Recent trends in liquid chromatography-tandem mass spectrometry to determine pesticides and their metabolites in food

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The applications of liquid chromatography-mass spectrometry (LC-MS) have exploded in innumerable analytical fields, including pesticide-residue determination. There is no doubt that LC-MS is currently competing with gas chromatography (GC)-MS for the status of 'reference' analytical technique to determine pesticide residues and that its ever-increasing application is bound to the evolution of modern instruments and their growing performance qualities. We highlight the large number and variety of pesticides that can be readily determined using such instruments, the respective merits of the different mass analyzers, and the improvements brought about by tandem MS (MS^2). We also discuss new applications of LC-MS in this field, such as "non-target" screening procedures and unknown identification by MS or MS^n presenting the different solutions proposed to solve this challenging problem. Finally, we address the opportunities afforded by the most recent instrument designs.

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1. Introduction

Pesticide residues in food continue to be the target of many studies due to the uncertainty about the adverse effects that those residues may have after a lengthy exposure at low doses. More than 1000 active ingredients have been employed and are currently formulated in thousands of different commercial products. These residues comprise a variety of compounds, mainly insecticides, herbicides and fungicides, as well as their metabolites, with very different physico-chemical characteristics and large differences in polarity, volatility and persistence [1,2]

Since the early 1970s, most routine pesticide-residue determination has been conducted by gas chromatography (GC) with different detectors, including mass spectrometry (MS). However, times change and there is now a clear trend to increase the number of liquid chromatography (LC) applications in pesticide-residue analysis, both in specific/individual and in multi-residue methods, especially after the introduction into the market of robust, easily operated LC-MS instruments that provide a new way of analyzing pesticides more sensitively and efficiently, and with greater scope [2–5]. In recent years, different reviews published, which cover totally or in part the subject of the analysis of pesticide residues in food by LC-MS or LC-tandem MS ($LC\text{-}MS^2$), document this movement from GC to LC [2–8].

This trend is closely bound to the evolution of modern LC-MS instruments and the improvements in the quality of their performance. MS research is focused mainly on expanding the implementation of mass analyzers instead of on ionization sources, as it was 10 years ago [2,6,9–12]. The sensitivity and specificity of pesticide analysis in food matrices have advanced by combining different designs of analyzer in order to enlarge versatility and to increase application of MS^2 (triple quadrupole (QqQ), quadrupole ion trap (QIT), quadrupole linear ion-trap (QLIT),

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quadrupole time-of-flight (QqTOF), ion-trap time-of-flight (IT-TOF)) [2,7,13–16].

These LC-MS instruments are well suited to solve difficult problems of identification, quantification and confirmation of pesticides and their metabolites. The selection of the mass analyzer, apart from its accessibility, is determined by the required sensitivity and selectivity and the general objective, taking into account their complementarity [16]. As a result of all these innovations, rather interesting advances and practical applications in pesticide-residue determination are available for a wide range of compound classes and matrices [4,15]. However, there is no comprehensive information about the relationship between widening the scope of these analyses and instrument developments.

This article reviews typical applications of the most common LC-MS^n mass analyzers used in pesticide-residue laboratories today, summarizing recent trends in this field. We highlight the large number and variety of pesticides that can be readily determined using such instruments, the respective merits of the different mass analyzers, and the improvements brought about by MS^2. We discuss new applications of LC-MS in this field, such as ''non-target'' screening procedures and unknown identification by MS or MS^2, and present different solutions proposed to solve this challenging problem. Finally, we address the opportunities afforded by the most recent instrument designs.

2. Opportunities

The aims of pesticide residue determination in food are related to quality enhancement in the agricultural production and governmental scrutiny of food safety. These analysis are required for the following reasons:

(i) to verify that no unexpected residues are occurring;
(ii) to check that statutory maximum residue levels (MRLs) are not exceeded;
(iii) to carry out enforcement programs; and,
(iv) to ensure that human dietary intakes of residues are at acceptable levels [5,10].

Analytical methods employed must be rapid and capable of screening for and identifying pesticides accurately to fulfill the aims of these analyses [2,3,5]. Both characteristics are quite contradictory – it is difficult to have both rapidity and accuracy in the same method [5]. However, the high specificity of MS^2 detection is expected to eliminate most of the problems arising from the presence of endogenous compounds, so reducing method-development time significantly [2,10]. We discuss below in detail how the application of LC-MS^2 offers outstanding possibilities to combine speed with accuracy by:

(i) simplifying sample preparation;
(ii) simultaneously screening and quantifying an increased number of target pesticides; and,
(iii) searching for non-target pesticides and unknown compounds in the same extracts.

2.1. Simplification of the extraction procedure

One analytical challenge involved in pesticide-residue analysis is that reliable results should be presented as fast as possible – often within 24 hours – without damaging method characteristics, such as sensitivity, selectivity, precision and specificity [10]. Several sample pre-treatment steps are necessary between sampling and chromatography. This phase has traditionally been considered the limiting factor, or ''bottleneck'', because sample pre-treatment occupies more than 60% of the analyst's time [2,3,10]. Matrix components co-extracted with pesticides produce several additional signals in chromatograms that can lead to false-positive identifications. While such interferences are not odd if extracts of complex matrices (e.g., herbs or tea) are analyzed by GC-MS or by LC-MS using selected ion monitoring (SIM), this is not so when MS^2 is used [7,15,16]. For this reason, LC-MS^2 methods do not require either extensive clean-up or sophisticated chromatographic separation. Different molecules that share the same transition are found more rarely than molecules producing fragments of identical mass. As a consequence, peak identification is easier and faster in LC-MS^2 than in GC-MS or LC-MS [8,17,18].

As an image is said to be worth more than 1000 words, Fig. 1 compares the extraction schemes proposed by Mills et al. [19] in 1963 and the QuEChERS (quick, easy, cheap, effective, rugged and safe) method recently proposed by Anastassiades et al. [20]. The Mills method was the first important quantitative multi-residue GC method reported in the literature. At present, this multi-residue method is undoubtedly one of the most commonly used for analysis of pesticides in non-fatty food, considered official by AOAC for a number of pesticides (AOAC, 1984,1990), and it is widely applied in governmental monitoring programs to determine pesticide levels in food products and to trace possible residue tolerance-level excesses [2,3,10,21]. This method and many others similar usually consist of common steps, such as extraction, clean-up by liquid-liquid partitioning, adsorbent column chromatography and/or gel permeation chromatography, determination by GC and confirmation of identity, most often by MS. Typically, in addition to the many steps included, the method is restricted to extract non-polar pesticides, such as organochlorine and organophosphorus pesticides [21]. In contrast to these methods developed a long time ago, the principal objective of the multi-residue methods being developed now is to obtain methods for the extraction of a large number of different pesticides using

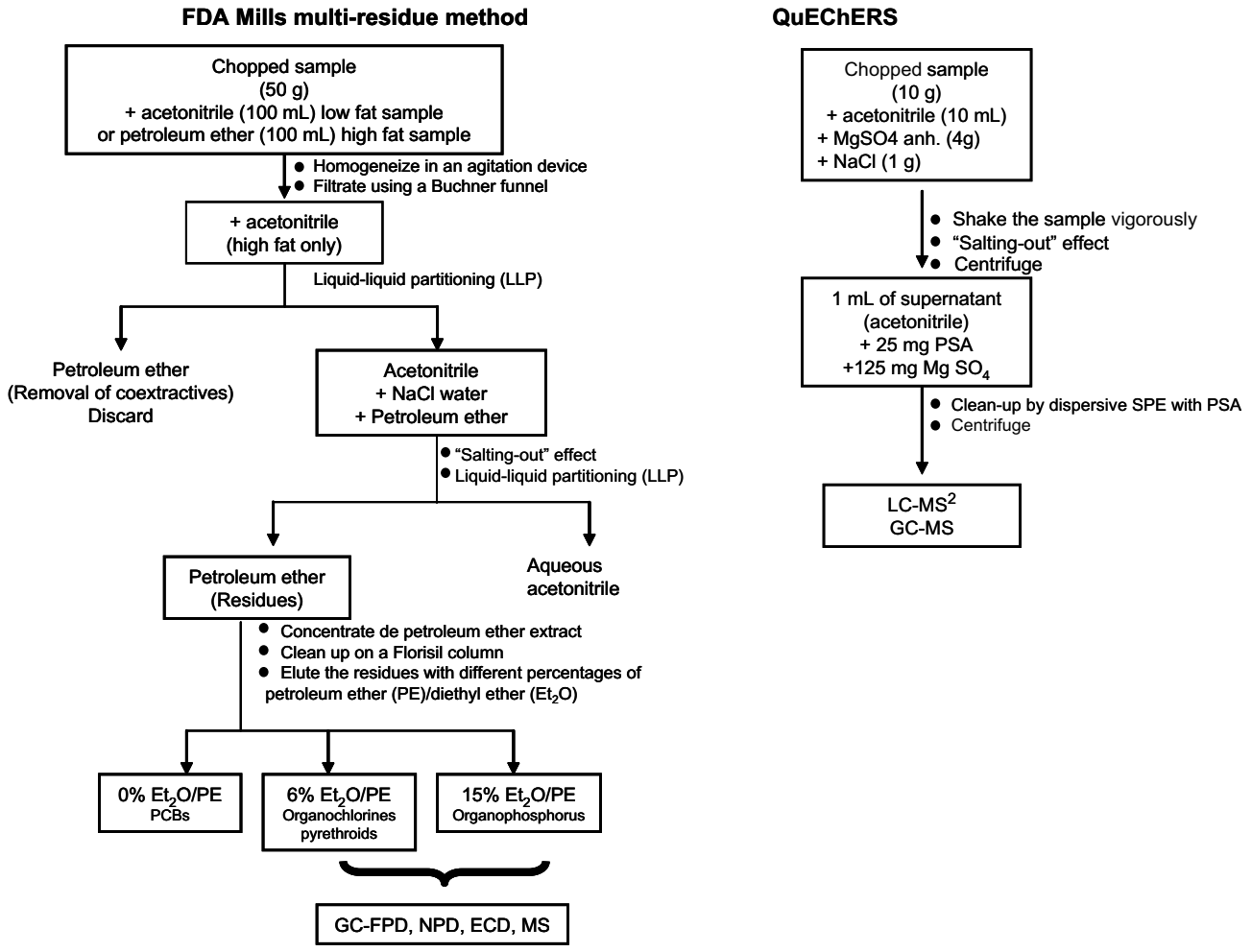

Figure 1. Scheme of the multi-residue methods developed by Mills et al. [19] and Anastassiades et al. [20].

a simple extraction scheme, preferably without the need for a clean-up step. The evolution of the extraction procedures has strongly reflected the need for simplification.

Solvent-extraction (SE) methods, such as that previously mentioned, have largely been employed with satisfactory results [22–26]. Fig. 2 shows the results of an SE procedure involving extraction of residues with methanol-water and partition into dichloromethane for the analysis of 98 pesticides that belong to 20 different groups in fruits and flour. The chromatogram obtained by LC-MS^2 is free of any interfering compound [17].

Preliminary clean-up procedures before determination by LC-MS^2 are used in few studies. While liquid-liquid partitioning (LLP), solid-phase extraction (SPE), dispersive SPE, solid-phase microextraction (SPME) and stir-bar sorptive extraction (SBSE) are still widely reported [17,25–30], complicated fractionation schemes or long, tedious combinations of several procedures have disappeared.

Other alternatives to SE that are also reported with some frequency are matrix solid-phase dispersion (MSPD) and pressurized liquid extraction (PLE). MSPD has shown very good characteristics for analyzing both fatty samples [31,32] and fruits and vegetables [33–35]. PLE has been especially recommended for grain and cereal samples with low moisture content [36] but has also been used for fruits and vegetables [37,38]. The possibility of using water as extractant reduces the use of organic solvents and provides good results [39].

Table 1 shows some characteristics of the extraction methods reported for the analysis of pesticides in fruits and vegetables; their advantages and disadvantages and some applications are also summarized.

As a consequence of the greater specificity, endogenous components are no longer observed but they may still affect quantitation because of the matrix effect, which is induced by matrix components co-eluting with the analyte from the LC column and interfering in the ionization response of the analytes, affecting the

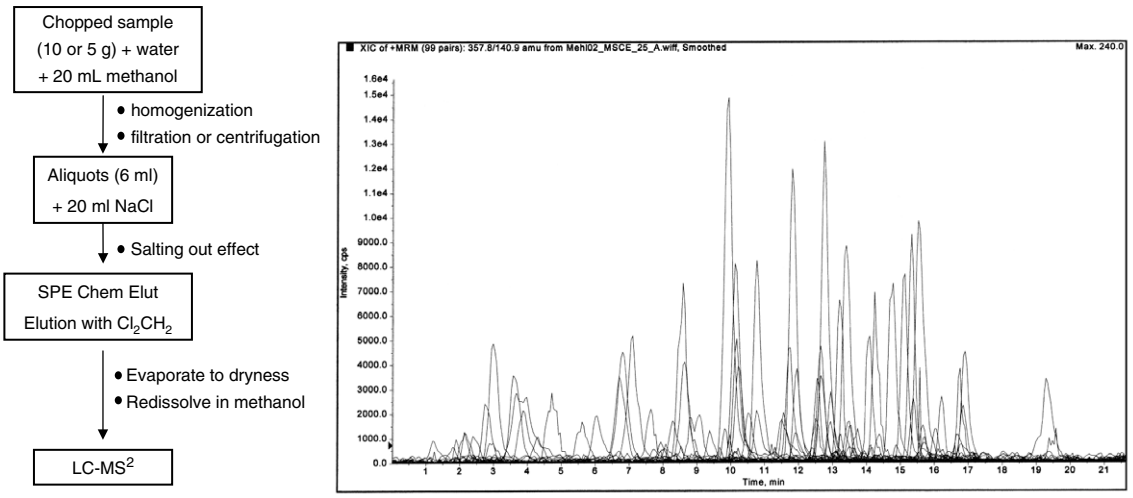

Figure 2. Chromatogram of a standard with 98 pesticides at a level of 0.025 mg/kg prepared in a blank extract of wheat flour. Flow chart of the SE-SPE method employed for the extraction. (Adapted from [17] with permission from AOAC International, © 2003).

Table 1. Extraction procedures combined with LC-MS^2 to determine pesticide residues in food

Method	Protocol	Cost (consumption/time)	Comments	Ref.
SE	Extraction with an organic solvent (methanol, acetonitrile, acetone, ethyl acetate and dichloromethane) by shaking, homogenizing or sonication. This step can be followed or not by a simple clean-up, such as LLP, SPE, dispersive SPE, MSPD or SBSE	5–25 g of sample 10–50 mL of organic solvent < 1 g of solid phase for SPE or dispersive SPE 1 fiber for SPME 1 stir bar for SBSE batches 2–10 samples < 30 min Approximately 2 € in reactives per sample	• Specially recommended for food with high water content (mostly fruits and vegetables) • Universal for any type of sample and a wide range of pesticides • Risk of emulsion formation	[22–26,30,32,40]
MSPD	Sample dispersion with solid phase (C_{18} or Florisil) until a homogeneous mixture is obtained. This dispersion is placed onto a column. Pesticide residues are eluted with a few milliliters of organic solvent	0.5 g of C_{18} 10 mL dichloromethane 5 mL methanol 1 sample < 15 min Approximately 1 € in reactives per sample	• Limited amount of sample because it is difficult to process more than 0.5 g and maintain the method	[32–34]
PLE	Extraction with an organic solvent or water at high pressure and temperature. Sample is homogenized with a solid-phase and inserted into a stainless-steel cell of the commercial system	For a 22-mL extraction cell 5 g sample, 4 g of diatomaceous earth or 12 g of Na_2SO_4 or other desiccants 22 mL of organic solvent or water 24 samples < 45 min Approximately 3 € in reactives per sample	• High temperatures can degrade some labile compounds • Specially recommended for food with low moisture content (cereals) • Able to substitute organic solvents by water. Ecofriendly	[10,37–39]

reproducibility and accuracy of the determined compound. To prevent enhancement or suppression problems during food analysis, the matrix effect should be investigated first and compensated for during method development and validation [41]. Although the matrix effects reported using these simpler, more rapid and shorter procedures are as low and consistent as those reported using the traditional ones, accurate quantification still needs to correct for the matrix effect by using matrix-matched standards (most common procedures) or by other compensating techniques, such as the ECHO Peak [22,24,42,43].

Table 2. Multi-residue methods for the analysis of pesticide in food using QqQ in SRM mode

Pesticides	Food	Extraction	Recoveries (%)	Concentration factor	Determination	LOQ (mg/kg)	N° IP	Ref.
43 compounds, mainly carbamates	Grain	ASE: acetonitrile and SPE with acetonitrile-toluene	54–170	5	ESI positive, Atlantis dC_{18} (150 × 2.1 mm, 3μm), gradient water-acetonitrile at 0.2 ml/min	0.001–0.1	4	[36]
144 pesticides, organophosphorus and carbamates	Fruit and vegetables	QuEChERS: acetonitrile, salting out and dispersive SPE	70–120	2	ESI positive, Alltima C_{18} (150 × 3 mm, 5 μm), gradient MeOH-water (5 mM formic acid) at 0.3 ml/min	0.01	2.5	[25]
74 polar pesticides, mainly carbamate, conazole, benzimidazole and pyrimide fungicides and insecticides	Fruit and vegetables (20 g)	SE with water (pH 6–7) and ethyl acetate	63–133	2	ESI positive, Nucleosil 100-5 C_{18}, (70 × 2 mm, 5 μm), gradient MeOH-water (0.1% formic acid) at 0.3 ml/min 11 acquisition groups as function of time	0.01–1	4	[24]
57 pesticides and 16 TPs, mainly carbamates	Fruit and vegetables	SE: acetonitrile and redissolved in water	77–124	1	ESI positive, HyPURITY C_{18} (150 × 2.1 mm, 5 μm), gradient 10 mM aqueous ammonium acetate-MeOH at 0.2 ml/min Three time windows	0.01–0.1	4	[23]
52 non GC-amenable pesticides	Fruit and vegetables	LLP-SPE: acidified mixtures MeOH-water and MeOH-MTBE	70–110	1	ESI positive, Atlantis C_{18} (100 × 2.1 mm, 5 μm), gradient MeOH-water (0.01% formic acid) at 0.2 ml/min	0.01–0.1	2.5[a]	[40]
32 pesticides and 25 TPs benzimidazoles and carbamates	Fruit and vegetables	SE: ethyl acetate	70–100		ESI positive and negative mode, Genesis C_{18} (100 × 3 mm, 4 μm), gradient MeOH-ammonium formate at 0.3 ml/min	0.01	2.5	[22]
98 pesticides, 20 different chemical groups	Fruit and flour	SE-LLP: methanol, NaCl solution and dichloromethane	70–120	1	ESI positive, Aqua C_{18} (50 × 2 mm, 125 Å) gradient MeOH-water (5 mM ammonium formate) at 0.2 ml/min, ionization mode	0.01	2.5[a]	[17]

[a]For confirmation purposes, a second transition was identified for each analyte in a different injection.

2.2. Development of multi-residue methods for target pesticides

These analyses highlight the large number of pesticides in use, which, as result of agricultural treatments, can be present in a sample. Accordingly, a suitable method should simultaneously be applicable to a wide range of pesticides and matrices, and capable of providing unambiguous identification at the low concentration levels required to quantify pesticide residues below the strict MRLs established by current legislations [44–48].

Classical routine screening methods, which were previously carried out by GC-MS, involve the screening, identification, quantification and confirmation of as many target pesticides as possible, with only one sample preparation method and one chromatographic technique. The enhanced selectivity afforded by MS^2 detection also attains discrimination between pesticides that are marginally separated [43]. A recent comparison of the scope and the sensitivity of GC-MS and LC-MS^2 really illustrates the better performance of LC-MS^2. Due to the small peak width in GC, the cycle time in GC-MS methods must be 1 s or shorter. Since all ions are recorded using a dwell time of 40 ms, no more than 25 characteristic ions can be recorded in one time window. Assuming 10 time windows in one GC run, 250 ions or 83 pesticides with 3 characteristic ions each can theoretically be analyzed in parallel. The peak width in LC measurements is usually higher, often allowing a typical cycle time of 2.5 s. Considering a typical dwell time of 20 ms, approximately 125 SRM transitions can be acquired simultaneously in one time window. Assuming 5 time windows per LC run, in that case, 625 SRM transitions are obtained with one injection. Since two SRM transitions are often sufficient to quantify and to confirm a result, up to 312 pesticides can theoretically be analyzed in one run. In practice, the theoretical numbers calculated above cannot be reached because usually more pesticides elute in the middle than in the beginning or the end of the chromatograms. However, irrespective of this limitation, the number of analytes covered in one LC-MS^2 run is at least two or three times greater than the number of pesticides measured in parallel by GC-MS in the SIM mode [8]. Another particular feature of MS^2 detection, which can be exploited in these LC-MS^2 methods, is the capability to differentiate between coeluting isobaric analytes (same nominal mass, different structure). Differences in the product-ion mass spectra yielded by common precursor ions were decisive in the unhindered determination of the following pairs of pesticides: (i) aldicarb and butocarboxim, (ii) aldicarb sulfoxide and butocarboxim sulfoxide, and (iii) carbaryl and thiabendazole [43].

The use of multi-residue methods would be ideal, especially if optimized conditions could be established in compliance with European Union (EU) regulations [44]. Recent improvements in LC-MS^2 instrumentation, which have improved the sensitivity, the selectivity and the robustness of the method, make it possible to increase the number and the diversity of pesticides that can be included in a single LC-MS^2 analysis [10]. As a consequence, LC-MS^2 methods for determining a large number of pesticides in food have flooded into current literature, showing that this technique can be comparable or even superior to GC for the development of multi-residue methods [8,25,36]. Table 2 summarizes the methods developed for multi-residue pesticide determination in food by LC-MS^2 that are suitable for screening more than 50 pesticides simultaneously.

As an example, Pang et al. [36] established an analytical method using GC-MS and LC-MS^2 for simultaneous determination of 405 pesticides worldwide used in

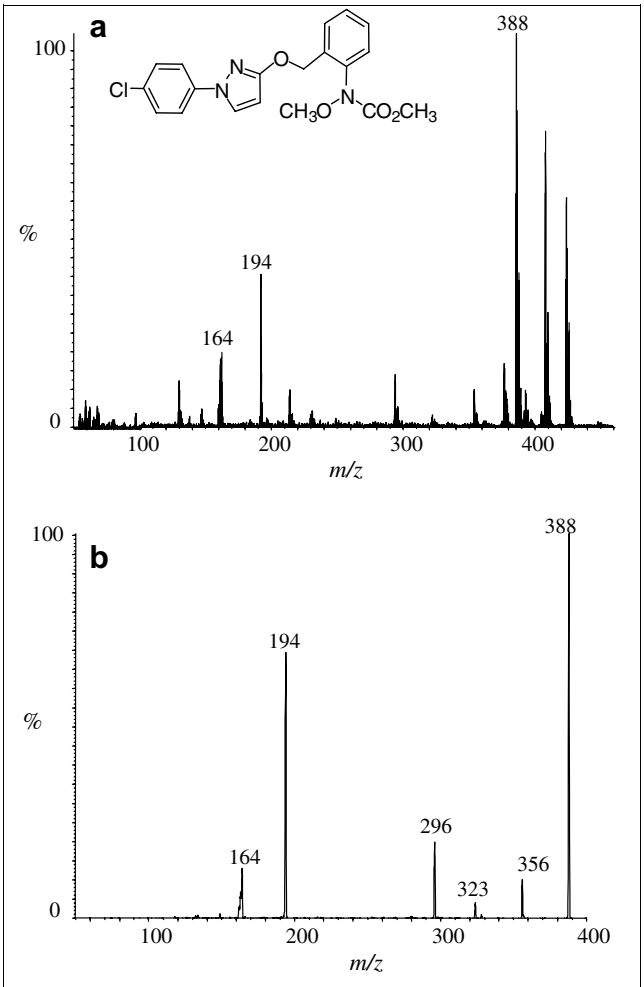

Figure 3. (a) Mass spectrum of the pesticide pyraclostrobin. The structure of the molecule is shown as an insert. Spectrum recorded by infusion of a standard solution (5 μg/ml in water/methanol 1:1, v/v) to the electrospray (ES) ionization source in positive mode. (b) Product-ion spectrum of the $[M+H]^+$ ion of pyraclostrobin (precursor ion *m/z* 388). (Reproduced from [23] with permission from Wiley InterScience, © 2004).

grain. After its optimization, based on the characteristics of the compounds, only 35 pesticides could not be determined by LC-MS^2, because recoveries were less than 40%. These low recoveries are linked to the matrix effects because the extraction procedure is the same, independent of the determination technique. In spite of this, finally only 43 pesticides of the total were analyzed by LC-MS^2.

Lehotay et al. [25] studied the combination of LC-MS^2 and GC-MS to quantify and identify 229 pesticides in two representative commodities (lettuce and orange). After comparing the results obtained for each technique, as values of recoveries and RSD, the authors concluded that the results do not differ greatly. However, LC-MS^2 is the preferred approach for those pesticides that are compatible in both analytical systems (GC and LC) because of the inherent advantages of the technique, such as: its better intrinsic instrument sensitivity and selectivity; its better reproducibility in the injection process, which provided better consistency in the results; its capability of detection at low concentrations spiked; and, unlike GC, its avoidance of degradation problems with thermolabile pesticides.

Ortelli et al. [24] developed a multi-residue method for the determination of 74 pesticides commonly used in crop protection, mainly including carbamate, conazole, benzimidazole and pyrimidine fungicides and insecticides. All pesticides studied were determined with high sensitivity and selectivity in one single injection, making it possible to apply the method satisfactorily to routine analysis; more than 2500 fruit and vegetables samples were analyzed as a part of a pesticide-monitoring program.

Hetherton et al. [23] proposed a multi-residue screening method for the simultaneous analysis of 73 pesticides and their metabolites employing LC-MS^2. The method was successfully used to determine a large number of target pesticides in apple, orange and lettuce, demonstrating its potential for multi-residue applications. In spite of the enhanced specificity, the possibility of MS^2 techniques achieving two transitions (one transition for screening and the other one for confirmation) can help to confirm the identity of analytes. As an example, Fig. 3 shows the MS^2 spectrum of a recently developed pesticide, pyraclostrobin, whose structure is shown in Fig. 3A. The mass spectrum

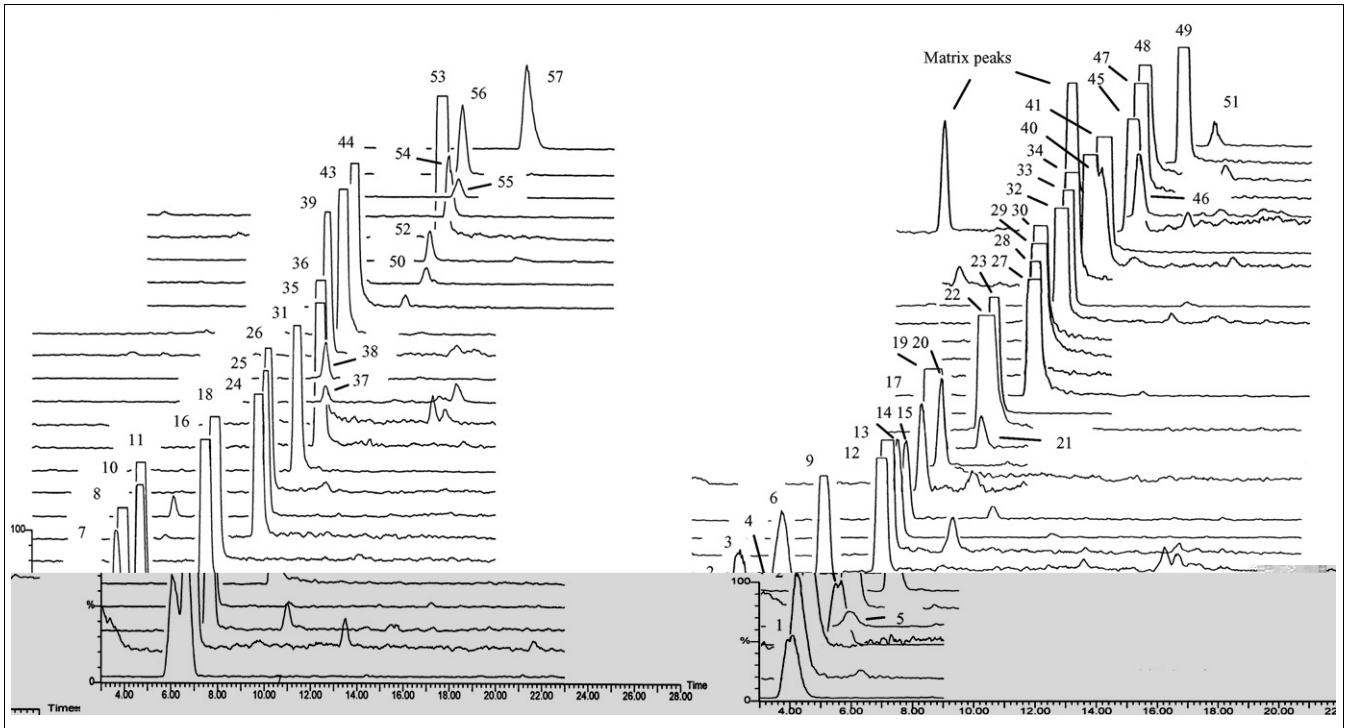

Figure 4. Chromatogram of orange spiked with pesticides studied at 0.01 mg/kg. One MS^2 fragment for each pesticide. Identification of peak: (1) Butocarboxim sulphoxide, (2) Oxamyl-oxime, (3) Aldicarb sulphoxide, (4) Butoxycarboxim, (5) Aldicarb sulphone, (6) Oxamyl, (7) Vamidothion sulphoxide, (8) Demeton-S-methyl sulphoxide, (9) Methomyl, (10) Demeton-S-methyl sulphone, (11) Vamidothion sulphone, (12) Ethiofencarb sulphone, (13) Ethiofencarb sulphoxide, (14) Imidacloprid, (15) Methiocarb sulphoxide, (16) Vamidothion, (17) Carbofuran-3-OH, (18) Acetamiprid, (19) Carbendazim, (20) Methiocarb sulphone, (21) Butocarboxim, (22) Thiabendazole, (23) Aldicarb, (24) Thiometon sulphoxide, (25) Terbufos-O-sulphone, (26) Thiometon sulphone, (27) Propoxur, (28) Thiophanate methyl, (29) Carbofuran, (30) Bendiocarb, (31) Demeton-S-methyl, (32) Carbary, (33) Thiodicarb, (34) Ethiofencarb, (35) Disulfoton sulphoxide, (36) Phorate sulphoxide, (37) Thiometon, (38) Disulfoton sulphone, (39) Phorate sulphone, (40) Isoprocarb, (41) Trimethacarb-2,3,5 (42) Trimethacarb-3,4,5, (43) Phorate-O-analogue, (44) Demeton, (45) Imazalil, (46) Linuron, (47) Methiocarb, (48) Promecarb, (49) Fenoxycarb (50) Phorate, (51) Clofentezine, (52) Disulfoton, (53) Furathiocarb, (54) Terbufos, (55) Dinocap, (56) Hexythiazox, (57) Carbosulfan. (Reproduced from [22] with permission from Elsevier, © 2003).