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Monitoring multi-class pesticide residues in fresh fruits and vegetables by liquid chromatography with tandem mass spectrometry☆

A. Garrido Frenich[a], J.L. Martínez Vidal[a,*], T. López López[b], S. Cortés Aguado[b], I. Martínez Salvador[a,b]

[a] *Department of Analytical Chemistry, University of Almería, 04071 Almería, Spain*
[b] *Laboratory of Pesticide Residues CUAM, 04700 El Ejido Almeria, Spain*

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Abstract

A new analytical method was developed using liquid chromatography with tandem mass spectrometry for the routine analysis of 31 multi-class pesticide residues and applied to approximately 50 fresh fruit and vegetable samples (green bean, cucumber, pepper, tomato, eggplant, watermelon, melon and zucchini). Extraction of the pesticides with ethyl acetate was carried out. The optimal ionisation conditions were selected for each pesticide in the same run. The procedure was validated and the values of some merit figures, such as recovery, precision, linear range, detection limit and quantification limit for each pesticide were calculated together with its calculated expanded uncertainty (*U*). The average recoveries in cucumber obtained for each pesticide ranged between 74 and 105% at two different fortification levels (n = 10 each) that ranged between 9 and 250 ng g^{-1} (depending on the pesticide). The uncertainty associated to the analytical method was lower than 23% for all compounds tested. The calculated limits of detection and quantitation were typically <1 ng g^{-1} that were much lower than the maximum residue levels established by European legislation.

Keywords: Fruits; Vegetables; Food analysis; Pesticides; Liquid chromatography; Tandem MS; Uncertainty

1. Introduction

The application of phytosanitary products in vegetables is an usual practice in modern agriculture. They help to control a wide range of pest and plant diseases, and consequently an increase in the harvest productivity is obtained. The toxicity of these compounds makes necessary the monitorization of vegetable quality in order to avoid risks to consumers, as well as to regulate international trade. This has led to the development of many multi-residue analytical methods, which allow the simultaneous determination of a several number of pesticides in food at very low concentration in response to the legislation in many countries. In most instances, capillary gas chromatography (GC) has been the technique selected to the analysis of pesticide residues in vegetables [1–8].

However, it is observed in the last years a tendency towards the use of more polar pesticides, which present lower persistence and toxicity than the apolar compounds. Polar compounds are less suitable for analysis using gas chromatography methods that implies the use of alternative techniques. Liquid chromatography (LC) coupled to mass spectrometry (MS) is the preferred approach for analysing these kind of compounds of low volatility or thermal lability. LC is very effective in separating analytes, while MS allows their identification and/or confirmation at trace levels. In the last few years, LC–MS has been widely used for analysis of pesticide residues in fruits and vegetables [9–12]. More recently, the coupling LC with tandem mass spectrometry

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* Corresponding author. Tel.: +34 950015429; fax: +34 950015483.
E-mail address: jlmartin@ual.es (J.L. Martínez Vidal).

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detection (MS–MS) is gradually becoming important for pesticide residue analysis [13–16]. Monitoring of secondary fragmentation products provides greater discrimination from matrix interferences than the use of primary fragmentation products, that is single-stage MS operation mode. In fact, this technique enables analysis of pesticides at trace levels in the presence of many interfering compounds. The controlled MS fragmentation is an essential tool for obtaining confidence in pesticide identification. In addition, this fragmentation generates cleaner signals improving the signal to noise ratio and decreasing the lower limits.

In spite of high sensitivity and selectivity of the technique, this is still not high enough to directly determine the trace amounts of pesticides in food commodities at the level required by the legislation. Therefore, an adequate extraction procedure for the analytes, by which compound enrichment is achieved, followed in certain cases by clean-up, must be applied. Extraction with solvents, such as acetone, ethyl acetate, or acetonitrile, is commonly chosen for this purpose [13,14,17,18], as it is very simple way of pre-concentration, although some matrix interferences can be also extracted that often require sample purification procedures. The presence of matrix interferences in extracts may result in the occurrence of false positive results or inaccurate quantitation. Aiming at developing methods that can be used for routine analysis, a high degree of selectivity is required. So, sample preparation and clean-up procedures must be avoided in order to reduce the costs of materials and labours.

The aim of this paper has been: (i) the development and validation of a new LC–MS–MS analytical method to determine 31 multi-class pesticide residues, frequently used in food commodities, at trace levels and (ii) the application of the method to real greenhouse samples from an agricultural area on the South Spain (El Ejido, Almería). Until now, another analytical methods have been published for determining pesticide residues in foods by LC–MS–MS [11,13,19,20]. The LC–MS–MS method described in this work is simple (no clean-up), and fast (analysis time lower than 40 min), and allows the simultaneous determination of a higher number of compounds (31) than the previous published methods. In addition, this is the first LC–MS–MS method in vegetables that includes the estimation for each pesticide of its expanded uncertainty (*U*).

2. Experimental

2.1. Chemicals and solvents

Analytical standards were obtained from Dr. Ehrenstorfer (Augsburg, Germany) always with the purity certified between 85.5 and 99.5%. Methanol, ethyl acetate, anhydrous sodium sulphate, formic acid and ammonium formiate for pesticide residue analysis were obtained from Scharlau (Barcelona, Spain). Distilled water provided by a Milli-Q water purification system from Millipore (Bedford, MA, USA) was used. Mobile phases were filtered through a 0.45 μm cellulose acetate (water) or politetrafluoroethylene (methanol). Stock solutions of individual pesticides at 300 μg ml^{-1} were prepared by exact weight and solution in methanol. The storage was in a refrigerator at 4 °C for a period of time not longer than 1 year. The standard working solutions were obtained by appropriate dilutions with methanol and, they were stored in a refrigerator at 4 °C, being used with an expiry date of 2 months to prepare calibration standards and spike the blank samples.

2.2. Liquid chromatography–mass spectrometry

The high-performance liquid chromatograph was a Varian (Walnut Creek, CA, USA), composed of two Prostar pumps, a 410 autosampler with a 10 μl sample loop, a column oven, and a Polaris 3 μm C_{18}-A (150 mm × 2.0 mm i.d., 5 μm particle size) reversed-phase column with a Metaguard cartridge 30 mm × 2.0 mm Polaris 3 μm C_{18}-A, both for Varian (Walnut Creek, CA, USA). The mobile phase was methanol:buffer (2 mM ammonium formate, pH 2.8) at a flow-rate of 0.2 ml min^{-1}. The initial composition was 20% methanol held for 3.5 min, followed by linear gradient to 35% methanol from 3.5 to 4 min. This was held at 35% methanol from 4 to 5 min, then raised to 85% methanol in 20 min, held at 85% methanol for 7 min, followed by a linear gradient to 20% methanol from 32 to 33 min and then the system was re-equilibrate at initial conditions (20% methanol) from 33 to 40 min. All chromatographic solvents were on-line degassed with a vacuum degasser (Varian).

The triple quadrupole system used was a Variant 1200 L Quadrupole MS–MS spectrometer fitted with an electrospray ionization (ESI) interface. The ESI–MS interface was operated in the positive ion detection mode. Calibration of the mass analyzer was performed by infusion (10 μl min^{-1}) of a commercial mixture of polypropylenglycol (Varian) using a 1000 μl Hamilton syringe and monitoring eight mass-to-charge ratios (*m*/*z*) in the 55–2300 μm mass range. The ESI source conditions were: capillary voltage, 5000 V in positive-ion (PI) mode; drying gas temperature, 300 °C; nebulizer gas pressure, 18 psi (both nebulizer and drying gas were high-purity nitrogen; 1 psi = 6894.76 Pa); electron multiplier voltage, 1800 V.

MS–MS experiments were carried out with an argon pressure of approximately 1.8 mTorr in the collision cell. Cone voltage and collision energy values optimized for each of the compounds selected were used. Full-scan product-ion spectra of the $[M + H]^+$ were collected with the first quadrupole (Q1) scanning from *m*/*z* 40 up to 50 amu above the molecular mass of the compound (scan time, 1 scan/s). For selected ion monitoring (SIM) experiments, both Q1 and Q3 were set at fixed *m*/*z* values, viz. $[M + H]^+$ for Q1 and two of the most intense product ions for Q3. For each analyte, the most abundant and characteristic fragment ion was chosen for quantitation and two fragment ions selected for confirmation (Table 1). These mass-to-charge ratios were carefully selected to avoid

Table 1
MS–MS conditions

Number	Compound	Capillary CID (V)	Parent ion (*m*/*z*)	Collision energy	Quantition ion 1 (*m*/*z*)	Collision energy	Quantition ion 2 (*m*/*z*)	Collision energy
1	Pymetrozine	40	218	−11	**105**	−17	79	−29
2	Oxamyl	40	237	−4	**72**	−9	90	−6
3	Carbendazime	40	192	−10	**160**	−15	132	−29
4	Methomyl	40	163	−4	**88**	−7	106	−8
5	Thiabendazole	40	202	−21	**175**	−23	131	−31
6	Imidacloprid	40	256	−8	**209**	−11	175	−15
7	Acetamiprid	45	223	−9	**126**	−18	56	−11
8	Cymoxanil	45	199	−3	**128**	−6	111	−17
9	Metoxuron	45	229	−11	**72**	−13	156	−23
10	Carbofuran	45	222	−7	**165**	−10	123	−20
11	Carbaryl	40	202	−2	**145**	−6	127	−25
12	Monolinuron	40	215	−9	**126**	−17	148	−13
13	Fluometuron	40	233	−11	**72**	−13	46	−7
14	Metobromuron	40	260	−8	**148**	−13	170	−15
15	Isoproturon	40	207	−11	**72**	−15	165	−12
16	Diuron	40	234	−9	**72**	−13	46	−11
17	Dimetomorph	40	388	−4	**301**	−17	165	−26
18	Linuron	40	250	−9	**182**	−12	160	−15
19	Methiocarb	40	226	−4	**169**	−8	121	−17
20	Promecarb	40	208	−4	**151**	−7	109	−15
21	Cyprodinil	55	226	−26	**93**	−31	108	−22
22	Tridemorph	55	298	−26	**130**	−24	116	−22
23	Diflubenzuron	55	311	−8	**158**	−12	141	−26
24	SpinosinA	55	733	−8	**142**	−15	98	−37
25	Prochloraz	55	377	−2	**308**	−8	310	−8
26	SpinosinD	55	747	−10	**142**	−15	98	−35
27	Triflumuron	55	359	−6	**156**	−12	139	−24
28	Hexaflumuron	45	461	−6	**158**	−11	141	−24
29	Tebufenpyrad	45	334	−22	**145**	−24	117	−31
30	Hexythiazox	45	353	−8	**228**	−12	168	−22
31	Lufenuron	45	512	−4	**158**	−12	141	−25
32	Flufenoxuron	45	489	−7	**158**	−13	141	−27

Quantitation ion is indicated in bold.

all those belonging to other pesticide residues of the same class.

A Pentium III personal computer using a Varian (Varian) software (Version 6.10) was used for acquisition and treatment of data.

A Model VV2000 LIF rotary vacuum evaporator (Heidolpf) thermostated by water circulation with a N-010 KN-18 vacuum pump (Telstar) was used to evaporate the extracts. A Model PT 2100 Polytron (Kinematica, Luzern, Switzerland) and a Model BV-401C blender (Fagor, Guipuzcoa, Spain) were used for blending the samples.

2.3. Sample collection and storage

Fresh vegetables from greenhouses located in the province of Almería (Spain) were sampled and transported following the 2002/63/CE Directive. Samples analysed within 24 h being stored at 4 °C until the moment of the extraction.

2.4. Extraction procedure

A sample (10 g) of blended vegetable was placed in a glass and homogenized with 50 ml ethyl acetate for 2 min with Polytron. Then, 20 g of sodium sulphate were added and the mixture was homogenized for 1 min. The extract was filtered through a 12 cm Büchner funnel and washed with two successive 15 ml portions of ethyl acetate. The rinsing were added to the combined extraction fractions. The filtered liquid was collected in a 250 ml spherical flask and evaporated to dryness in a rotating vacuum evaporator with a water bath at 60 ± 1 °C. The obtained residue was redissolved in 5 ml methanol; 0.5 ml of which were taken to 2 ml with distilled water. The background obtained from chromatograms of real samples was very low and thus the extracts did not require further clean-up.

2.5. Validation

Linear dynamic range, precision, recovery, lower limits, selectivity and uncertainty were evaluated for the analytical methodology developed.

For linear dynamic range, the calibration samples were prepared by appropriate dilution of the stock solution in blank matrix extract in order to avoid matrix effects. Calibration solutions, at concentrations ranged between 7 and 750 ng g^{-1} were used.

Intra-assay precision and recovery were assessed using spiked blank samples (10 g) at two concentration levels, at 30% over the first point of the calibration curve and at the level of the second point of the calibration curve. The spiked samples were made adding 130 μl (low concentration level), or 500 μl (medium concentration level), of the mixture standard working solution prepared in methanol containing the pesticides at concentrations about 1 mg l^{-1}. Samples were spiked after homogenizing whole fruit. The spiked samples were allowed to stand for 1 h before extraction to allow the spiked solution to penetrate the test material. Replicated (n = 10) samples were all run and the R.S.D. and recovery values were calculated for each.

Lower limits of detection (LOD) and quantitation (LOQ) were determined as the lowest pesticide concentration injected that yielded a signal-to-noise (S/N) ratios of 3 and 10, respectively, when the quantitation ion was monitored.

The presence of potential interferences in the chromatograms from the analysed samples was monitored by running control blank samples in each calibration. The absence of any chromatographic components at the same retention times as target pesticide suggested that no chemical interferences occurred.

The uncertainty of measurement obtained by applying the analytical method was estimated using the bottom-up approach on the basis of in-house validation data [21]. The combined relative uncertainty (u_{rel}) can be expressed in function of the following expression:

$$u_{rel} = \sqrt{u_{rel}^2(C_c) + u_{rel}^2(F_d) + u_{rel}^2(C_s)}$$

where $u_{rel}(C_c)$ represents the contribution of estimation of the analyte concentration from the calibration curve (C_c); $u_{rel}(F_d)$ represents the contribution of the dilution factor of the sample extract (F_d) and $u_{rel}(C_s)$ represents the contribution of the calculation of the sample concentration (C_s).

The expanded uncertainty, U, is obtained by multiplying u_{rel} by a coverage factor k, assuming a normal distribution of the measurand. Usually, k is 2 [22], which provides an approximate level of confidence of 95%.

3. Results and discussion

3.1. Optimization of LC–MS–MS method

In order to select an LC eluent composition that would provide an overall optimum response for MS detection, different modifiers were selected, acetonitrile or methanol, and several aqueous solutions, 10 mM phosphate buffer (pH 3), a 10 mM ammonium acetate (pH 4) solution and a 10mM ammonium formiate (pH 2.8) solution. It was found that the analyte responses vary widely with the LC eluent composition. Gradient LC with aqueous ammonium formiate–methanol gave the best results. However, analyte responses with an aqueous phosphate buffer, which probably is the buffer most frequently used in LC, were, typically, at least 10-fold lower than with ammonium formiate, both with methanol and acetonitrile as organic modifier. Fig. 1A shows the chromatogram of a spiked sample with the target compounds obtained in the conditions described in the experimental section. As it can be seen, a complete resolution for all the pesticides was not reached. MS–MS allow the analysis without chromatographic resolution between compounds, and therefore, low analysis time can be used.

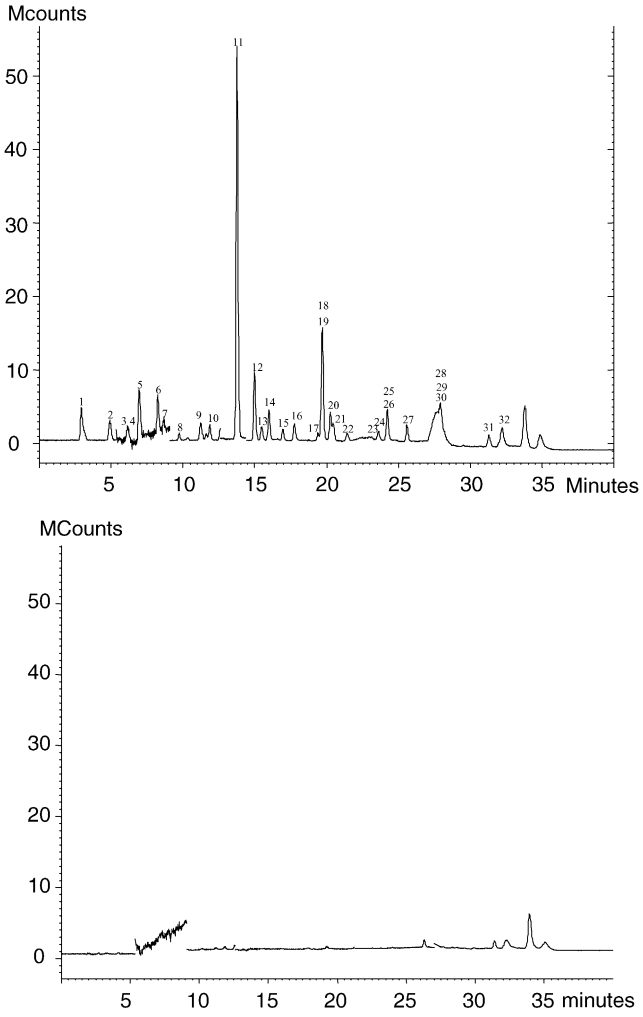

Fig. 1. (A) Typical chromatogram in the SIM mode of a spiked (at the second level in Table 3) blank cucumber sample (numbers in figure corresponding with the order in Table 1), and (B) chromatogram in the SIM mode of a blank cucumber sample.

To optimise the MS–MS conditions, experiments by flow injection analysis (FIA) were carried out by direct infusion of 1 ml of a standard solution of each target compound. The solutions were prepared in a 50% mixture of 10 mM ammonium formiate (pH 2.8) and 50% methanol, and injected in the ESI source at a flow rate of 0.02 ml min^{-1}. At the first place, both positive and negative modes were tested for the ESI source. Since the majority of the compounds showed maximum sensitivity in the positive ionisation (PI) mode, it was decided to continue the experiments in this way. The

exception was the benzoylureas group (diflubenzuron, triflumuron, hexaflumuron, lufenuron, flufenoxuron) that showed a higher signal in the negative mode. However, this sensitivity was still enough to reach the maximum residue limits provided by the legislation. Although, it is possible the use of both ionisation modes in a method, it has to be in different segments (i.e. separated acquisition periods). The limitation is that the switching of mode must be carried out in a time where there is no elution of analytes. As the above conditions were not present in our method, we did not use the negative mode.

Another parameters influencing mass spectra as: gas temperature, drying gas flow, nebulizer gas pressure, and capillary voltage were investigated with the aim of obtaining an intense peak for each compound. This allows to select the precursor ion for each pesticide taking into account its relative abundance and the mass to charge ratio (m/z), both as high as possible. The precursor ion selected in the first quadrupole is submitted to collision-induced dissociation (CID) in the second quadrupole to obtain the product ions monitored in the third quadrupole. The most influence parameter was the capillary voltage. It was varied from 10 to 100 V to find the maximum response for each pesticide in the optimum LC–MS conditions. The precursor ion obtained for each pesticide and the optimum voltage are given in Table 1. The optimum working conditions for the other studied parameters were those described in Section 2.2. Table 1 also shows the optimised collision energy values in the second quadrupole for each compound. Always, the aim was to generate spectra where the parent ion was present (between 10 and 20% of relative abundance). Two values were selected for each pesticide in order to reach an accurate identification in the analysis of samples. The most abundant product ion was selected for quantitation purposes.

3.2. Validation of the method

All validation experiments were performed using cucumber as representative sample matrix from the crop group with high water content.

The selectivity of the method was tested by the analysis of unspiked samples. The absence of any chromatographic signal at the same retention times as target pesticide suggested that there were no matrix compounds that might give a false positive signal (Fig. 1B). The background obtained from chromatograms of real samples was low, indicative of that the extracts did not require further clean-up (Fig. 1B). However, in Fig. 1B there were background peaks at the retention times of lufenuron and flufenoxuron, which disappeared when the quantitation ions of the above mentioned pesticides were monitored. That means that although the precursor ion of the blank and of the mentioned pesticides, respectively, coincided, the quantification ions or the fragment ions selected for confirmation were different and for that there was no interferences in the determination of any of the pesticides. On the other hand, it can also observed in Fig. 1B the baseline drift in the 5–8 min range, which can be explained taking into account the increase of methanol in the gradient of the mobile phase.

The identification of the target pesticides was carried out by searching in the appropriate retention time windows (RTWs), retention time average ±3 standard deviations of the retention time of 10 blank samples spiked at a mid-level calibration standard for each compound, Table 2.

The quantitation of the samples was carried out by injecting daily blank sample extracts spiked with the pesticides at four different concentration levels to perform the calibration curves. The concentrations of the calibration levels were selected for each pesticide according to the maximum residue limits (MRLs) established by European Union legislation [23]. The first calibration level was always equal or lower than the MRLs established. As an example, Table 3 summarizes the MRLs values for cucumber matrix. The linearity of the calibration curves was studied without including the origin point and, better quantitation results were obtained when peak area rather than peak height was considered. The calibration data obtained are shown in Table 2. Good linearity of the response was found for all pesticides at concentrations within the tested interval, with linear determination coefficients higher than 0.984.

The LOD and LOQ values obtained are shown in Table 3. In general, excellent values were obtained, with LOQ values in the range of a few $\mu g\,kg^{-1}$. The exception was methomyl that showed poor LOD and LOQ values, probably due to its poor chromatographic response and relatively high background presented for their ions.

The accuracy of the method was calculated through the recovery of each pesticide. For that an extraction procedure was optimised using ethyl acetate. Different solvent volumes (50, 60 and 70 ml) were used but not better results were obtained using higher volumes, so 50 ml were selected. However, it was necessary to wash with two successive 15 ml portions of ethyl acetate the Büchner funnel to obtain good recoveries. In the extraction procedure, the evaporation to dryness was specially controlled in order to avoid loss of more volatile compounds.

The recovery rate of each pesticide at two different fortification levels was evaluated in order to assess the extraction efficiency of the proposed method. For this, 10 g of uncontaminated cucumber samples were spiked with the pesticides at each fortification level (at 30% over the first calibration level and, at the second calibration levels of the calibration curves). Satisfactory results were found in both instances, with recoveries between 72 and 104% (Table 3). Settling down as a criterion for validation recoveries of the compounds ranged between 70 and 110%, all the pesticides gave acceptable recoveries within the mentioned validation interval.

The intra-assay (repeatability) precision was assessed, at the two concentration levels of the recovery studies, by extraction and analysis on the same day of 10 fortified vegetable samples for each level (Table 3). For the intermediate ("inter-assay" or reproducibility) precision a set spiked samples at the two concentration levels were analyzed each week for 5

Table 2
Retention times windows (RTWs) and typical calibration parameters of the method in cucumber matrix

Compound	RTW (min)	Calibration range ($mg\,kg^{-1}$)	Calibration parameters		
			Slope	Intercept	R^2
Pymetrozine	2.9–3.1	0.014–0.210	4.5*E* + 08	2.5*E* + 06	0.998
Oxamyl	4.8–5.1	0.035–0.525	4.5*E* + 08	5.5*E* + 06	0.997
Carbendazime	6.2–6.4	0.035–0.525	1.3*E* + 09	2.4*E* + 07	0.997
Methomyl	6.1–6.3	0.050–0.750	2.0*E* + 08	4.2*E* + 06	0.997
Thiabendazole	7.9–8.0	0.035–0.525	6.9*E* + 08	9.2*E* + 06	0.995
Imidacloprid	8.6–8.7	0.035–0.525	1.5*E* + 08	1.6*E* + 06	0.997
Acetamiprid	9.7–9.9	0.007–0.105	7.5E + 08	2.0*E* + 06	0.998
Cymoxanil	10.3–10.4	0.035–0.525	1.1*E* + 08	2.1*E* + 06	0.997
Metoxuron	11.8–12.1	0.007–0.105	6.8*E* + 08	−1.3*E* + 06	0.997
Carbofuran	13.7–14.0	0.035–0.525	3.7*E* + 09	2.7*E* + 07	0.997
Carbaryl	15.0–15.3	0.035–0.525	3.5*E* + 09	3.8*E* + 07	0.998
Monolinuron	15.5–15.7	0.035–0.525	4.6*E* + 08	5.6*E* + 06	0.998
Fluometuron	16.0–16.2	0.035–0.525	7.6*E* + 08	8.6*E* + 06	0.998
Metobromuron	16.5–16.7	0.007–0.105	2.9*E* + 08	5.9*E* + 05	0.997
Isoproturon	17.0–17.2	0.007–0.105	1.5*E* + 09	2.8*E* + 06	0.999
Diuron	17.8–18.0	0.035–0.525	4.2*E* + 08	7.7*E* + 06	0.998
Dimetomorph	20.5–20.7	0.014–0.210	1.0*E* + 09	3.4*E* + 06	0.999
Linuron	19.7–20.0	0.035–0.525	1.4*E* + 08	4.2*E* + 06	0.995
Methiocarb	19.7–19.9	0.035–0.525	2.7*E* + 09	1.7*E* + 07	0.999
Promecarb	20.3–20.5	0.007-0.105	3.2*E* + 09	9.4*E* + 05	0.999
Cyprodinil	21.6–21.8	0.014-0.210	2.4*E* + 08	−9.6*E* + 05	0.993
Tridemorph	22.6–23.7	0.035–0.525	4.6*E* + 08	5.8*E* + 06	0.997
Diflubenzuron	23.7–23.9	0.035–0.525	1.8*E* + 08	1.2*E* + 06	0.992
SpinosinA	24.1–24.4	0.007–0.105	5.0*E* + 08	−1.9*E* + 05	0.998
Prochloraz	24.4–24.6	0.035–0.525	3.7*E* + 08	7.9*E* + 06	0.997
SpinosinD	25.4–25.8	0.007–0.105	1.2*E* + 08	9.0*E* + 04	0.999
Triflumuron	25.7–25.9	0.035–0.525	1.7*E* + 08	3.7*E* + 06	0.992
Hexaflumuron	27.9–28.2	0.035–0.525	6.1*E* + 06	1.6*E* + 05	0.997
Tebufenpyrad	28.0–28.3	0.035–0.525	2.5*E* + 08	5.5*E* + 06	0.993
Hexythiazox	29.6–30.0	0.007–0.105	2.8*E* + 08	1.1*E* + 06	0.991
Lufenuron	30.5–31.1	0.02–0.300	8.0*E* + 06	6.8*E* + 04	0.984
Flufenoxuron	32.1–32.8	0.007–0.105	1.8*E* + 08	3.6*E* + 05	0.991

weeks. Repeatability and intermediate precision values, expressed as relative standard deviation, lower than 19 and 22%, respectively, were obtained.

The uncertainty (U) values measured for the LC–MS–MS method at two concentration levels (at 30% over the first calibration point and, at the second calibration levels of the calibration curves) were evaluated. For that the identification of the main sources of uncertainty of the analytical method was performed on the basis of a previous study performed in our laboratory [21], finding that the dispersion of results around the true value depended upon the following steps: (i) estimation of the analyte concentration from the calibration curve (C_c); (ii) dilution factor of the sample extract (F_d) and (iii) calculation of the sample concentration (C_s). Conclusions obtained in reference [21] can be applied in the current study because the general procedure of both papers is similar, the only differences between both were the analytical technique used, GC instead of LC, the detection system, electron capture detector instead of MS, and the extraction solvent, dichloromethane instead of ethyl acetate. Uncertainty values ranged from 15% for tridemorph to 23 % for imidacloprid and cyprodinil (Table 3) were obtained for both calibration levels.

After the identification by retention time window, a compound is confirmed comparing the MS–MS spectra obtained from an analysed sample with those previously stored as reference spectra obtained in the same experimental conditions. Such reference spectra are checked daily by matching they with the spectra obtained from the second calibration level injected to quantify the results. The comparison results (fit parameter) are scaled to 1000 for the best match (identical spectra). To set the fit threshold for each pesticide, 10 spectra are obtained at the second calibration level, during the validation step, under the same analytical conditions. One of these spectra is selected as "reference validation spectrum" and the other nine spectra are compared with it. The product of the comparison is nine fit values (from 0 to 1000 for best match) and an average fit value. A threshold fit value defined as the average fit value minus 250 units is considered admissible for identification purposes. The differences in the fit values can be explained taking into account the spectral variations in routine analysis of samples, as consequence of maintenance operations that would slightly affect to the detector response or due to the pesticide concentration, which would affect the proportion of product ions in the spectra.

The software of the instrumental system would confirm the presence of the pesticide if fit exceeded the threshold value and the signal-to-noise ratio was greater than 3 in the chromatographic peak obtained, when the quantitation ion is

Table 3
Recovery, precision (R.S.D.), LOD, LOQ, uncertainty (*U*) and maximum residue level (MRL) values in cucumber matrix[a]

Compound	First level (n = 10)			Second level (n = 10)			LOD ($\mu g\,kg^{-1}$)	LOQ ($\mu g\,kg^{-1}$)	*U* (%)	MRL ($mg\,kg^{-1}$)
	Concentration ($\mu g\,kg^{-1}$)	Recovery (%)	R.S.D. (%)[b]	Concentration ($\mu g\,kg^{-1}$)	Recovery (%)	R.S.D. (%)[b]				
Pymetrozine	18	82	12	70	74	6	0.280	0.950	18	0.5
Oxamyl	46	84	9	175	87	6	0.097	0.320	17	0.05
Carbendazime	46	104	15	175	88	5	0.170	0.560	20	0.5
Methomyl	65	97	16	250	86	8	6.400	21.000	22	0.05
Thiabendazole	46	91	12	175	87	8	0.036	0.120	19	0.05
Imidacloprid	46	98	19	175	85	6	0.250	0.840	23	0.05
Acetamiprid	9	104	7	35	89	7	0.064	0.210	17	0.01
Cymoxanil	46	85	17	175	86	11	0.790	2.600	22	0.05
Metoxuron	9	102	11	35	89	5	0.086	0.290	19	0.01
Carbofuran	46	90	14	175	86	4	0.031	0.100	18	0.1
Carbaryl	46	86	16	175	87	6	0.046	0.150	21	1
Monolinuron	46	87	13	175	84	6	0.081	0.270	20	0.05
Fluometuron	46	90	15	175	86	5	0.100	0.340	21	0.01
Metobromuron	9	97	15	35	86	8	0.140	0.460	21	0.05
Isoproturon	9	93	13	35	88	4	0.110	0.370	22	0.05
Diuron	46	91	14	175	87	6	0.380	1.300	19	0.05
Dimetomorph	18	98	12	70	90	6	0.012	0.040	20	0.02
Linuron	46	89	15	175	80	8	0.160	0.540	20	0.05
Methiocarb	46	81	17	175	93	11	0.410	1.400	21	0.05
Promecarb	9	93	15	35	83	6	0.032	0.110	21	0.01
Cyprodinil	18	91	18	70	83	7	0.280	0.930	23	0.02
Tridemorph	46	75	5	175	75	4	0.530	1.800	15	0.05
Diflubenzuron	46	105	10	175	82	16	0.320	1.100	19	0.05
SpinosinA	9	105	13	35	85	10	0.011	0.038	21	0.01
Prochloraz	46	83	11	175	79	8	0.028	0.094	18	0.05
SpinosinD	9	95	15	35	81	9	0.027	0.089	21	0.01
Triflumuron	46	94	14	175	80	8	0.110	0.370	20	0.01
Hexaflumuron	46	91	20	175	82	13	0.410	1.400	23	0.01
Tebufenpyrad	46	98	15	175	83	13	0.080	0.270	21	0.05
Hexythiazox	9	92	15	35	83	8	0.043	0.140	21	0.05
Lufenuron	26	97	13	100	94	15	0.570	1.900	20	0.01
Flufenoxuron	9	91	14	35	86	12	0.092	0.310	20	0.01

[a] Mass spectral match factor.
[b] Intra-assay precision values: *U* values estimated at the first level of concentration.

monitored. The instrument software can be programmed to automatically carry out detection, confirmation and quantitation of the positive results. Nevertheless, these operations can be supervised by the analyst or be manually carried out.

3.3. Internal quality criteria

To assure the quality of results when the proposed method is applied to routine analysis various internal quality criteria have been established. The set of samples analyzed each day was processed together with:

(i) A blank extract that eliminates a false positive by contamination in the extraction process, instrument or chemicals used.
(ii) A blank extract spiked at the concentration of the second calibration level in order to assess the extraction efficiency. Recovery rates between 60 and 120% are accepted if (a) the majority of recoveries are within 70–110% range and (b) samples which contain residues in a batch are re-analyzed and the results reported are within the 70–110% range.
(iii) Calibration curves prepared daily to check both, sensitivity and linearity in the working range of concentrations in order to avoid quantitation mistakes caused by possible matrix effects of instrumental fluctuations ($R^2 > 0.9$ are requested).

In addition, to control the evolution of the analytical process with the time quality control charts are constructed representing the results obtained from the analysis of quality control (QC) samples each week. The QC samples are prepared by spiking blank cucumber matrices with the target compounds at the second concentration level.

3.4. Application of the method to real samples

The proposed method has been applied to the routine analysis of approximately 50 real vegetable samples of different matrices (green bean, pepper, cucumber, eggplant, tomato,

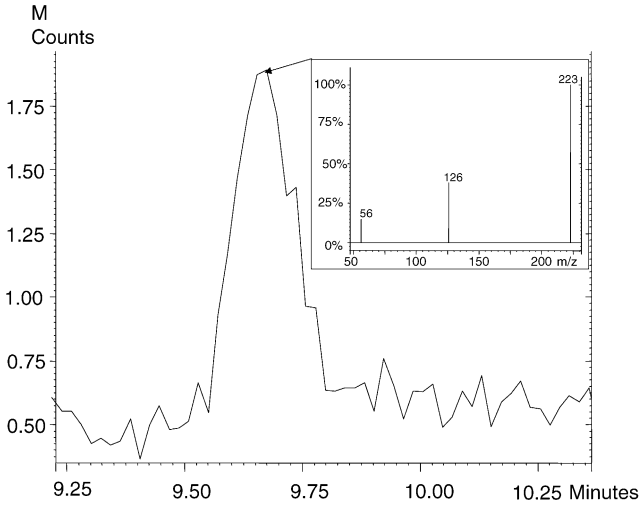

Fig. 2. Acetamiprid chromatogram and spectrum in a positive sample of pepper. Concentration found 0.01 mg kg^{-1}, ions used 56 and 126, LOD and LOQ 0.064 and 0.210 μg kg^{-1}, respectively.

zucchini, watermelon and melon) in the laboratory CUAM (El Ejido, Almería, Spain). For this aim, matrix matched calibrations were prepared in each one of the analysed matrices in order to avoid possible matrix effects. The laboratory is accredited by UNE-EN-ISO/IEC 17025 for pesticide residue analysis, and so, internal quality control procedures are routinely applied in order to check if the system is under control.

The results showed that the 35% of the analyzed samples gave positive values (higher than the routine quantitation limit). Only 16% of them overcame the levels established by the European legislation (MRL). The pesticides most frequently found over EU MRLs were acetamiprid and spinosad with one and two cases, respectively. Traces of other compounds (imidacloprid, carbendazime and oxamyl, overall) were detected. This relatively low number of samples rejected for their consumption is due to the proper usage of pesticides in agricultural matrices in the studied area. Fig. 2 shows a positive of acetamiprid in pepper as an example of a real sample analyzed.

4. Conclusions

The present method based on a rapid and non-selective extraction with ethyl acetate allows the simultaneous determination of 31 pesticides in vegetables by LC–MS–MS. It combines the advantages of LC and MS for the separation and unequivocal identification of pesticides belonging to different classes in real matrices. It showed satisfactory validation parameters, such as accuracy, precision, lower limits or selectivity. For all the pesticides, the sensitivity of the method was good enough to ensure a reliable determination at levels lower than the respective MRL.

In addition, the analysis time is short allowing the routine analysis of large number of samples. Finally, it is complementary to a GC–MS–MS method previously developed for the determination of pesticides of good thermal stability and low polarity in vegetables [2]. The coupling of the chromatographic techniques with MS provided us with an efficient and reliable methodology for the pesticide residue analysis in vegetable and fruit matrices.

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