Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1045 (2004) 125–135
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

One-year routine application of a new method based on liquid chromatography–tandem mass spectrometry to the analysis of 16 multiclass pesticides in vegetable samples☆

Ana Agüera[a], Susana López[a], Amadeo R. Fernández-Alba[a,*], Mariano Contreras[b], Juan Crespo[b], Luis Piedra[b]

[a] *Pesticide Residue Research Group, University of Almería, 04071 Almería, Spain*
[b] *Laboratorio de Análisis Agrícolas de COEXPHAL, Cosecheros-Exportadores de Hortalizas de Almería, 04070 Almería, Spain*

Received 8 March 2004; received in revised form 1 June 2004; accepted 17 June 2004

Abstract

An analytical procedure has been developed for determining a group of 16 multiclass pesticides most commonly used in crop protection. The extraction step is performed with a mixture of ethyl acetate and sodium sulphate, in the presence of 6.5 M NaOH. After evaporation, a final extract containing 1 mg sample/ml extract, is obtained in methanol. Analysis of the methanolic extract, without additional clean-up steps, is performed by liquid chromatography–electrospray ionisation-tandem mass spectrometry combining positive and negative ion mode. The analytical performance of the method has been validated for three different matrices: pepper, lettuce and aubergine. Mean recoveries obtained were between 70 and 110% in most of the cases with a precision of <28%. Linearity of response over 2 orders of magnitude was demonstrated ($r \geq 0.99$) with limits of detection ≤0.01 mg/kg in all the cases. No significant matrix effects were observed with the exception of triflumizol that presented a drastic decrease in response as a consequence of an ion formation suppression effect in the presence of pepper matrix. The method has been applied to the analysis of 560 vegetable samples, as a part of the monitoring programme of the Association of Producers and Exporters of Fruits and Vegetables of Almería (COEXPHAL). The unambiguous confirmation of the positive findings by comparison of the product ion mass spectra of the peaks in samples and standards, demonstrated the applicability of the method in routine analysis.

Keywords: Vegetables; Food analysis; Pesticides

1. Introduction

Gas chromatography (GC) with selective detection or, more recently, coupled to mass spectrometry (GC–MS) has been the analytical option more frequently applied to the analysis of pesticide residues in foods [1–5]. However, the strict regulations imposed by the European Union (Directive 98/82/CE), with maximum residue levels (MRLs) each time more restrictive, and the appearance in the market of new formulations containing active ingredients no amenable to GC have made necessary the application of alternative analytical techniques. Among these techniques liquid chromatography coupled to mass spectrometry (LC–MS) is growing in importance, especially with the appearance of atmospheric pressure (API) interfaces, electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI), which provide an improved sensitivity and selectivity compared with classical detectors [6]. Although the enormous potential of LC–MS has contributed to the development in the last years of numerous applications, specially in the field of food [7–17] and environmental analysis [18–20], a certain number of disadvantages and limitations have been also evidenced affecting both qualitative and quantitative determination [21]. One of these disadvantages is the limited fragmentation obtained under API conditions that can induce to uncertainty in pesticide confirmation. Some strategies are available to partially compensate this

☆ Presented at the 3rd Meeting of the Spanish Association of Chromatography and Related Techniques and the European Workshop: 3rd Waste Water Cluster, Aguadulce, Almeria, 19–21 November 2003.

* Corresponding author. Tel.: +34 950 01 50 34; fax: +34 950 01 54 83.
E-mail address: amadeo@ual.es (A.R. Fernández-Alba).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.06.039

drawback. Therefore, the increase of the fragmentor voltage using a single quadrupole or the use of tandem mass spectrometric detection with ion trap or triple-quadrupole instruments can yield more fragmentation [22,23].

Another limitation is the ion suppression effect, observed as a consequence of the presence of sample matrix during the ionisation of the target analytes that can reduce drastically the chromatographic signal and, thus, affect both quantitation and detectability of the pesticide residues in real samples [21,24]. This problem has a more difficult solution because of its unpredictability and the high dependence of the kind of matrix considered.

All these considerations have made that, despite the potentiality of the LC–MS technique, some doubts exist about its applicability in routine analysis. Thus, the aim of this study has been: (i) to develop a sensible and specific analytical method to determine 16 highly used pesticides in a variety of food commodities at concentration levels of $\mu g\,kg^{-1}$; and (ii) to apply the method in a 1 year monitoring programme under strict quality assurance conditions to demonstrate the ruggedness of the total procedure.

2. Experimental

2.1. Chemicals

Ethyl acetate and cyclohexane for pesticide residue analysis were obtained from Scharlau. Anhydrous sodium sulphate was purchased from Panreac (Barcelona, Spain). Sodium hydroxide, formic acid and ammonium formiate were from Fluka. Methanol and acetonitrile (HPLC grade) was purchased from Scharlau. A Milli-Q-Plus ultra-pure water system from Millipore was used to obtain the HPLC-grade water used during the analyses.

Pesticide analytical standards (purity > 96%) were provided by Dr. Ehrenshtorfer (Augsburg, Germany). Pesticide selected in the study and their chemical structures are exposed in Fig. 1. Individual pesticide stock solutions (250–300 μg/mL) were prepared in methanol and stored in the dark at −18 °C. Following a quality control procedure, an exhaustive weight control of theses solutions is applied to detect possible losses by evaporation. Weight changes higher than 0.2 g are not allowed. Working standard mixtures (10 mg/L for each pesticide) were prepared in methanol and stored at 4 °C. Both, stock and working solutions are stored for a maximum time of 1 year, provided that no changes in their concentrations are detected. A solution of triphenyl phosphate (TPP) 99% (Merk, Darmstadt, Germany) at a concentration of 25 mg/L, was also prepared in cyclohexane and used as internal control during the sample preparation and analysis.

2.2. Sample preparation

Samples of the different matrices used in this study came from greenhouses located in the province of Almería, in the southeast of Spain. Samples found to contain no detectable residues of the target pesticides were used for method validation assays and for the preparation of matrix-matched calibration solutions.

The extraction procedure is described as follows. Fifteen gram portion of sample previously homogenised was weighed in a 200 mL PTFE centrifuge tube. For the recovery tests, the samples were fortified with aliquots of the 10 μg/mL spiking solution and around 3 h were allowed to favour the interactions pesticide/sample the solvent evaporation. A 0.2 mL aliquot of the TPP solution, prepared as it was described in Section 2.1, was added to the sample just prior to extraction to give a final concentration in the analysis of 0.33 μg/mL. Then, 90 mL ethyl acetate and 1 mL NaOH (6.5 M) were added and blended in Polytron for 30 s at 21,000 rpm. After this time, 13 g anhydrous Na_2SO_4 were added repeating the extraction again for 30 s. The extract was then filtered through a thin layer of 20 g of anhydrous Na_2SO_4. After that, the solid was washed with 50 mL of ethyl acetate and the combined extracts were evaporated to dryness on a vacuum rotary evaporator using a water bath at 45 ± 5 °C. The remaining residue was dissolved by sonication in 15 mL of methanol. The extracts so obtained, containing 1 g of sample per mL, were filtered through 0.2 μm PTFE filters (Millex FG, Millipore) before LC–MS analysis.

2.3. Instrumentation

The triple quadrupole system used was a Varian 1200 L Quadrupole MS–MS spectrometer (Varian, Lake Forest, CA, USA) fitted with an ESI interface and controlled by Kodiak software V2.1.023. Typical interface conditions were optimised for maximum intensity of the precursor ions as follows: nebulizer and desolvation (drying gas) N_2 pressures were set to 48 and 25 psi, respectively, source block and desolvation temperatures were 40 °C and 300 °C. The ESI polarity ionisation mode was set individually for each target compound.

MS–MS: Argon was used as collision gas at a pressure of 2 mTorr (1 Torr = 133.322 Pa). A Version 6.10 Varian Mass Spectrometry Workstation software was used for data acquisition under multiple reaction monitoring (MRM) mode. Selection and tuning of MRM transitions were performed individually for each analyte. For optimising the mass spectrometer, direct infusion of 1 mL of a standard solution of each analyte was used. The standard solutions were prepared in a 50% mixture of Solvent A (2 mM NH_4COOH/0.1% HCOOH) and Solvent B (acetonitrile–methanol, 3:1 (v/v)), and introduced in the ESI source at a flow of 1 mL/min. The scan time was set to 1 s/scan.

LC separations were performed in a Varian Prestar liquid chromatography system by using a Polaris C18-A column (150 mm × 2 mm, with 3 μm particle size) and a precolumn (30 mm × 2 mm) supplied by Varian. A sample volume of 10 μL was injected with a Varian 410 autosampler (loop volume of 100 μL) in the micropick-up mode. The mobile

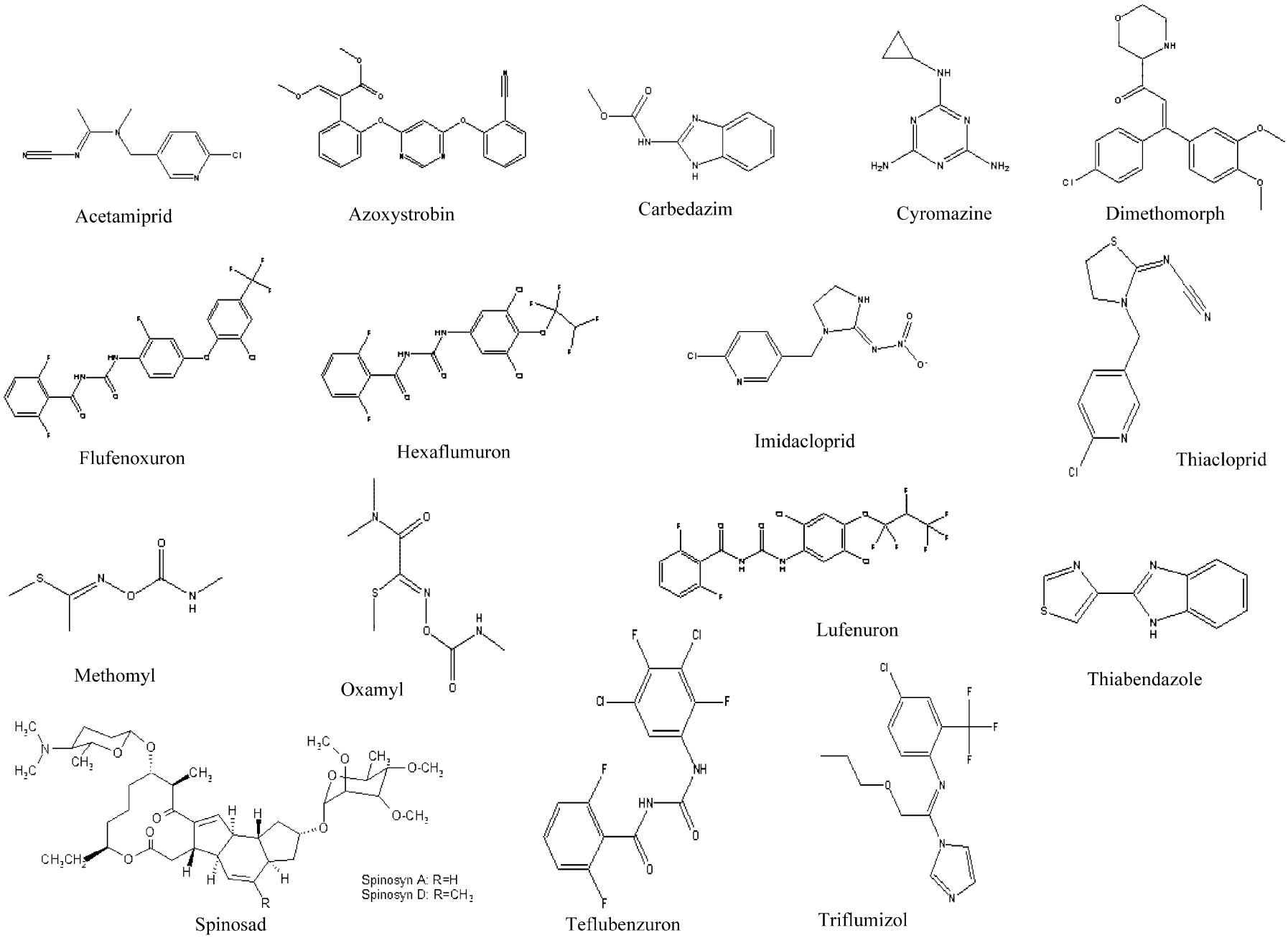

Fig. 1. Chemical structures of the pesticides studied.

phase was composed of Solvent A (2 mM NH_4COOH/0.1% HCOOH) and Solvent B (acetonitrile–methanol, 3:1 (v/v)) at a constant flow of 0.2 mL/min. The gradient was programmed to increase the amount of B from an initial 5–35% in 3 min, to 42% in 10 min, to 65% in 12 min, to 70% in 25 min, to 100% from 25.01 to 27 min, returning to the initial conditions in 27.06 min. Total time of analysis was 40 min including an equilibration time of 13 min prior to the next analysis.

2.4. Validation studies

The method development was performed at University of Almería. All validation studies were performed by using pesticide-free samples previously analysed. The linearity in the response was studied by using pure methanol and matrix blank extracts solutions (from pepper, lettuce and eggplant) to evaluate possible matrix effects. Calibration solutions, at concentrations of 10, 25, 50 100, 250, 500 and 1000 μg/L were used for this purpose and the integrated peak area data of the selected quantification masses (see Table 1) were used to construct the curves. The calibration curves generated with matrix-matched standards were used for quantification purposes. Recovery studies were determined in triplicate at two concentration levels, 0.05 and 0.50 mg/kg for the three matrices studied. The limit of detection (LOD) was determined as the analyte concentration that gave a S/N of 3, as calculated by the instrument software, and empirically verified by analysing pesticide mixtures at these concentration levels in matrix extracts to check the presence of all the diagnostic ions at their correct relative abundances.

2.5. Quality control

Routine analyses were performed in COEXPHAL, an association of fruits and vegetables producers and exporters of Almería (Spain). The laboratory works under a quality system and therefore quality control procedures are routinely applied to assess the quality of the results.

With each analytical set analysed, the calibration was checked by the injection of three calibration solutions. The first one contains the pesticides at a half of the concentration correspondent to the lower MRL established in the EU Directives or Spanish regulation, among the different pesticide/commodity combinations. This amount is considered as the reporting limit. The second calibration solution contains pesticides at 0.25 mg/kg and the third one at 0.50 mg/kg. Calibration curves are recalibrated and reviewed to check if all the points have been correctly detected and quantified and if the new curves are linear ($r > 0.99$). These new curves are used for the quantitative analyses of the samples.

Control of the extraction step in all the samples is done by the addition of TPP to the samples before the extraction. The presence of the TPP peak at the correct retention time and with the expected recovery (70–110%) is considered as the evidence of an efficient sample handling process. If TPP response falls out from the interval allowed, the sample is reanalysed and if the difference persists the whole extraction procedure is repeated. Additionally duplicate samples were also analysed fortnightly. These were samples, coming from the monitoring program, which yielded positive results for one or more of the pesticides studied.

To monitor the variation of the analytical process with the time, quality control charts are plotted representing the results obtained from weekly analyses of quality control (QC) samples. These QC samples are prepared into the laboratory by spiking "blank" matrixes (lettuce) with thiabendazole and teflubenzuron (at 100 μg/kg).

A procedural blank analysis is also performed in the absence of matrix to check possible interferences derived from the application of the method, such as impurities presents in solvents and reagents, possible sources of contamination, etc.

3. Results and discussion

3.1. Performance of the extraction procedure

A simple method based in the use of ethyl acetate and sodium sulphate was developed for the extraction of the group of pesticides selected. The method was based in a previously reported extraction procedure that was validated for the GC–MS determination of 55 multiclass pesticides (organophosphorous, organochlorinated and pyrethroids) in fruits and vegetables [5]. This method demonstrated to be simple, rapid and efficient for such a group of compounds but direct application to the new pesticides selected (see Fig. 1) yielded recoveries lower than 70%. Therefore, some modifications had to be introduced to improve the efficiency of the method. The pesticides studied were selected based on their extensive application in crops from the Mediterranean area and belong to different chemical classes, benzoylureas, benzimidazoles, carbamates and others, presenting different physico-chemical properties. One of the modifications introduced was the addition of a 6.5 M sodium hydroxide solution that contributed to the stabilization of the analytes and improved significantly the extraction efficiency for all the compounds with the exception of oxamyl that exhibited recoveries lower than 50%. Another critical point in the extraction method was the selection of the solvent used to redisolve the final extract before the LC analysis. That is because when the ethyl acetate extract is finally evaporated to dryness, a residue of vegetal material remains in the bottom of the container. The addition of acetonitrile–water and methanol–water mixtures at different ratios (50:50 and 75:25) was assayed but in all the cases and with all the matrices studied the formation of a precipitate was observed, giving rise to a competition between the vegetal residue and the dilution solvent for the analytes. Pure methanol demonstrated to be the most efficient and an aliquot of the final extract was directly injected in the chromatographic system.

Table 1
LC–ESI-MS–MS operational parameters optimised for the pesticides studied

Pesticide	M_w	Capillary (V)	(Q1) precursor ion	(Q2) collision energy (V)	(Q3) product ions (RA (%))[a]	Ionization mode
Cyromacine	166	60	167	−16	**85** (**39**)	Positive
				−16	125 (9)	
				−5	167 (100)	
Carbendazime	191	40	192	−28	132 (19)	Positive
				−14	**160** (**73**)	
				−5	192 (100)	
Oxamyl	219	40	237	−10	**72** (**100**)	Positive
				−9	90 (27)	
				−5	237 (15)	
Methomyl	162	40	163	−10	**88** (**100**)	Positive
				−10	106 (84)	
				−4	163 (59)	
Thiabendazole	201	40	202	−30	**131** (**24**)	Positive
				−5	202 (100)	
Imidacloprid	255	40	256	−14	128 (9)	Positive
				−15	**175** (**35**)	
				−5	256 (100)	
Acetamiprid	**222**	40	223	−30	90 (29)	Positive
				−38	99 (21)	
				−19	**126** (**100**)	
Thiacloprid	252	40	253	−34	90 (18)	Positive
				−20	**126** (**100**)	
				−13	186 (8)	
Azoxystrobin	403	60	372	−38	172 (39)	Positive
				−23	**329** (**100**)	
				−18	344 (88)	
Dimethomorf	387	60	388	−30	139 (21)	Positive
				−28	165 (55)	
				−18	**301** (**100**)	
Spinosin A	732	60	733	−33	98 (14)	Positive
				−16	**143** (**100**)	
				−5	733 (93)	
Spinosin D	746	40	747	−40	98 (13)	Positive
				−20	**142** (**100**)	
				−5	747 (78)	
Triflumizol	345	40	346	−15	73 (49)	Positive
				−8	**278** (**100**)	
				−5	346 (93)	
Hexaflumuron	460	40	459	18	276 (21)	Negative
				8	**439** (**66**)	
				5	459 (100)	
Teflubenzuron	380	40	379	18	196 (43)	Negative
				9	**338** (**67**)	
				5	379 (100)	
Lufenuron	510	40	509	14	**325** (**35**)	Negative
				7	339 (46)	
				5	509 (100)	
Flufenoxuron	488	40	487	12	**156** (**26**)	Negative
				16	329 (6)	
				5	487 (100)	

[a] Quantification masses in bold.

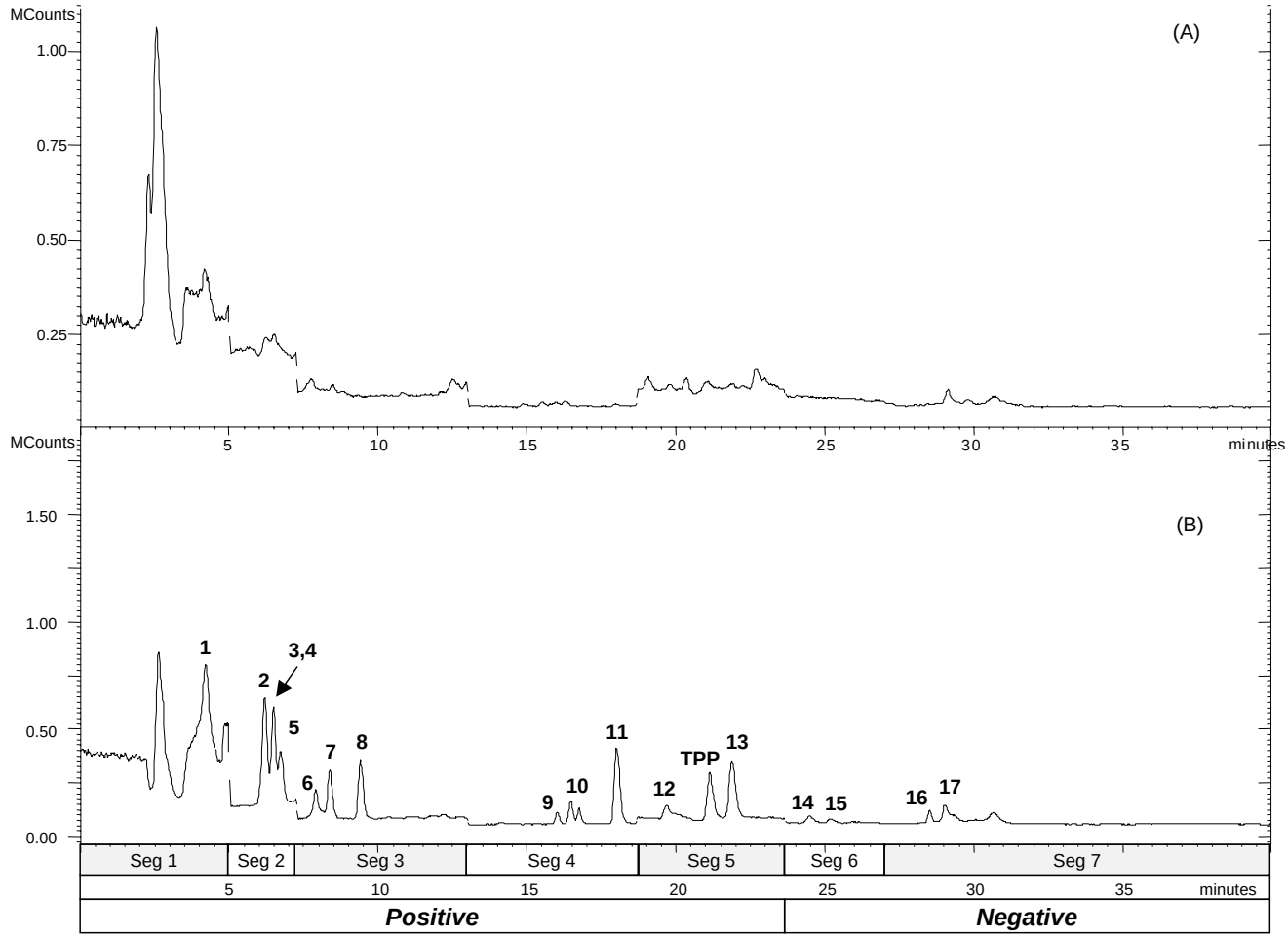

Fig. 2. LC–ESI(+/−)-MS–MS analysis of (A) a blank of lettuce and (B) a lettuce sample spiked with the standard mixture at 25 μg/kg.

To avoid possible losses in the analytes resolution derived from the injection of the methanolic extract into a mobile phase with a very high content of water, the autosampler worked in the micropick-up mode. With this mode the autosampler loop (100 μL volume) was filled with the following sequence: 45 μL mobile phase—10 μL sample—45 μL mobile phase. Peaks band-broadering was minimized, especially for the first eluting peaks (1–8 in the Fig. 2) that eluted with a high water content in the mobile phase.

The high selectivity reached by the use of the MS–MS target compound technique made unnecessary the application of laborious and time-consuming clean-up steps. As it can be observed in the chromatogram represented in Fig. 2A, no interfering peaks coming from the lettuce matrix are detected. A similar circumstance was observed in the analysis of other current vegetal matrices. On the other hand, the use of the MRM mode also reduced the probability of spectral interferences allowing an unambiguous identification. The MRM mode also provides a high sensitivity and so the application of very extensive preconcentration factors is not required.

The efficiency of the extraction method was evaluated by using spiked samples at two concentration levels, 50 and 500 μg/kg. The method was validated for the three matrices studied (pepper, eggplant and lettuce). Recovery data obtained are showed in Table 2. Recoveries ranging from 70 to 105% with relative standard deviations lower than 20% were obtained in most of the cases, with the exception already mentioned of oxamyl.

3.2. Optimisation of the LC–MS–MS chromatographic method

3.2.1. LC separation

The application of the gradient elution described in Section 2 did not provide a complete resolution for all the pesticides studied. In Fig. 2B, the chromatogram obtained from the analysis of a matrix matched standard solution is presented. The peak numbers in the figure correspond to the compounds listed in Table 2, in which are also included the retention times obtained. As it can be observed, first eluting analytes (carbendazim, oxamyl, thiabendazol and methomyl) were not completely separated and cyromazine showed a peak splitting as a consequence of an inadequate retention in the analytical column. Both problems could be overcome, as it will be explained below.

Table 2
Retention times, limits of detection and recovery data of the studied compounds

No.	Compound	t_r (min)	LODs (μg/kg)			Recovery (R.S.D.) (%)					
			Pepper	Lettuce	Eggplant	Pepper		Lettuce		Eggplant	
						50 μg/kg	500 μg/kg	50 μg/kg	500 μg/kg	50 μg/kg	500 μg/kg
1	Cyromazine	3.47	5.0	5.0	5.0	100 (14)	71 (17)	98 (13)	79 (7)	80 (7)	93 (10)
2	Carbendazime	6.23	0.5	0.5	0.5	102 (10)[a]	97 (4)	95 (5)[a]	93 (4)	71 (9)[a]	93 (7)
3	Oxamyl	6.45	2.5	1.5	2.5	35 (28)	41 (17)	38 (20)	45 (19)	37 (18)	47 (23)
4	Thiabendazol	6.53	1.5	1.5	1.5	94 (28)	95 (17)	87 (8)	91 (12)	83 (7)	84 (5)
5	Metomyl	6.75	2.5	2.5	2.5	88 (9)	87 (6)	85 (7)	89 (4)	76 (11)	80 (4)
6	Imidacloprid	7.92	2.5	2.5	2.5	101 (3)	95 (5)	93 (7)	95 (3)	84 (6)	84 (5)
7	Acetamiprid	8.39	1.5	1.5	1.5	103 (4)	96 (4)	102 (3)	97 (4)	82 (8)	93 (7)
8	Thiacloprid	9.43	1.5	1.5	1.5	97 (3)	95 (5)	93 (5)	95 (5)	83 (8)	92 (8)
9	Dimethomorf	16.23[b]	2.5	2.5	1.5	102 (8)[c]	100 (4)	83 (10)[c]	89 (3)	80 (11)	85 (5)
10	Azoxystrobin	16.74	0.5	0.5	1.0	89 (4)	93 (5)	80 (6)	81 (4)	70 (6)	77 (5)
11	Spinosin A	17.93	1.0	1.5	1.5	91 (7)	108 (10)	82 (7)	95 (5)	82 (11)	88 (8)
12	Spinosin D	19.55	1.5	2.5	2.5						
13	Triflumizol	21.74	5.0	1.5	1.5	105 (5)[a]	104 (6)	88 (10)	85 (12)	78 (10)	85 (6)
14	Hexaflumuron	24.29	2.5	2.5	2.5	87 (6)	99 (2)	79 (8)	83 (5)	79 (8)	75 (6)
15	Teflubenzuron	25.03	5.0	2.5	5.0	96 (11)	95 (3)[d]	77 (10)	81 (5)	76 (9)	78 (4)
16	Lufenuron	28.43	5.0	5.0	2.5	62 (14)	78 (9)	70 (9)	73 (5)	75 (6)	80 (4)
17	Flufenoxuron	28.95	2.5	2.5	2.5	77 (19)[c]	91 (6)	83 (13)	89 (10)	79 (10)	80 (6)

[a] Spiking level 100 μg/kg.
[b] As an average of the two isomers.
[c] Spiking level 20 μg/kg.
[d] Spiking level 250 μg/kg.

3.2.2. Selection of MS–MS conditions

The first aspect to optimise in the method was the polarity of the ESI source. Most of the compounds showed maximum sensitivity operating in the positive ionisation mode (ESI+) with the exception of the benzoylureas group (hexaflumuron, teflubenzuron, lufenuron and flufenoxuron) that exhibited an increased response in the negative mode (ESI−). It is known that continuously switching the polarity during the same acquisition method can decrease the time effectively available for analytes detection and so reduce the intensity of the peaks observed [25]. However, if two separated acquisition periods are used in the way that the switching of the polarity is performed in a part of the chromatogram where no elution of the analytes is observed, the sensitivity is not significantly affected. In our case, the change from ESI+ to ESI− is produced at 23 min.

Another characteristic source parameters were also optimised to get an only and very intense peak. This peak (precursor ion) will be filtered in the first quadrupole Q1 and further submitted to collision-induced fragmentation in Q2 to obtain the correspondent product ions monitored via Q3. The most important analyte-dependent parameter in this case is the capillary voltage. It is important to consider that, although ideally each pesticide should be analysed in a different segment, the presence of coeluting or closely eluting peaks make this unviable. In these cases, an ideal capillary voltage cannot be applied to each pesticide making necessary to adopt a compromise solution. Such is the case of carbendazim, oxamyl, thiabendazol and methomyl for which a voltage of 40 V was applied. Other optimised values obtained for the compound studied are showed in Table 1. Under these conditions the quasi-molecular ion, $[M + H]^+$ or $[M - H]^-$, was obtained for most of the compounds with the exception of oxamyl and azoxystrobin. In the first case, the adduct ion $[M + NH_4]^+$ (*m/z* 237), formed as a consequence of the presence of ammonium formate in the mobile phase [26], showed a higher intensity. Azoxystrobin yield fragmentation in the selected conditions giving the $[M - CH_3O]^+$ (*m/z* 372) as the most abundant ion, that it was selected as precursor ion in this case. Table 1 summarize the precursor ions selected.

Suitable transitions from these precursor ions to product ions were automatically optimised by the instrument software by increasing the collision voltage in Q2 to get the optimum collision energy values. Table 1 shows the ions used for MRM and the optimised collision energy values. Whenever it was possible, the complete disappearance of the parent ion was avoided to get more confidence in the identification. Two transitions were selected in most of the cases, except for acetamiprid, thiacloprid, azoxystrobin and dimethomorph for which three transitions were possible. This allowed obtaining an accurate identification of the target compounds in the samples. The most abundant product ion was selected for quantification purposes provided that this ion not correspond with the precursor ion, in order to avoid possible contribution of the matrix in the quantification.

When two or more pesticides coelute or are included into the same segment the characteristic parameters selected for each compound are applied in successive scans along the segment. The information obtained for each analyte is

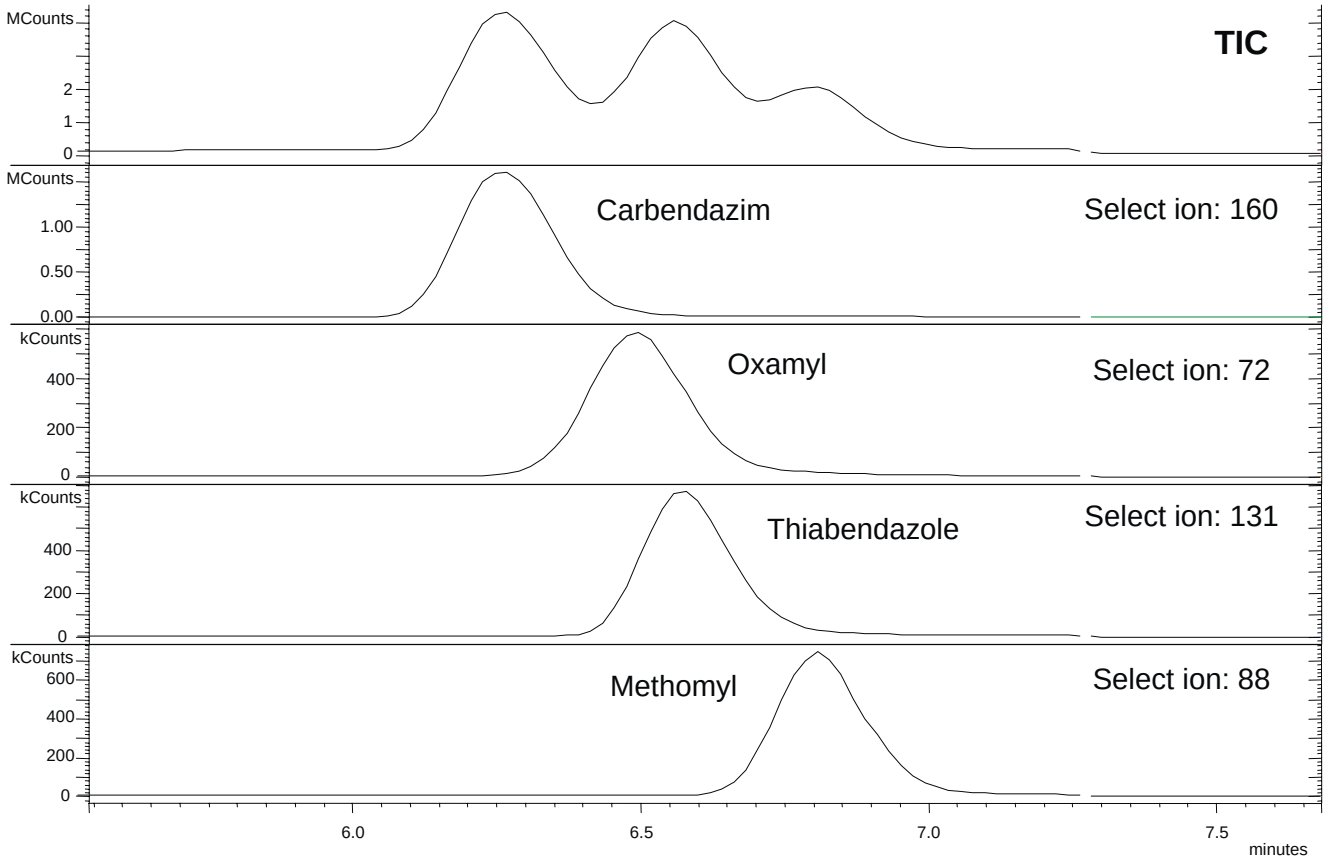

Fig. 3. Example of resolution of four coeluting peaks (carbendazim, oxamyl, methomyl and thiabendazole) by selecting the correspondent selected ion chromatograms for the quantitation masses and MS–MS spectra.

stored in different channels allowing the obtaining of the corresponding spectra separately for each compound. In this way, it is possible to resolve coeluting peaks according with adequate identification and quantification criteria. Fig. 3 shows the case of carbendazim, oxamyl, thiabendazol and methomyl, where the selected ion chromatograms for the quantification masses (160 *m*/*z* for carbendazim, 72 *m*/*z* for oxamyl, 131 *m*/*z* for thiabendazole and 88 *m*/*z* for methomyl) are represented.

3.3. Method performance and matrix effect

One significant drawback, especially when using electrospray ionisation [27], is that presence of matrix components may affect the ionisation of the target analytes producing an ion formation suppression effect. To evaluate the presence and extension of this effect sample extracts of lettuce, pepper and eggplant were spiked at different concentration levels (see Section 2.4) and analysed. Comparison of the calibration graphs obtained in each case with those obtained with the standards in pure solvent allows observing possible deviations in the response. In most of the cases, the matrix calibration was not significantly (>40%) different from the standard calibration. Suppression of the analytes response was the effect more frequently observed with variations in the slope of the calibration curves ranging from 3 to 33%. Higher variations were detected in cyromazine in pepper (41%) and especially in the triflumizol–pepper pair that presented a drastic decrease in response. This matrix suppression effect is illustrated in Fig. 4 where triflumizol peak is represented in the different matrices studied. Matrix enhancement effects were also observed for dimethomorf, azoxystrobin and spinosin A in pepper and for lufenuron and flufenoxuron in aubergine, in a different extension, being

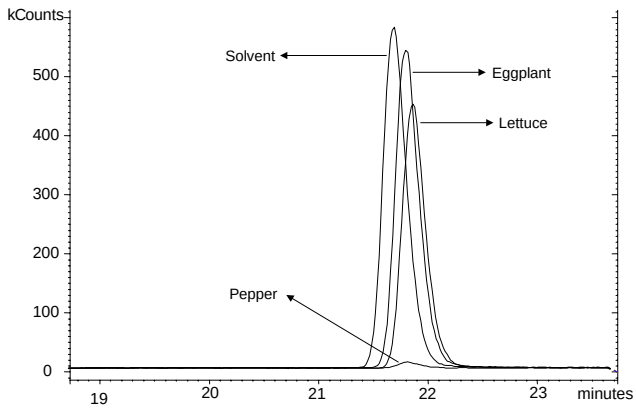

Fig. 4. Matrix suppression effect observed in the response of triflumizol in different matrices.

especially significant for dimethomorf (46%) and flufenoxuron (53%). As it can be derived from these observations, the intensity of this effect depends on the matrix nature and on the pesticide considered. Although many hypotheses have been proposed in the literature a definitive explanation cannot be given up to now [12,24]. Due to the unpredictability of these effects, previous studies have to be done when new matrices or new pesticides are going to be included in the method, in order to avoid quantification errors.

Calibration curves prepared in the different matrices were linear over the entire range studied (10–1000 μg/L) with correlation coefficients higher than 0.990 in most of the cases, except flufenoxuron in lettuce ($r = 0.985$). The LODs obtained for each pesticide are listed in Table 2. As can be seen the LODs are in the range of 0.5–5.0 μg/kg which guaranties a correct identification of the pesticides even if the application of the most exigent MRL fixed in the EU legislation (0.01 mg/kg) is required. For quantification purposes, a practical limit of determination is considered for each pesticide–matrix combination at a concentration equal to the MRL × 1/2. This amount is considered as the reporting limit, the level at which the laboratory guaranties that residues have been identified and quantified satisfactorily.

3.4. Reliability of the identification criteria

As the identification of the pesticides in the samples was based on the comparison of the retention times and product ion spectra in the samples and standards, reliability of these criteria was conveniently verify. Repeatability in the retention time was studied by the injection (10 times) of a sample extract fortified with the pesticides at a concentration of 250 μg/kg. Relative standard deviations lower than 0.5% were obtained in most of the cases with the exception of cyromazine that showed a higher variability (0.7%) as a consequence of the very early elution and the presence of two peaks. As it was previously commented, the elution conditions are not the most adequate for this compound. However, it has been included in the multi-residue method as a consequence of the good reproducibility observed in the response and in the mass spectrum that guaranties a quantification and confirmation with an acceptable reliability.

With respect to the stability of the mass spectra, the influence of the matrix in the pesticide spectra was also evaluated. For this purpose, variations in the relative intensities of the diagnostic product ions were observed at different concentration levels (25 and 250 μg/kg) and with different matrices (pepper, lettuce and aubergine). Generally speaking, the relative abundance of the ions was more stable at the higher concentration level with coefficients of variation that ranged between 2 and 11%. At the low level, a higher variation 3–20% was observed. In all the cases, the mass spectra were considered adequate for an accurate identification.

3.5. Application to real samples

The applicability of the method in routine was tested with the analysis of 560 real vegetable samples coming from the

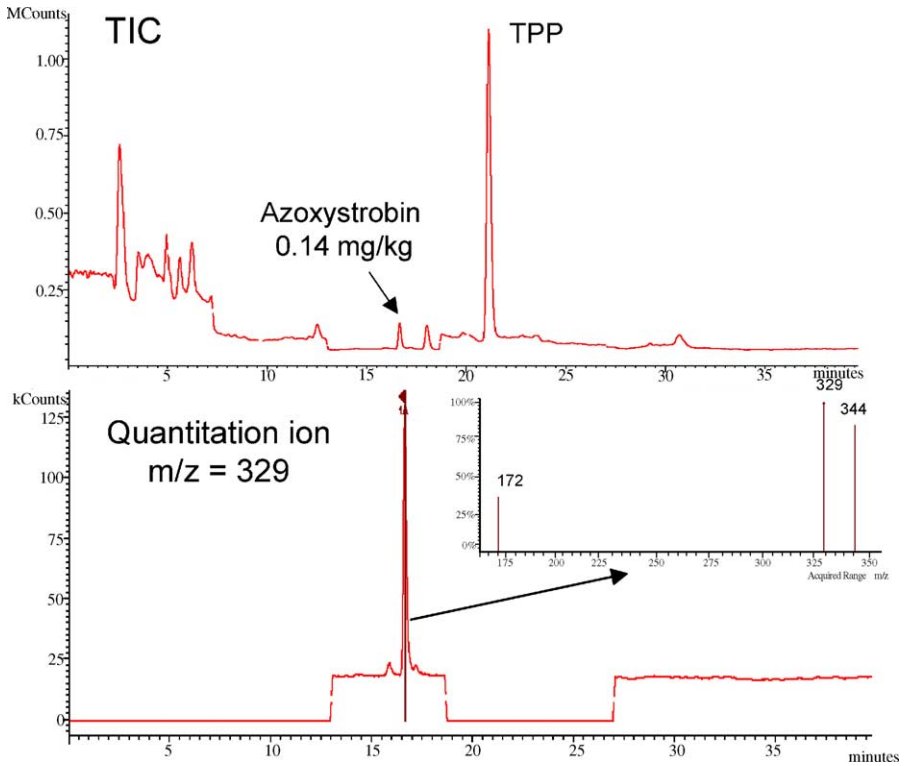

Fig. 5. LC–MS–MS chromatogram of a real sample where azoxystrobin has been detected at a concentration of 0.14 mg/kg.

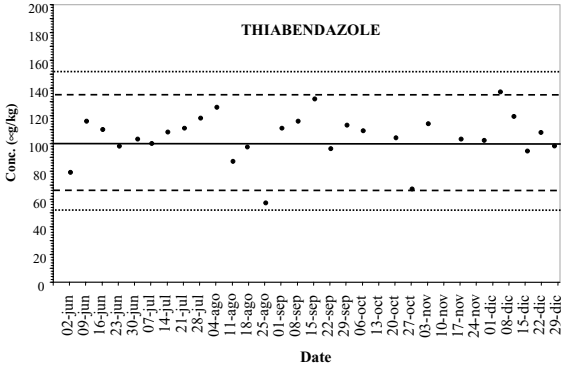

Fig. 6. Shewart chart obtained after the weekly analysis, during a period of 7 months, of a QC sample containing thiabendazole at a theoretical concentration of 100 μg/kg.

monitoring programme of COEXPHAL. Samples were analyses during a 1-year period. The correct retention time and a fit value ≥800 (fit ≥700 at lower concentrations) were used as identification criteria. For quantification purposes, the single ions chromatograms for the quantitation mass selected were obtained and the peaks were quantified by using the calibration equation previously stored and daily checked. As an example, Fig. 5 shows the total ion current (TIC) and selected ion LC–ESI-MS–MS chromatograms obtained for one of the samples where azoxistrobin was detected at a concentration of 0.14 mg/kg.

Confirmation of the applicability of the developed method was obtained by the application of quality control procedures (Section 2.5) during the routine analysis of the samples. The presence of the TPP peak in all the samples guarantied the effectiveness of the entire method, including extraction and chromatographic analysis. During the period in which the analyses were performed a 5% of them presented differing results. In most of the cases the source of the mistake could be detected and it was not directly attributable to the analytical method but to the operator (no TPP addition to the samples, concentration of the TPP solution by evaporation). Procedural blank analysis did not reveal in any case possible interferences that could affect analytical results. Duplicate samples also yield results within the tolerances previously specified, that is 30% of variation for concentrations lower than 50 μg/kg, 25% for concentrations in the range from 50 to 200 μg/kg and 20% for concentrations higher than 200 μg/kg.

Control charts plotted did not evidence discrepant results or drifts of the data, demonstrating the good reproducibility of the method with the time. As an example, Fig. 6 shows the control chart obtained for the case of thiabendazole during a period of 7 months, from July to December 2003. All the results obtained fell inside the action limits and corrective actions were not necessary. It is necessary, however, clarify that the reproducibility of the chromatographic system it is only obtained when a systematic maintenance is performed. Thus, the ESI chamber must be cleaned in order to obtain the best results in terms of sensitivity. The solvent used for this purpose is a mixture of Milli-Q water–MeOH–isopropanol (1:1:1). Once cleaned all the chamber surface, the end plate, the ceramic insulator and the capillary must be removed and sonicated with the solvent mixture described for 15 min. Finally, the end plate must be cleaned with a thin moisten sandpaper using MeOH as wetting agent until the end plate has its normal silver color.

Finally, the efficiency of the method was externally checked by the participation in a proficiency test organized by TestQual (http://www.TestQual.com). This test was specifically design for LC amenable pesticides. All the compounds present in the samples and cover by the analytical method (carbendazime, methomil, imidacloprid and hexaflumuron) were correctly identified. Quantification data were provided only for those compounds present at a concentration level higher than the official detection limit established for each pesticide (Directive 90/642/CEE). *Z*-score values obtained in these cases were: 1.1 for carbendazime and −0.4 for methomilo. These results provided a further confirmation of the applicability of the analytical method.

The results obtained with the screened samples are shown in Table 3. Among the samples analysed, a total of 139 positive findings, corresponding to 11 from the 16 pesticides studied, were detected. Nearly 45% of them corresponded to concentrations <0.10 mg/kg, the same proportion to concentrations between 0.10 and 0.50 mg/kg and <10% presented concentrations higher than 0.50 mg/kg. These results corroborate the necessity of using more sensitive and specific methods able to determine, with a high grade of accuracy, low levels of pesticide residues to get an adequate

Table 3
Application to 560 real samples, including tomato, pepper, eggplant, lettuce, cucumber, strawberry and melon. Pesticides and number of positive residues founded at different concentration levels

Pesticides	No. of residues founded/conc. level (mg/kg)			
	<0.10	0.11–0.50	0.51–1.00	>1.00
Cyromazine	0	0	0	0
Carbendazim	6	13	0	0
Oxamyl	1	4	0	0
Methomyl	0	0	0	0
Thiabendazole	2	1	3	6
Imidacloprid	12	19	0	1
Acetamiprid	20	14	2	1
Thiacloprid	2	0	0	0
Azoxystrobin	10	8	0	0
Dimethomorph	0	0	0	0
Spinosad (Spinosin A + B)	4	0	0	0
Triflumizol	0	2	0	0
Hexaflumuron	0	0	0	0
Teflubenzuron	3	0	0	0
Lufenuron	3	2	0	0
Flufenoxuron	0	0	0	0
Total	63	63	5	8

control even in complicated matrixes with a high content of interferences.

4. Conclusions

The multi-residue method developed complies with the performance characteristics requested for the analysis of this group of pesticides in vegetable samples. This means good selectivity, linearity in the response, recovery values higher than 70% in most of the cases with good precision in the response (R.S.D., <28%), good sensitivity (LODs in the range of 0.5–5.0 μg/kg) and confident identification criteria. The extraction method applied is simple, rapid and efficient and LC–MS–MS provides characteristic parent to product ion transitions, enough for a suitable pesticide confirmation. The applicability of the method to routine analysis was tested in real samples with good results and quality control systems applied demonstrated a good performance and stability in the time.

References

[1] T. Cairns, K.S. Chiu, K.S. Navarro, E. Siegmund, Rapid Commun. Mass Spectrom. 7 (1993) 971.
[2] J. Fillion, F. Sauvé, J. Selwyn, J. AOAC Int. 83 (2000) 698.
[3] H.-J. Stan, J. Chromatogr. A 892 (2000) 347.
[4] M. Gamón, C. Lleó, A. Ten, F. Mocholí, J. AOAC Int. 84 (2001) 1209.
[5] A. Agüera, M. Contreras, J. Crespo, A.R. Fernández-Alba, Analyst 127 (2002) 347.
[6] J. Abian, J. Mass Spectrom. 34 (1999) 157.
[7] G.S. Nunes, R.M. Alonso, M.L. Riveiro, D. Barceló, J. Chromatogr. A 888 (2000) 113.
[8] M. Fernández, Y. Picó, J. Mañez, J. Chromatogr. A 871 (2000) 43.
[9] M. Careri, F. Bianchi, C. Corradini, J. Chromatogr. A 970 (2002) 3.
[10] M. Hiemstra, D. De Kok, J. Chromatogr. A 972 (2002) 231.
[11] A.R. Fernandez-Alba, A. Tejedor, A. Agüera, M. Contreras, J. Garrido, J. AOAC Int. 83 (2000) 748.
[12] C. Jansson, T. Pihlstroem, B.G. Ostedahl, K.E. Markides, J. Chromatogr. A 1023 (2004) 93.
[13] M.J. Taylor, K. Hunter, K.B. Hunter, D. Lindsay, S. Le-Bouhellec, J. Chromatogr. A 982 (2002) 225.
[14] Y. Picó, C. Blasco, G. Font, Mass Spectrom. Rev. 23 (2004) 45.
[15] H.G.J. Mol, R.C.J. van Dam, O.M. Steijger, J. Chromatogr. A 1015 (2003) 119.
[16] D. Perret, A. Gentili, S. Marchese, M. Sergi, G. D'Ascenzo, J. AOAC Int. 85 (2002) 724.
[17] A.C. Hogenboom, M.P. Hofman, S.J. Kok, W.M.A. Niessen, U.A.Th. Brinkman, J. Chromatogr. A 892 (2000) 379.
[18] O. Pozo, E. Pitarch, J.V. Sancho, F. Hernández, J. Chromatogr. A 923 (2001) 75.
[19] T. Reemtsma, J. Chromatogr. A 1000 (2003) 477.
[20] T. Reemtsma, Trends Anal. Chem. 20 (2001) 500.
[21] T. Reemtsma, Trends Anal. Chem. 20 (2001) 533.
[22] J. Hau, S. Riediker, N. Varga, R.H. Stadler, J. Chromatogr. A 878 (2000) 77.
[23] A. Lagana, G. Fago, A. Marino, V.M. Penazzi, Anal. Chim. Acta 415 (2000) 41.
[24] K. Bester, G. Bordin, A. Rodriguez, H. Schimmel, J. Pauwels, G. Van Vyncht, Fresenius J. Anal. Chem. 371 (2001) 550.
[25] J. Klein, L. Alder, J. AOAC Int. 86 (2003) 1015.
[26] J.M.F. Nogueira, T. Sandra, P. Sandra, J. Chromatogr. A 996 (2003) 133.
[27] R. King, R. Bonfiglio, C. Fernandez-Metzler, C. Miller-Stein, T. Olah, J. Am. Soc. Mass Spectrom. 11 (2000) 942.