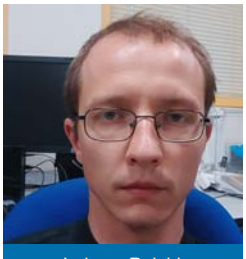
An Executive Summary

Utilizing the Power of LC-Orbitrap MS Technology for the Multi-residue Analysis of Pesticides

Amadeo Fernandez-Alba, Ph.D.



Łukasz Rajski, Ph.D. student

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

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 to 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.

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

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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.

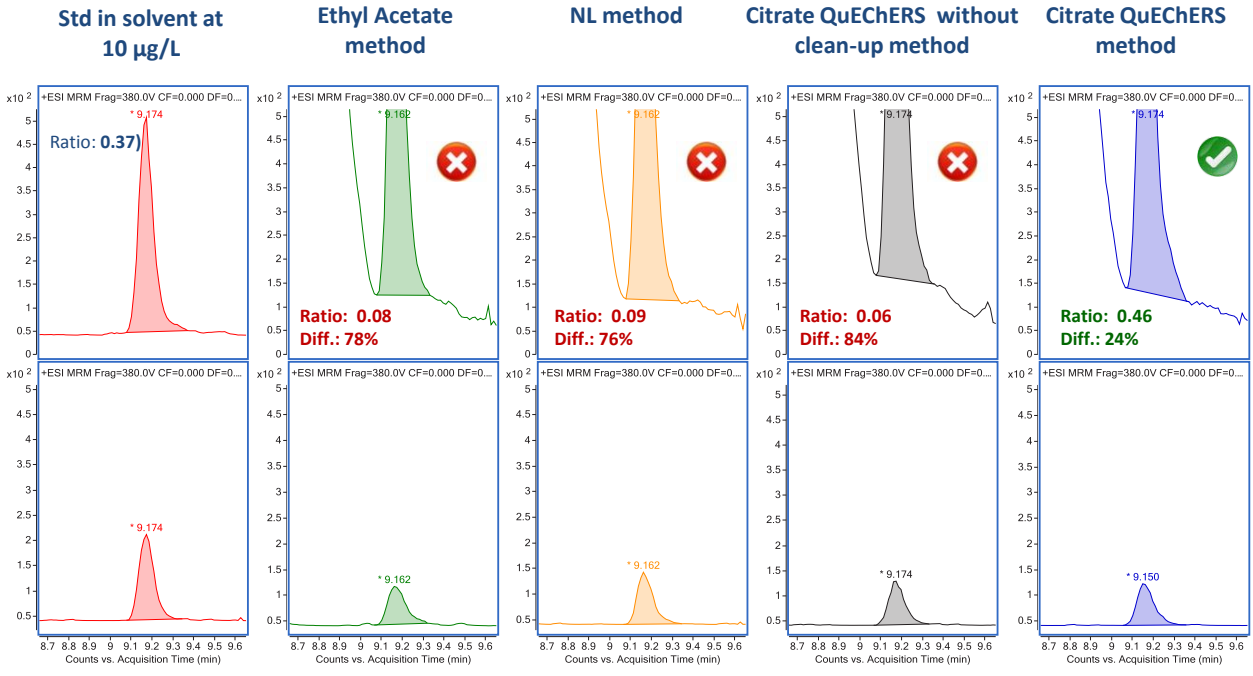
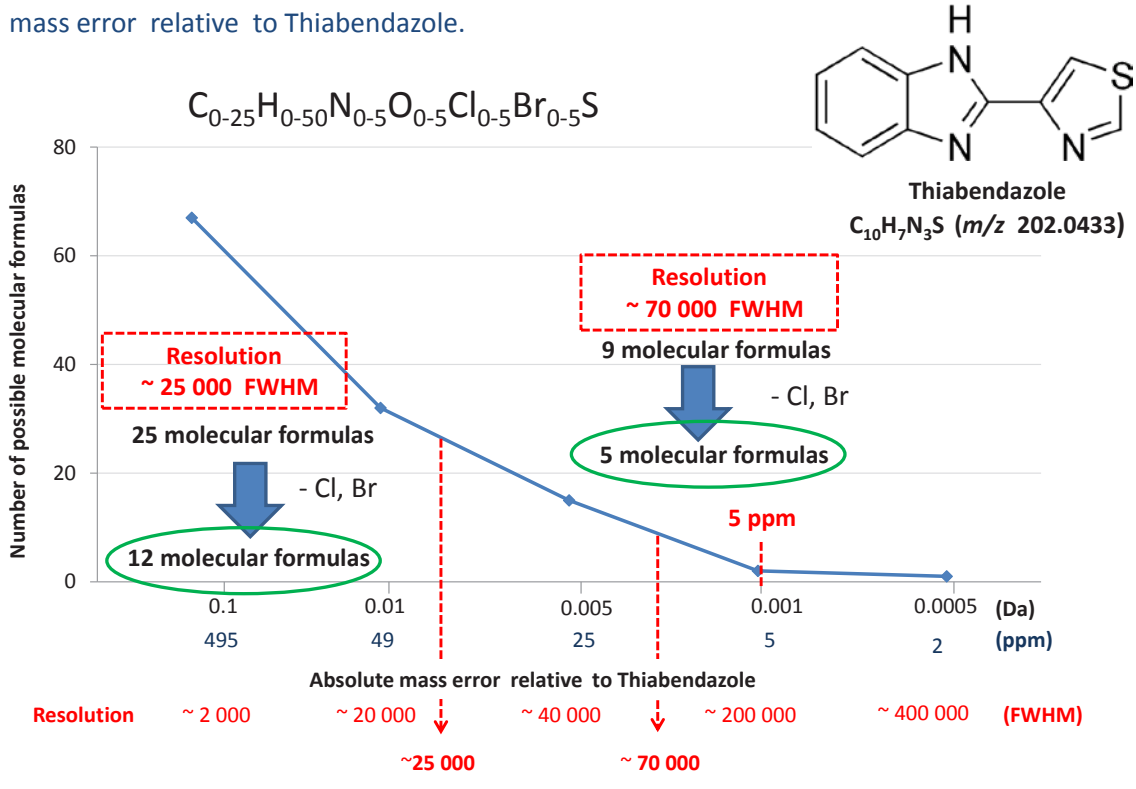


Figure 2: Plot of the number of possible molecular formulas against absolute mass error relative to Thiabendazole.



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 a resolution of 70,000, we obtain 5 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.

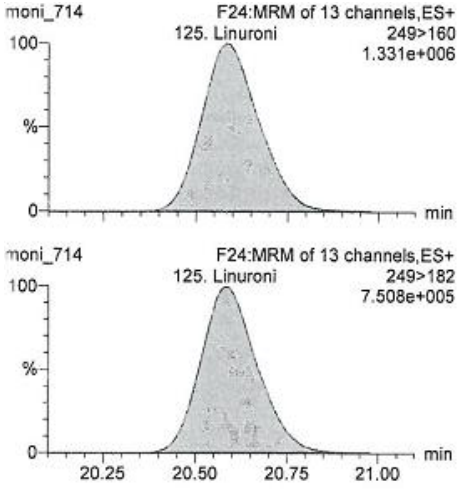
This is further demonstrated by the example of the determination of linuron in coriander for which the nominal mass MS/MS ion ratio in the sample is very different from the ratio obtained in the solvent standard, as shown in **Figure 3**. Fortunately, using a mass resolution of 70,000, the linuron ion 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.

Q Exactive™ Focus™ hybrid quadrupole-Orbitrap Mass Spectrometer

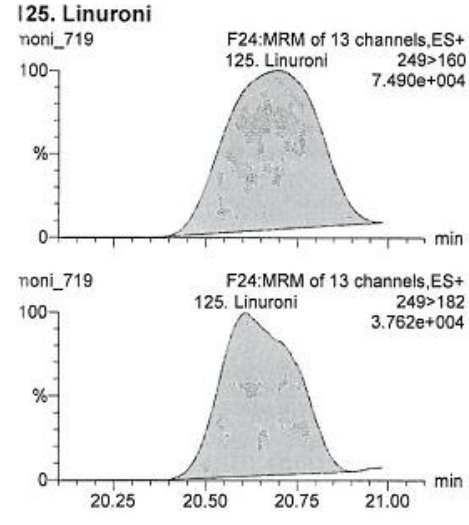
When we are analyzing pesticides we have to fulfill two criteria for detection. The first criterion is retention time and the second one is mass error. It is our experience that for positive identification, the mass error has to be lower than 5 ppm. Fortunately, using the Thermo Scientific™ Q Exactive™ 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 & 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 which were not spiked. In all 3 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

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.



Linuron
Standard in solvent
Ion ratio: 1.8



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

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

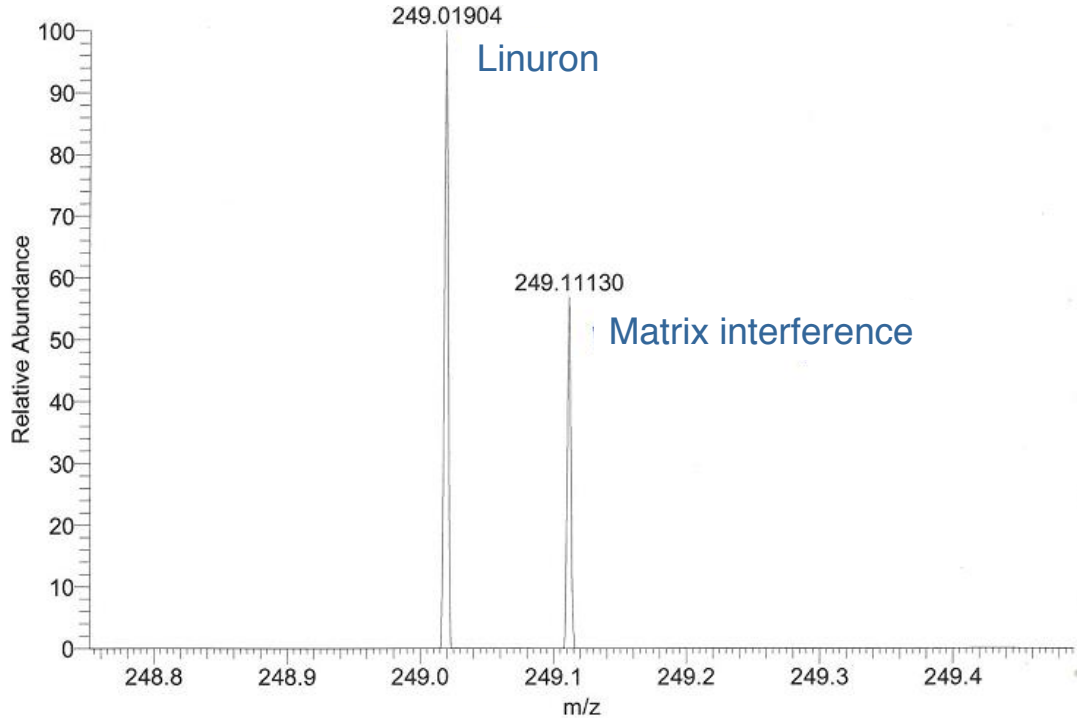


Figure 5: Mass errors in full scan MS mode are below 2 ppm, even for orange, which is considered a difficult sample matrix containing a high number of co-extracted compounds.

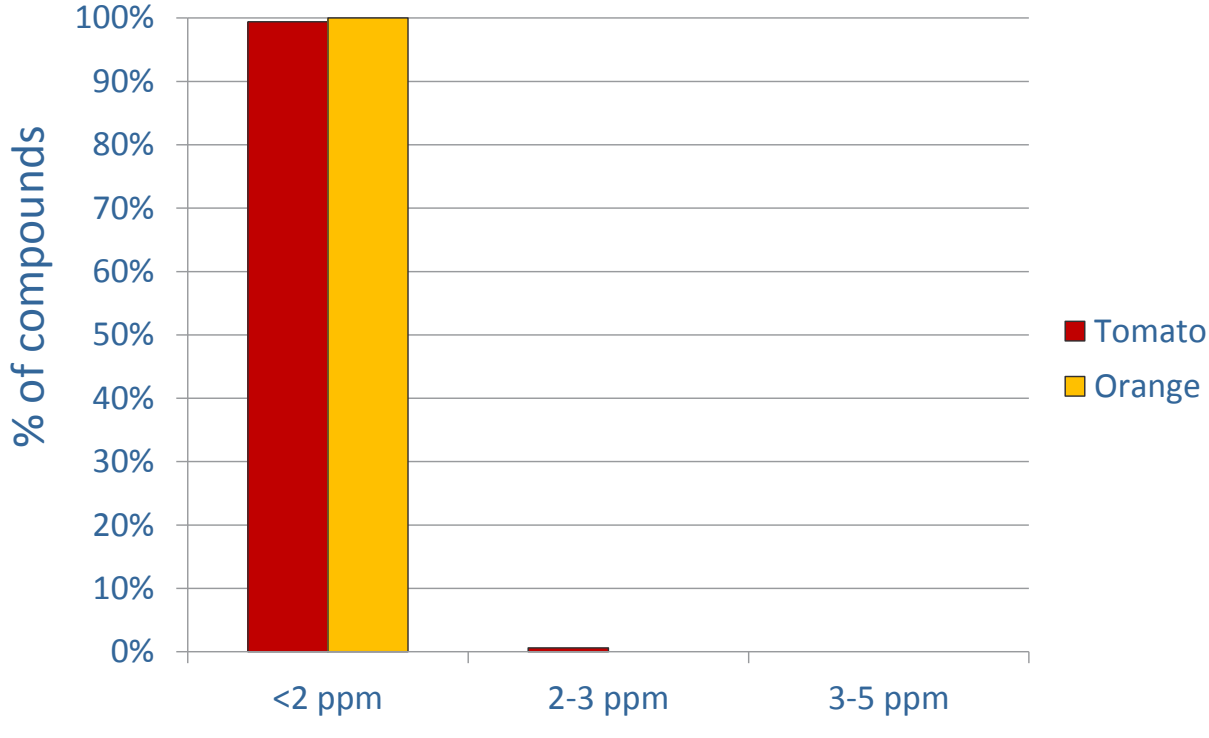


Figure 6: Determination of the fungacide, Metalaxyl-M in pepper and grapefruit in full mass scan mode.

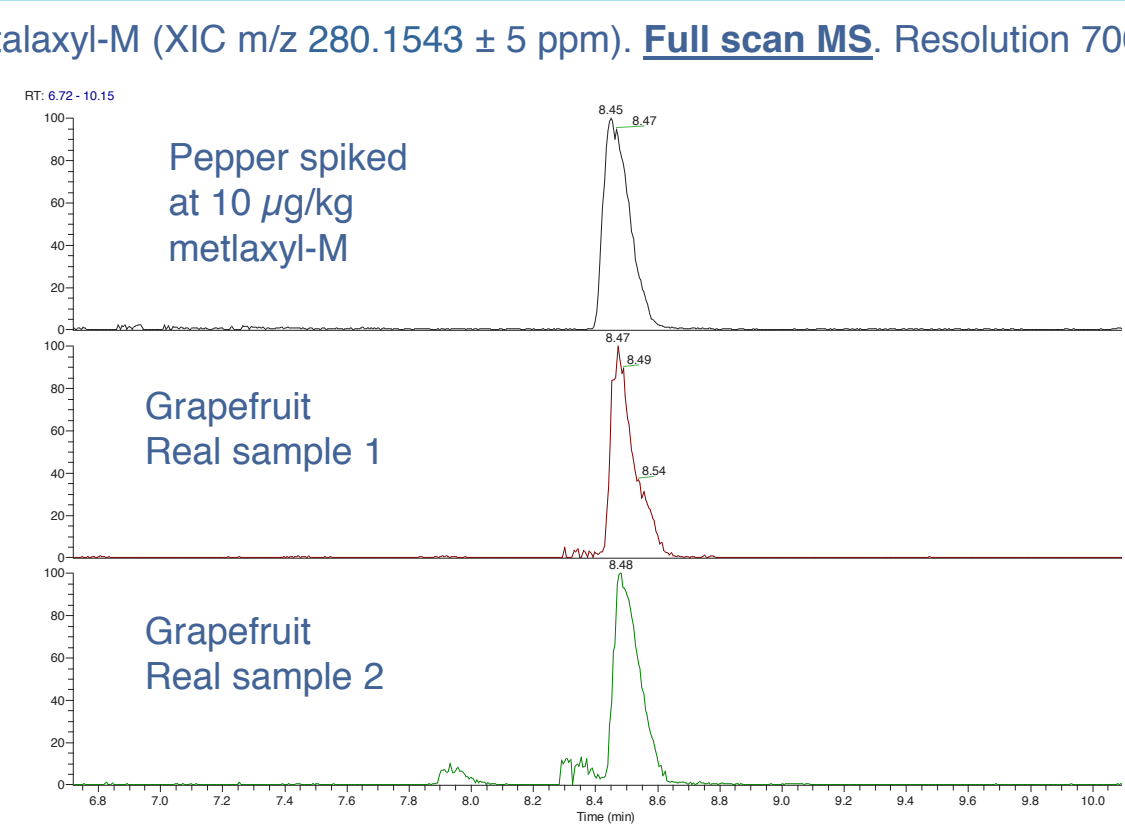
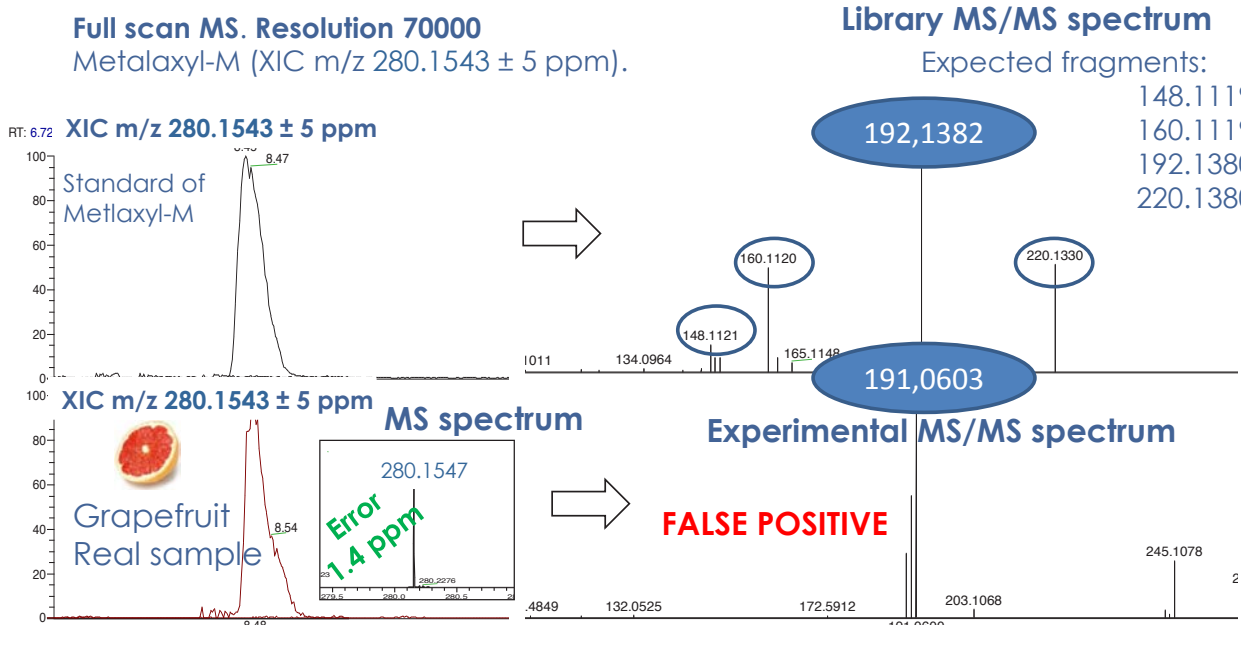


Figure 7: Fragments of Metalaxyl-M confirm its presence in the standard, but no fragments in the grapefruit sample show the full mass scan gave a false positive for the compound.



time as metalaxyl-M in the standard. An evaluation of the MS2 data in **Figure 7**, show 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 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) 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 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

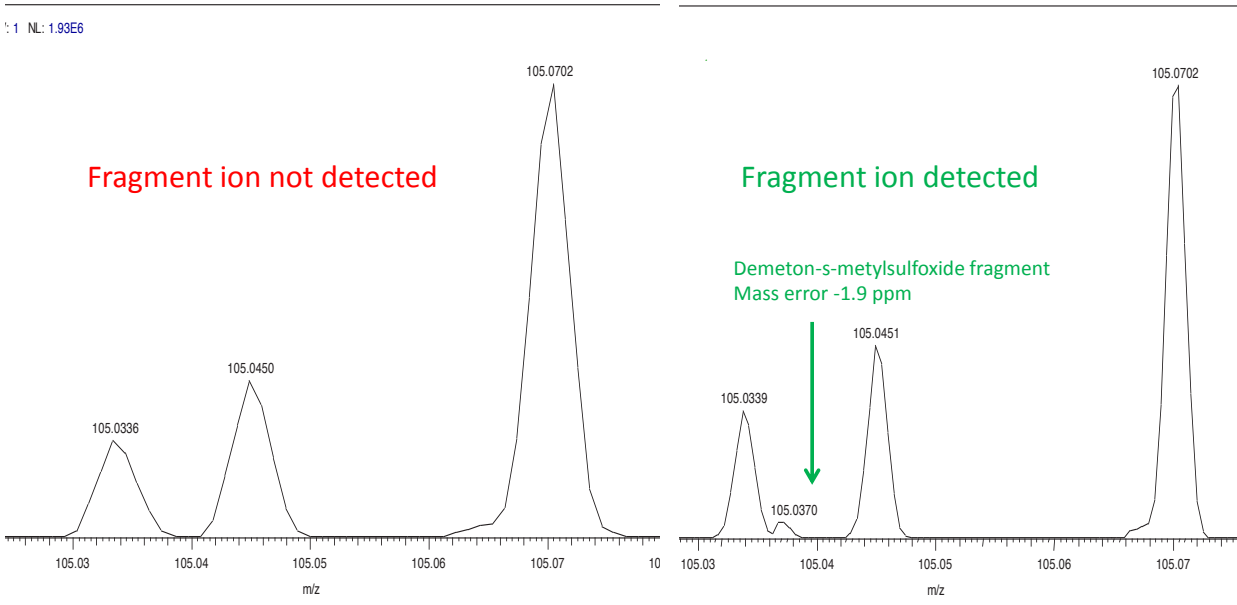
For this evaluation we selected 11 representative matrices of different kinds of fruit and vegetables. Some of them were very straightforward such as tomato, apple and cucumber, but we also selected very complex matrices like orange, leek and onion. We spiked the fruit and vegetable extracts with 166 pesticides at two concentrations – 100 and 10 µg/kg. We obtained almost 2,000 results at each spiking concentration, for each of the 3 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 percent were identified. The compounds which 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 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 3 and 5 segments respectively at a resolution of 35,000. We can see that in the case of the 3 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 sample contained co-extractives with mass peaks between 120 and 195 Daltons, which produced fragment ions with the same mass as dodine.

† vDIA is not available in the U.S.

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 MS2 spectrum
3 mass segments with resolution of 17,500

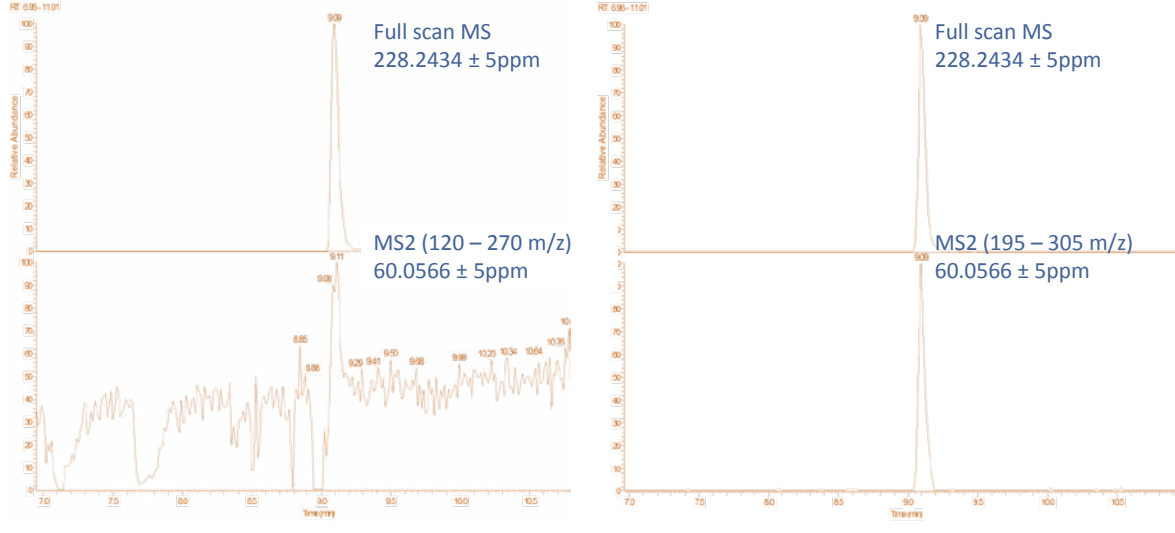
vDIA MS2 spectrum
3 mass segments with resolution of 35,000

vDIA is not available in the U.S.

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.

0.01 mg/kg of dodine in orange extract

Resolution 70,000



3 mass segments

Resolution 35,000

5 mass segments

vDIA is not available in the U.S.

In another example (propargite in leek) 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. As well as 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 is demonstrated by **Figure 12**, which shows that linearity up to 1.6 ppm for 3 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 which 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 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

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

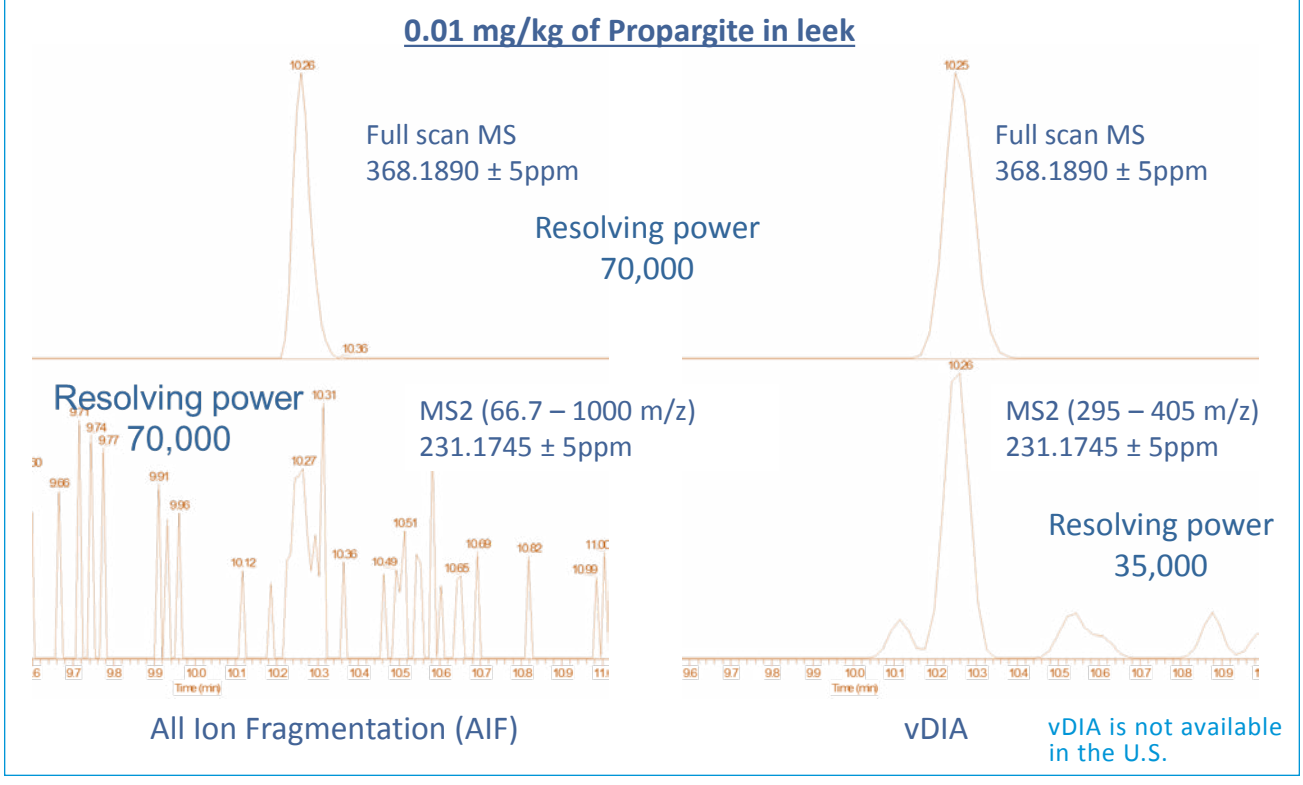


Figure 11: Mass errors in MS2 mode are below 2 ppm for 70% of compounds extracted using three different workflow techniques.

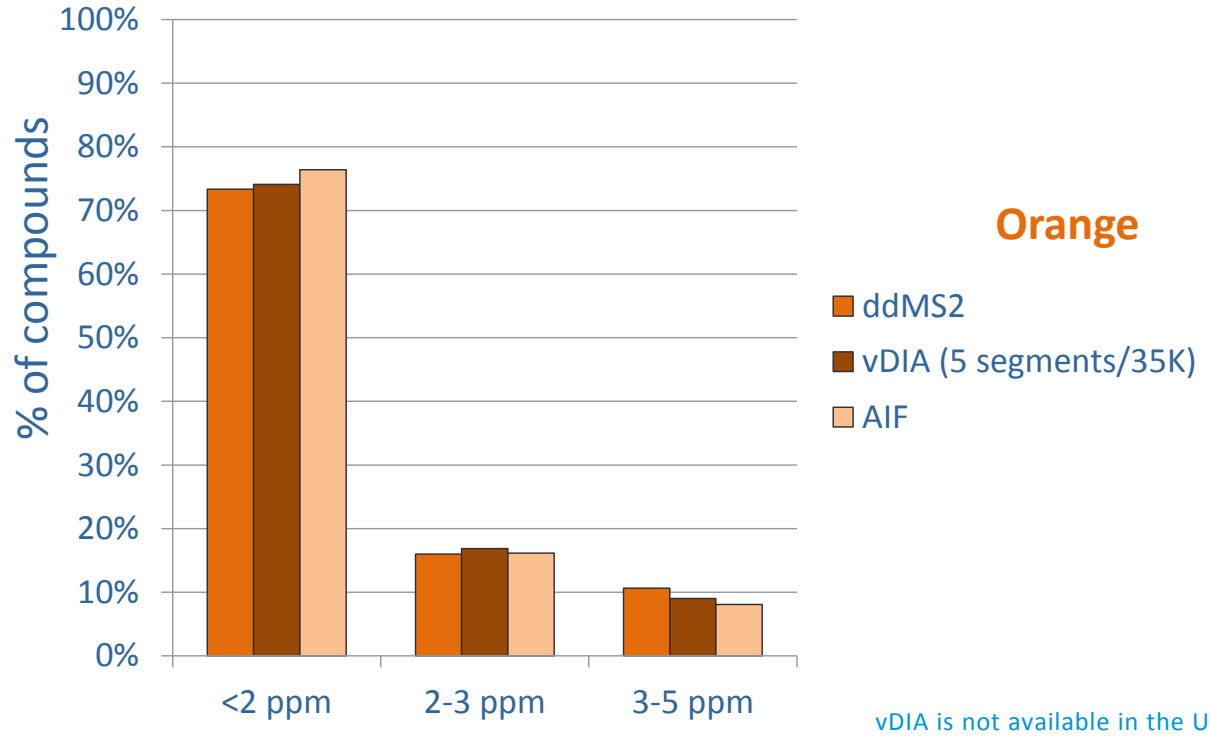
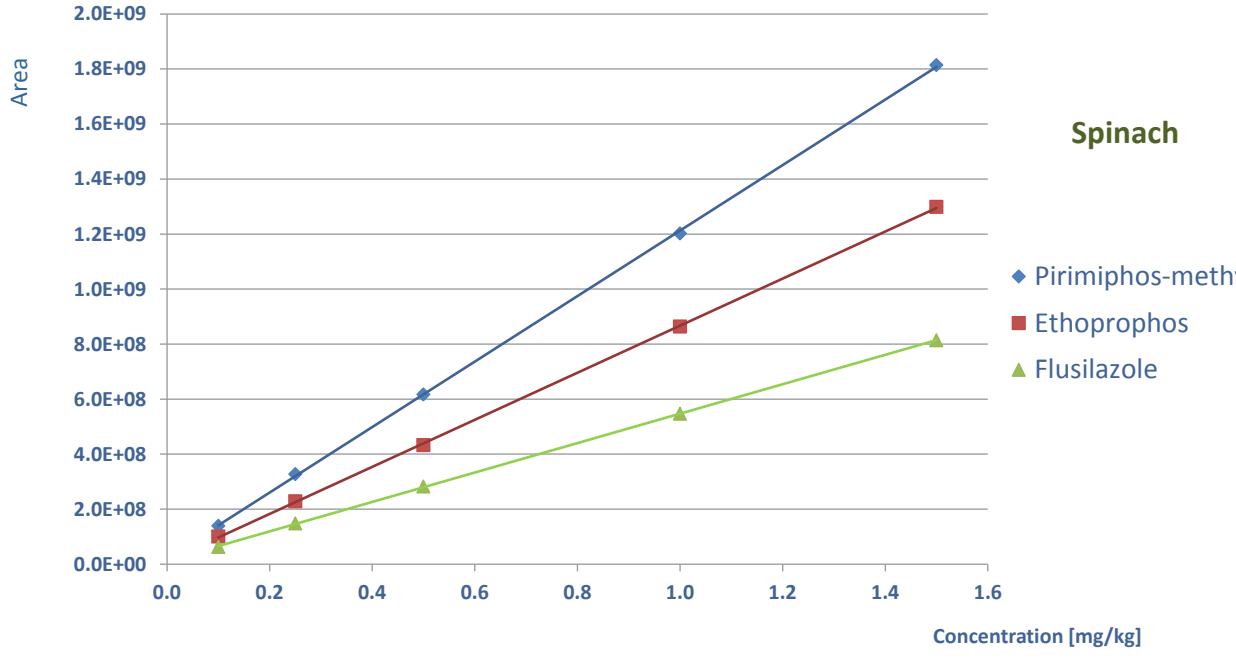


Figure 12: Linearity for three pesticides in spinach by dd-MS2.



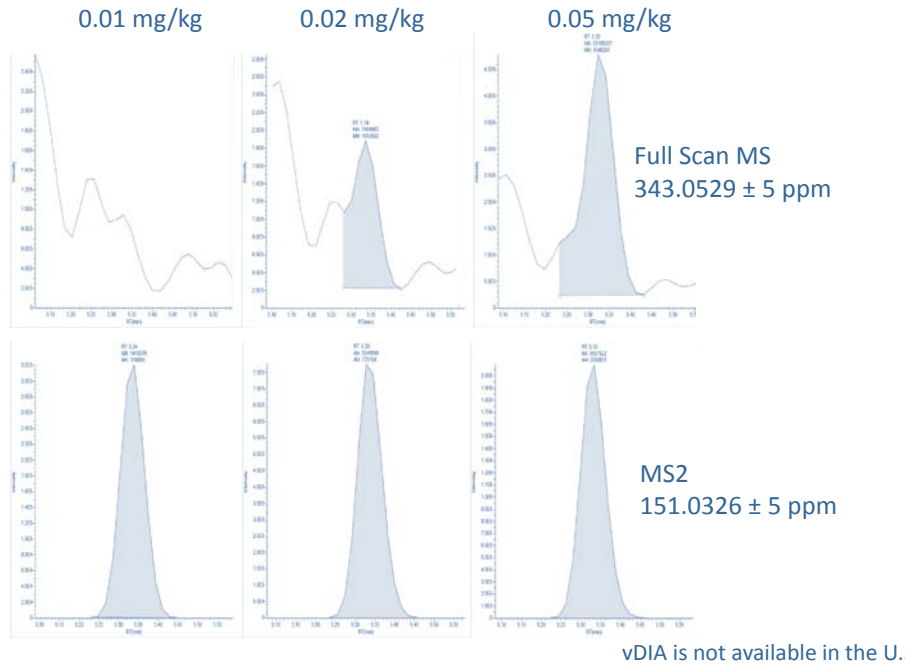
used for quantitation. This is shown in the lower scans in **Figure 13**, where we see peaks free from the interferences because the compounds present in the onion extract do not produce the same fragments as thiophanate methyl.

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

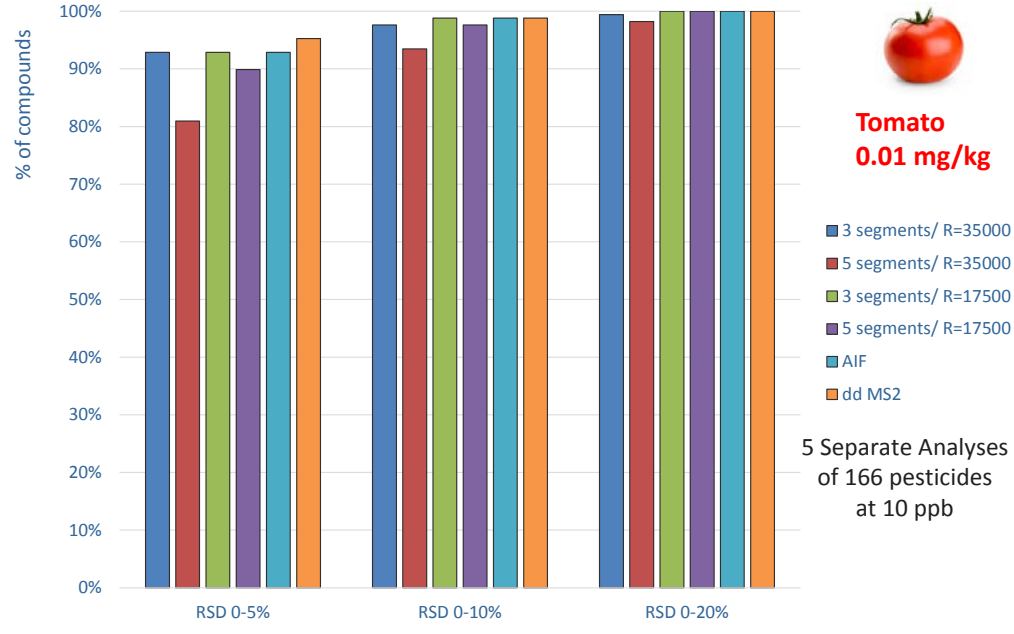
Another very important parameter of quantitative analysis is peak area repeatability of the molecular ion (not the fragment ions). In general we want to obtain precision below 20 %. The histogram shown in **Figure 14**, illustrates the results obtained for a tomato extract spiked with 166 different pesticides at 10 µg/kg, and analyzed using dd-MS2, AIF, and vDIA using four different settings. Almost 100% of

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



vDIA is not available in the U.S.

Figure 14: Comparison of measurement repeatability between different workflows.



vDIA is not available in the U.S.

the pesticides are below 20% RSD. However, if we look 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 3 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.

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 data bases to possibly detect new compounds of interest. We can not only detect compounds, we can also identify those detected compounds using their fragmentation products, because we previously obtained fragments from all compounds present in the sample.

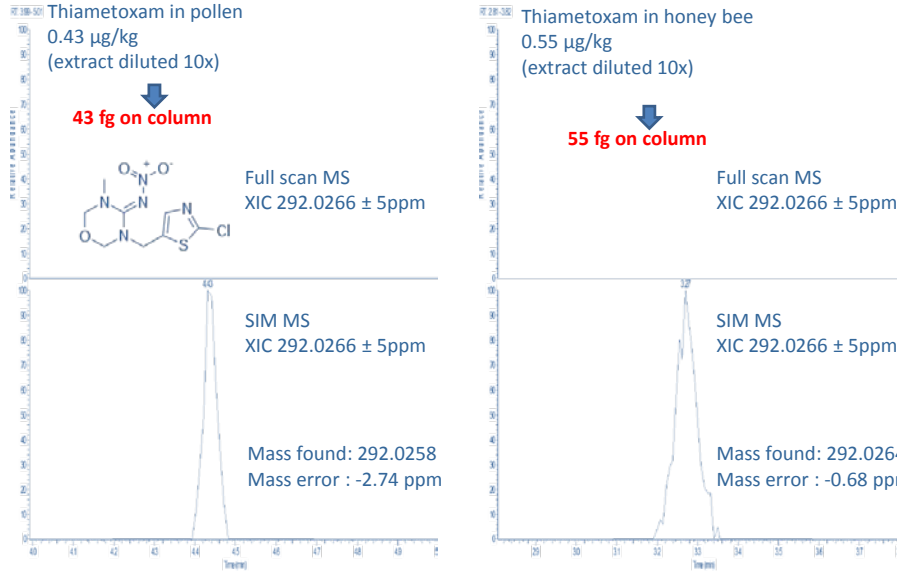
Another very interesting application is operation of the Q Exactive Focus instrument in selected ion monitoring (SIM mode) for the analysis of analytes at very low concentrations. In our experience, SIM mode is 5-10 times more sensitive than full mass scan mode, as demonstrated by the detection of the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

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.

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



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 percent 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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