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Study of the effects of operational parameters on multiresidue pesticide analysis by LC–MS/MS

Béla Kmellár[a,b], Lucía Pareja[a,c], Carmen Ferrer[a], Péter Fodor[b], Amadeo R. Fernández-Alba[a,*]

[a] Pesticide Residue Research Group, EC Community Reference Laboratory for Pesicide Residues in Fruits and Vegetables Chemistry, University of Almeria, 04120, La Cañada de San Urbano, Almeria, Spain
[b] Cornivus University of Budapest, Faculty of Food Science, Department of Applied Chemistry, 1118 Villany str. 29-43, Budapest, Hungary
[c] Farmacognosia y Productos Naturales, Facultad de Quimica, General Flores 2124, Montevideo, Uruguay

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ABSTRACT

In this paper, the influence of several operational parameters on a well established multiresidue LC–MS/MS method has been studied in relation to the analysis of 150 pesticides commonly present in vegetable samples. The operational parameters investigated are: (i) the influence of different modifiers (0.1% formic acid; 5 mM ammonium formiate; 5 mM ammonium acetate in aqueous phase) – both on the retention time and on the analytical response of the studied compounds; (ii) the effect of the analytical column's temperature on the retention time and on the analytical response of the pesticides investigated; (iii) the effects of co-elution in mixture containing 150 pesticides and, additionally, (iv) the carrying out of a study about the common transitions obtained by LC–MS/MS. Various common transitions were found among the 150 pesticides, but there were only two problematic cases, the pairs diuron–fluometuron and prometryn–terbutryn, which have common scanned transitions and have very close retention times. The use of ammonium salts as modifier instead of formic acid reports enhancement or suppression of the response depending on the pesticides. No great influence on the retention time or on the response of the pesticides and commodities studied was observed with relation to the column temperature. Two different columns: an HPLC (5 μm particle size) and an UHPLC analytical column (1.8 μm particle size) have been used. As was expected, shorter run times and lower peak width was achieved with the UHPLC column.

In this paper, the effect of the compounds on each other in the MS analysis when the number of co-eluting compounds is quite high is also described. Mainly small suppression or enhancement co-elution effect was observed, but some particular pesticides presented high sensitivity (>±60% effect) when they elute together with others. This is an important factor and it has to be taken into account when performing multiresidue pesticide analysis.

1. Introduction

Pesticides are widely used chemicals in agricultural practice, not only during cultivation but also in post-harvest storage. Various organizations have set stringent regulatory controls on pesticide use in order to minimize exposure on the general population to pesticide residues in food. The great variety of applied pesticides both within European Union countries (EU) and outside of the EU, as well as the arrival of new plant protectors and chemicals, has needed an ever-expanding list of pesticides along with their accompanying maximum residue limits (MRLs). The list of MRLs for a wide variety of commodities and pesticides is updated from time to time and is part of the EU Plant Protection Products Directive (1107/2009 EEC) [1], which is the update of the former directive (91/414/EEC) [2].

Multiresidue analysis, which determines a range of multi-class pesticides as wide as possible, is the primary need for food control laboratories. This is because the great variety of products applied following the different agricultural practices in fruit and vegetable and the international trade.

Regarding the analytical methods, there is no doubt that liquid chromatography–mass spectrometry (LC–MS) currently competes with gas chromatography–mass spectrometry (GC–MS) for the status of the reference technique in this field [3–5]. There are different mass analyzers that can enhance tandem mass spectrometry (MS/MS) capabilities, such as quadrupole ion-trap (QLIT), triple quadrupole (QqQ) and quadrupole time-of-flight (QTOF) – each one has different features [6]. The main advantage of QqQ instruments is their very good quantitative capabilities and their great sensitivity in the selected reaction monitoring (SRM) mode, in addition

* Corresponding author at: EURL-FV, University of Almeria, 04120, La Cañada de San Urbano, Almeria, Spain. Tel.: +34 950015034; fax: +34 950014102.
E-mail address: amadeo@ual.es (A.R. Fernández-Alba).

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to the capability of simultaneously selecting multiple transitions [7–9]

The low EU MRLs have encouraged the development of more sensitive analytical methods to meet the requirements of complex samples. Therefore, sensitive and reliable confirmatory methods are required to monitor pesticide residues in foods. In this sense, liquid chromatography/tandem mass spectrometry (LC–MS/MS) with a triple quadrupole in selected reactions monitoring (SRM) mode has become, to date, the most widely used technique for the monitoring and the quantification of pesticides in food, as reported extensively in the literature [3,10–18]

The most common stationary phases in the analysis of pesticides in food by HPLC are C8 and C18 with a particle size from 3 μm to 5 μm and a column length from 10 cm to 25 cm. Flow rate, injection volume and run time among others depend on the characteristics of the column and especially on the number of the measurable pesticides. The common flow rates used for these types of columns in pesticide residue analysis are between 0.2 and 0.6 mL min^{-1}. The flow rate is naturally limited by the maximum pressure tolerance of the column or the pump used. The total run time, depending on the number of compounds to be analyzed, typically varies between 15 and 35 min. The injection volume varies from 5 to 20 μL [19–22] – set to achieve a compromise by taking into account two criteria; (i) to introduce the highest volume to ensure adequate sensibility and (ii) to inject the lowest volume to protect the column and the system from any extracts, especially from dirty samples.

Nowadays, there is a clear trend in decreasing the diameter and the particle size of the chromatographic columns in order to reduce the run time and/or enhance resolution.

Over recent years, liquid chromatography–mass spectrometry (LC–MS) and liquid chromatography–mass spectrometry in tandem (LC–MS/MS) have experienced impressive progress, both in terms of technology development and application. More recently, alternative strategies have been developed to obtain increased efficiency, together with short analysis times by using 1.7 μm porous stationary phases, mobile phases at high linear velocities and instrumentation that operates at high pressures (ca. 15,000 psi). This new technology has been commonly called ultra performance liquid chromatography (UPLC, trademark of the Waters company) and rapid resolution liquid chromatography (RRLC, trademark of the Agilent company) [23,24], that is why we call this technique ultra high performance liquid chromatography (UHPLC) to distinguish this procedure from conventional HPLC.

In order to increase the speed of chromatographic separation in HPLC, different strategies based on the increase of the mobile phase flow rate, faster gradients, the use of short columns and/or the use of normal-sized columns with smaller particles size (<2 μm) can be considered. The use of shorter columns, with 1.8 μm particle size, provides faster analysis and improves resolution compared to columns of 3.5 or 5.0 μm particle size. It might be, for instance, a good alternative when the laboratory equipment is not provided with a UHPLC system: its additional resolving capability makes it a powerful technology for those laboratories which do not have UHPLC systems [25,26].

For the chromatographic separation, not only the analytical column but also the mobile phase is of great importance. Two type of eluent are typically used as mobile phase: the most common being pure water (or high content water) eluent as the aqueous phase and methanol or acetonitrile as the organic phase [11,27–29].

Additives and buffers are used in LC mobile phases to improve sensitivity, resolution and reproducibility. Chemical properties and concentration of the additive, as well as pH, have a significant effect on analytes response in ESI. Unfortunately, many of the additives and buffers commonly used in LC are not compatible with ESI/MS. In general, non-volatile buffers such as phosphate and borate tend to cause increased background signal suppression, and rapid contamination of the ion source resulting in decreased sensitivity and stability. Although various volatile additives have been employed in LC–(ESI+)MS, the most common modifiers employed in the analysis of pesticides in food samples are: formic acid (0.01–0.2%) [20,23,28,30–32]; ammonium formiate (2–10 mmol L^{-1}) [24,33,34] and ammonium acetate (1–20 mmol L^{-1}) [12,14,15,23,35]. The addition of the modifier can be performed in both eluent or only in the aqueous phase depending on the analyst preference.

These parameters – and others like column temperature during the chromatographic analysis and the number of compounds included in a mixture – are parameters that must be carefully investigated in order to control all variables that may influence the effectiveness of a multiresidue method. And as well, these are in some cases system dependent and therefore difficult to extrapolate from one method/system to other.

In this paper, the influence of the main operational parameters commented above in an established LC–MS/MS method has been studied. This work is linked to our previous study about the measurement of 160 pesticides by LC–MS/MS [36]. As it was explained in that publication, 10 pesticides were insensitive and/or problematic in detection thus we followed the study with the rest of 150 pesticides. Generally speaking food control laboratories apply very similar extraction and analytical procedures but in many cases there is not clear evidence about how those differences can affect the efficiency of the analysis. Obviously it is expected that pro and contras can be appear with the selection of different parameters but only a good balance can be done if the adequate technical information is available. Consequently, the aim of this work is to evaluate a range of operational-technical parameters those commonly affect the efficiency of the LC–MS/MS measurement in multiresidue pesticide analysis to serve useful information for routine analysis.

2. Experimental

2.1. Chemicals and reagents

Pesticide analytical standards were purchased from Dr. Ehrenstorfer (Ausburg, Germany) and Riedel-de-Haën (Seelze, Germany). HPLC-grade acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). Formic acid, anhydrous magnesium-sulfate, ammonium formiate and ammonium acetate were obtained from Fluka (Buchs, Switzerland). Acetic acid was purchased from Merck (Darmstadt, Germany), and sodium acetate 3-hydrate from Panreac (Barcelona, Spain). PSA (primary–secondary amine) was obtained from Supelco. A Milli-Q-Plus ultrapure water system from Millipore (Milford, MA) was used throughout the study to obtain the HPLC-grade water used during the analyses.

2.2. Pesticide solutions

Individual stock solutions (1000–4000 μg mL^{-1}) were prepared in pure organic solvent depending on their solubility and stored in the dark at −18 °C. When the pesticide was not easily soluble in acetonitrile, 10% of dimethyl sulfoxide (DMSO) was added. After preparation, all the data concerning the preparation of the solution were recorded on a Register Form, on which the weight of the container was recorded as a quality control measure. Each time that the solutions were used, they were equilibrated to room temperature and weighed to check for losses caused by evaporation. If the weight had changed, the differences observed were taken into account when calculating the new concentrations.

Mixtures of pesticides containing medium number (15 compounds) and large number (150 compounds) of compounds were prepared in acetonitrile, containing 10 μg mL^{-1} of each pesticide.

These mixtures were used as working solutions. Furthermore, they were used to study the influence of the presence in the mixture of medium or large number of pesticides in the analytical response of each compound. To perform this study, the 10 μg mL^{-1} mixtures were diluted to 100 ng mL^{-1}.

2.3. Sample treatment

Fruit and vegetable samples were purchased from different local markets. The "acetate buffered QuEChERS" methodology (described elsewhere [37,38]) was applied. It comprised the following steps: a representative 15 g portion of previously homogenized sample was weighed in a 50 mL PTFE centrifuge tube; followed by the addition of 15 mL of acetonitrile (containing 1% acetic acid) and 200 μL 25 μg mL^{-1} TPP surrogate standard (in MeCN) were added, and the tube was vigorously shaken for 1 min manually. After this time, 2.5 g of Na-acetate-3-hydrate and 6 g of anhydrous $MgSO_4$ were added, and the shaking process was repeated for 4 min. The extract was then centrifuged (3700 rpm) for 5 min. 5 mL of the supernatant (acetonitrile phase) was transferred to a 15 mL centrifuge tube containing 250 mg of PSA and 750 mg of $MgSO_4$; this was then shaken energetically for 20 s. Afterwards, the extract was centrifuged again (3700 rpm) for 5 min. In this way, an extract of 1 g sample/mL was obtained. A 1 mL of extract was then evaporated to dryness and reconstituted to 1 mL of 20% acetonitrile in water and filtered through a 0.45 μm PTFE filter (Millex FG, Millipore, Milford, MA), prior to LC–MS/MS analysis. This last step is not a part of the "acetate buffered QuEChERS" methodology, but based on our experience this improves the chromatography, as peak distortions are generally reduced because the extracts are injected using the initial mobile phase.

Sample extracts were fortified by adding appropriate amount and concentration of standard solution after evaporation of the sample acetonitrile extract.

1 mL of blank extract was evaporated to dryness and redissolved it in standard solution prepared in 10% acetonitrile in water.

2.4. Liquid chromatography

The separation of the pesticides was carried out using an HPLC system (working pressure maximum 400 bar), consisting of a vacuum degasser, auto sampler and a binary pump (Agilent Series 1200, Agilent Technologies, Santa Clara, CA) equipped with two different reversed phase XDB-C8 analytical columns: an Agilent Zorbax Eclipse 150 mm × 4.6 mm, 5 μm particle size HPLC column (working pressure maximum 400 bar) and an Agilent Zorbax 100 mm × 2.1 mm 1.8 μm particle size UHPLC column (working pressure maximum 600 bar).

Working with the HPLC column, three different modifiers were tested in mobile phase A (aqueous phase): (i) 0.1% formic acid, (ii) 5 mM ammonium formiate and (iii) 5 mM ammonium acetate. Mobile phase B was acetonitrile. The chromatographic method held the initial mobile phase composition (20% B) constant for 3 min, followed by a linear gradient up to 100% B over 30 min and then maintained for 3 min at 100% B. After that, a post time of 12 min was set. The flow rate used was 0.6 mL min^{-1}. A 10 μL amount of the sample was injected in each run.

Working with the UHPLC column, the mobile phase A was milliQ water (0.1% formic acid) while mobile phase B was acetonitrile. In this case, the chromatographic method held the initial mobile phase composition (20% B) constant for 2 min, followed by a linear gradient up to 100% B over 15 min and then maintained for 3 min at 100% B. After that, a post time of 12 min was set. The flow rate used was 0.3 mL min^{-1}. A 5 μL amount of the sample extract was injected in each run.

2.5. Mass spectrometry

Mass spectrometric analyses were performed using a 6410 Triple Quad LC/MS (Agilent Technologies, Santa Clara, CA, USA) equipped with an electro spray ionization (ESI) source operating in positive ionization mode setting with the following operating parameters: capillary voltage 5000 V; nebulizer pressure 50 psi; gas temperature 325 °C; gas flow 12 L min^{-1}. SRM experiments were carried out in order to obtain the maximum sensitivity for the detection of the target molecules. Dwell times of 15 and 5 ms were set for HPLC and UHPLC analyses, respectively. Five time windows with a ±1 min overlapping range around the window limits were used when analyses were performed with the HPLC column (the start time of the individual segments were 0, 15.5, 20.2, 21.9 and 24.2 min, respectively), whereas for the UHPLC column the retention time windows were 0, 8.7, 11.5, 12.8 and 14.3 min. Nitrogen was used as the nebulizer and collision gas. Agilent Mass Hunter Data Acquisition; Qualitative and Quantitative Analysis software was used for method development and data acquisition.

3. Results and discussion

3.1. Liquid chromatography

In this article, peak width was calculated under the IUPAC definition as the base width: "the segment of the peak base intercepted by the tangents drawn to the inflection points on either side of the peak" [41].

The high efficiency of the separation using 1.8 μm particle size column results in reductions of the peak width from 0.70 to 0.30 min on average.

The use of small particle size columns (1.8 μm) can increase column efficiency with better baseline separation and narrower peaks than standard particle size columns (3.5–5 μm). On the other hand, the sensitivity achieved in small particle size columns could be limited by the volume of sample injected.

3.1.1. HPLC column with 5 μm particle size

3.1.1.1. Effect of the modifiers in the retention time. Three different modifiers were used: matrix match solutions of 150 pesticides at the 50 ng mL^{-1} level were analyzed employing formic acid, ammonium formiate and ammonium acetate. Analyses were performed keeping the flow rate and the gradient constant and employing the same column in all the experiments. The total run time was 45 min and it was divided into five time windows. Different retention times were obtained for the selected pesticides depending on the modifier employed. In Table 1 the target pesticides and their values obtained under different operational parameters are shown. The detection parameters of the pesticides studied were included in our previous article [36].

Theoretically, the optimal detection parameters could differ when different eluent are used. For that reason, we optimized all compounds by injecting them to the flow of acetonitrile:water (+ modifier) 50:50 in order to test whether all the modifiers used can drift the ionization optimum. No difference on optimal parameters was observed for all the modifiers studied, this supports the fact that the hydrogen adducts will behave exactly the same during ionization independently from the modifier used.

The average in the peak width was 0.50 min, and varied from 0.99 min in cambendazole to 0.26 min in cyromazine.

Differences in the retention time caused by the use of ammonium formiate and ammonium acetate are in the range of ±1 min in 93% of the cases, greater differences (e.g. up to 14.6 min) are observed in emamectin benzoate, flazasulfuron, spinosyn A, spinosyn D, spiroxamine, thiabendazole and thiocyclam (see

Table 1
Obtained values under different operational parameters.

Pesticide	Number of the "15"-mix	Mixture-effect (%) – response differences: 150 vs 15 pesticides in mix	Retention times (min) using different modifiers in the mobile phase (water)			
			HPLC; 0.1% HCOOH	HPLC; 5 mM ammonium formiate	HPLC; 5 mM ammonium acetate	UHPLC; 0.1% HCOOH
Acephate	3	1	3.17	3.15	3.17	1.88
Acetamiprid	6	−8	11.30	11.30	11.33	4.83
Aclonifen	7	–	23.50	23.40	23.33	14.14
Alachlor	4	3	23.18	23.00	22.93	13.83
Albendazole	9	0	13.60	17.50	17.36	8.62
Aldicarb	1	31	13.90	13.90	13.86	7.79
Aldicarb sulfone	4	19	5.28	5.30	5.33	1.82
Aldicarb sulfoxide	8	−4	3.40	3.40	3.44	1.25
Anilofos	8	−26	24.70	24.60	24.54	14.72
Atrazine	4	−1	17.30	17.20	17.17	10.32
Azinphos-ethyl	3	−13	23.10	23.00	22.94	13.95
Azoxystrobin	3	−8	21.06	21.00	20.90	12.95
Benalaxyl	2	1	24.10	23.90	23.90	14.39
Bendiocarb	2	30	16.60	16.60	16.57	10.09
Bromacil	3	−31	14.23	14.20	14.14	8.43
Bromuconazole[a]	6	−3	20.50 and 21.58	20.50 and 21.40	22.79	12.55 and 13.08
Bupirimate	7	−5	19.85	23.80	23.71	11.83
Buprofezin	10		24.90	28.80	28.75	14.00
Butocarboxin	7	0	13.00	13.00	12.96	6.75
Butoxycarboxin	1	−15	4.80	4.90	16.50	10.02
Cambendazole	9	−3	9.10	14.10	13.80	3.22
Carbaryl	3	−18	17.20	17.20	17.15	10.53
Carbendazim	7	−11	3.38	10.10	10.00	1.28
Carbofuran	10	−30	16.58	16.50	16.49	10.01
Chlorbromuron	2	−31	21.00	20.90	20.85	12.76
Chlorfenvinphos[a]	10	−36	23.70 and 24.50	23.6	23.47 and 24.29	14.19 and 14.60
Chloridazon	1	−16	9.98	10.00	10.00	3.61
Chlorotoluron	1	–	16.59	16.60	16.58	10.21
Chloroxuron	4	−1	20.40	20.30	20.27	12.57
Chromafenozide	6	−2	22.70	22.50	22.45	13.73
Clofentezine	1	−9	24.90	24.80	24.72	14.82
Cyproconazole	9	−3	20.13	20.00	19.94	12.30
Cyprodinil	9	−8	18.80	23.10	23.06	11.14
Cyromazine	1	27	2.30	3.00	3.09	0.93
Deet	9	−9	17.60	17.50	17.41	10.66
Demeton-S-Metil	1	−28	15.27	15.20	15.18	9.21
Desethylterbutylazine	10	−6	15.20	15.20	15.18	9.13
Diazinon	3	−3	25.30	25.20	25.18	14.84
Dichlorvos	2	−19	15.50	15.40	15.40	9.26
Dicloran	5	−8	19.60	14.20	14.12	8.45
Diethofencarb	7	−31	20.60	20.40	20.39	12.48
Difenoconazole[b]	8	−42	23.59 double peak	23.6	23.38 and 23.54	14.25
Difenoxuron	8	−7	17.56	17.60	17.57	11.03
Diflubenzuron	2	3	22.20	23.20	22.00	13.48
Dimethoate	5	−2	11.20	11.20	11.24	4.58
Dimethomorph[b]	10	−10	18.80 and 19.10	19.1	18.59 and 19.00	11.70 and 11.93
Dimethylvinphos	4	6	21.10	21.00	20.94	12.67
Diuron	1	−21	17.70	17.6	17.57	10.85
Edifenphos	5	−3	23.30	23.20	23.14	14.00
Emamectin benzoate	6	−8	18.50 and 19.20	24.40	27.02	12.80
Ethiofencarb	3	−7	17.80	17.80	17.73	10.86
Ethion	8	−4	28.60	28.40	28.38	16.80
Ethiprole	8	−2	20.10	20.10	19.96	12.42
Ethoxyquin	4	–	15.70	23.90	23.79	9.23
Fenamiphos	1	−7	20.80	20.60	20.58	12.71
Fenarimol	5	−26	20.50	20.40	20.35	12.56
Fenazaquin	6	−5	27.80	28.00	28.05	16.19
Fenbendazole	8	−8	16.47	18.90	18.80	10.46
Fenhexamid	7	−13	21.30	21.10	20.94	12.96
Fenobucarb	8	−9	20.60	20.40	20.39	12.45
Fenoxycarb	5	−6	22.60	22.50	22.42	13.71
Fenuron	2	−11	10.02	10.00	9.98	3.55
Fipronil	5	109	24.30	24.20	24.10	14.61
Flazasulfuron	2	−29	19.00	11.20	7.29	11.81
Fluacrypyrim	2	−5	26.60	26.40	26.35	15.73
Fluazifop	1	2	20.30	12.30	11.75	12.52
Flufenoxuron	5	−7	27.50	27.30	27.27	16.31
Fluometuron	5	−31	17.30	17.2	17.18	10.55

Table 1 (*Continued*)

Pesticide	Number of the "15"-mix	Mixture-effect (%) – response differences: 150 vs 15 pesticides in mix	Retention times (min) using different modifiers in the mobile phase (water)			
			HPLC; 0.1% HCOOH	HPLC; 5 mM ammonium formiate	HPLC; 5 mM ammonium acetate	UHPLC; 0.1% HCOOH
Fluquinconazole	8	−2	21.50	21.40	21.32	13.11
Fluroxypyr	4	−23	14.50	4.30	4.02	8.94
Hexaflumuron	2	−33	25.06	24.90	24.83	15.01
Hexythiazox	7	−1	28.30	28.10	28.09	16.56
Imazalil	2	−5	13.90	22.10	21.87	9.50
Imidacloprid	3	−9	10.32	10.40	10.35	3.86
Indoxacarb	9	8	26.20	26.00	25.93	15.58
Iprodione	7	90	22.70	22.50	22.46	13.67
Isocarbofos	2	11	20.30	20.20	20.17	12.42
Isofenfos Metil	5	−27	25.40	25.20	25.19	15.04
Isoprocarb	8	2	18.70	18.60	18.62	11.35
Isoproturon	10	−8	17.60	17.50	17.44	10.78
Kresoxim-methyl	4	−7	24.20	24.00	23.96	14.48
Lenacil	8	−15	15.11	15.00	14.97	9.31
Linuron	9	−6	20.60	20.50	20.49	12.54
Lufenuron	3	−6	26.80	26.60	26.55	15.93
Malathion	2	4	23.03	22.90	23.00	13.86
Mebendazole	10	−4	13.70	15.20	15.10	8.59
Metalaxyl	6	−31	17.50	17.40	17.34	10.78
Metamitron	10	−19	9.20	9.30	9.31	3.11
Methamidophos	4	−49	3.20	3.10	3.13	1.16
Methidathion	10	−7	20.70	20.60	20.59	12.60
Methiocarb	10	2	20.27	20.20	20.11	12.36
Methiocarb sulfoxide	8	12	8.14	8.1	21.74 (wrong)	9.99
Methomyl	5	−10	6.20	13.5	6.18 (wrong)	2.09
Methoxyfenozide	3	−3	22.30	22.20	22.13	13.54
Metobromuron	7	−16	18.58	18.50	18.48	11.29
Metolachlor	10	−29	22.96	22.80	22.73	13.68
Metolcarb	9	33	15.15	15.10	15.12	8.88
Miconazole	2	−13	17.70	27.30	27.08	12.00
Monocrotophos	2	−35	4.80	4.80	4.77	1.64
Monolinuron	4	−3	17.90	17.90	17.85	10.88
Monuron	7	−28	14.58	14.60	14.53	8.65
Myclobutanil	6	−6	21.20	21.00	21.02	12.93
Neburon	6	−2	23.30	23.10	23.09	13.98
Nitempyram	10	−18	4.57	4.40	4.36	1.63
Omethoate	6	−32	3.30	2.30	3.29	1.21
Oxadixyl	5	−9	14.50	14.50	14.43	8.88
Oxamyl	3	−4	5.05	5.00	5.01	1.73
Oxfendazole	4	−17	10.34	11.60	11.60	4.10
Parathion-methyl	7	–	24.67	24.50	24.46	14.69
Penconazole	4	−6	22.40	22.30	22.25	13.47
Pirimicarb	6	−8	6.60	16.30	16.30	2.28
Pirimiphos-methyl	4	−13	25.30	26.20	26.17	14.72
Prochloraz	3	−14	19.50	22.90	22.79	12.09
Procymidone	1	1	23.00	22.90	22.82	13.82
Promecarb	5	5	21.14	21.00	20.95	12.78
Prometryn	6	4	16.35	21.50	21.43	9.82
Propamocarb	5	−17	3.20	3.80	4.29	1.19
Propargite	9	4	29.00	28.80	28.77	17.00
Propazine	6	−2	19.78	19.60	19.59	11.87
Pyridaben	10	−8	29.60	29.40	29.38	17.28
Pyridaphenthion	1	−6	21.70	21.60	21.52	13.26
Pyrimethanil	3	−5	15.60	19.90	19.83	8.56
Pyriproxyfen	4	−15	27.40	27.30	27.25	16.12
Quinalphos	3	−31	24.10	23.90	23.88	14.37
Quinoxyfen	7	−14	25.90	26.30	26.24	15.18
Simazine	6	−4	14.50	14.40	14.38	8.20
Spinosyn A	7	7	17.50	25.70	31.06	11.93
Spinosyn D	5	0	18.30	27.80	32.90	12.46
Spiroxamine	5	2	16.17	22.50	27.27	10.91
Tebuconazole	10	−7	21.67	21.50	21.47	13.19
Tebufenozide	9	−84	23.70	23.50	23.48	14.24
Tebufenpyrad	10	−20	26.30	26.10	26.07	15.47
Teflubenzuron	6	−6	25.40	25.30	25.21	15.10
Terbuthylazine	3	−3	20.30	20.20	20.14	12.24
Terbutrin	9	83	16.48	21.80	21.77	9.97
Thiabendazole	9	−14	3.60	13.50	11.44	1.36
Thiacloprid	8	−7	13.28	13.30	13.32	7.73
Thiametoxam	7	−4	7.48	7.60	7.51	2.48

Table 1 (*Continued*)

Pesticide	Number of the "15"-mix	Mixture-effect (%) – response differences: 150 vs 15 pesticides in mix	Retention times (min) using different modifiers in the mobile phase (water)			
			HPLC; 0.1% HCOOH	HPLC; 5 mM ammonium formiate	HPLC; 5 mM ammonium acetate	UHPLC; 0.1% HCOOH
Thiocyclam	2	−41	2.90	10.30	15.63	1.19
Thiophanate-ethyl	1	8	18.90	18.80	18.80	11.70
Tolfenpyrad	8	−18	26.10	26.00	25.91	15.47
TPP	6	−3	24.60	24.40	24.40	14.67
Triadimefon	9	−2	21.60	21.50	21.45	13.11
Triadimenol[a]	4	−5	19.50 and 19.90	19.40 and 19.90	19.31 and 19.79	12.00 and 12.26
Triazophos	8	−13	22.80	22.70	22.65	13.79
Triclocarban	9	−28	24.80	22.70	24.80	14.88
Trifloxystrobin	1	0	26.40	26.20	26.18	15.66
Triflumizol	9	−17	22.90	25.00	24.93	13.67
Triflumuron	1	−11	24.08	23.90	23.64	14.48
XMC[b]	7	−20	16.40 and 16.90 and 17.50	17.4	16.90; 17.40; 17.60	9.87; 10.19; 10.35; 10.59

[a] Separated isomers.
[b] Not well-separated isomers.

Table 1). No correlation between the pKa values of the pesticides and the retention times were found. The differences in the retention time obtained with formic acid as modifier instead of ammonium salts are in the range of ±1 min in majority of the cases.

Fluroxypyr, miconazole and pirimicarb show differences in the retention times in the range of ±1 min using ammonium acetate and ammonium formiate as modifiers, and more than 7 min when formic acid is employed.

The retention time of spinosyn A and spinosyn D differed greater than 5 min using formic acid, ammonium formiate and ammonium acetate.

3.1.1.2. Effect of temperature on the retention time and peak response. Generally, the column is thermostated. Therefore, as probably this is not the main analytical parameter that can affect the determination of the target compounds, it could be interesting to evaluate whether there is an optimal temperature in order to get the best peak shape.

Different temperatures in the analytical column were assayed to investigate their effect on the retention time of the pesticides and on its peak responses. Analyses were performed with the HPLC column using a gradient program as it was described in section 2.4. The assayed temperatures were 25, 30, 35, 40, 45 and 50 °C.

The retention time average of five injections, at each different temperature, was compared with the retention time average of five injections at 20 °C. The comparison was performed as follows: the retention time average at 20 °C was subtracted from the retention time average at the assayed temperatures (25–50 °C). These differences did not exceed the ±1 min range even at 50 °C (comparing to 20 °C) but in general, higher temperature causes earlier elution for the compounds. No variations in the peak widths were observed increasing the temperature.

The influence of the temperature in the peak response was also investigated, the signal obtained at 20 °C was divided by the signal obtained when at each different investigated temperatures – from this value, one was subtracted and then was multiplied by 100 to be expressed the difference in percentage. The majority of the investigated compounds showed just ±10% difference even when 50 °C was applied. At this temperature, nine compounds showed signal suppression and seven pesticides presented more than 20% of signal enhancement compared to the results obtained at 20 °C. This indicates that retention time and peak response are not very influenced by column temperature. High temperatures must be controlled otherwise reproducibility problems can arise in some compounds.

3.1.2. UHPLC column with 1.8 μm particle size

Flow rate, injection volume and total run time were optimized for the use of the UHPLC column. A flow rate between 0.2 and 0.4 mL min^{-1} is recommended as laid out in the columns' specifications. However, the flow set in this application was 0.3 mL min^{-1} – working with this flow rate, the pump pressure achieved (330 bar) near to the limit established in the method as the safety pressure value (350 bar), when the composition of the eluent is acetonitrile:water (0.1% formic acid) 20:80. Well, the column can tolerate higher pressure (up to 600 bar), but the HPLC pump could not (up to 400 bar). Whereas the usage of UHPLC pumps is increasing now, still the majority of laboratories who uses LC pumps, have only HPLC and conducting these to an UHPLC column filled with small particles (e.g. <2 μm) is an alternative to reach better throughput under normal HPLC pressure.

The UHPLC columns' specification recommend an injection volume from 1 to 5 μL. Shapes and peak widths were compared by injecting 1, 2 and 5 μL amounts of standards, and 5 μL was chosen as the optimum in order to achieve the best sensitivity.

The most important advantage of using UHPLC columns is the better separation efficiency which allows the reduction in the analysis time without losses in peak capacity.

To optimize the run time, 20 pesticides were selected, eluting in different segments of the HPLC optimized method, and different gradient times were assayed – 33, 20, 15 and 12 min. In run times of 12 and 15 min, some pesticides remained on the column;

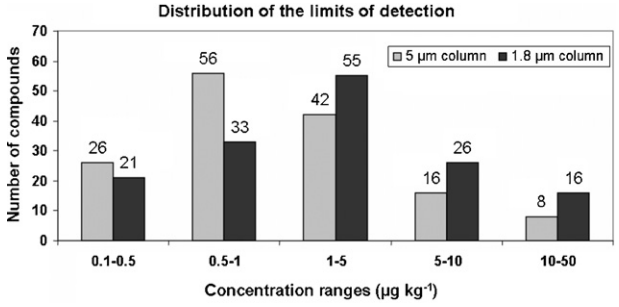

Fig. 1. The distribution of limits of determination of pesticides studied, analyzed by 5 and 1.8 μm column.

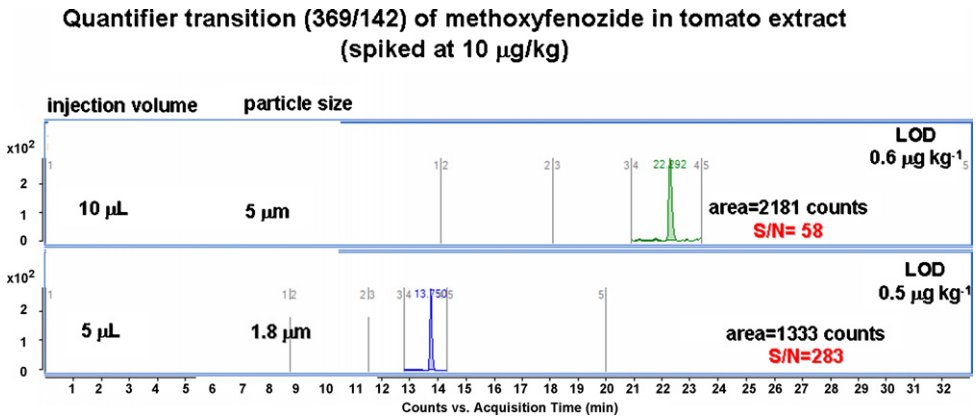

Fig. 2. Similar LODs of methoxyfenozide in tomato extract using two types of columns.

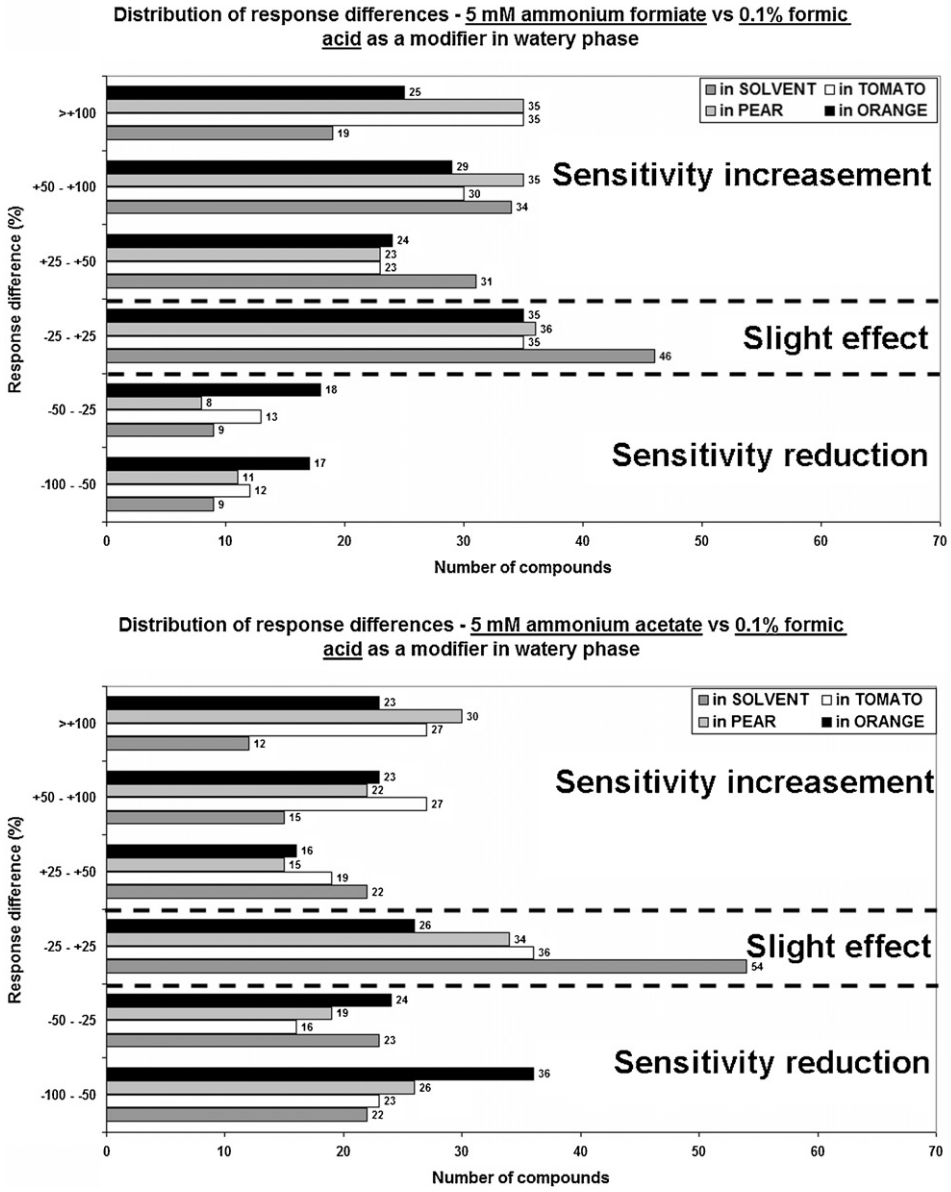

Fig. 3. Distribution of response differences: comparison of modifiers.

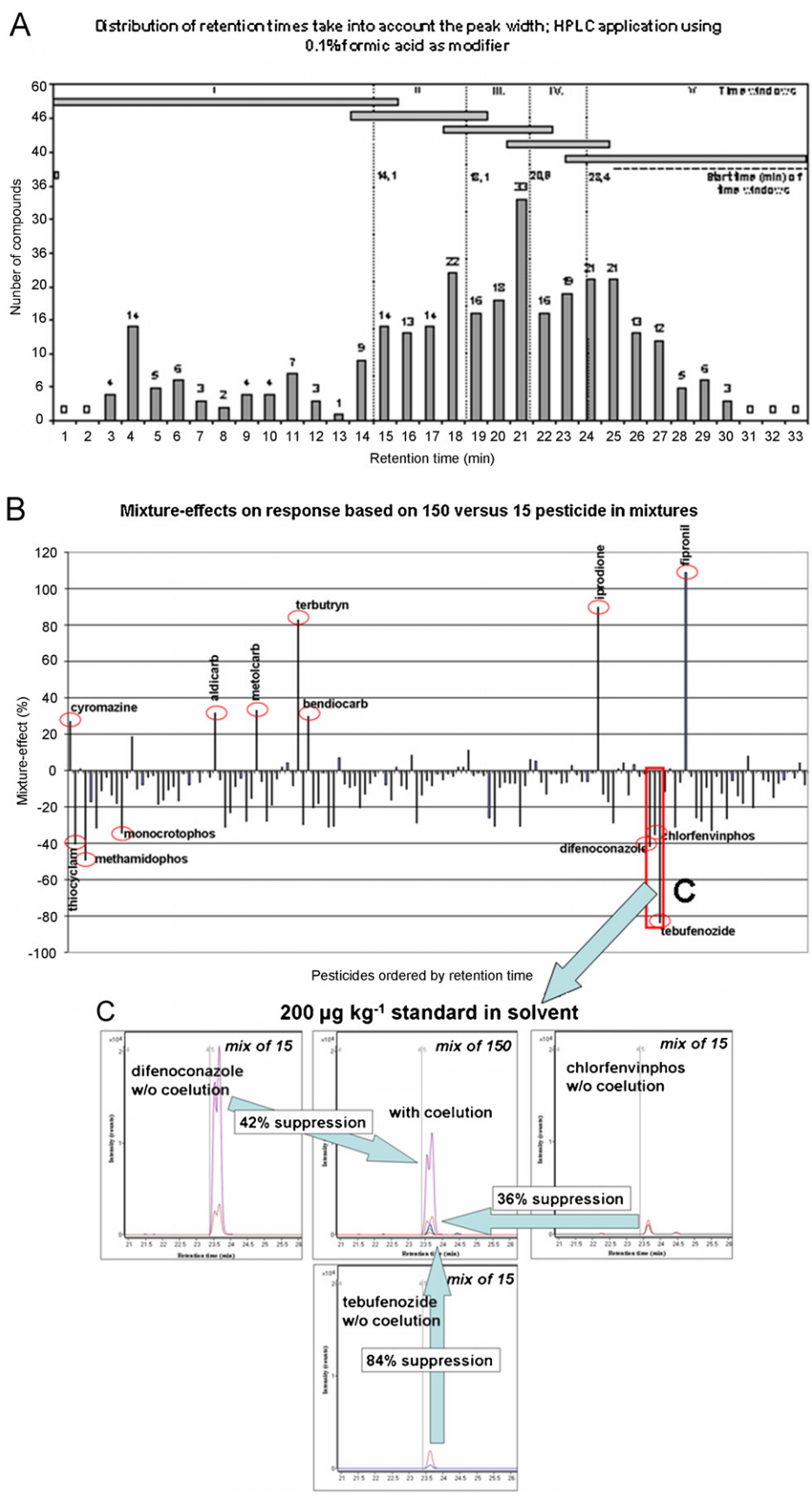

Fig. 4. Mixture effect due to co-elution. (A) Distribution of co-eluting compounds in 1-min slices. (B) Mainly suppression because of competitive effect in the source (some salient values are highlighted). (C) Example of mutual suppression of three co-eluting compounds.

Table 2
Groups of compounds with common transitions. The common fragments are underlined.

Pesticide	HPLC retention time (min)	Peak width (min)	Precursor ion	Quantitation transition (SRM1)	Confirmation transition (SRM2)	Other (unscanned) transitions using 5–20 V as collision energy				
						1	2	3	4	5
Butoxycarboxim	4.8	0.41	223.0	106.0	166.0	86.1	63.0	–	–	–
Acetamiprid	11.3	0.36	223.0	126.0	56.0	90.1	–	–	–	–
Aldicarb sulfone	5.3	0.37	223.0	86.0	148.0	165.9	81.0	76.0	59.1	–
Ethiofencarb	17.8	0.60	226.0	107.0	164.0	143.9	–	–	–	–
Methiocarb	20.3	0.54	226.0	169.0	121.0	93.0	–	–	–	–
Cyprodinil	18.8	0.48	226.2	92.9	76.9	193.1	184.1	167.1	118.1	108.2
Propazine	19.8	0.60	230.0	146.0	188.0	–	–	–	–	–
Terbuthylazine	20.3	0.60	230.0	174.0	146.0	131.9	96.0	79.0	–	–
Dimethoate	11.2	0.48	230.0	199.0	171.0	124.9	88.0	–	–	–
Diuron	17.7	0.44	233.0	72.0	160.0	–	–	–	–	–
Fluometuron	17.3	0.44	233.0	72.0	160.0	–	–	–	–	–
Methiocarb sulfoxide	8.1	0.21	242.0	185.0	170.0	121.9	–	–	–	–
Terbutrin	16.5	0.63	242.0	186.0	158.0	91.0	–	–	–	–
Prometryn	16.4	0.60	242.0	200.0	158.0	109.9	68.0	–	–	–

however in a 20-min run time all the compounds eluted from the column.

A mixture of 150 pesticides was injected five times under the optimized conditions and using formic acid as the modifier. The chromatographic analysis was divided into five retention time segments. Lower peak widths were achieved working with this type of column compared to the ones obtained with the HPLC column: in the range of 0.17 for cyromazine and 0.53 for DEET (diethyl-M-toluamid, N,N-). The average peak width for the 150 pesticides was 0.30 min.

Formic acid was the only modifier used in the analyses performed with the UHPLC column; ammonium formiate and ammonium acetate were also assayed but high pressure was achieved even when the flow rate was 0.2 mL min^{-1}. For this reason, the comparison of different modifiers was studied just in the HPLC column. Probably these can work well by conducting them to an UHPLC pump that can ensure higher pressure (up to 1200 bar).

The injection volume in the 1.8 μm column (5 μL) is half of the injected volume in the 5 μm column (10 μL). The concentration where the signal-to-noise ratio of the qualifier transition was ≥3 was accepted as the limit of detection. The sensibility of both columns was studied and compared in tomato matrix: the limits of detection showed small differences, ±0.15 μg kg^{-1} on average, in 75% of the pesticides. Fig. 1 shows the distribution of the pesticides in different groups, depending on their limit of detection in both columns; each group include a range of concentrations, with limits of detection between 0.1 and 0.5 μg kg^{-1}; 0.5 and 1 μg kg^{-1}; 1 and 5 μg kg^{-1}; 5 and 10 μg kg^{-1} and between 10 and 25 μg kg^{-1}.

Although the volume injected in the 1.8 μm column is half of the volume injected in the 5 μm column, similar limits of detection were achieved working with both columns. More than 90% of the pesticides presented limits of detection lower than 10 μg kg^{-1}.

Fig. 2 shows an example of a tomato extract spiked with a pesticide methoxyfenozide at a concentration 10 μg L^{-1} measured using a 5 μm (10 μL) and a 1.8 μm column (5 μL). The figure shows the area of the quantifier transition in the 1.8 μm column is half of the area in the 5 μm column but, the signal-to-noise ratio is better for the small particle size column, yielding similar LODs for these compounds in both columns.

3.2. Mass spectrometry

3.2.1. MS/MS parameters

The optimization process and all the optimized MS parameters are included in our previous article [36].

3.2.2. Effect of modifiers in the ionization

To study how the modifiers affect ionization, extracts of tomato, pear and orange matrices, spiked with the selected pesticides at the 50 μg kg^{-1} level and the standards in pure solvent were injected five times. These results were compared by calculating the quotient between the signal obtained with ammonium formiate and the signal with formic acid for all the target analytes. One was subtracted from the obtained value, which was then multiplied by 100 to get the enhancement or suppression in percentage. All compounds were categorized into six groups (between −100% and −50%; −50% and −25%; −25% and 25%; 25% and 50%; 50% and 100%; and higher than 100%) according to their sensitivity increasement or reduction.

Fig. 3 shows their distribution in solvent, tomato, pear and orange. Ammonium formiate mainly increased the response in the majority of the compounds, about 75% of the pesticides showed signal enhancement with small differences between different matrices and solvent. It is important to remark that strong signal enhancement (>100%) occurred for acetamiprid, carbaryl, thiacloprid and thiamethoxam in all the studied matrices and in solvent; whereas for fenoxycarb and fenobucarb was only observed in pear matrix. In contrast, strong signal suppression (<50%) occurred with butocarboxin, aldicarb, kresoxim-methyl, thiocyclam and diflubenzuron in all the studied matrices and in pure solvent.

The same method was followed to calculate the percentage of suppression or enhancement when ammonium acetate was used instead of formic acid. In this case the number of compounds presenting suppression was higher than when ammonium formiate was used. Strong signal enhancement (>100%) was observed for butoxycarboxim, emamectin benzoate and prometryn in solvent and in all matrices; however, indoxacarb presented these behaviour only in the studied matrices. Strong signal suppression (<50%) was observed for diazinon, fluometuron, metolcarb and difenoconazole. As it is summarized in the figure, we can consider that a clear difference exists between using ammonium formiate and ammonium acetate as the modifier. Compared to formic acid, using ammonium formiate as the modifier, most of the studied pesticides showed more than 25% signal enhancement.

As mentioned beforehand, no great differences were observed between matrices, the signal suppression in orange [37] is enlarged when ammonium formiate or acetate is employed instead of formic acid. As it is shown in Fig. 3, the number of compounds presenting signal suppression was higher in orange than in other matrices or in solvent.

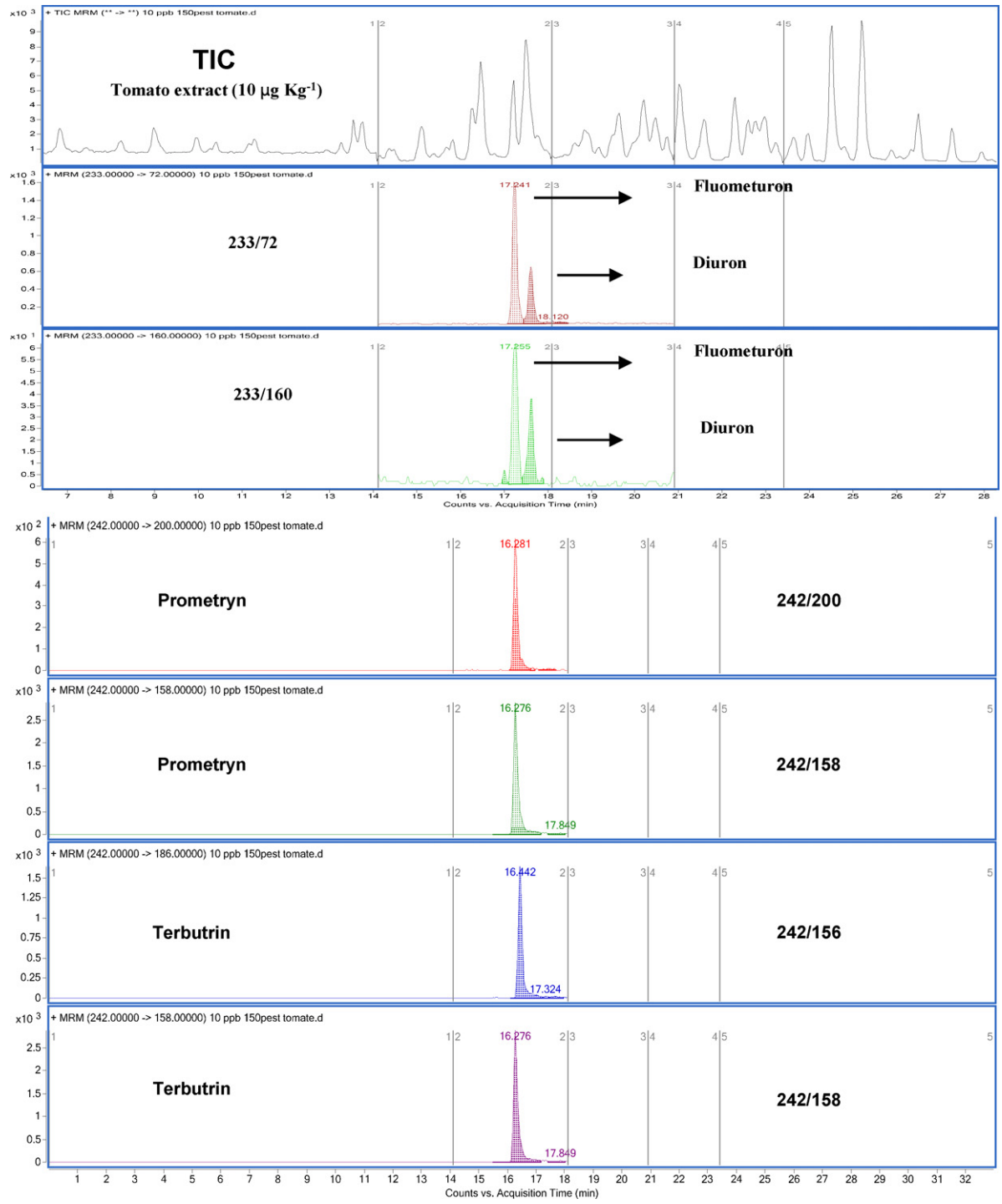

Fig. 5. Total ion chromatogram of a tomato extract spiked at 10 μg kg^{-1} and the extracted common transitions selected for the partners of pesticides fluometuron–diuron and prometryn–terbutryn.

Although the enhancement of the signal for some compounds involves an increase in the sensitivity, some pesticides showed more than 50% suppression, resulting in an increment on the LODs. Nevertheless, all the studied pesticides presented LODs in the range 0.5–10 μg kg^{-1} [36] which are low enough to meet the current established MRLs. The sensitivity increment is an advantage, but not indispensable. Despite the fact the overall sensitivity produced with the studied modifiers was better than the one obtained with formic acid, in the present study formic acid was used because we think it is the most suitable for the development of multiresidue methods in ESI (+) mode.

3.2.3. *Effect of mixtures of standards*

A mixture containing 150 pesticides and 10 mixtures containing 15 pesticides were each analyzed with the proposed method. The signal for each pesticide in the 150 mixture was compared with the signal from the most adequate 15 compound mixture: the purpose of this study was to confirm if any undesirable ionization occurred when the number of compounds being analyzed in the same run changed from 15 to 150.

To compare the signals, the difference of area obtained for each pesticide in the 150 and 15 mixture was expressed in percentage.

Fig. 4A represents the number of compounds eluting in the same 1-min time slices of the chromatogram. It is clear that the majority of the compounds elute in the middle and second half of the chromatogram. Therefore we expected significantly more effect between pesticides in this range. Facing to this, in Fig. 4B, mainly small suppressive effect on the signal was observed when 150 pesticides were in the mix instead of 15, independently on the part of the chromatogram. Besides this, some particular pesticides were more sensitive for co-elution, these analytes are highlighted in Fig. 4B. An example of mutual suppression effect is presented in Fig. 4C, where the signals of difenoconazole, chlorfenvinphos and tebufenozide are suppressed when they co-eluted; consequently, it is an evidence for the presence of competitive effect in the ion source. This cannot be the consequence of the lack of positive charges in the source, because the applied modifiers ensure a concentration of charges much higher than pesticide concentration.

3.2.4. Common transitions

For the identification and quantification of pesticides in multiresidue methods, two transitions are needed as it is recommended by the DG SANCO directive [39].

The advantage of working in tandem mass spectrometry mode versus single mass spectrometry mode is the possibility of scanning specific fragment ions from a parent ion instead of scanning less specific parent ions – as is the case with single mass spectrometry.

The transitions are specific for a particular compound in most of the cases, but when a multiresidue method is created to identify and quantify a large number of compounds, common transitions can occur between them.

Taking into account that (1) formic acid is the modifier most commonly used; (2) ammonium adducts are not very common, even when ammonium formiate or ammonium acetate are used as the modifier; and (3) sodium adducts usually lose the sodium atoms together with the charge – except some particular *N-methylcarbamate* pesticides [40] – during fragmentation; then the common transitions considered in this work are originated only from hydrogen adduct ions. Two transitions of one compound are scanned, but other unscanned transitions could be in the background. It is not disturbing until it is interfering with another transition. From this perspective, depending on the analytical sense, common transitions can be classifed into three groups:

(i) 1–1 (isobaric) scanned transition of different molecules is the same. If these molecules are co-eluting, the quantification must be performed using the second selected transition. In any other cases (w/o co-elution), it is not a problem.
(ii) 1 (scanned) transition and 1 unscanned transition of different molecules is the same. If these compounds are co-eluting, the unscanned transitions can increase the analytical response of the scanned one, therefore the value of the SRM ratio varies depending on the abundance of the isobaric unscanned transition. In any other cases (w/o co-elution), it is not a problem.
(iii) (To sake of completeness) 1–1 unscanned transition from different molecules is the same. This has no analytical sense, because both are unscanned and are in the background, consequently these do not affect any analytical responses.

In order to study the ocurrence of common transitions originating from the 150 pesticides chosen for this work, those with the same molecular mass were selected. Naturally, common transitions can be derived from matrices also, but that was not included in this study. As real samples normally contains none or just a few pesticides, the origin of common transitios could be the calibration mixtures that turns the identification and quantification process more difficult.

A total of 32 groups of compounds with the same parent ion were studied, most of the groups did not presented common transitions. The five groups of compounds which have common transitions are shown in Table 2. Butocarboxim-aldicarb sulfone and cyprodinil-methiocarb have a scanned and an unscanned transition as unscanned isobaric transition (group ii, see above); this does not represent any analytical problem. The pair propazine-terbuthylazine showed two scanned common transitions (group i, see above). Although they have very close retention times, they are not co-eluting compounds, as the difference between their retention times is 0.5 min. The common scanned transition for propazine is the one selected for quantification whereas for terbuthylazine, it is the transition selected for confirmation, which is m/z 146, thus it was not considered as an analytical problem.

Fig. 5 shows the total ion chromatogram and the selected transitions of diuron–fluometuron and prometryn–terbutryn (group i, see above) of a tomato extract spiked at 10 $\mu g\,kg^{-1}$.

Prometryn–terbutryn, are co-eluting compounds and they have the selected confirmation transition in common. Therefore, the confirmation of these two pesticides must be performed by other techniques or by the analysis of these compounds under other conditions. Working with a different collision energy such as 5 eV other transitions can be monitored, i.e., for terbutryn (m/z 91) and for prometryn (m/z 110).

Diuron and fluometuron presented both the quantification and confirmation transitions in common (group i, see above), no other possible transitions were found for these two compounds even working with different conditions. Nevertheless, the retention time difference between them was 0.4 min; thus they are distinguishable by their retention time.

4. Conclusions

The use of ammonium salts as the modifier instead of formic acid reports the enhancement of some compounds but also the suppression in the signal of other pesticides. Any of the three modifiers studied is considered a good choice for multiresidue analysis of pesticides in fruits and vegetables. The obtained results using formic acid meet with the established MRL for all the studied compounds [36]. For that reason, the sensitivity enhancement provided by ammonium-based modifiers is not necessary and considering that the signal of some pesticides can be drastically suppress, then formic acid seems somewhat a better choice than the other two from the aspect of general ionization for multiresidue pesticide analysis.

The column temperature had no great influence on the retention time nor response of the studied pesticides. Only small differences in the retention times were observed when the temperature varied between 25 °C and 50 °C. Moreover, few compounds presented signal suppression at higher temperatures. A possible explanation could be that the pressure is affected by temperature: the viscosity of the mobile phase will decrease as temperature increases.

A fast gradient elution using a column with a small particle size has allowed the analysis of 150 compounds with the reduction of the run time (15 min), without a loss in resolution – compared with a run time of ~30 min in conventional HPLC analysis.

The phenomenon of common transitions can be controlled easily by evaluating the possible interferences between pesticides. Matrix-pesticide isobaric transitions could be an identification problem. In these cases – if it is possible – scanning three transitions could be necessary.

Mixtures of medium and large numbers of standards can show mainly small auto-supression when the number of co-eluted compounds is high. The analyst has to pay attention to some particular pesticides which are sensitive for the competitive effect present in the ESI source due to co-elution.

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