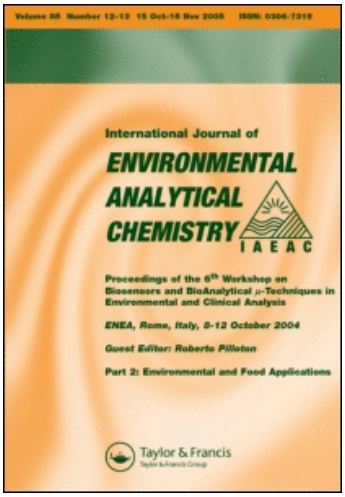

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Celine Lesueur[ab]; Michael Gartner[a]; Axel Mentler[c]; Maria Fuerhacker[b]
[a] Gartner & LVA GmbH, Blaasstrasse 29, 1190 Vienna, Austria [b] Department of Water, BOKU-University of Natural Resources and Applied Life Sciences, Atmosphere and Environment, Institute of Sanitary Engineering and Water Pollution Control, Muthgasse 18, 1190 Vienna, Austria [c] Department for Forest and Soil Science, BOKU-University of Natural Resources and Applied Life Sciences, Institute of Soil Research, Peter Jordanstrasse 82, 1190 Vienna, Austria

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Qualitative and quantitative analysis of polar pesticide multiresidues in leaf samples with a liquid chromatography–ion-trap mass-selective detector

CELINE LESUEUR*†‡, MICHAEL GARTNER†, AXEL MENTLER§ and MARIA FUERHACKER‡

†Gartner & LVA GmbH, Blaasstrasse 29, 1190 Vienna, Austria
‡Department of Water, BOKU-University of Natural Resources and Applied Life Sciences, Atmosphere and Environment, Institute of Sanitary Engineering and Water Pollution Control, Muthgasse 18, 1190 Vienna, Austria
§Department for Forest and Soil Science, BOKU-University of Natural Resources and Applied Life Sciences, Institute of Soil Research, Peter Jordanstrasse 82, 1190 Vienna, Austria

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Since 1995, in Austria, an agricultural programme (ÖPUL) has promoted an environmentally friendly and extensive production with restricted pesticide use. To observe the achievement of this goal, the pesticides in leaf samples are monitored. This study aimed to develop a multiresidue method for the qualitative and quantitative analysis of 46 pesticides in leaf samples with HPLC-IT-MS equipped with an electrospray ionization in positive mode after extraction with the QuECheRS method. The method has been validated for leaf samples based on the SANCO European Guideline at two fortification levels (LOQ and 10 times LOQ). The recoveries of the pesticides, with a few exceptions, were between 70 and 110% at both fortification levels and modes (full scan and selected reaction monitoring, SRM) with acceptable precision (RSD < 16%). For most pesticides, the method was linear over two orders of magnitude, repeatable, and accurate. Although the matrix effect was relevant for only a few pesticides, matrix-matched standards were used. The quantification of real samples in both modes fitted well, but a confirmation in the SRM mode was always necessary to avoid false-positive samples. Unfortunately, the method is not yet sensitive enough for organic farming foodstuff, since the limits of detection and quantification are still too high (between 1.5 and 218 $\mu g\,kg^{-1}$ and between 4.8 and 725 $\mu g\,kg^{-1}$ in full scan, respectively) compared with the Austrian authorized value of 100 $\mu g\,kg^{-1}$ fresh leaf sample defined in the ÖPUL programme.

Keywords: Polar pesticides; Liquid chromatography; Ion-trap mass spectrometry; Multiresidue method

1. Introduction

As pesticides are potentially harmful to humans, the European Community established directives and maximum residue levels (MRLs) in water (Directive 2000/60/EC [1]) and

*Corresponding author. Fax: +43-13689949. Email: celine.lesueur@boku.ac.at

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foodstuff (Directive 90/642/EEC [2]). Nevertheless, the plants themselves should also be taken into consideration, since pesticide uptake can occur directly from the leaves to the fruits or vegetables during the vegetation and florescence period, as in the case of systemic pesticides for instance.

Since 1995, in Austria, the Federal Ministry of Agriculture, Forestry, Environment and Water Management has devised a programme called ÖPUL to promote an environmentally friendly and extensive agriculture that would protect our natural environment [3]. In this sense, a new method of farming, called 'organic farming' is increasingly being used. Defined in the Directive 2092/91/EEC [4], organic farming implies that 'only products composed of substances mentioned in Annex I and Annex II of the Directive 2092/91/EEC i.e. not chemically synthesized substances may be used as plant protection products, fertilizers or soil conditioners'.

This implies the control of pesticide use by farmers by pesticide residue monitoring in the plants during the growing time of the cultures. Although there are no MRLs available for plants, pesticides authorized for agricultural use in Austria are listed in section 11 and section 12 of the pesticide national law [5]. Non-used pesticides are defined for residue concentrations in leaf samples lower than $100\,\mu g\,kg^{-1}$ fresh weight. The list entails many gas-chromatography (GC) amenable pesticides but also an increasing number of pesticides with physico-chemical properties making them more amenable for liquid-chromatography (HPLC).

Whereas apolar and middle polar pesticide residues are commonly analysed with GC coupled to single quadrupole (SQ) and, less frequently, triple quadrupole (TQ) mass spectrometers, polar pesticides in contrast are determined by HPLC. With the use of these polar pesticides, a need for new detection methods has emerged. SQ detectors are easy to use, stable, and cheap but do not offer the possibility of confirmation and can support false-positive results. More elaborate mass spectrometers like TQ, time-of-flight (TOF) and ion-trap (IT) detectors allow this confirmation, since they can carry out tandem spectrometry [6]. Until recently, TQ detectors were the detectors of choice for the quantification of pesticide multiresidues in environmental and food samples, since they are stable, sensitive, and selective, and have a wide linear range in SRM mode [7–14]. The other MS detectors in the market were until recently principally reputed as confirmation and identification systems because they can measure exact masses (TOF) or operate in MS^n mode (IT) but are less sensitive and linear. TOF has been studied in the recent years, mainly as an identification tool to discover unknown pesticides in environmental and food samples, as intensively published by Hernández *et al*. [9] and Thurman *et al*. [15–17]. Regarding IT, several studies have been published on the detection of up to 14 pesticides in water [18, 19], detection of quaternary ammonium pesticides in foodstuff [20–22], and detection of up to 17 pesticides in food samples, especially citrus fruits [14, 23–29].

One of the latest successful foodstuff preparations for the analysis of pesticide multiresidues to our knowledge is the QuECheRS (quick, easy, cheap, rugged, and safe) method [30], which interestingly can be combined with GC but also with LC. It has already been applied to GC-MS for the determination of pesticides from fruit and vegetable matrices, which are difficult to detect and quantify [30–32]. The QuECheRS method principally relies on the extraction of the pesticides from the matrices with acetonitrile followed by a salting effect with $MgSO_4$ and NaCl. The actual clean-up step of the matrices is achieved through the addition of a dispersive sorbent in the bulk liquid followed by its removal.

The goal of our work was to apply the QuECheRS method to leaf samples (apple trees, cornstalks, grapevines, hop plants, strawberry bushes, potato plants, and vegetable plants) in a combination of LC-MS using an IT analyser to develop a multiresidue method for the analysis of 46 polar pesticides.

2. Experimental

2.1 *Reagents and chemicals*

Pesticide standards were purchased either from Dr. Ehrenstorfer (Germany) or from Sigma-Aldrich (USA) with the highest available purity. Ultra-residue reagent acetonitrile, HPLC/MS grade methanol, ultra HPLC/MS grade water and HPLC/MS grade formic acid were purchased from J.T. Baker (USA).

Anhydrous magnesium sulfate, sodium chloride, and sodium citrate dihydrate were purchased from J.T. Baker (USA), di-sodium hydrogen citrate sesquihydrate was provided from Fluka (Germany), and 40 μm Bondesil-PSA was from Varian (Germany).

Single standard stock solutions were prepared by dissolving 10 mg of standard in 10 mL of acetonitrile to yield a concentration of 1000 $\mu g\,mL^{-1}$ and further diluted with acetonitrile down to 10 $\mu g\,mL^{-1}$. Multicompound standard stock solutions were prepared, dissolving 10 mg of each standard in 1000 mL of acetonitrile to yield a concentration of 10 $\mu g\,mL^{-1}$ and further diluted with acetonitrile to achieve concentrations of 5 $\mu g\,mL^{-1}$, 2 $\mu g\,mL^{-1}$, 0.5 $\mu g\,mL^{-1}$, 0.2 $\mu g\,mL^{-1}$, and 0.05 $\mu g\,mL^{-1}$. Matrix-matched standards were obtained by evaporating 10 mL of the acetonitrile multicompound standard and redissolving it in 10 mL of blank leaf matrix extract. Further dilution with the same extract brought the solution to the same concentrations as mentioned above. The single and multicompound standards were stored at 4°C in the dark.

2.2 *Sample preparation*

The real samples were at first analysed in the full scan mode (MS mode) before confirmation in the SRM mode (MS^2 mode) and comparison of the quantification in the full scan and SRM modes. We analysed 1400 leaf samples over a period of 3 months from production sites in Austria originating from organic farming (22%) and conventional farming (88%). They consisted of apple tree leaves (42%), grape tree leaves (38%), cornstalks (9%), potato plant leaves (7%), vegetable plant leaves (cabbage, cucumber, pumpkin) (3%), hop plant leaves (1%), and strawberry-bush leaves (1%).

The samples were prepared with the QuECheRS method described by Anastassiades [30] and adapted for leaf samples. Roughly summarized, it consisted of (1) homogenize around 500 g leaf samples, (2) weigh 10 g previously chopped fresh sample into a 50 mL Teflon centrifuge tube; (3) add 20 mL acetonitrile and shake the sample vigorously for 1 min using a vortex mixer; (4) add 4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate dihydrate and 0.5 g di-sodium hydrogen citrate sesquihydrate and vortex immediately for 1 min; (5) centrifuge the extracts for 3 min at 5000 rpm; (6) transfer a 6 mL aliquot of the upper layer into a 15 mL

Teflon centrifuge tube containing 150 mg PSA and 950 mg $MgSO_4$; (7) centrifuge the extracts for 3 min at 5000 rpm; (8) filter through 0.45 µm filter; (9) transfer 1.5 mL of the extract into an autosampler vial for HPLC/MS analysis.

Triphenylphosphate (TPP) was used as internal standard and spiked at the initial step to reach a 0.5 µg mL^{-1}-concentration in the final extract.

2.3 *Analyses*

The high-performance liquid chromatography system was an Agilent Technologies HP-1100 Series (Agilent Technologies, Waldbronn). Chromatographic separation was achieved using a Zorbax SB-C18 analytical column 2.1 × 150 mm (3.5 µm particle size) from Agilent Technologies at a flow rate of 300 µL min^{-1}. The mobile phases consisted of A: H_2O–MeOH, 90–9.95% (v/v) with 0.05% HCOOH and B: H_2O–MeOH, 9.95–90% (v/v) with 0.05% HCOOH. The gradient was 100% A at 0 min, 100% A at 1 min, 0% A at 10 min, 0% A at 17 min, and 100% A at 20 min. The post time was 2 min with 100% A and the stop time 22 min. The HPLC was controlled with the Agilent Technologies Chemstation for LC 3D System Software.

The HPLC system was interfaced to an Agilent Technologies mass spectrometer LC/MSD trap XCT Plus (Agilent Technologies, Waldbronn) equipped with an electrospray ionization (ESI) interface operated in positive mode and controlled with the Agilent Technologies LC/MSD trap software 5.3. Parameters were optimized by continuous injection of a standard solution of 10 µg mL^{-1} via a syringe pump, at a flow rate of 6 µL min^{-1}, mixed with the mobile phase at 50 µL min^{-1} by means of a T piece. The IT detector operating conditions were set as shown in table 1. The IT mass detector operated in full scan and SRM modes. The precursor ions (MS mode) were isolated and fragmented with an amplitude of 0.6 V to produce a first set of product ions (MS^2 mode) and so on.

Table 1. IT operating conditions.

ESI source	Nebulizer gas (nitrogen) pressure	40 psi
	Drying gas flow rate	9 mL min^{-1}
	Drying gas temperature	350°C
	Capillary voltage	4500 V
	Endplate offset	Fixed at −500 V
Detector and block voltages	Multiplier voltage	1900 V
	Dynode voltage	7 kV
	Skimmer block	40.0 V
	Lens 1 block	−200.0 V
	Octopole RF amplitude Block	0 Vpp
	Partition block	12.0 V
	Lens 2 block	0 V
	Capillary exit block	0 V
Ion Charge Control (ICC)	Target (ion counts)	150 000
	Maximum accumulation time	50 ms
	Scan (m/z)	From 50 to 500, from 0 to 15 min From 500 to 1000 from 15 to 22 min
	Scan averages	5

2.4 *Pesticide selection*

We selected a total of 49 pesticides representing 24 insecticides, 11 herbicides, six fungicides, five chitin synthesis inhibitor, two mite growth inhibitors, and one acaricide (tables 2 and 3). Derivates of aldicarb, demeton-S and fention were not selected in these sets of experiments. After the first experiments, the list was reduced to 46 pesticides, as explained in section 3, leaving acephate, benomyl, and chlorfluazuron out of the method.

2.5 *MS optimization*

We first worked with single standards to determine the retention time (RT), the characteristic m/z ions in the MS and SRM modes (i.e. precursor and product ions) of each substance (tables 2 and 3). In the MS mode, we obtained the ions $[M+H]^+$ and $[M+Na]^+$ for each pesticide, as well as the ion $[M+K]^+$ for 20 substances and in 13 cases fragmentation ions, which were product ions also found in the SRM mode, or isotope ions. The most abundant ion (i.e. with the highest intensity(I)) was in 28 cases the ion $[M+H]^+$, in 14 cases the ion $[M+Na]^+$, in four cases another ion, and in three cases a fragmentation ion.

We always used $[M+H]^+$ as precursor ion in the SRM mode when it was the most abundant ion. When $[M+H]^+$ was not the most abundant ion, product ions of the main ions in the MS mode were collected in the SRM mode, and their signal to noise (S/N) ratios were compared. In this way, we finally used $[M+H]^+$ as a precursor ion in the SRM mode in 44 cases, since it produced in each case product ions with a better S/N ratio. For avermectin B1a, demeton-S, and thiofanox, we used $[M+Na]^+$, and for tebufenozide, the fragmentation ion at m/z 297 as precursor ion in the SRM mode. Metobromuron showed two main precursor ions at m/z 259 and m/z 261 due to the presence of the two isotopes of the bromide atom $^{79}Br/^{81}Br$ in the molecule. Since they both presented the same main product ion at m/z 148 (resulting in the loss of a molecule containing the bromide atom) but with a better intensity in the case of the ion at m/z 261, they were selected as the precursor ion in the MS^2 mode.

Consequently, we worked with multicompound standards at decreasing concentrations down to $0.05\,\mu g\,mL^{-1}$ to determine the ratio between the characteristic m/z in the full scan and the SRM modes as well as the LOQ and LOD of each pesticide. The substances were identified, as recommended by the SANCO European Guidelines [33, 34] relying on three ion criteria for permitted substances and four ion criteria for banned substances, i.e. one precursor ion and two product ions with an IT detector. Collecting two product ions was possible for 40 of the substances (only one product ion for acephate, benomyl, carbaryl, dimethoate, fenpyroximate, metamitron, methabenzthiazuron, tebufenozide, and thiofanox) with a capillary voltage of 4500 V and an amplitude fragmentation in MS^2 mode of 0.6 V. The ion ratios in the full scan and SRM modes were compiled in a library and referred to as (first but not sufficient) identification in MS mode and as complete identification (together with the retention time) in MS^2 mode.

2.6 *LC optimization*

Due to the high amount of samples to analyse, we wanted to use the selectivity of the IT detector to develop a short-run-time method as detailed in section 2.3. With the given

Table 2. Pesticide class, most abundant ions and LOQ/LOD in MS mode in matrix-matched standards (μg kg^{-1} fresh weight).[a]

Name	Pesticide class	RT (min)	Molecular weight	$[M+H]^+$ ion (m/z)	I (%)	$[M+Na]^+$ ion (m/z)	I (%)	Other ion (m/z)	I (%)	Fragment ion (m/z)	I (%)	LOQ (μg kg^{-1})	LOD (μg kg^{-1})
Acephate	Insecticide	0.7	183.0	183.8	16.8	205.9	74.3	243.1	100.0			298	90
Acetamiprid	Insecticide	8.6	222.0	222.9	100.0	244.9	47.4	282.0	31.4			54	16
Aldicarb	Insecticide	9.3	190.0			212.9	100.0					56	16
Avermectin B1a	Insecticide	16.7	872.5	873.5	21.1	895.5	52.6			871.7	100.0	396	118
Bendiocarb	Insecticide	9.8	223.0	223.9	67.5	245.9	100.0	283.2	5.5	166.9	97.8	66	20
Benomyl	Fungicide	12.5	290.0	291.0	100.0			313.1	3.5			524	158
Butocarboxim	Insecticide	9.3	190.0			212.9	100.0					48	14
Carbaryl	Insecticide	10.1	201.0	201.9	21.7	223.9	99.2	261.0	32.4	144.9	100.0	92	28
Carbendazim	Fungicide	6.6	191.0	191.9	100.0							44	12
Carbofuran	Insecticide	9.8	221.0	221.9	100.0	243.9	61.1	281.0	54.8			24	7.3
Chlorfluazuron	Chitin synthesis inhibitor	14.6	539.0	539.9	100.0	561.9	60.1	541.9	77.3			20 600	6 180
Clomazone	Herbicide	11	239.0	239.9	100.0	261.9	61.7	299.0	13.3			40	12
Clothianidin	Insecticide	8.1	249.0	249.9	100.0	271.9	92.7			168.9	35.8	46	14
Cyromazine	Chitin synthesis inhibitor	1.4	166.0	166.9	100.0							28	8.5
Demeton-S	Insecticide	11	258.0	258.9	16.0	280.9	100.0	318.0	39.5			40	12
Dictrotophos	Insecticide	7.8	237.0	237.9	73.5	259.9	26.2	297.0	100.0			64	20
Diflubenzuron	Chitin synthesis inhibitor	11.7	310.0	311.0	43.0	332.9	100.0					74	22
Dimethoate	Insecticide	8.4	229.0	229.8	100.0	251.8	94.3	288.9	24.4	198.8	78.3	94	28
Diuron	Herbicide	10.7	232.0	232.9	100.0	254.9	44.6					60	18
Ethiofencarb	Insecticide	10.3	225.0	225.9	28.2	247.9	100.0	285.0	49.1	168.9	41.9	60	18
Ethirimol	Fungicide	8.8	209.0	210.0	100.0							18	5.1
Fenamiphos	Insecticide	11.8	303.0	304.1	100.0	326.0	90.1	363.1	37.6			32	9.4
Fenpyroximate	Acaricide	14.5	421.0	422.3	100.0	444.2	26.6			366.2	13.5	32	9.4
Fenthion	Insecticide	12.4	278.0	278.9	100.0	300.9	90.7					128	38

Flufenoxuron	Chitin synthesis inhibitor	13.4	488.0	489.1	42.1	511.1	100.0					166	50
Furathiocarb	Insecticide	13.4	382.0	383.2	51.5	405.2	100.0	442.2	96.5			26	7.9
Hexythiazox	Mite growth regulator	13.4	352.0	353.1	56.7	375.1	100.0					104	32
Imazalil	Fungicide	10.26	296.0	296.9	100.0							10	2.8
Imidacloprid	Insecticide	8.01	255.0	255.9	100.0	277.9	52.1					104	30
Indoxacarb	Insecticide	12.5	527.0	528.0	41.3	550.0	100.0					244	74
Linuron	Herbicide	11.1	248.0	248.9	100.0	270.9	59.4	250.9	67.4			86	26
Metamitron	Herbicide	8.4	202.0	202.9	100.0							36	12
Methabenzthiazuron	Herbicide	10.7	221.0	221.9	100.0	243.9	11.4					30	10
Metobromuron	Herbicide	10.5	258.0	258.9	94.9	280.9	53.5	260.9	100.0			64	20
Metoxuron	Herbicide	9.3	228.0	228.9	100.0	250.9	32.7	288.0	28.9			36	10
Monocrotophos	Insecticide	7.4	223.0	223.9	91.3	245.9	100.0	283.0	71.4			312	94
Monolinuron	Herbicide	10.3	214.0	214.9	100.0	236.9	40.3					40	12
Omethoate	Insecticide	5.4	213.0	213.9	100.0	235.9	49.4	272.9	28.3			390	118
Oxycarboxim	Fungicide	8.7	267.0	267.9	78.5	289.9	100.0	327.0	29.5			42	12
Paclobutrazol	Mite growth regulator	11.3	293.0	294.0	100.0							14	4.4
Pencycuron	Herbicide	11.8	328.0	329.1	100.0	351.1	56.9	388.1	56.6			42	12
Propamocarb	Herbicide	4.8	188.0	188.9	100.0							58	18
Pyraclostrobin	Herbicide	12.2	387.0	388.1	84.5	410.0	100.0					22	6.7
Rotenone	Insecticide	11.9	394.0	395.1	100.0	417.1	87.4					56	16
Tebufenozide	Insecticide	11.9	352.0	353.1	14.1	375.1	76.5	412.2	80.6	297.1	100.0	26	7.8
Thiabendazole	Fungicide	7.5	201.0	201.9	100.0							46	14
Thiamethoxam	Insecticide	7.2	291.0	291.9	84.5	313.9	100.0	351.0	35.7			260	78
Thiofanox	Insecticide	10.3	218.0	218.9	8.8	240.9	67.2	278.1	100.0	183.9	20.6	58	18
Triflumuron	Chitin synthesis inhibitor	12.1	358.0	359.2	100.0	381.0	3.1					4.8	1.5

[a]The intensity, I (%), was set to 100 for the peak presenting the higher MS ion count response.

Table 3. Product ions and LOQ/LOD in MS^2 mode in matrix-matched standards ($\mu g\,kg^{-1}$ fresh weight).[a]

Name	RT (min)	Precursor ion (m/z)	Product ion (m/z)	I (%)	Product ion (m/z)	I (%)	Product ion (m/z)	I (%)	LOQ ($\mu g\,kg^{-1}$)	LOD ($\mu g\,kg^{-1}$)
Acephate	0.7	183.8	143.1	100					8.1	2.4
Acetamiprid	8.6	222.9	126.1	100	196.1	55.9	187.1	48.7	50	14
Aldicarb	9.3	212.9	212.9	100	98.0	22.4	89.1	7.5	24	7.0
Avermectin B1a	16.7	895.5	751.5	100	607.5	50.7	752.5	11.1	210	64
Bendiocarb	9.8	223.9	167.1	100	109.2	8.5	391.4	0.1	38	12
Benomyl	12.5	291.0	192.1	100					42	12
Butocarboxim	9.3	212.9	212.9	100	155.8	33.3	75.2	24.2	24	7.2
Carbaryl	10.1	201.9	145.1	100					14	4.0
Carbendazim	6.6	191.9	159.9	100	192.9	31.8			20	6.2
Carbofuran	9.8	221.9	165.1	100	123.2	8.7			24	7.4
Chlorfluazuron	14.6	539.9	382.9	100	383.8	13.6			32	9.4
Clomazone	11	239.9	124.9	100	127.9	22.3	239.9	10.3	28	8.3
Clothianidin	8.1	249.9	169.1	100	168.1	41.9	132.1	35.0	30	9.2
Cyromazine	1.4	166.9	125.2	100	85.4	81	139.1	37.9	80	24
Demeton-S	11	280.9	253.0	100	281.1	83.5	89.3	78.7	32	9.6
Dictrotophos	7.8	237.9	193.1	100	112.3	41.4			32	9.8
Diflubenzuron	11.7	311.0	157.9	100	140.9	19.4			42	12
Dimethoate	8.4	229.8	199.0	100					14	4.0
Diuron	10.7	232.9	232.9	100	72.3	15.2			30	8.8
Ethiofencarb	10.3	225.9	168.9	100	107.0	42.9	163.9	18.4	36	12
Ethirimol	8.8	210.0	210.0	100	211.0	41			22	6.6
Fenamiphos	11.8	304.1	262.1	100	234.1	76.1	217.1	71.4	52	16
Fenpyroximate	14.5	422.3	366.3	100					18	5.6
Fenthion	12.4	278.9	247.0	100	169.0	22.4			40	12

Flufenoxuron	13.4	489.1	158.1	100	141.1	36			20	6.2
Furathiocarb	13.4	383.2	194.8	100	251.9	98.9	383.1	76.9	88	26
Hexythiazox	13.4	353.1	227.8	100	270.8	48.3	167.8	14.2	44	14
Imazalil	10.26	296.9	255.1	100	201.0	63.3	163.1	31.2	24	7.0
Imidacloprid	8.01	255.9	209.1	100	175.1	64.6	210.1	55.1	40	12
Indoxacarb	12.5	528.0	496.2	100	249.1	71	223.1	58.2	62	18
Linuron	11.1	248.9	182.0	100	160.0	48.3	249.1	18.5	46	14
Metamitron	8.4	202.9	202.9	100					20	5.7
Methabenzthiazuron	10.7	221.9	164.9	100					30	8.9
Metobromuron	10.5	260.9	147.9	100	171.8	28.6	258.9	28.2	24	7.0
Metoxuron	9.3	228.9	228.9	100	229.8	50.1	72.3	39.7	40	12
Monocrotophos	7.4	223.9	193.0	100	98.3	21	167.1	13.5	3.9	1.2
Monolinuron	10.3	214.9	214.8	100	147.9	39.9	125.8	15.5	30	9.0
Omethoate	5.4	213.9	183.0	100	196.1	20.7	143.1	11.4	10	2.9
Oxycarboxim	8.7	267.9	175.0	100	120.2	13.3			26	7.6
Paclobutrazol	11.3	294.0	294.0	100	206.9	4.6			38	12
Pencycuron	11.8	329.1	329.1	100	124.8	81.8	217.8	70.7	46	14
Propamocarb	4.8	188.9	144.2	100	102.3	66.1			12	3.3
Pyraclostrobin	12.2	388.1	194.1	100	296.1	90.5	164.1	38.1	22	5.8
Rotenone	11.9	395.1	395.1	100	213.1	55.9	192.1	16.8	60	18
Tebufenozide	11.9	297.1	132.9	100					20	5.8
Thiabendazole	7.5	201.9	175.0	100	205.1	11.9			24	7.3
Thiamethoxam	7.2	291.9	211.1	100	210.1	27.6			10	3.2
Thiofanox	10.3	240.9	183.8	100					30	9.1
Triflumuron	12.1	359.2	156.0	100	139.1	26.9			16	4.9

[a]The intensity, I (%), was set to 100 for the peak presenting the higher MS ion count response.

flow and gradient, we achieved a good separation of all substances except for aldicarb and butocarboxim. These two substances have the same retention time and are different only in regard to the position of one methyl group (figure 1). Although they have different intensities, they present the same product ions (m/z 213, m/z 156, m/z 116, and m/z 98) in the SRM mode. The only difference is the presence of the ion at m/z 89 only produced from aldicarb and the ion at m/z 75 only produced from butocarboxim. Since only ions with an m/z larger than around one-third of the precursor ion m/z can be efficiently stored in the IT for MS^2 detection [6, 14, 18], the two ions at m/z 75 and m/z 89 are not reliable for the quantification of aldicarb and/or butocarboxim. This forced us to develop a method with a lower flow rate (200 μL min^{-1}) and a smoother gradient (100% A at 0 min, 100% A at 3 min, 0% A at 25 min, 0% A at 35 min, 100% A at 40 min; post-time 5 min with 100% A; stop time 45 min), which allowed their partial separation and quantification (with the product ion at m/z 156) when necessary. This gradient was only applied in case aldicarb and/or butocarboxim were suspected.

2.7 *Validation study*

The method was validated for the 46 substances in the MS mode and for ten selected substances (i.e. those found in real samples) in the SRM mode based on the European SANCO Guideline [33] testing the method for sensitivity, recovery, and precision. Linearity was studied for the standards in acetonitrile and in the matrix by analysing in quintuplicate six concentration levels between 0.05 and 10 μg mL^{-1}.

The limits of detection (LOD) and limits of quantification (LOQ) were estimated for the ion with an m/z at the highest intensity (denoted as I in tables 2 and 3) as the lowest concentration injected that yielded to an S/N ratio of 3 and 10, respectively.

The accuracy and precision (i.e. repeatability expressed in term of relative standard deviation (RSD,%)) of the method were tested with recovery experiments, performed with seven replicates of leaf blank samples spiked at the LOQ and 10 times the LOQ (after obtaining the LOQ as explained below). The spiked samples were allowed to stand for 30 min before extraction to allow the pesticides to penetrate into the matrix. Two different blank matrices were used for the fortification experiments.

2.8 *Quantification of real samples*

For substances with a linear calibration curve, the quantification was done within the linear range including the origin in the calibration curve. For substances with a quadratic calibration curve, a linear interpolation between two calibrations around the sample concentration was achieved, as recommended elsewhere [28].

3. Results and discussion

3.1 *Validation of the method*

3.1.1 Linearity of the standard curve and matrix matched standards. Table 4 shows that most of the 46 substances were linear, covering a range between 0.05 and 5 μg mL^{-1} or

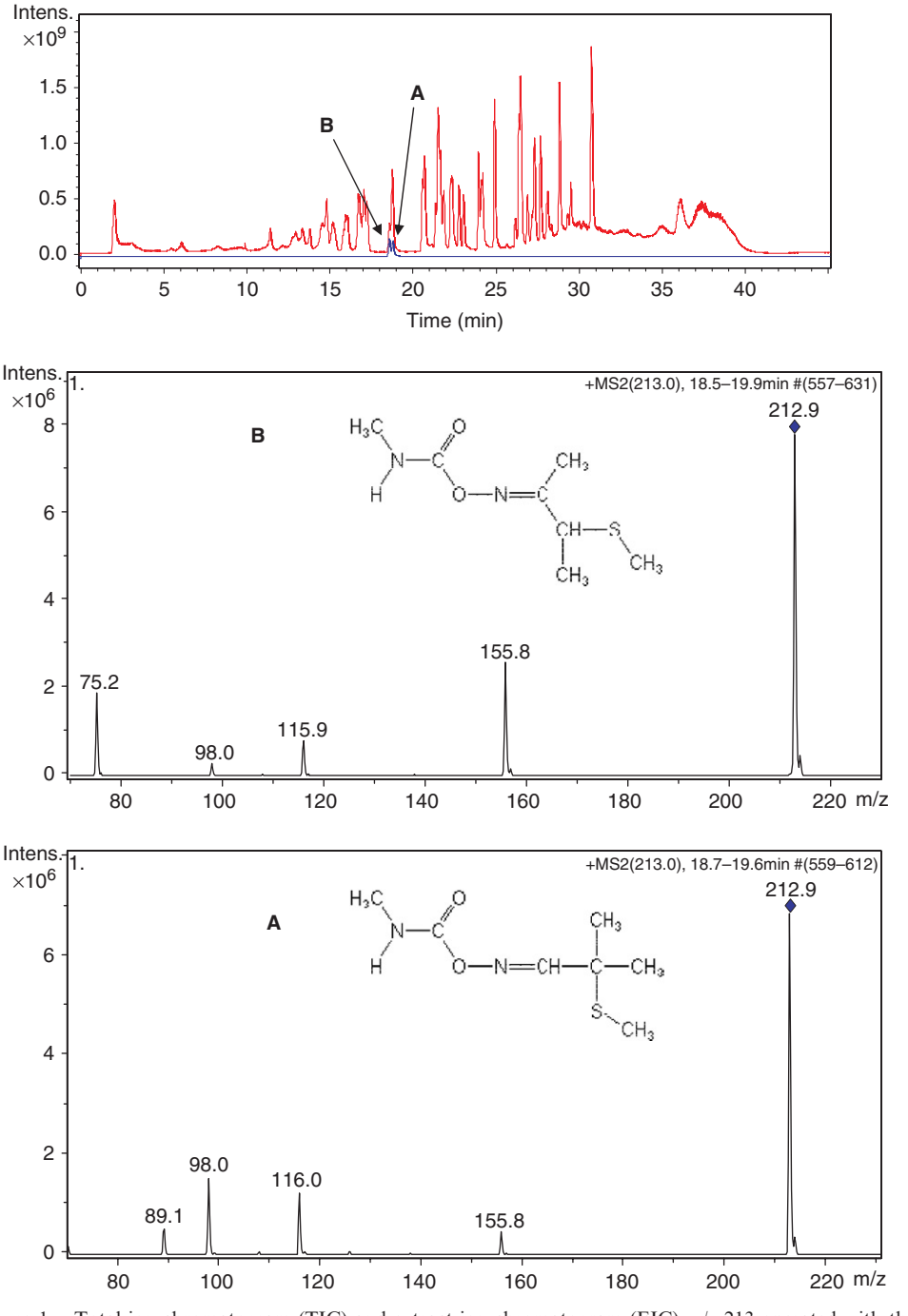

Figure 1. Total ion chromatogram (TIC) and extract ion chromatogram (EIC) *m/z* 213 operated with the smoother gradient coupled to MS^2 spectra of a 5 µg mL^{-1} multicompound standard containing (a) aldicarb and (b) butocarboxim.

Table 4. Linearity of the method in the MS and SRM mode in solvent and in matrix-matched standards ($\mu g\,mL^{-1}$).

Substance	m/z	Function	Linear range ($\mu g\,mL^{-1}$)	R^2 solvent MS mode	R^2 matrix MS mode	Matrix/solvent MS mode	R^2 solvent SRM mode	R^2 matrix SRM mode	Matrix/solvent SRM mode
Acetamiprid	222.9	Linear	0.05–5	0.9998	0.9990	1.018			
Aldicarb	212.9	Linear	0.05–5	0.9992	0.9942	0.947			
Avermectin B1a	895.5	Linear	0.2–10	0.9940	0.9971	0.933			
Bendiocarb	245.9	Linear	0.05–2	0.9923	0.9946	0.995			
Butocarboxim	212.9	Linear	0.05–2	0.9939	0.9966	0.928			
Carbaryl	223.9	Linear	0.05–5	0.9992	0.9961	0.907			
Carbendazim	191.9	Linear	0.05–5	0.9993	0.9984	0.998	0.9994	0.9991	0.990
Carbofuran	221.9	Linear	0.05–5	0.9997	0.9961	0.938			
Clomazone	239.9	Linear	0.05–5	0.9998	0.9958	0.905			
Clothianidin	249.9	Linear	0.05–5	0.9973	0.9981	0.930			
Cyromazine	166.9	Linear	0.05–5	0.9987	0.9960	0.859			
Demeton-S	280.9	Linear	0.05–5	0.9953	0.9971	0.976			
Dicrotophos	297.0	Linear	0.08–7.5	0.9992	0.9977	0.912			
Diflubenzuron	332.9	Linear	0.05–2	0.9924	0.9965	1.015	0.9966	0.9984	0.904
Dimethoate	229.8	Linear	0.05–5	0.9970	0.9946	0.922			
Diuron	232.9	Linear	0.05–5	0.9981	0.9973	0.972			
Ethiofencarb	247.9	Linear	0.05–5	0.9931	0.9972	0.861			
Ethirimol	210.0	Linear	0.05–5	0.9984	0.9979	0.904			
Fenamiphos	304.1	Linear	0.05–2	0.9707	0.9967	0.976			
Fenpyroximate	422.3	Linear	0.05–2	0.9971	0.9993	0.643	0.9999	0.9973	0.813

Fenthion	278.9	Linear	0.08–3.5	0.9998	0.9986	0.873				
Flufenoxuron	511.1	Linear	0.05–2	0.9910	0.9402	0.974	0.9987	0.9993	0.895	
Furathiocarb	405.2	Quadratic	0.05–10		0.9955	–				
Hexythiazox	375.1	Linear	0.05–2	0.9987	0.9978	1.126	0.9994	0.9978	0.918	
Imazalil	296.9	Linear	0.05–5	0.9975	0.9955	0.963				
Imidacloprid	255.9	Linear	0.05–5	0.9985	0.9936	0.896	0.9995	0.9922	0.784	
Indoxacarb	550.0	Quadratic	0.05–10		0.9989	–	–	0.9975	–	
Linuron	248.9	0.05–5	0.05–5	0.9996	0.9976	0.950				
Metamitron	202.9	Linear	0.05–5	0.9987	0.9986	0.951				
Methabenzthiazuron	221.9	Linear	0.05–5	0.9976	0.9979	1.002				
Metobromuron	260.9	Linear	0.05–5	0.9991	0.9971	0.992				
Metoxuron	228.9	Linear	0.05–5	0.9982	0.9979	0.923				
Monocrotophos	245.9	Linear	0.2–5	0.9987	0.9986	0.982				
Monolinuron	214.9	Linear	0.05–5	0.9995	0.9969	0.957				
Omethoate	213.9	Linear	0.2–5	0.9994	0.9963	0.893				
Oxycarboxin	289.9	Quadratic	0.05–10		0.9977	–				
Paclobutrazol	294.0	Linear	0.05–5	0.9985	0.9859	0.885				
Pencycuron	329.1	Linear	0.05–2	0.9953	0.9988	1.095				
Propamocarb	188.9	Linear	0.05–5	0.9957	0.9978	0.594	0.9890	0.9994	0.592	
Pyraclostrobin	410.0	Quadratic	0.05–10		0.9907	–	–	0.9952	–	
Rotenone	395.1	Linear	0.05–2	0.9956	0.9937	1.026				
Tebufenozide	297.1	Linear	0.05–2	0.9974	0.9978	0.972	0.9980	0.9980	1.004	
Thiabendazole	201.9	Linear	0.05–5	0.9983	0.9988	0.988				
Thiamethoxam	313.9	Linear	0.1–5	0.9985	0.9968	0.957				
Thiofanox	240.9	Linear	0.05–2	0.9976	0.9933	0.980				
Triflumuron	359.2	Linear	0.05–2	0.9910	0.9825	0.815				

between 0.05 and 2 $\mu g\,mL^{-1}$. Avermectin B1a, which had a higher LOQ, was linear between 0.2 and 10 $\mu g\,mL^{-1}$. Most correlation coefficients, R^2, were higher than 0.99 in both modes. Furathiocarb, indoxacarb, oxycarboxim, and pyraclostrobin did not show any linear range but quadratic functions. Linear ranges of three orders of magnitude have even been reported in MS^3 mode for pesticides in orange matrix-matched standards [14]. Nevertheless, it seems that TQ detectors are more linear, although many studies report linear ranges of two orders of magnitude, too [7, 8, 18].

The influence of the matrix on the detector response for the substances with linear calibration functions was also studied. For 23 of the 46 substances, the difference for the signal in solvent and in the matrix was less than 5%. For hexythiazox and pencycuron, the signal in the matrix was enhanced by 12.6 and 9.5%, repectively. For 21 substances, we observed a decrease in the signal between 5.0 and 40.6%. Most of them presented a decrease between 5 and 15% except fenpyroximate, propamocarb, and triflumuron with signal decreases of 35.7, 45.6, and 18.5%, respectively. The influence of the matrix relies on the competition between the analyte ions and the matrix components [26, 28] and is seemingly more obvious when working with IT [14] than with TQ. As a consequence, we used matrix-matched standards.

3.1.2 LOD and LOQ. The LOD and LOQ are given in $\mu g\,kg^{-1}$ product (fresh weight). Chlorfluazuron could hardly be detected at a 10 $\mu g\,mL^{-1}$ $\mu g\,mL^{-1}$ (20 000 $\mu g\,kg^{-1}$) level (LOD and LOQ in full scan 6180 $\mu g\,kg^{-1}$ and 20 600 $\mu g\,kg^{-1}$, respectively), which was far off the necessary authorized value of 100 $\mu g\,kg^{-1}$, and was consequently removed from the present method.

LOD and LOQ are presented in tables 2 and 3 in MS mode and SRM mode, respectively. The lowest LOD and LOQ were as low as 1.4 $\mu g\,kg^{-1}$ and 4.8 $\mu g\,kg^{-1}$, respectively in the MS mode and 1.2 $\mu g\,kg^{-1}$ and 4.0 $\mu g\,kg^{-1}$, respectively in the SRM mode. In the full scan mode, 45 substances showed LODs lower than 100 $\mu g\,kg^{-1}$, i.e. satisfying the required 100 $\mu g\,kg^{-1}$ fresh leaves for organic farming. Only three substances (avermectin B1a, benomyl and omethoate) gave LODs higher than 100 $\mu g\,kg^{-1}$. In the same way, 37 substances were below 100 $\mu g\,kg^{-1}$ and nine substances were between 100 and 520 $\mu g\,kg^{-1}$. The LOD and LOQ in SRM mode were better, as illustrated with 47 substances with an LOD lower than 30 $\mu g\,kg^{-1}$ and only avermectin B1a with a LOD of 63 $\mu g\,kg^{-1}$. The LOQ in the SRM mode was below 100 $\mu g\,kg^{-1}$ for 47 substances. Only avermectin B1a showed an LOQ higher than 100 $\mu g\,kg^{-1}$ (at 210 $\mu g\,kg^{-1}$). To decrease the LOD and LOQ and match the 10 $\mu g\,kg^{-1}$ required for foodstuff, we are now trying to optimize our IT detector. Another idea is the improvement in the clean-up step, although this could be quite tedious and time-consuming.

Since benomyl was rapidly converted to carbendazim [35, 36] and acephate, which is thermally labile, pH-labile, very polar, not retained on the column (RT: 0.7 min), and rapidly undetectable in the standard solution, these two substances were removed from the present method, which finally resulted in a multiresidue method for 46 pesticides.

3.1.3 Recovery. The recovery of the method was tested at the two fortification levels LOQ and 10 times the LOQ in MS mode (figure 2a and b) for all the substances and in SRM mode for ten of them (figure 2c). The method was found to be precise and accurate, with recoveries between 70 and 110% for almost all the substances and a

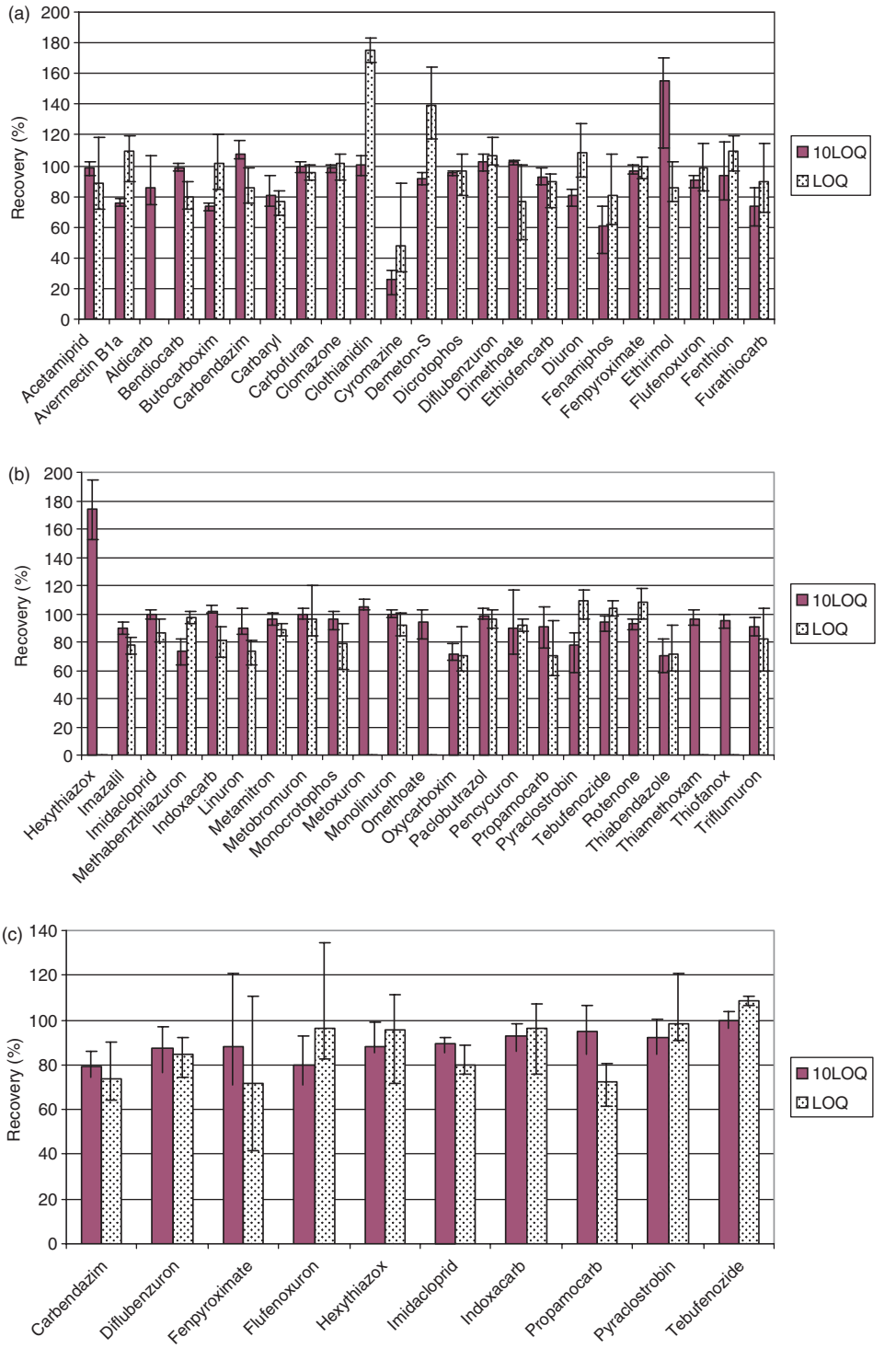

Figure 2. Recovery (average value and min/max in%) of the method in (a) and (b) MS mode at LOQ and 10LOQ and (c) SRM mode ($n = 7$).

relative standard deviation (RSD) lower than 16% at both modes and fortification levels.

At the LOQ fortification level in MS mode, we found recoveries between 70.4% (thiabendazole) and 107.8% (carbaryl) and repeatabilities between 1.1% (dicrotophos) and 16.0% (pencycuron) for all the substances except for fenpyroximate (154.9%, RSD 30.9%), hexythiazox (174.3%, RSD 30.5%), ethirimol (60.5%, RSD 6.1%) and cyromazine (26.1%). At ten times the LOQ fortification level, we obtained recoveries between 70.1% (propamocarb) and 109.7% (aldicarb) coupled to repeatabilities between 2.4% (methabenzthiazuron) and 14.9% (ethiofencarb) except for clothianidin (174.9%, RSD 9.9%), demeton-S (139.2, RSD 20.7%), and cyromazine (48.2%, RSD 9.6%).

The two blank matrices used for the two fortification levels presented different interference peaks of the same m/z ratio and RT than the analytes, resulting in recoveries for these substances higher than 110% in the full scan mode as shown for hexythiazox in figure 3. It could be certified in the SRM mode that these interfering substances in the blank matrices were not our analytes.

It is stated in figure 3(b) that the identification of hexythiazox in MS mode can be easily achieved in an interference-free sample but is not relevant in an interference-containing blank. When considering this same interference-containing blank in the SRM blank, the interference can be completely removed and the right concentration measured. This was confirmed when analysing the fortification samples at LOQ in SRM mode, where the recoveries for fenpyroximate and hexythiazox decreased to 71.9% (RSD 15.8%) and 95.5% (RSD 16.0%), respectively. In the SRM mode at LOQ, we achieved recoveries between 71.9% (fenpyroximate) and 108.4% (tebufenozide) with repeatabilities ranging from 1.5% (tebufenozide) to 17.9% (flufenoxuron). At ten times the LOQ, we obtained recoveries between 79% (carbendazim) and 100% (tebufenozide) coupled with repeatabilities between 2.1% (imidacloprid) and 14.9% (fenpyroximate).

Cyromazine showed at both fortification levels poor recoveries (26.1% at LOQ and 48.2% at ten times the LOQ). It is a small polar basic molecule ($pK_a = 5.22$), easily hydrolysed at an extreme pH owing to an ionic behaviour. Because of its particular properties, only a few analytical methods have been reported. Sancho *et al.* [13] recommends not only an acidic extraction solvent to promote its protonation and thus increase the extraction efficiency but also ion-pair reversed-phase liquid chromatography for its analysis. We obtained the same m/z precursor ion 167 and product ions 125, 85, and 139 at RT 1.4 min, but the extraction took place at pH 5.5, which can thus deplace cyromazine as its weak base and reduce its recovery.

3.2 *Application to real samples*

All the organic farming samples were below LOD in the full scan mode against 88% of the conventional farming samples. Of the remaining conventional samples, 12% presented pesticide residues above LOQ in full scan mode; 74% of the contaminated samples (residues > LOQ) contained one residue; 21% contained two residues; and 5% contained three residues. Samples with residue concentrations outside the linear range were diluted with acetonitrile before the second analysis. Figure 4 shows the occurrence of the pesticide residues in the contaminated samples after confirmation in the SRM mode. Indoxacarb was the most encountered pesticide (51.5% of the

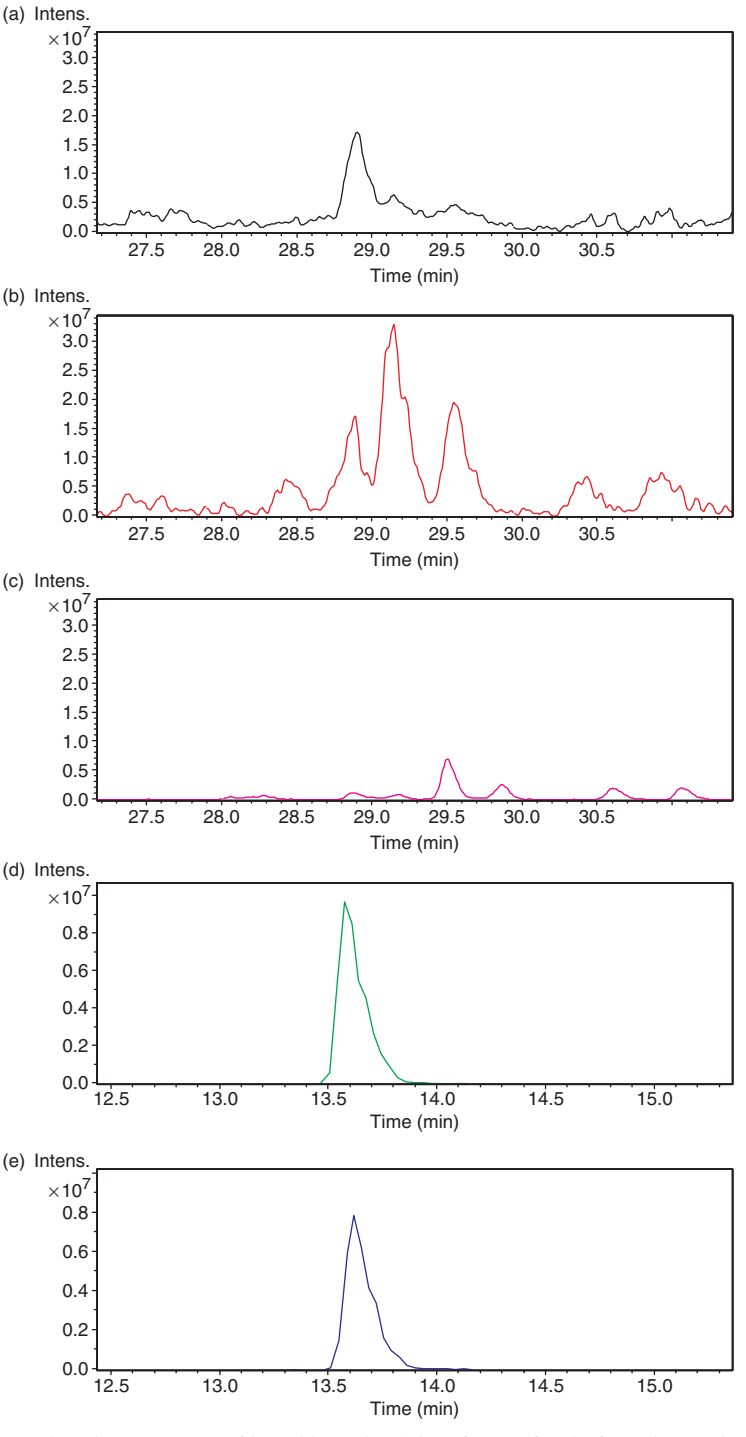

Figure 3. Extract ion chromatogram of hexythiazox in (a) interference-free leaf matrix-matched standard at 0.5 μg mL^{-1} in MS mode, (b) leaf blank with interference peak at ca 0.5 μg mL^{-1} in MS mode, (c) interference-free leaf blank in MS mode, (d) interference-free leaf matrix-matched standard at 0.5 μg mL^{-1} in MS^2 mode, and (e) leaf blank with interference peak at ca 0.5 μg mL^{-1} in MS^2 mode.

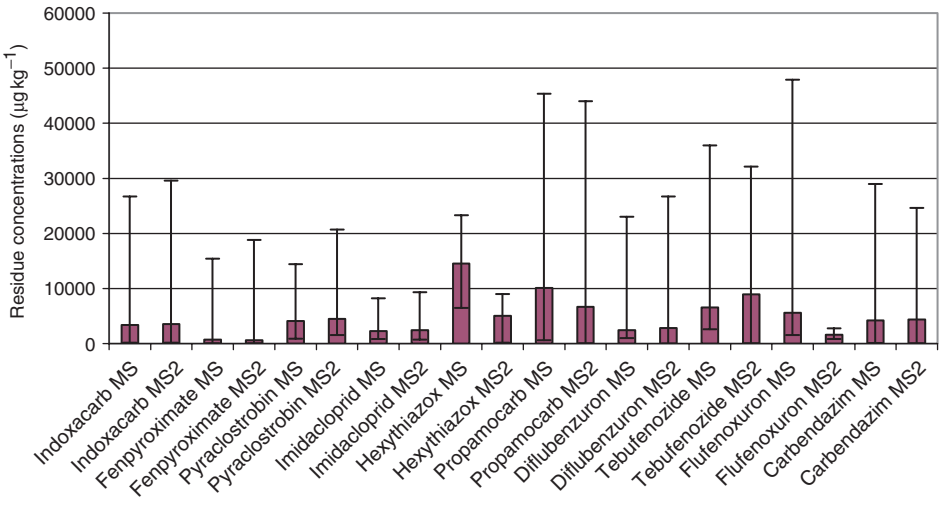

Figure 4. Occurrence of the pesticide residues in the contaminated leaf samples (minimum concentration and maximum concentration in bars; mean concentration in blocks) ($\mu g\,kg^{-1}$).

contaminated samples) followed by fenpyroximate (17.6% of the contaminated samples) and tebufenozide (15.2% of the contaminated samples). The seven other detected pesticides represented between 2 and 11% of the contaminated samples. Fenpyroximate was found in grape and in apple leaves; indoxacarb, tebufenozide, and pyraclostrobin were found only in grape leaves; diflubenzuron, carbendazim, and flufenoxuron were typical for apple leaves; potato and vegetable leaves were only contaminated with propamocarb, whereas hexythiazox and imidacloprid were characteristic for hop leaves. As already mentioned, there is no MRL established for pesticide residues in leaves but there is a list of pesticides authorized for use. As a matter of fact, none of the samples analysed presented unauthorized residues.

For hexythiazox and flufenoxuron, the quantification in the SRM mode gave concentrations always much lower than in the full scan mode as seen when comparing the min value, max value, and median value. The explanation is the same as that for the fortification experiments, since some samples showed interference peaks at the same ion and RT than hexythiazox and flufenoxuron, respectively (figure 3). In addition, we suspected nine samples of being contaminated with flufenoxuron based on the MS results, which was confirmed in the SRM mode only in three cases.

4. Conclusions

This study presents a multiresidue method for the analysis of 46 non-easily GC amenable pesticides in leaf samples with HPLC-IT-MS. The method is repeatable and accurate in full scan and SRM mode, and allows the quantitative and qualitative analysis of samples within 24 min. The method showed good recoveries at the LOQ and 10 times the LOQ fortification levels. When applied to around 1400 real leaf samples, it showed a good correlation for the concentrations in MS and SRM modes, apart

for hexythiazox and flufenoxuron due to the presence of interfering componds in MS mode, implying a possible quantification of the samples in both modes. Unfortunately, for some pesticides this method shows LOD and LOQ values (up to 218 $\mu g\,kg^{-1}$ and 725 $\mu g\,kg^{-1}$ in full scan, respectively) that are still too high to match the authorized values of 100 $\mu g\,kg^{-1}$ fresh weight required for organic farming leaf samples in the ÖPUL programme. Future work will involve (1) decreasing the sensitivity of the method by optimizing the IT detector; (2) testing the method for other matrices as recommended in the SANCO European Guidelines [33] for high-water-content matrices, high-sugar-content matrices, high-acidic matrices and high-sulfur-content matrices; and (3) broadening the spectrum of pesticide analysed with this method.

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