New LC-MS Approaches to Overcome Analytical Difficulties Deriving from the Application of EU Pesticide Regulations for Fruits and Vegetables

The effectiveness of EU enforcement and the application of risk assessment under Regulations (EC) No: 396/2005, 882/2004, 7882/2012 etc. relies, to a large extent, on adequate performance on the part of the food control laboratories.

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A number of conflict issues between these regulations and their application in routine laboratories have been highlighted by our experience as a European Union Reference Laboratory. Some of these issues have been satisfactorily resolved but others remain significant analytical challenges. This article is focused on some of those analytical concerns that still seriously challenge the laboratories as well as the importance of using new mass spectrometry instrumentation to overcome them. Three of these issues have been selected for commentary in this article: sensitivity, matrix effect and analytical scope.

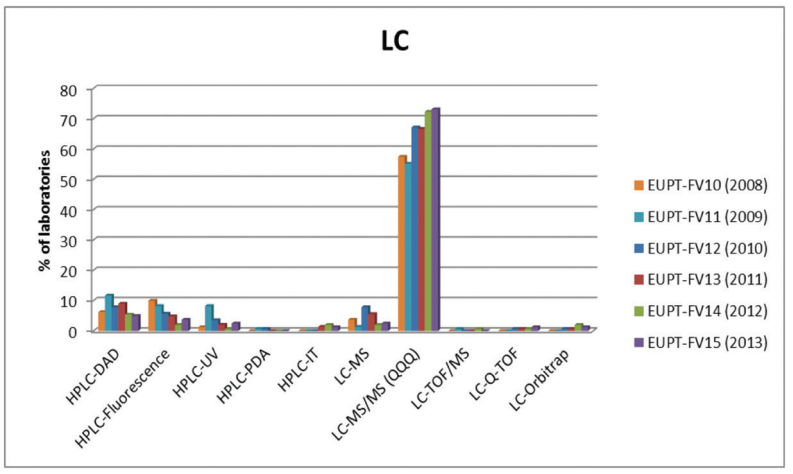

Figure 1: Overview of the analytical techniques (data reported) used by the EU official laboratories 2008-2013

Sensitivity

The abundance of data obtained from organising the European Union Proficiency Tests for Pesticides in Fruits and Vegetables -EUPTs- (www.eurl-pesticides.eu) has, over recent years, revealed difficulties that typically appear in food control laboratories.

In Figure 1, we collected information regarding the analytical techniques employed by official laboratories from EUPT-FV10 (2008) to EUPT-FV15 (2013). As is shown, when liquid chromatography is used, triple quadrupole is the analyzer of choice. This is as a consequence of its excellent quantification and identification properties for a group of target compounds.

Nonetheless, the evolution and improvement of this technology over these years has been very important, mainly as a consequence of the sensitivity obtained being dependent on using different levels of upgrade. Considering specific cases, achieving low quantification limits is not possible in all commodities simply by applying the common extraction procedures. Bearing in mind some of the restrictive legislation, such as the baby food legislation (Regulation (EC) Nº 125/2006) - where, as an example, the Maximum Residue Limit for fipronil in baby food is 4 $\mu g \cdot kg^{-1}$ - this very low level is considerably challenging. It is in such cases where highly sensitive instruments come into their own and significantly help to achieve this.

In Figure 2, the identification of fipronil is shown at 4 $\mu g \cdot kg^{-1}$. In this example, the advantage of using an upgraded system in order to meet with current legislation is significant.

The HPLC system used was the 1200 series from Agilent Technologies, equipped with an Eclipse XDB C8 analytical column of 150×4.6mm; and a 5 µm particle size.

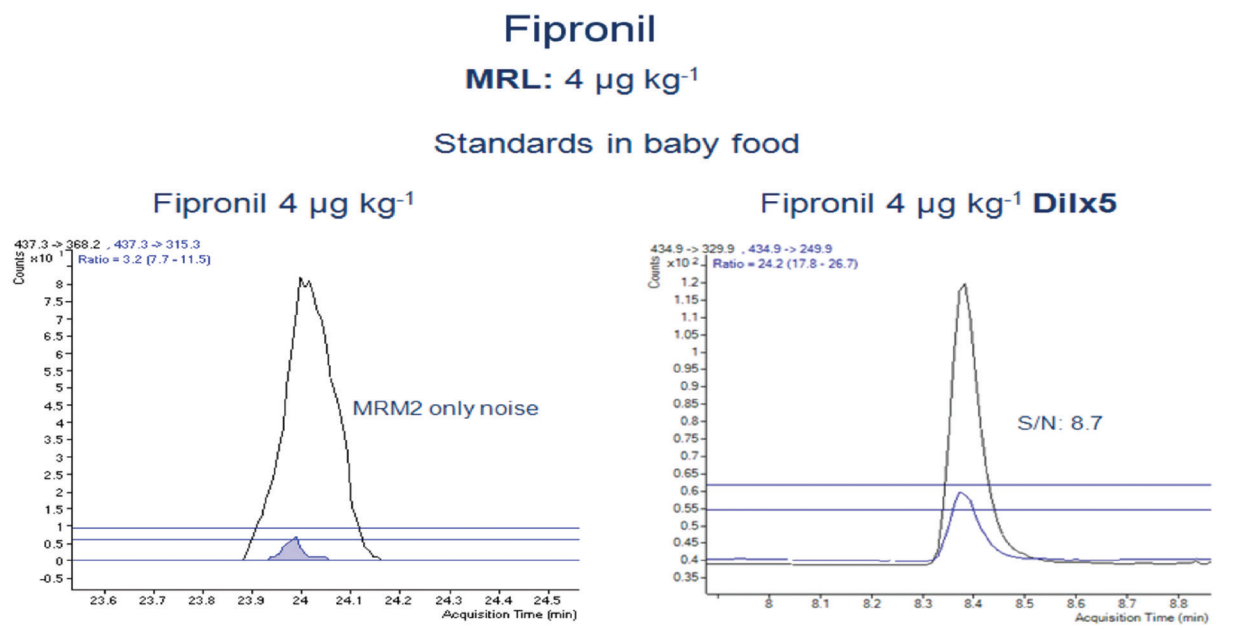

Figure 2: HPLC-MS/MS chromatograms of fipronil at 4µg·kg-1 in fruit-based baby food using: a) a 6410 Agilent LC-QQQ-MS/MS system and b) a 6490 Agilent LC-QQQ-MS/MS system.
The HPLC system used was the 1200 series from Agilent Technologies, equipped with an Eclipse XDB C8 analytical column of 150×4.6mm; and a 5 µm particle size

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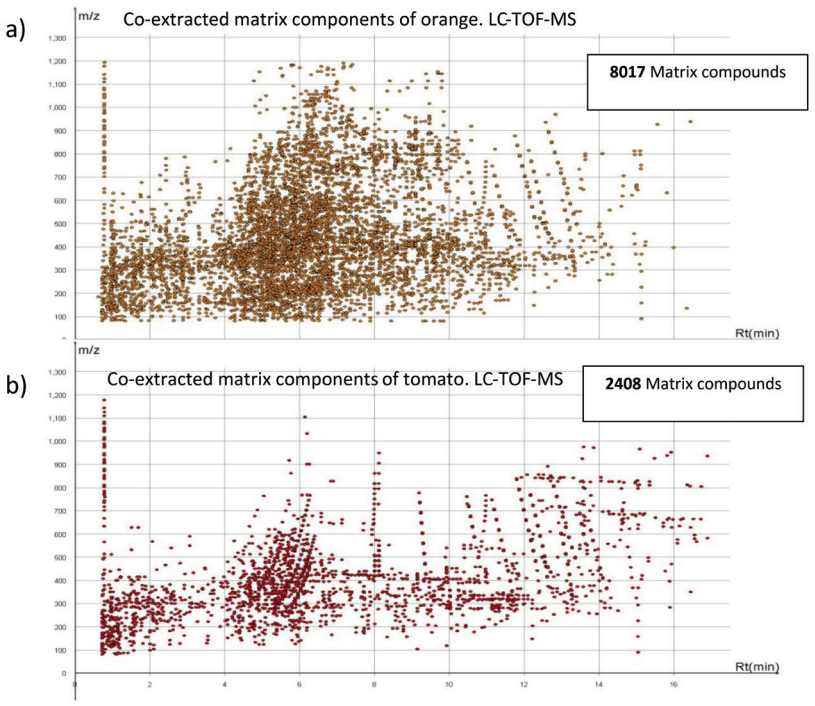

Figure 3: Matrix compounds of orange (a) and tomato (b) extracts analyzed using a 6530 Agilent LC-QTOF-MS. The HPLC system used was the 1200 series from Agilent Technologies, equipped with a XDB-C18 analytical column of 4.6 mm × 50 mm and 1.8 µm particle size

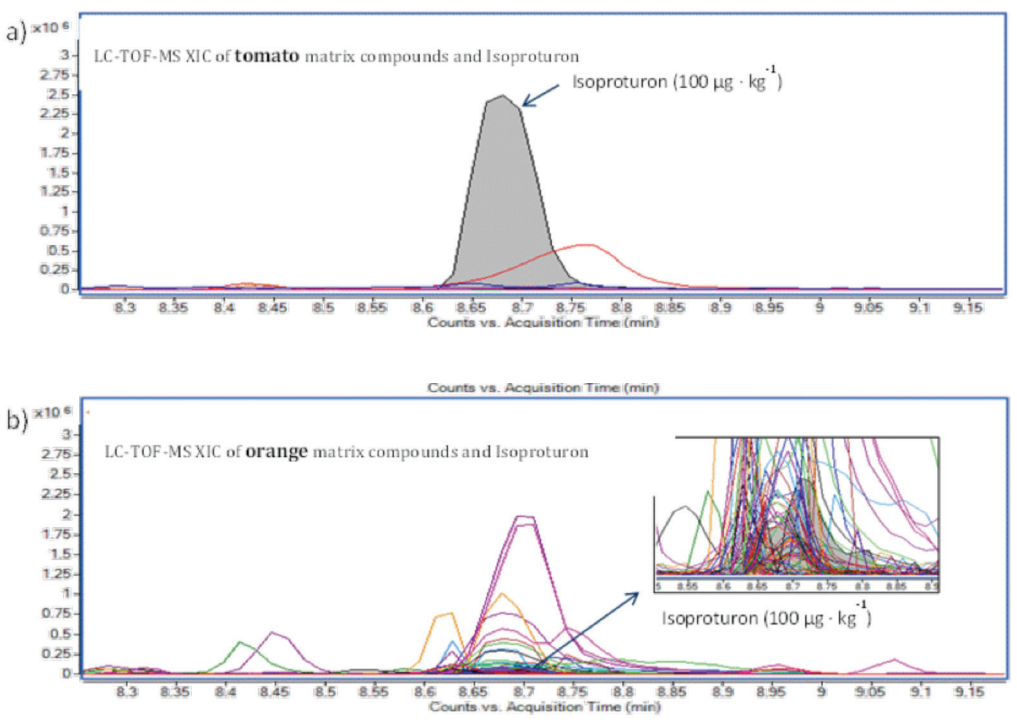

Figure 4: Extracted ion chromatogram of Isoproturon (m/z 207.1492) and co-eluting matrix compounds in tomato (a) and orange (b). Analyzed by a 6530 Agilent LC-QTOF-MS. The HPLC system used was the 1200 series from Agilent Technologies, equipped with a XDB-C18 analytical column of 4.6 mm × 50 mm and a 1.8 µm particle size

Matrix Effects

Over recent years, matrix effects in liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) have become of increasing concern in food analysis. The ion suppression phenomenon can lead to errors in the quantification of the analytes of interest, as well as affect detection capability, precision, and method accuracy.

Matrix effects occur because of co-eluting compounds interacting with the analytes in the ionization process, producing, in some cases, signal suppression. This is as a consequence of the great competition between different compounds (matrix/analytes) to become ionized from the surface of the ESI droplets. Thus, the complexity of certain matrices can significantly affect ionization efficiency and, therefore, the sensitivity of the analyzed compounds. The number and distribution of interfering matrix components varies greatly depending on the particular vegetable matrix; even sometimes those included in the same commodity category. In Figure 3, all the matrix components of two matrices, orange and tomato, are represented – these are considered to be a "difficult" and an "easy" matrix, respectively.

The number of co-extracted matrix compounds is clearly higher in the orange extract (8,017 compounds) compared to the tomato (2,408 compounds). As a result, the target analyte response is expected to be worse in orange than in cleaner matrices such as tomato. As an example, Figure 4 shows the difference in sensitivity of the pesticide Isoproturon analyzed in either orange or tomato. In orange, due to the large number of co-eluting matrix compounds, the sensitivity of the pesticide is much lower than in tomato, with a signal suppression of more than 80 %.

Consequently, depending on the pesticide and the matrix being analyzed, detection problems (false negatives) or different responses may occur.

Sample dilution is an easy and effective method in reducing interfering compounds and, therefore, to diminish matrix effects. A sample dilution decreases the number of these competing molecules per micro droplet, the ionization efficiency increases and, thus, the analyte signal also increases; this strategy to overcome the matrix effect, if only partially, has gone hand-in-hand with the instrumentation development.

The application of micro-liquid chromatography can provide very high sensitivity allowing greater matrix dilution (20-50 times dilution) decreasing signal suppression caused by matrix effects. The implementation of this technique is a sound alternative to using highly sensitive mass spectrometers. New, commercially-available narrow columns and micro pumps facilitate the effectiveness of its application in routine work. This instrumentation has been evaluated by a µLC-MS/MS method validation for fruits and vegetables.

Figure 5 shows the signals for the pesticide propiconazole in tomato, pepper and orange at 10 µg·kg^{-1}, diluted 30 times. As is observed, the signals are very similar in the three matrices.

With a 30-times dilution factor, the slope obtained for the calibration curves in tomato, pepper and orange in the linear range from 5 to 500 µg·kg^{-1} was calculated; and then compared with the slope in solvent so as to study the matrix effect. The results are shown in Figure 7 - in general, signal suppression was observed. The behaviour of the compounds has been divided into three groups, those compounds with less than 20% ion suppression, those with 20% to 50% ion suppression and those with more than 50 % ion suppression.

As we can see in the figure, even in orange, which is considered a "difficult" matrix, less than 10 % of pesticides showed a matrix effect higher than 50% - with no dilution this effect would have reached far higher values.

Sample dilution has proven to be very advantageous, given that it is simple to implement and highly effective with regard to diminishing signal suppression.

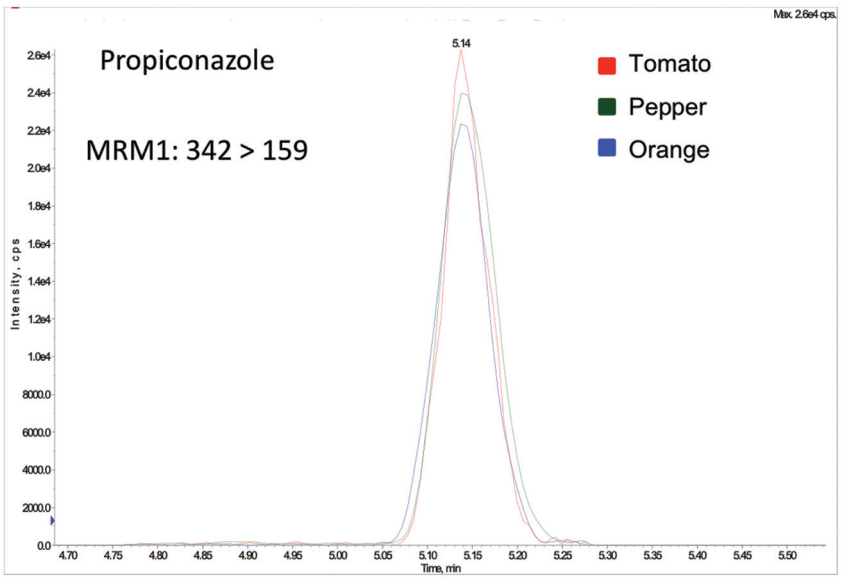

Figure 5: Propiconazole at 10 µg·kg^{-1}, diluted 30 times, in three different matrices analyzed by a 4500 AB SCIEX QTRAP LC-MS/MS system. The HPLC system used was the Eksigent ekspert™ microLC 200, equipped with a Halo C18 column, 0.5 x 50 mm, and a 2.7µm particle size at 30 °C (Eksigent, AB SCIEX Instruments, Foster City, CA)

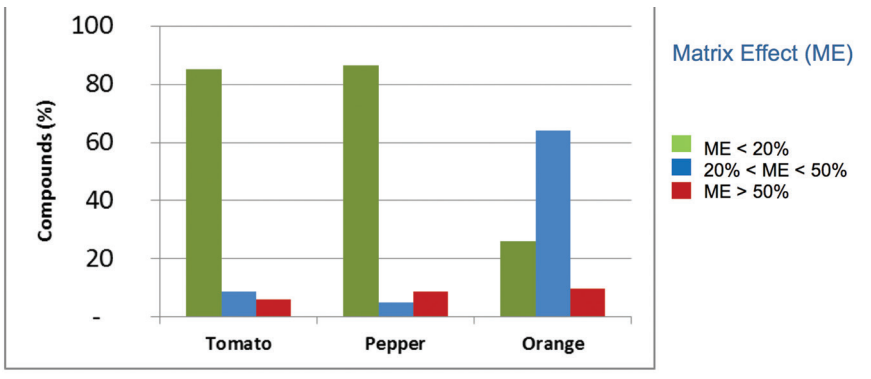

Figure 6: Percentage of compounds which showed a matrix effect in tomato, pepper and orange

Analytical scope

The extension of the analytical scope to a large number of compounds (e.g. 300) is necessary for a proper evaluation of pesticide residue content in food. However, the high work load necessary to develop a fully-validated multiresidue method with a wide scope using LC-QQQ-MS/MS poses a considerable challenge to control laboratories.

Screening methods applied to low frequency compounds, which have fewer validation procedure requirements, can facilitate that extension. Full scan screening methods represent a promising approach, given the introduction of new LC-HRMS technologies based on high resolution mass spectrometry and mass accuracy. Mass accuracy single ion detection, typically the (de)protonated molecule with low mass errors (e.g. 5 ppm), and operating at higher resolution values (e.g. 50,000) permit rapid, adequate screening of a large number of compounds.

A combination of both LC-QQQ and HR-MS in routine food laboratories can cover the main legal requirements with regard to pesticide residues, enlarging the analytical scope in a very cost-effective way while avoiding false positives and negatives;

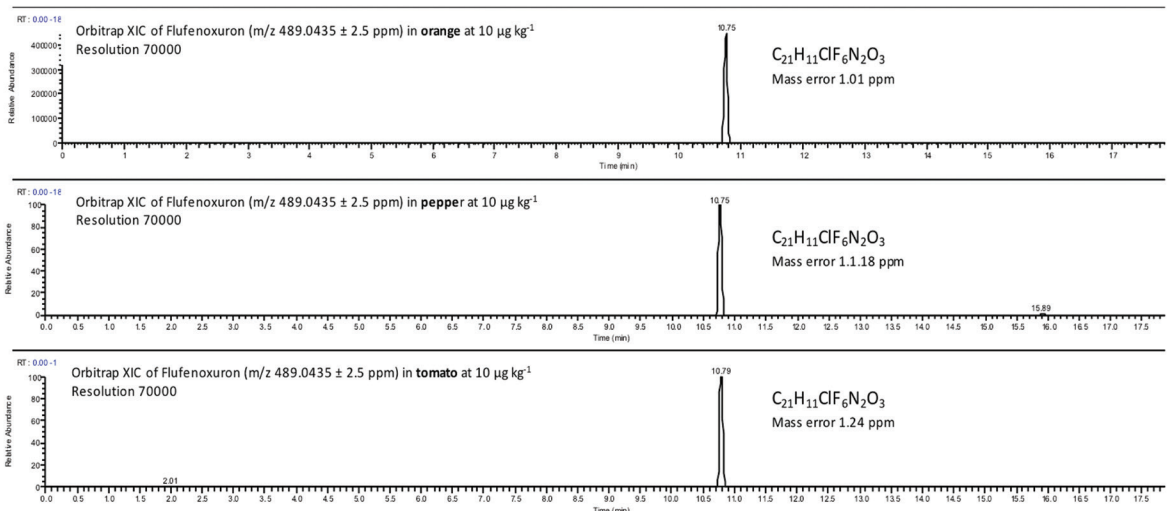

Figure 7: Detection of pesticide flufenoxuron at 10 µg·kg^{-1}in tomato, pepper and orange. Resolution 70000, mass tolerance of 5 ppm. Analyzed by Orbitrap QExactive (ThermoFisher Scientific, Bremen, Germany) mass spectrometer. The UHPLC system used for these tests was the Dionex Ultimate 3000. It was equipped with an Thermo Accucore C18 column 150×2.1mm; 2.6 µm particle size held at 25 °C

and additionally allowing retrospective evaluation.

High resolution mass spectrometers operated in full scan mode can theoretically register an unlimited number of compounds. Optimisation of analysis parameters is far less laborious than with triple quadrupoles and retrospective analysis is also possible.

A good example of this is the application of an LC-Orbitrap-MS to the evaluation of pesticide residues in vegetable matrices.

Mass accurate results (e.g. 5ppm) with an increase in resolution improves mass measurements. It allows one to set a narrow mass extraction window and discard isobaric compounds present in the matrix. Consequently, the number of false positives decreases. Figure 7 presents 10 µg·kg^{-1}of flufenoxuron in tomato, pepper and orange. With a resolution of 17,500, an isobaric matrix compound with a similar retention time is present. Thanks to better mass resolution measurement of 70,000, only peak corresponding flufenoxuron appears in the different commodities evaluated. High resolution also can help to avoid false negatives and false positives. This is possible because with higher resolution, masses of analytes and matrix compounds are better separated, and as a consequence, fewer accurate mass errors occur. Analytical performance parameters such as linearity range, reproducibility and robustness makes these new technologies (HRMS) also adequate for quantification purposes. Furthermore simultaneous or sequential combination of HR-MS and HR-MS/MS can provide adequate identification standards.

Even when more information about the adequacy in implementing these new LC-MS advanced approaches they can clearly facilitate the overcoming of analytical difficulties in routine laboratories producing faster analysis in a more cost effective way.