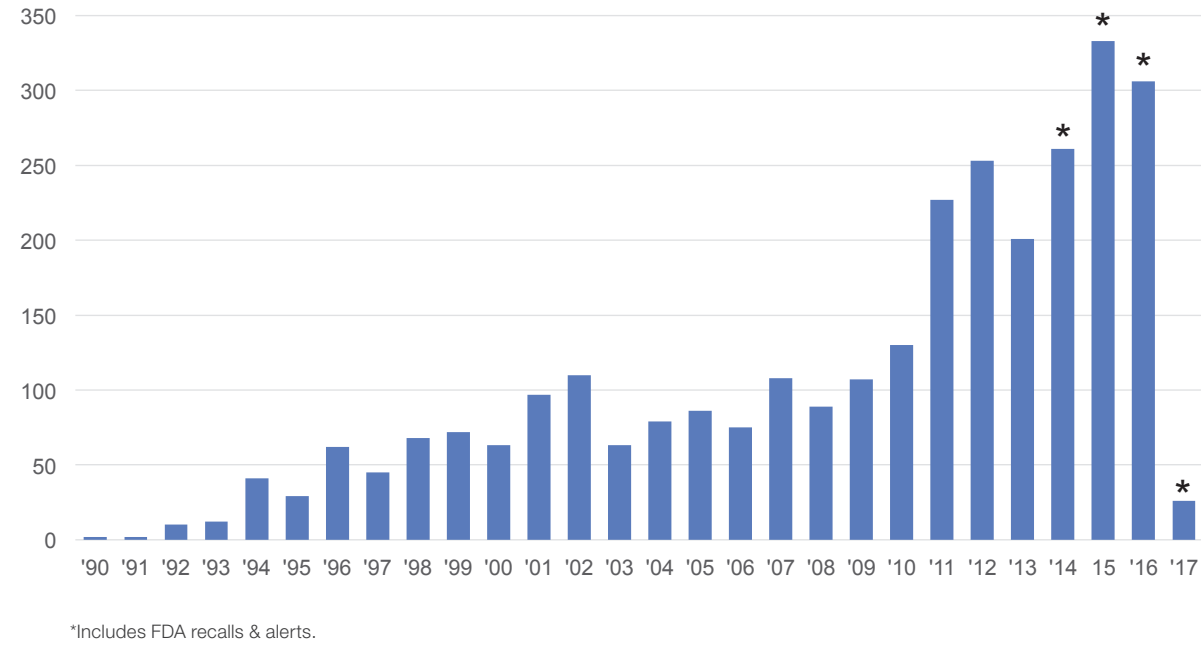

Figure 1: U.S. FDA food allergen recall incidents, 1990–2017.

- Are ingredient suppliers providing accurate information about the presence of food allergens?
- Should products use precautionary allergen labeling?
- Should consumers be notified of a potential risk?

Allergen detection methods also must be specific, identifying only the peptide of interest and be sensitive to the low parts per million (total protein or total food, depending on units used) level. Due to the complexity of foods and food processing, analysts should ideally be able to detect and quantify all forms of protein—containing, allergen-derived ingredients present in any type of food matrix that has undergone any kind of processing.

The most common methods in use are ELISA (or related immunoassay-type methods) and polymerase chain reaction (PCR). These methods perform well under a variety of different conditions, but they also have some limitations. ELISA methods can struggle with certain types of ingredients and they can have decreased quantitation following thermal or hydrolytic processing such as chemical hydrolysis or fermentation. ELISA method performance can also be influenced by certain food matrices, resulting in false negatives. Not being able to detect food allergens when they are actually present can be particularly problematic.

PCR does not detect the clinically relevant food component, which can

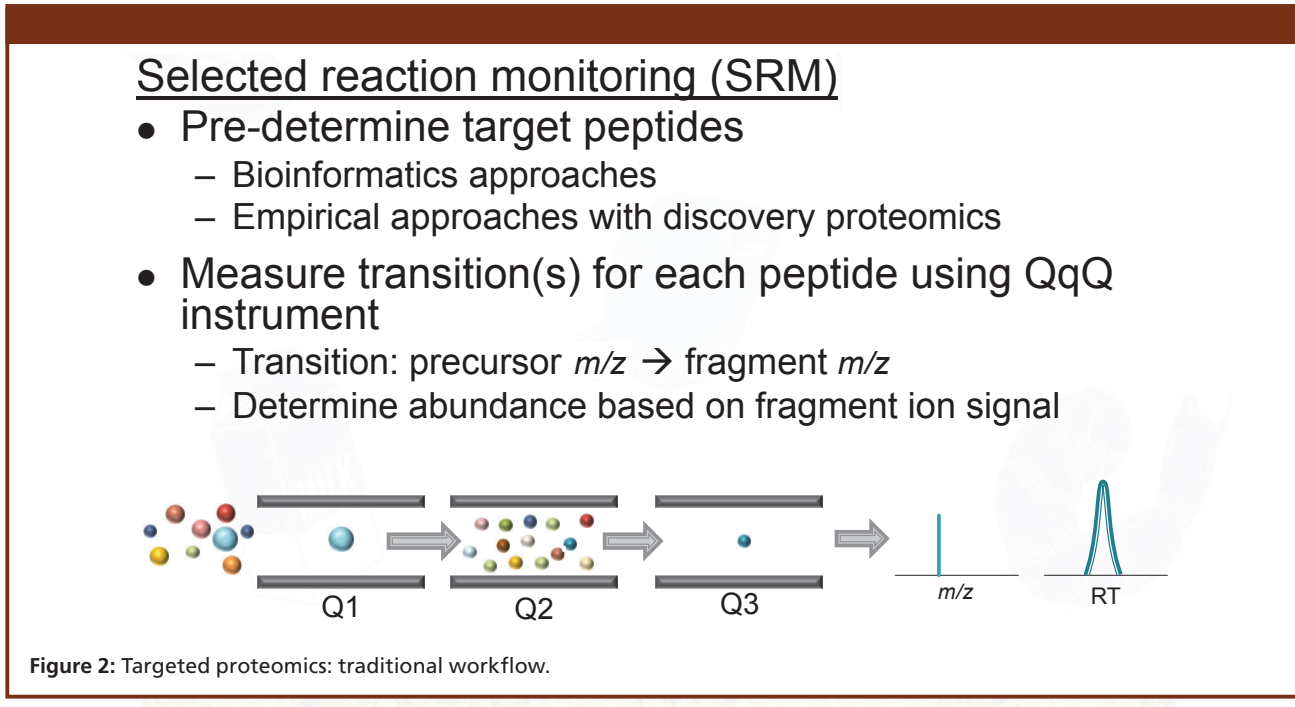

Figure 2: Targeted proteomics: traditional workflow.

make it problematic to use PCR results for quantitative risk assessment purposes and hence to determine the level of risk associated with the product. PCR can also have difficulty detecting certain ingredients derived from allergenic foods if limited DNA is present or if the DNA has been affected by processing.

Liquid chromatography (LC)-mass spectrometry (MS)/MS methods offer many advantages. The technique allows direct detection of the allergenic food protein component of concern and, more specifically, a peptide derived from those proteins. LC-MS/MS methods are also more reliable because they permit the use of more rigorous extraction techniques to overcome issues caused by food processing. LC-MS/MS methods are not dependent on protein conformation like some immunoassays, which is a benefit when analyzing thermally processed or modified protein systems. Another advantage of LC-MS/MS methods is the high level of specificity, which facilitates in-depth characterization of proteins and peptides.

LC-MS/MS methods are not without their challenges. For example, the current lack of protein sequence knowledge (libraries) for certain target allergens (e.g., tree nuts) or different background food matrices can negatively affect the method's specificity. There are also some challenges in developing reference standards for quantitation strategies and reporting units.

That said, overall LC-MS/MS methods may be ideal for allergen detection as they are specific, sensitive, accurate, and

- Untargeted, bottom-up proteomics
- Identify peptides/proteins without prior selection
  - Full-scan MS, typically high-resolution, accurate-mass (HRAM) mass analyzer
  - Selection and fragmentation of top n precursor ions
  - MS/MS, typically collected at lower resolution
  - Protein database searching

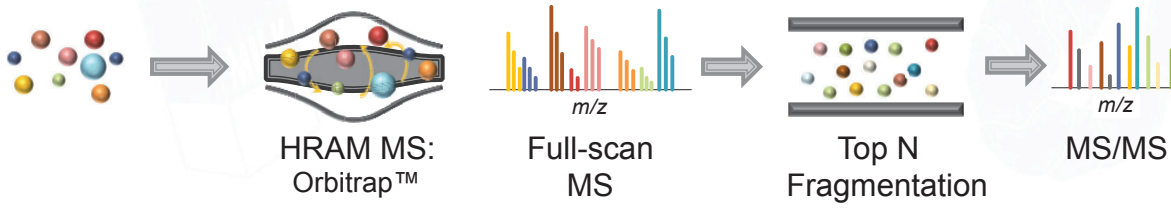

Figure 3: Discovery proteomics: traditional workflow.

precise with broad application across a variety of allergen sources and food matrices, and with potentially fewer impacts of food processing.

Traditional Target Peptide Selection Proteomic Workflows

The traditional workflow for targeted proteomics is selected reaction monitoring (SRM) or multiple reaction monitoring (MRM), as shown in **Figure 2**. The target peptides are first determined using discovery proteomics or bioinformatic approaches. The target peptide is then measured using a triple-quadrupole (QqQ) instrument to acquire the precursor to product ion transitions, specific for the target peptide. The detector response is determined by the fragment ion signal. SRM methods using QqQ are sensitive, accurate, and precise for quantitative measurements. On the downside, nominal mass QqQ have low resolving power and mass accuracy and are thus prone to ion interference from matrix co-extractives. The optimization of SRMs and system maintenance to sustain reproducible retention times can be time consuming.

High-resolution accurate mass (HRAM)-MS systems offer some distinct advantages over QqQ. The acquisition of full-product ion spectra and product ion spectra at high resolving power and with excellent mass accuracy provides high selectivity for confident peptide identifications. HRAM-MS instruments have many applications in terms of food

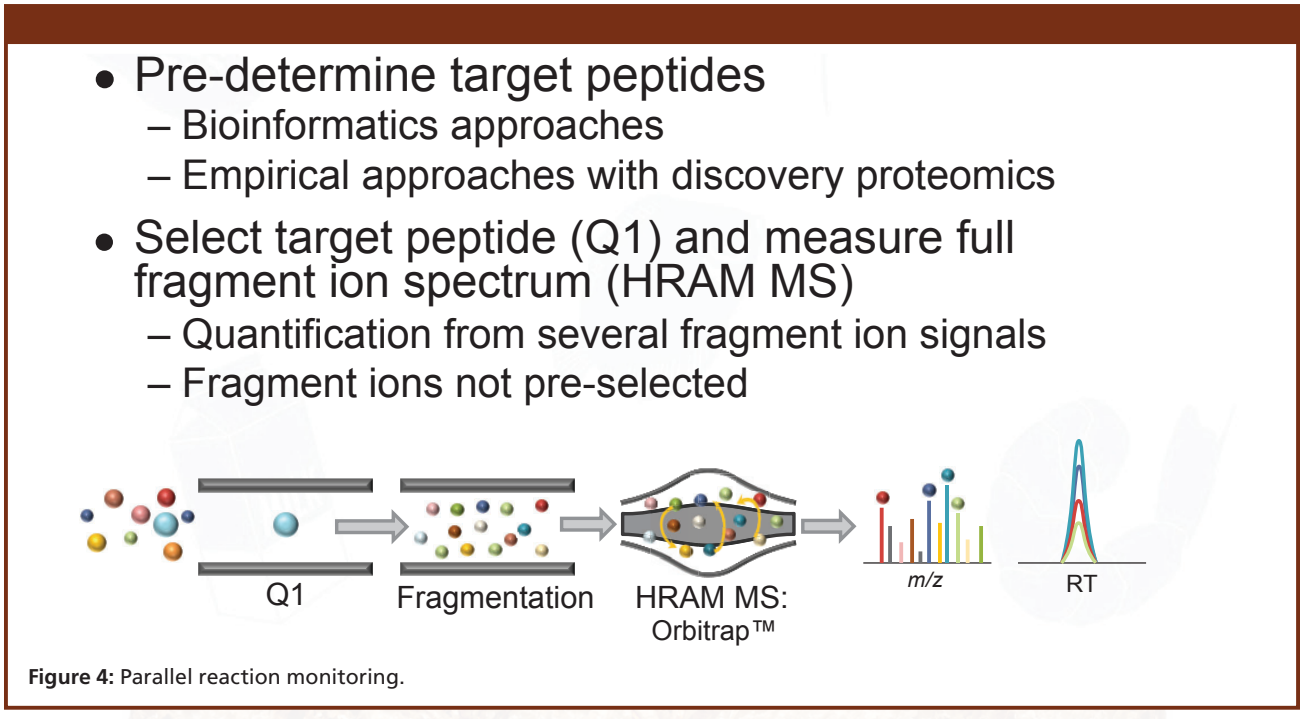

Figure 4: Parallel reaction monitoring.

allergen analysis including precursor ion monitoring, which can also include full-scan acquisition of precursor ions as well as selected ion monitoring, and full-scan product ion spectra with or without precursor ion selection. If a target ion transition suffers interference, it is likely that an alternative product can be selected from the raw data retrospectively.

Discovery proteomics for target peptide selection. An example discovery proteomic workflow can be seen in **Figure 3.** Discovery proteomics typically follow an untargeted bottom-up workflow and using protein database searching, where peptides are identified without any prior selection. This is almost always performed using a HRAM instrument because of the complexity of the samples and to obtain high-quality identification data using protein database searching. Conducting discovery proteomics can be used to determine if target peptides are present in all forms of ingredients derived from an allergenic source. It is possible to have many different ingredients derived from an individual allergenic food. They may have different protein compositions because of having been processed in different ways, and predicting whether those target peptides are present can then become problematic. Examples are liquid milk, whey protein isolates, sodium caseinate, lactose, butter, enzyme-ripened cheese, and caramel color—all milk-derived ingredients processed in very different ways.

Discovery proteomics: soy. Parallel reaction monitoring (PRM) development

	Protein Groups	Non-heated flour		Roasted flour		Isolate-A	Isolate-B		Concentrate-A		Concentrate-B	
7S globulin	Basic 7S globulin	2	3	1	1	1	1	1	1	1	1	1
	β-conglycinin beta chain	10	7	10	11	10	6	6	6	8	8	7
	β-conglycinin alpha prime chain	6	4	5	7	8	5	7	5	9	6	6
	β-conglycinin alpha chain	7	7	7	9	9	6	7	7	10	7	6
11S globulin	Glycinin G5 (A3B4)	5	2	4	6	4	ND	2	3	7	3	3
	Glycinin G4 (A5A4B3)	8	6	7	11	10	4	6	3	12	3	6
	Glycinin G3 (A1bB2)	4	1	2	1	3	2	3	3	4	2	2
	Glycinin G2 (A2B1a)	5	3	2	5	4	3	4	1	7	3	4
	Glycinin G1 (A1aB1b)	7	6	6	9	7	6	6	6	6	6	6
Protease inhibitor	Bowman-Birk type proteinase inhibit	1	1	1	1	ND	ND	ND	ND	1	1	1
	Kunitz trypsin inhibitor	7	5	5	5	4	5	5	5	6	5	4
	2S albumin	2	2	2	2	2	2	2	2	2	2	2
	Lectin	4	2	2	2	ND	ND	ND	ND	3	2	2
	Grand Total	68	49	54	70	62	40	49	42	76	49	50

Figure 5: Number of high-confidence peptide identifications conserved among isoforms in each protein group.

can overcome these limitations by using the same instrument platform used to perform discovery proteomics on the allergen derived ingredients and then developing a targeted detection method on the same platform, as illustrated in **Figure 4.**

PRM uses target peptides predetermined with discovery proteomics. The target peptide is selected and then the full fragment ion spectrum of that peptide is measured by HRAM-MS. Unlike SRM methods, fragment ions are not preselected.

For example, the development of methods to detect soy protein can be problematic for both MS and immunoassay methods because soy products are so diverse, having undergone many different types of processing. Using discovery proteomics methods to guide target peptide selection for a PRM method using a Thermo Scientific™ Q Exactive™ hybrid quadrupole-Orbitrap™ MS system, **Figure 5** shows the results of the analysis of six different soy-derived ingredients in a data-dependent acquisition mode. There is a lot of variability in the number of peptide identifications in the different types of ingredients, particularly in the isolates and some of the concentrates. Individual peptides also varied across the different ingredients, but some peptides are clearly identified across the different types of ingredients, which would then make for good target peptides. In the case of soy target peptide selection, a pool of 15,332 detectable peptides was filtered down to 57 unique, conserved peptides present across all processed soy ingredients, which was helpful for PRM method development.

Discovery proteomics: milk-derived ingredients. Another discovery proteomic method was developed for milk-derived ingredients: both acid set and enzyme set products, which

include whey and casein proteins. A similar discovery analysis was performed; again, results showed variability in high-confidence peptide identifications across the different ingredients. Using the information from the discovery analysis, these peptides were then used for targeted PRM methods with a Q Exactive MS system. To have a targeted method, a good collection of peptides that represent different types of ingredients is required, so that it is possible to detect and quantify milk, for example, regardless of the source. It is possible to use this information to further refine what target peptides would be ideal to have for large and robust coverage of milk-derived ingredients. Having this information derived from using HRAM instrumentation and then having the easy transition over into the targeted methods is a major advantage.

Selecting Surrogate Peptides Using High-Resolution Instruments

Food allergen detection methods for whole foods do not exist; for instance, "peanut" or "milk" cannot be detected. Consequently, analysts rely on surrogacy, the detection of molecules that represent the presence of a food. Apart from PCR, all food allergen detection methods rely on the detection of one or more proteins or peptides from proteins as surrogates; PCR relies on DNA.

When developing a proteomic method for allergen detection, one must select peptides that can be used as surrogates for allergenic foods. Theoretically, any peptide could be used as a surrogate if it is both sensitive and specific (i.e., discriminatory) for the allergenic food in question. The choice of a surrogate molecule is very important, if it is not detected, it will be assumed that no allergenic food is present (i.e., a false negative).

On the other hand, if a surrogate peptide is not specific to the allergenic food, the peptide may be detected where the food is not present (i.e., a false positive). The surrogate molecule should always be present where the food is present, and absent where it is absent. It is for this reason that allergen detection methods have largely focused on using peptides from identified allergenic proteins as surrogate proteins. By using these peptides, the risk of "losing" the hazardous molecule through processes such as thermal degradation and fractionation, while still having allergenic proteins present and the potential to cause allergic reactions, is minimized. An additional complication is that families of proteins that are known to be allergenic are usually made up of several different protein sequences. Since the specific sequences that cause reactions are not known, one can try to select peptides that represent them all.

Traditionally, selecting peptide surrogates for the detection of food allergens is performed primarily with a bioinformatic screen followed by an experimental demonstration of the

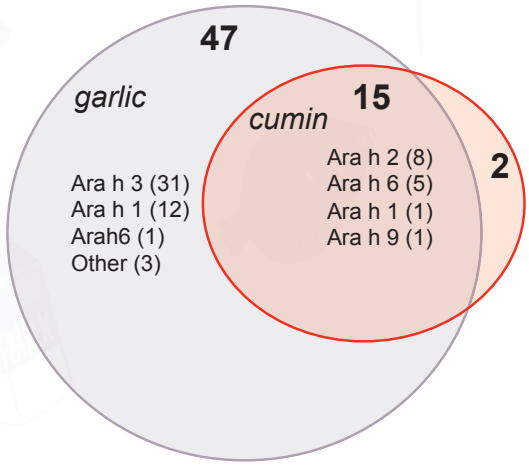

Figure 6: Peptides selected using HRAM-MS data for the detection of peanut in cumin and garlic and the proteins from which they derive. Peptides determined as being most suitable for the detection of peanut in cumin were suitable for garlic, and were derived from major peanut allergens.

presence of the selected peptides in the allergenic food. However, this type of target selection does not consider whether selected peptides will be detected in the complicated ranges of foodstuffs and processing conditions that constitute modern foods. Often, MS methods function well to detect allergens in simple, unchallenging foods, but fail to detect in foods that are complex, heavily processed, or contain chemicals that hinder protein extraction and detection.

To demonstrate this, researchers from the Food Allergy Research & Resource Program in the department of food science and technology at the University of Nebraska-Lincoln used an ELISA method to detect peanut in cumin that was spiked with either raw or roasted peanut. Recoveries for roasted peanuts were far lower, by as much as five times, than that of raw. This can make getting accurate quantitative results difficult, or even impossible, in situations where the processing method or the sample history is unknown. This might include, for example, a sample detected to contain 100 ppm of peanut, which could be either 100 ppm roasted peanut or 20 ppm raw peanut. This can also affect MS methods that do not account for recovery in matrices in the method development and target selection phase. Such ambiguous data can make decisions regarding food safety difficult for food manufacturers and regulatory agencies.

The University of Nebraska-Lincoln group decided to select its surrogate peptides using a different method. Essentially, their initial selection was based solely on the peptides that are detectable consistently across a range of foods and processing conditions. As a test case for this selection, the group looked at detecting peanut (both raw and roasted) in cumin or garlic powder. To make their selection, the University of Nebraska-Lincoln researchers relied on untargeted quantitative HRAM-MS experiments, using a Thermo Scientific™ Q Exactive™ Plus hybrid quadrupole-Orbitrap™ mass spectrometer, of peanut that was incurred into either cumin or garlic, as well as peanut on its own. Label-free quantitation allowed the researchers to select peptides that were recovered most robustly across all their samples, and were abundant enough to allow

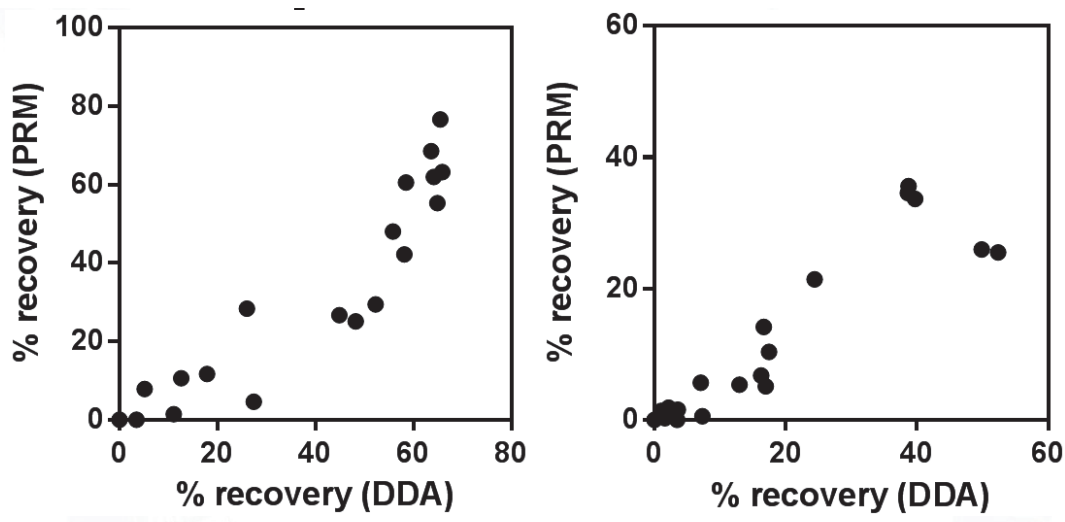

Figure 7: Robustness (recovery of peptide from peanut alone/from a cumin matrix) of peanut surrogate peptides in HRAM-MS (x-axis) is a good predictor of robustness in PRM experiments (y-axis) for both raw (A) and roasted (B) peanut.

for a sensitive detection method. The team followed this selection with further bioinformatics screening to ensure that its peptides were specific to peanut.

Researchers saw that roasting peanuts had a significant deleterious effect on the recovery of many peptides even in the absence of a food matrix. These peptides would be considered poor surrogates. When the team introduced a food matrix, even more peptides proved unsuitable. This information can be used when determining a peptide to select for a surrogate peptide in allergen detection. The relative recovery of peptides from a food matrix ("robustness") versus the overall abundance ("sensitivity") was plotted. Cut-offs can be set to each criterion allowing us to narrow the potential peptide surrogates. Doing so allowed the group to select 17 potential peptides for the detection of peanut in cumin, and 62 for garlic **(Figure 6).** Of the two spices, cumin appears to be the more challenging with regard to peanut detection as fewer peptides meet cutoffs. However, most peanut peptides that recover well from cumin are also suitable for use in garlic, allowing the researchers to develop a single method for both uses. Once this experimental screen is completed, the team can then go on to perform standard bioinformatics analyses to further narrow the focus on different target peptides. HRAM-MS data can also be used to predict lack of specificity to a certain degree by examining the occurrence of potential peanut surrogate peptides in spectra acquired from different foods.

The aim of this selection process is, of course, to choose surrogate peptides

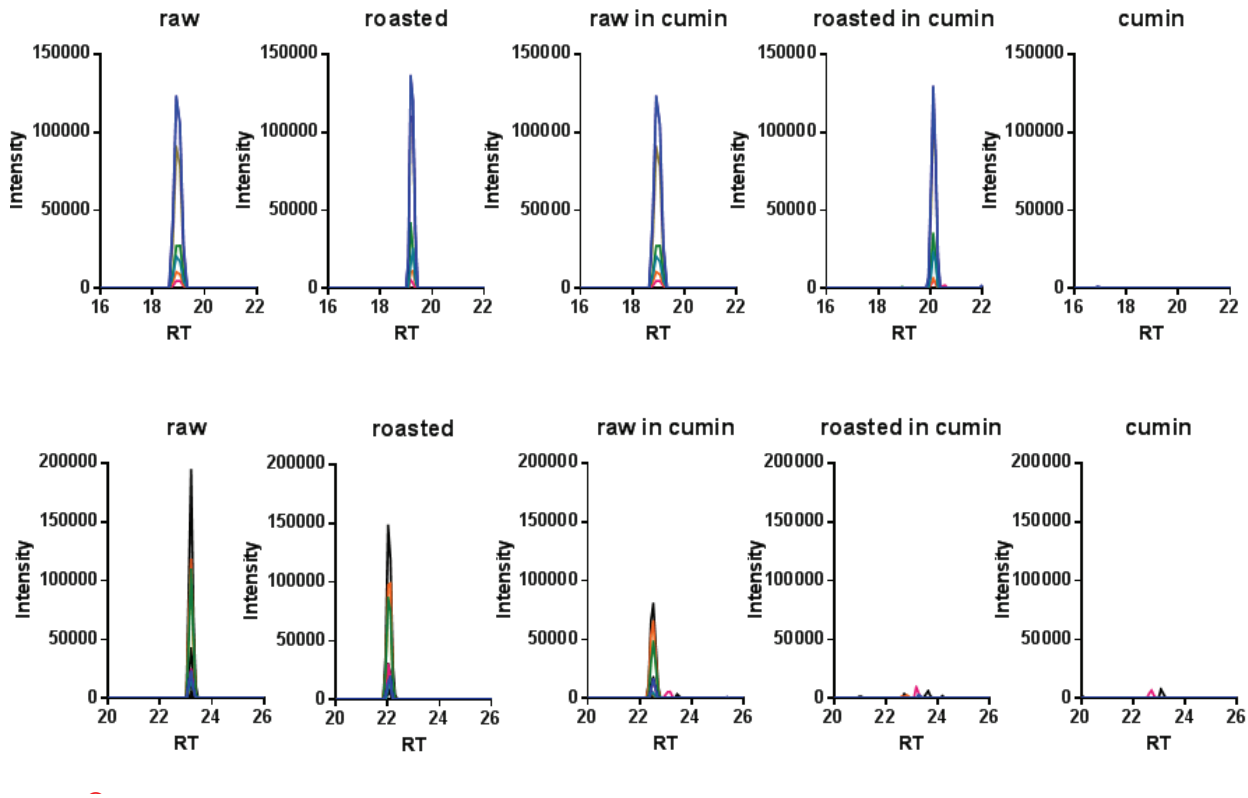

Figure 8: Performance of a peptide surrogate selected in the study (A) and the most commonly used peptide for peanut detection by MS (B) in PRM experiments across a range of matrix and processing conditions.

that can then be used in a targeted MS experiment. The group, therefore, went on to use its selected surrogates in PRM experiments. The researchers could then compare how well they predicted their peptides would work (from our HRAM-MS screening) versus how well they worked in a targeted method that would actually be used for allergen detection. This is illustrated in **Figure 7** with a comparison in robustness (recovery under different conditions) of surrogate peptides using HRAM-MS data (*x*-axis) to robustness in a PRM method (*y*-axis). This can demonstrate that HRAM-MS data is a good predictor of the robustness of surrogate peptides in a final targeted PRM method for both raw (A) and roasted (B) peanut.

The researchers can therefore go on to perform HRAM-MS experiments on peanut, or other food allergens, in broader ranges of food matrices with confidence that the data will allow them to develop robust, sensitive detection methods. The robustness of PRM detection of a peptide selected using the group's pipeline (NLPQQCGLR) (A)

compared to that of the most commonly used peptide for peanut detection by MS (SPDIYNPQAGSLK) (B) is shown in **Figure 8.** The newly selected peptide shows robust recovery in raw and roasted peanut in cumin, whereas the most commonly used peptide shows lower detection in roasted peanut and in raw peanut in cumin, and is undetectable under these conditions in roasted peanut in cumin.

Conclusion

The difference in the recovery of targets can significantly affect quantitation and thus methods developed for use in one situation, such as raw peanut, will not necessarily work well for roasted peanuts or for roasted peanut in various food matrices. This is a common issue with many current methods, and with MS methods developed using traditional surrogate peptide selection strategies. One can leverage the ability of HRAM-MS to detect and quantify large numbers of peptides from allergenic foods spiked into food matrices to choose peptides which overcome this limitation, and allow consistent results in different types of foods.

This executive summary is based on a material presented in a webcast by Melanie Downs, PhD, Assistant Professor, and Phil Johnson, PhD, Assistant Professor, both of Food Allergy Research & Resource Program in the Department of Food Science and Technology at the University of Nebraska-Lincoln. To view a recording, please click **here**.

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IMPORTANT CONSIDERATIONS REGARDING MATRIX EFFECTS WHEN DEVELOPING RELIABLE ANALYTICAL RESIDUE METHODS USING MASS SPECTROMETRY

Alexander J. Krynitsky, Jon W. Wong, Kai Zhang, and Hudan Safarpour

Liquid chromatography–mass spectrometry (LC–MS) using electrospray ionization (ESI) is subject to matrix effects when analyzing complex matrices, such as food samples, for trace organic residues and contaminants. Even though a sample extract has gone through extensive cleanup, there are still enough coeluted compounds to possibly cause signal suppression or signal enhancement when analyzing a complex matrix, thus adversely affecting quantitation. Likewise, gas chromatography–mass spectrometry (GC–MS) is also subject to matrix effects that can hinder accurate MS quantification. This article shows some practical examples of how to correct for matrix effects to obtain reliable quantitative data using LC–MS and GC–MS.

With the availability of sensitive and selective techniques such as mass spectrometry (MS), proper use of sample preparation is often overlooked. For example, many pesticides registered over the last decade are primarily polar compounds and are not amenable to gas chromatography (GC) techniques, thus liquid chromatography–mass spectrometry (LC–MS) is the methodology of choice to monitor these contaminants in foods for enforcement purposes. Likewise, LC–MS is needed for the reliable determination of other polar food contaminants such as mycotoxins and veterinary drugs. Although LC–MS offers several advantages in terms of sensitivity, selectivity, and overall speed of analysis, there are many important considerations, such as matrix effects, which must be considered when developing analytical methods. Matrix effects are either observed as suppression or enhancement of analyte signal in the electrospray ionization (ESI) source and have been studied by many researchers since the mid-1990s (1–7). Ion suppression is typically observed in atmospheric pressure ionization processes, and the causes for ion suppression in LC–MS are discussed elsewhere (8). Most of the studies have been focused on the ESI suppression rather than the enhancement because the former is the more commonly observed phenomenon (9). Quantitative analysis by GC–MS is also subject to matrix effects since matrix-induced enhancement has been observed. This enhancement results in improved chromatographic peak intensities and peak shapes because matrix components protect the analytes by covering the active sites in the GC inlet system (10–13). This article shows some practical examples of how to correct for matrix effects to obtain reliable quantitative data using ESI LC–MS and capillary GC–MS. The examples given include some of the more popular areas in food safety, such as pesticides, mycotoxins, melamine, perchlorate, active pharmaceutical ingredients (APIs) found in food, herbal dietary substances, and personal care products. The validation procedures used for these methods were similar to the ones described in the US Food and Drug Administration's (FDA) "Guidelines for the Validation of Chemical Methods for the FDA FVM Program, 2nd Edition" (14). The common tools used to correct for matrix effects include stable isotope dilution, matrix-matched or method-matched standard calibration, sample dilution, method of standard additions, sample cleanup, alternative ionization sources other than ESI used in LC–MS, and analyte protectants used in GC–MS.

Stable Isotope Dilution Mass Spectrometry

The dilution of the native chemical by the addition of its stable isotopically labeled compound in a particular sample matrix is commonly referred to as *stable isotope dilution assay (SIDA)*. This step is usually

followed by LC–MS analysis since the mass spectrometer can easily separate and differentiate the native (and naturally abundant) compound from its labeled isotope because of differences in their molecular masses. The stable isotope can compensate for matrix effects since both the native compound and its isotope counterpart share the same physical and chemical properties so that they are chromatographically coeluted and interact with the same matrix components that may be responsible for any suppression effects during ionization. However, for analyzing many analytes, such as those encountered in multiresidue pesticide procedures, using SIDA-LC–MS is impractical because the stable isotopes are expensive and the isotopes for each native pesticide may not be readily available.

A procedure that was successfully performed at the FDA used SIDA and LC–tandem mass spectrometry (LC–MS/MS) to analyze mycotoxins in corn, peanut butter, and wheat flour (15). The method was single-laboratory validated by uniformly fortifying the 12 ^{13}C-labeled homologs for each of the targeted mycotoxins in the food sample, followed by extraction with 50:50 (v/v) acetonitrile–water, centrifugation, filtration, and analysis by LC–MS/MS. The method was simple to use and applicable to a wide variety of food matrices because of the effective and efficient compensation of matrix effects provided by the addition of the labeled mycotoxin standards.

The success of the validated procedure was followed with a collaborative study of six laboratories to evaluate SIDA and LC–MS/MS for the simultaneous determination of aflatoxins B1, B2, G1, and G2; deoxynivalenol; fumonisins B1, B2, and B3; ochratoxin A; HT-2 toxin; T-2 toxin; and zearalenone in foods. In addition to certified reference materials, the laboratories analyzed corn, peanut butter, and wheat flour fortified with the 12 mycotoxins at concentrations ranging from 1 to 1000 ng/g. Using their available LC–MS/MS platform, each laboratory developed in-house instrumental conditions for analysis. The majority of recoveries ranged from 80% to 120% with relative standard derivations (RSDs) < 20%. Greater than 90% of the average recoveries of the participating laboratories were in the range of 90–110%, with repeatability RSD_r (within laboratory) <10% and reproducibility RSD_R (among laboratory) <15%. All Z scores of the results of certified reference materials were between –2 and 2. The use of ^{13}C-internal standards eliminated the need for matrix-matched calibration standards for quantitation, and all participating laboratories were able to validate and implement a simple sample preparation procedure to achieve simultaneous identification and quantitation of these regulated mycotoxins using LC–MS/MS.

The second example where SIDA LC–MS/MS was successfully used was with the direct determination of glyphosate,

glufosinate, and aminomethylphosphonic acid (AMPA) in soybeans and corn (16). These two organophosphorus acidic herbicides and metabolites (AMPA) are amphoteric, low mass, highly water soluble, and do not possess any distinguished and recognizable chromophores that could be exploited for detection. They are very difficult to retain in reversed-phase high performance liquid chromatography (HPLC) and are poorly detected by ultraviolet (UV) or fluorescence detectors. An LC–MS/MS method was developed to determine these analytes in soybeans and corn using reversed-phase LC with weak-anion-exchange and cation-exchange mixed-mode columns. Three isotopically labeled internal standards, $^{13}C^{15}N$-glyphosate, glufosinate-d_3, and $^{13}C^{15}N$-aminomethylphosphonic acid corresponding to each analyte were used to counter matrix suppression effects when added to soybean and corn matrices. The samples were extracted with a buffer containing acetic acid and ethylenediaminetetraacetic acid (EDTA) to avoid recovery losses caused by metal ion (such as calcium) complexation with the three compounds. The supernatant was passed through an Oasis HLB solid-phase extraction (SPE) column (Waters Corporation) to retain suspended particulates and nonpolar interferences. The extract was directly injected and analyzed in 6 min by LC–MS/MS with no concentration or derivatization steps. The use of the isotope internal standard for each analyte resulted in linearity with a minimum coefficient of determination > 0.995 in the range of 10–1000 ng/mL, and accuracy (recovery %) and precision (RSD %) were evaluated at the fortification levels of 0.1, 0.5, and 2 µg/g in seven replicates in both soybean and corn samples.

The third example where SIDA LC–MS/MS was successfully used was with the determination of melamine and cyanuric acid in foods (17). In this procedure, both cyanuric acid and melamine are extracted from tissue and infant formula with a 50:50 (v/v) acetonitrile–water extraction solution, followed by centrifugation. The cleanup procedure for melamine involves mixed-mode cation-exchange SPE and that for cyanuric acid uses mixed-mode anion-exchange SPE. Consequently, aliquots of the same extract are individually processed with the two modes of SPE. The final cleaned up extracts for both melamine and cyanuric acid are in acetonitrile, making the procedure amenable to evaporate the excess solvent for sensitivity needs or solvent exchange (depending on the LC column used). Each compound is analyzed separately using a zwitterionic hydrophilic-interaction chromatography (HILIC) LC column. Electrospray ionization is used in both the negative-ion (cyanuric acid) and positive-ion (melamine) modes. Two selected reaction monitoring (SRM) transitions are monitored for both compounds. Commercially available, isotopically labeled internal standards,

$^{13}C_3{}^{15}N_3$-melamine and $^{13}C_3{}^{15}N_3$-cyanuric acid for each of the native compounds, were used to correct for any matrix effects. The method limit of quantitation (LOQ) for melamine was: 25 µg/kg for tissue and liquid formula and 200 µg/kg for dry infant formula powder. The method LOQ for cyanuric acid was 50 µg/kg for tissue and liquid formula and 200 µg/kg for dry infant formula powder. Fortified test portions were within 75–125% recovery. Determination of incurred residue in tissue agreed well with the results of an independent laboratory.

The final example where isotope dilution MS was successfully used was with the determination of inorganic perchlorate (**Figure 1a**) in foods (18,19). In these studies, a rapid, sensitive, and specific method was developed for the determination of perchlorate anion in foods. The foods included high-moisture fruits and vegetables, low-moisture foods (for example, wheat flour and corn meal), and infant foods. Improvements to existing procedures were made in sample preparation that reduced the sample test portion size from 100 g to 5 or 10 g and the extraction solvent volume from 150 mL to 20–40 mL, and replaced blending extraction–vacuum filtration and its associated large glassware with a simple shakeout centrifugation in a small conical tube. Procedures common to all matrices involved extraction, centrifugation, graphitized carbon SPE cleanup, and ion chromatography–tandem mass spectrometry (IC–MS/MS) analysis. A 75 mm × 4.6 mm Waters IC-Pak Anion HR column was used with a mobile phase consisting of 100 mM ammonium acetate in 50:50 (v/v) acetonitrile–water with a flow rate of 0.35 mL/min. IC–MS/MS, equipped with ESI in the negative ion mode, was used to detect perchlorate anion. An $^{18}O_4$-labeled perchlorate anion internal standard was used to correct for any matrix effects. Losses of the ^{16}O and ^{18}O atoms from perchlorate were used as transition product ions from the native and isotope perchlorate species, respectively (**Figure 1b**) and provided a stable calibration curve (**Figure 1c**) that can be used to quantitate a variety of different food commodities. The method LOQ was 1.0 g/kg in fruits, vegetables, and infant foods and 3.0 g/kg in dry products. Fortified test portions gave 80–120% recoveries. Determination of incurred perchlorate anion residues agreed well with results for comparable commodities or products analyzed by published methods.

The combined SIDA and LC–MS/MS procedure is very convenient for analyzing both organic, as well as inorganic (in the case of perchlorate) chemicals in difficult food matrices. In the four cases presented, the matrix effect was corrected and the isotopes were used to compensate for any loss or suppression in the quantitation of the chemical because consistent results were obtained when a stable isotope was used in the procedure. However, the native and isotope chemicals are equally

(a)

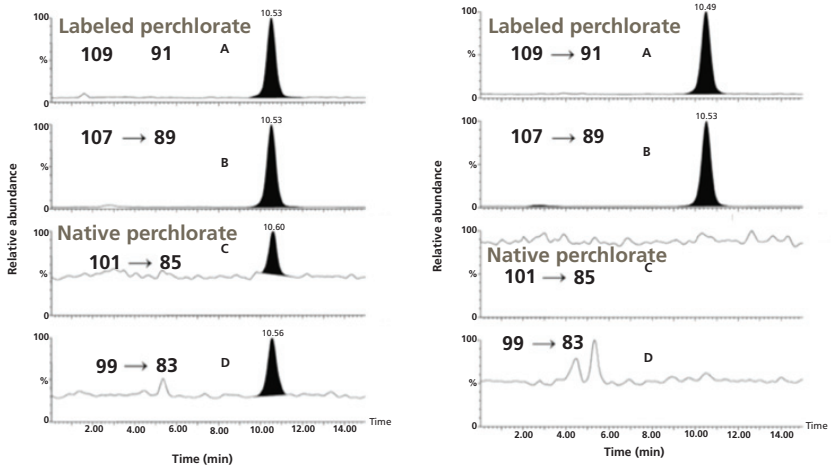
Native perchlorate

Avg. MW = 99 (^{35}Cl)
101 (^{37}Cl)

Isotope-labeled perchlorate

Avg. MW = 107 (^{35}Cl)
109 (^{37}Cl)

(b)



Incurred Lettuce

Blank Lettuce

(c)

Conc. (ng/mL)	Retention time (min)	Native *m/z* 99>83	Labeled IS *m/z* 107>89	Ratio (Native/IS)	Ratio (101/99)
0.25	10.00	108.33	3806.28	0.0285	32.80
0.50	10.28	234.10	4162.87	0.0562	31.98
1.00	10.28	477.65	4357.62	0.1096	36.11
2.00	10.21	1011.80	4377.76	0.2311	32.63
5.00	10.28	2607.89	4405.70	0.5919	33.35
10.0	10.28	5027.26	4188.06	1.2111	31.49
100	10.21	35207.60	2879.01	13.229	33.40

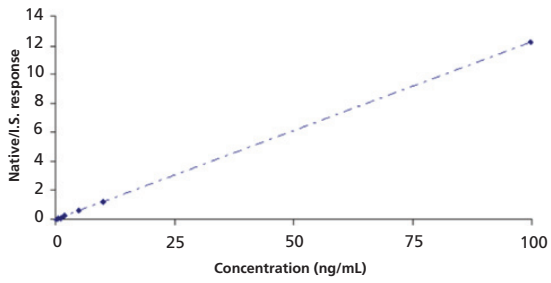


Figure 1: (a) Native and ^{18}O-labeled perchlorate; (b) LC–MS/MS chromatograms of native and ^{18}O-labed perchlorate in lettuce; (c) calibration curve of perchlorate using response ratio of (native–isotope labeled internal standard) versus native perchlorate concentration (ng/mL).

affected by matrix effects and both must be able to exceed the limits of detection (LOD) and LOQ for proper identification and quantitation of the native chemical. Whether the method includes different SPE procedures involving glyphosate, melamine, and perchlorate or no cleanup at all, as in the case of the mycotoxin analysis, the use of the stable isotope before sample extraction and cleanup demonstrates that SIDA with LC–MS/MS is both a robust and rugged procedure. There is a concern about whether the cost of the stable isotope may dissuade laboratory analysts from implementing SIDA and LC–MS/MS, but there are other cost dependent factors that also need to be evaluated as well to show the advantages of SIDA, which include increased productivity, the lack of a need for matrix-matched calibration (see the next section), and consistent results (near perfect accuracy and precision) over a wide range of sample matrices.

Matrix-Matched Standard Calibration

As mentioned earlier, when developing multiresidue methods for the determination of pesticide and veterinary drug residues in foods, the use of SIDA LC–MS is impractical because several hundred isotopically labeled standards would be needed, which is costly, and the labeled standards may not be commercially available. The method of choice for validation of multiresidue procedures would involve matrix-matched standard calibration, where analytical standards are fortified in a sample extract that has been treated exactly the same as the regular sample and is free of the residues of interest. The caveat is that the blank matrix (that is, avocado, blueberry, and so on) must not contain the compounds of interest and must be consistent with the sample matrices.

Matrix-matched calibration standards were used to validate a multiresidue method analyzing 209 pesticides in 24 agricultural commodities using the original quick, easy, cheap, effective, rugged, and safe (QuEChERS) procedure and LC–MS/MS analysis (20,21). Using solvent-only calibration standards and matrix-matched calibration standards, it was demonstrated that a minimal concentration of 5–10 µg/kg (ppb) of analytes in matrix is required for the consistent identification of targeted pesticides with two MRM transitions. Method performance was validated by the precision and accuracy results obtained from fortification studies at 10, 25, 100, and 500 ppb and matrix-matched calibration standards. The method was demonstrated to achieve an average recovery of 100 ± 20% (n = 4) for >75% of evaluated pesticides at the low fortification level (10 ppb) and improved to >84% at the higher fortification concentrations in all 24 matrices. Matrix effects in LC–MS/MS analysis were studied by evaluating the calibration curves (1.0–100 ng/mL) obtained from the solvent-only calibration standards and

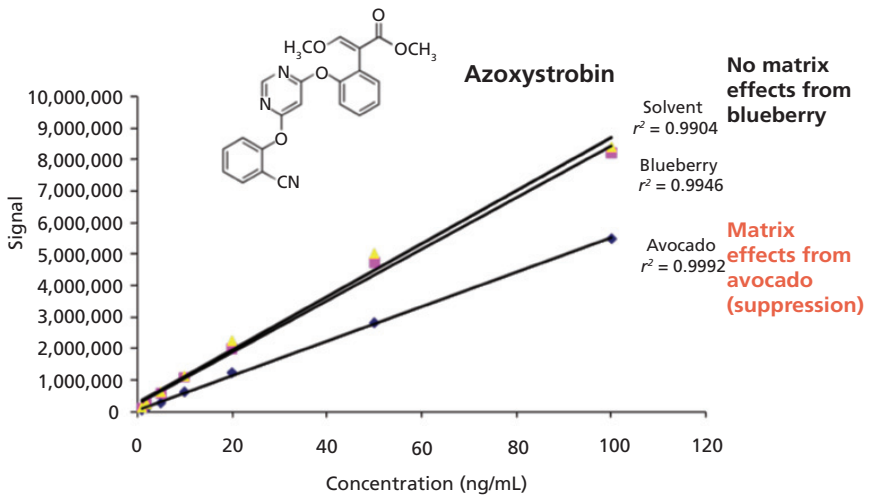


Figure 2: The effects of ESI LC–MS/MS matrix suppression. Comparisons of solvent-only, blueberry-matched, and avocado-matched calibration curves for the fungicide azoxystrobin, indicating the influence of the matrix on a specific pesticide.

matrix-matched calibration standards. The matrix effect is primarily dependent on the type and concentration of the matrix and pesticide. Matrix-matched calibration standards were needed to compensate for matrix effects, and the effects of the matrix on a particular pesticide are illustrated by the calibration curves of the fungicide azoxystrobin in solvent, avocado, and blueberry (**Figure 2**). In the case of blueberry, the matrix had very little effect on azoxystrobin since the calibration curves between blueberry-matched and the solvent-only standard of azoxystrobin are essentially equivalent. However, when comparing the avocado-matched calibration curve with the same solvent-only calibration, suppression is observed for azoxystrobin because of the lower signal intensities.

The matrix-matched (in this case, avocado-matched) calibration curve was used to identify and quantitate pesticide residues in avocado, and similar matrices are used to determine accuracy and precision results in validation studies. In this multiresidue pesticide validation, concentrations ranging from 2.5 to >1000 ppb in a variety of agricultural samples demonstrate fitness for screening, quantitation, and identification applications. The major drawbacks of matrix-matched (or *method-matched*, in which the matrix is fortified at the beginning of the procedure at the appropriate standard concentration and subsequently used for quantitation) standards are the need for analyte-free matrix (which may not be possible) and that additional work is required for

accurate quantitation if a wide range of matrices are to be evaluated. The difficulty of selecting matrices that represent certain food groups—for example, high or low moisture, high lipid, high lipid–low moisture, acidic, and high pigmentation—is also a challenge and generalization of these food groups may not be possible.

Method of Standard Additions and Sample Dilution

Although it is difficult to find a blank matrix that is consistent with the samples to be analyzed, it is not possible to compensate for matrix effects using matrix-matched standard calibrations. The only options left are sample dilution and using the method of standard additions. The obvious disadvantage with diluting the sample to compensate for matrix effects is that it will raise the limit of quantitation, which could affect the required sensitivity. Although the method of standard addition compensates for matrix effects, the disadvantages are that the approximate concentration of the analyte must be known to construct a proper calibration curve. Secondly, it requires at least three additional sample runs per sample in order to have sufficient data points for the calibration curve.

A demonstration of the use of standard addition and sample dilution to compensate for matrix effects involves the analysis of multiple pharmaceuticals, plant toxins, and other secondary metabolites in herbal dietary supplements by ultrahigh-pressure liquid chromatography (UHPLC)–quadrupole-orbital ion trap MS (22). A UHPLC–quadrupole-orbital ion trap MS method was developed for the simultaneous determination of 96 pharmaceuticals, plant toxins, and other plant secondary metabolites in herbal dietary supplements. Target analytes were extracted from samples using the QuEChERS procedure (20). With the exception of highly polar analytes, the optimized QuEChERS extraction procedure provided acceptable recoveries in the 70–120% range. Because of variations in matrix effects in extracts of herbal dietary supplements that differ in composition, the method of standard additions and an approach based on dilution of matrix components followed by quantification using solvent standards were applied for quantification. For the majority of compounds with signal suppression above 20%, a dilution factor of 25 or higher was used. Because the outcome of the dilution-based approach is largely determined by the dilution factor used and the actual concentration of an analyte, this procedure was successfully applied for quantification of selected pharmaceuticals (indomethacin, phenylbutazone, hydrochlorothiazide, metoprolol tartrate, chloropropamide, and glibenclamide) added to test matrices at therapeutic doses. At such high concentrations, which are often used in commercial adulteration,

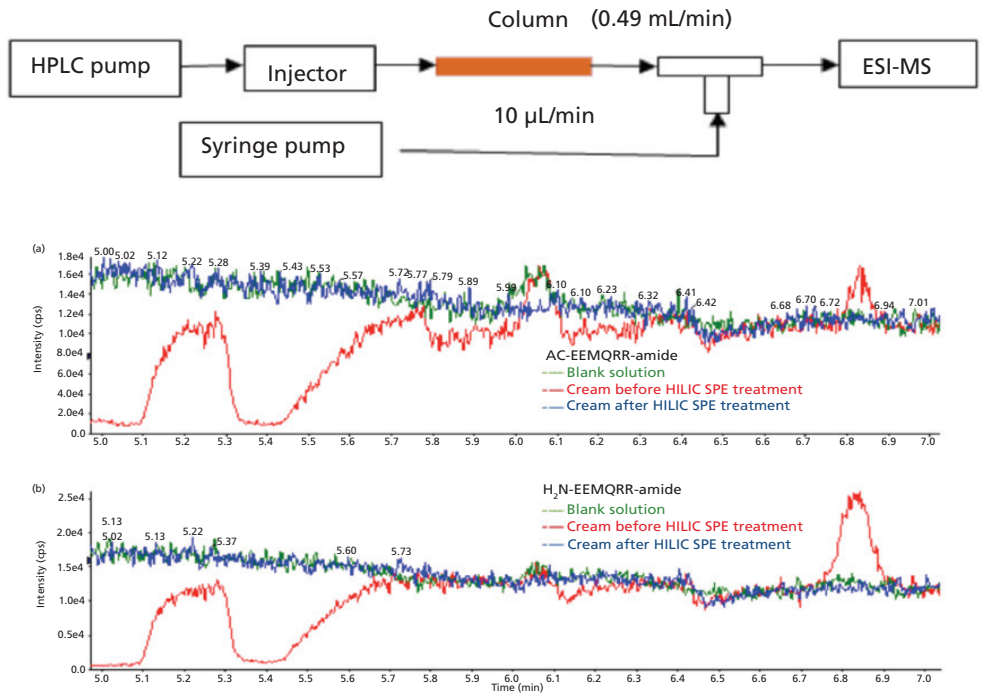


Figure 3: The evaluation of ion suppression caused by face cream matrix: (a) The infusion experimental protocol for detecting ion suppression; (b) the comparison of responses of Ac-EEMQRR-amide for injection of blank solution, cream sample before and after HILIC–SPE cartridge treatment; (c) the comparison of responses of H2N-EEMQRR-amide for injection of blank solution, cream sample before and after HILIC-cartridge treatment.

dilution factors as high as 10,000 can be commonly used. Under these conditions, the complete elimination of the effects of the matrix is possible without compromising the detectability of analytes. The recoveries calculated by this quantitative approach ranged from 82% to 98%.

Sample Cleanup

Sample cleanup can reduce, if not eliminate matrix effects. As discussed in the previous section, sample cleanup can benefit SIDA and LC–MS analysis by removing potential matrix components that may interfere with the analysis of the chemical of interest. A study involving the simultaneous determination of hexapeptides (Ac-EEMQRR-amide and H_2N-EEMQRR-amide) in antiwrinkle cosmetics by HILIC–SPE preparation and HILIC–MS/MS, involves a rapid method for the simultaneous determination of Ac-EEMQRR-amide and H_2N-EEMQRR-amide in cosmetic products (23). Samples showing serious ion suppression were further cleaned up using HILIC–SPE before HILIC–MS/MS analysis.

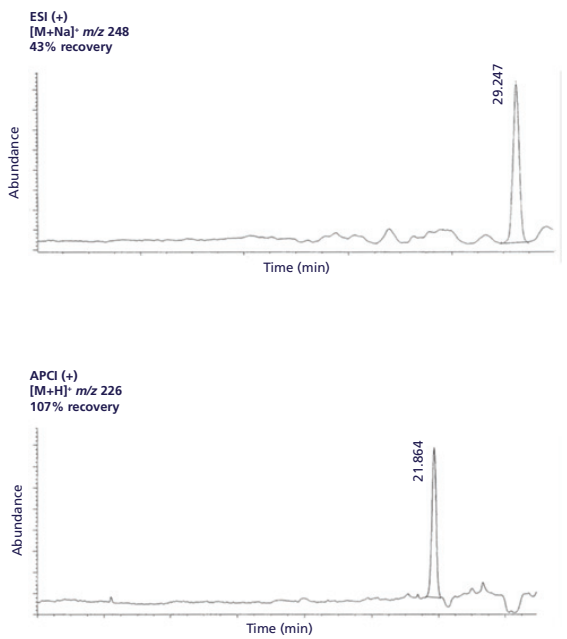


Figure 4: The analysis of 50 ppb methiocarb in orange via ESI (top) and via APCI (bottom). The retention times are different because of different separation conditions.

Stable isotopically labeled peptides, corresponding to the above two peptides, were used as internal standards to correct for loss of recovery and matrix effects.

An infusion experiment was designed to evaluate matrix effects similar to a study described in another publication (24). The experiment was carried out using a built-in tee union, a syringe pump, and LC pumps as shown in **Figure 3a**. The mobile phases were delivered by the LC pumps using a regular gradient program. A standard solution containing the peptides was continuously introduced into the ionization source using the syringe pump connected to the tee union. A blank solvent sample was first injected to define a "baseline," followed by an extracted "blank" sample. Since the dropped region in the baseline falls in the time window of peptides of interest (5.3 min and 6.1 min for Ac-EEMQRR-amide and H_2N-EEMQRR-amide, respectively) as shown in **Figures 3b** and **3c**, the eluted matrix components will suppress the ionization of the analytes. However, the baseline after HILIC–SPE cleanup does not show this signal suppression. Although internal standards can be used to compensate for the ion suppression of matrices, the sensitivity and detection limit would have been affected if the additional HILIC–SPE cleanup had not been used.

Changing from ESI to APCI

Another way to compensate for matrix effects is to change interfaces from ESI to atmospheric pressure chemical ionization (APCI). **Figure 4** shows an LC–MS chromatogram of 50 ppb methiocarb in an orange sample analyzed by both ESI and APCI. When analyzing the sample using external standard in solvent calibration, the apparent recovery was 43% when using ESI in positive mode (ESI+). However, the apparent recovery dramatically improved to 107% when changing from ESI+ to APCI+ (unpublished results). This result is consistent to Souverain and colleagues (1), where APCI appears to be less susceptible to matrix effects than ESI. However, ESI is more popular than

APCI because of its sensitivity, and ESI is typically 5- to 10-fold more sensitive than APCI for many pesticides.

Matrix Effects with GC–MS

GC–MS is subject to signal enhancement since the matrix is blocking active sites in the injection liner that protects the analyte from thermal degradation (10–13). The causes of signal enhancement in GC–MS are different from signal suppression in LC–MS because in the former case enhancement is a result of the longer residence times spent by the chemical in the injector liner, whereas in the latter case suppression is primarily because of ionization efficiency. In a study (10) that involved the analysis of organophosphorus pesticides (OP) in ginseng root using GC–MS using selective ion monitoring (SIM) and GC with flame photometric detection in phosphorus mode (GC–FPD), three methods for standard calibration were used and compared: external standard in solvent, matrix-matched standards, and standard addition. When using GC–MS with SIM, organophosphorus pesticides quantitatively determined using a calibration curve based on matrix-matched standards agreed with the results obtained from the method of standard addition. The GC–MS-SIM results obtained using a calibration curve based on external solvent standards resulted in significant enhancement and higher quantitative results. When using the GC–FPD, all three methods for standard calibration agreed. With the GC–FPD analysis, a megabore column (30 m × 0.53 mm) in conjunction with a faster flow rate (10 mL/min) was used (as opposed to the narrow-bore capillary column with a flow rate of ~1 mL/min typically used with GC–MS), which resulted in a reduction of the residence times these pesticides spent in the injection liner and reduced the ability of the pesticide to accumulate within the inlet, and therefore minimized enhancement effects.

Nowadays, the current and popular trend for capillary GC analysis is to use MS-based detectors and less use of element-selective detection methods such as the FPD. Anastassiades and colleagues (11,25) discussed several possible considerations to minimize matrix effects with GC–MS such as cold, on-column injection as well as other procedures such as extensive cleanup, method of standard addition, isotopically labeled internal standards, and matrix-matched standardization that have been discussed in the previous section for LC–MS. These possible approaches have their benefits and limitations. Anastassiades and colleagues (25) proposed the use of analyte protectants, chemical additives added to the GC extracts, and matrix-free standards to provide a standardized enhancement effect that would eliminate the need to prepare matrix-matched standards and compensate for matrix enhancement. Chemical compounds containing multiple hydroxyl groups were

shown to be effective protecting agents for a wide range of pesticides. The use of chemical compounds or "analyte protectants" in the GC–MS extracts and solvent calibration standards were shown to be effective in providing accurate quantitation. Since many of these protectants have multiple hydroxyl groups and high water solubility, the GC solvents used must be water soluble, which means that the GC–MS extracts and standards most likely need to be prepared in acetonitrile.

Summary

Although both LC–MS and GC–MS are highly selective, they are both vulnerable to matrix effects. Numerous chromatographic and sample preparation techniques are available to help reduce signal suppression or signal enhancement. Eliminating the risk of matrix effects is possible, but it involves careful optimization of sample preparation. Even though stable isotopically labeled standards are available, some effort in sample cleanup should be attempted so that severe signal suppression will not compromise the needed sensitivity. Several possible considerations have been provided in this report to minimize matrix effects for both LC–MS and GC–MS. With newer instrumentation being developed and technologies that will address the root cause of the matrix effects, it is quite possible to take advantage by just diluting the sample to eliminate or minimize matrix effects.

Finally, when developing new analytical methods it is recommended, if possible, to take advantage of proficiency testing programs to verify how well the newly developed analytical method compares to other existing methods with regard to incurred residues.

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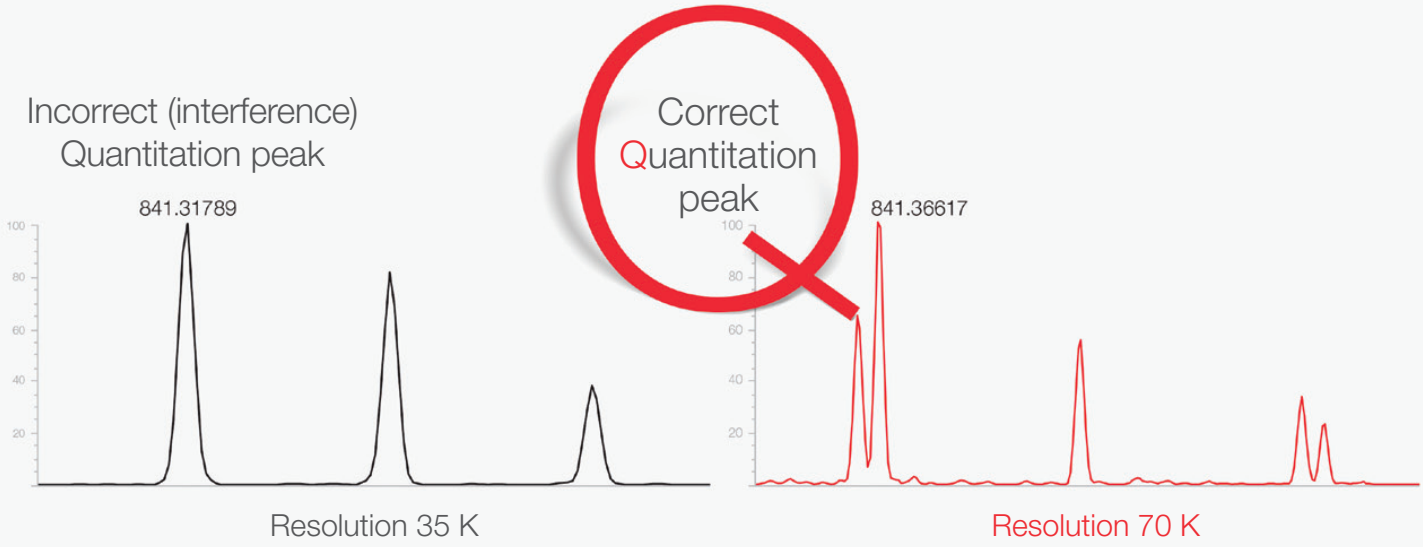

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