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Evaluation of various QuEChERS based methods for the analysis of herbicides and other commonly used pesticides in polished rice by LC–MS/MS☆

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ABSTRACT

Four different extraction and clean-up protocols based on the QuEChERS method were compared for the development of an optimized sample preparation procedure for the multiresidue analysis of 16 commonly applied herbicides in rice crops using LC-QqQ/MS. Additionally the methods were evaluated for the analysis of 26 insecticides and fungicides currently used in rice crops. The methods comprise, in general, the hydratation of the sample with water followed by the extraction with acetonitrile, phase separation with the addition of different salts and finally a clean-up step with various sorbents.

Matrix effects were evaluated for the 4 studied methods using LC-QqQ/MS. Additionally LC-TOF/MS was used to compare the co-extractants obtained with the four assayed methodologies. Thirty-six pesticides presented good performance with recoveries in the range 70–120% and relative standard deviations below 20% using 7.5 g of milled polished rice and the buffered acetate QuEChERS method without clean-up at both fortification levels: 10 and 300 $\mu g\,kg^{-1}$. The other six pesticides presented low recovery rates, nevertheless all these analytes could be analyzed with at least one of the other three studied procedures.

1. Introduction

Pesticides are widely used to prevent diseases and pests, and may adversely affect the production of fruits, vegetables, cereals and animal foodstuff. Residues of those compounds can sometimes be harmful to human health, as well as to the environment [1]. Therefore, in order to ensure consumer safety and international trade, pesticide residues in food products must be controlled and monitored. For these reason many countries and international organizations have established maximum residue limits (MRLs) to regulate pesticide residues in food products [2,3].

Rice is one of the most consumed foods in the world and its consumption has increased in the recent decades, with a consequent rise in the use of pesticides to improve its production yield, like pre and post-emergence herbicides, insecticides, and fungicides during various stages of cultivation [4]. The use of these pesticides affects the whole system of rice: the soil, water, and rice grain. For these reasons there is a clear need to develop fast methods for the multiresidue analysis of the most commonly used pesticide in rice crops.

In 2003, Anastassiades et al. reported an attractive method for sample preparation called as QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe). This method covers a very wide scope of analytes, including polar, semi-polar and non polar pesticide residues in various food matrices. The procedure involves initial single-phase extraction of the sample with acetonitrile, followed by liquid–liquid partitioning by the addition of anhydrous magnesium sulphate ($MgSO_4$) and sodium chloride. Removal of water and clean-up are performed simultaneously on an aliquot of the acetonitrile extract with dispersive solid phase extraction using $MgSO_4$ and primary secondary amine (PSA) sorbent [5].

Whereas several studies have been described for the analysis of pesticide residues in fruits and vegetables [5–11], there are only few methodologies reported for the determination of pesticides in polished rice, particularly for herbicides either by LC–MS/MS or GC–MS/MS [12–24].

In 2006, Pang et al. described the simultaneous determination of 405 pesticide in grain by accelerated solvent extraction [18]. In 2008, Koesukwiwat et al. described a method for the determination of phenoxy acids in rice by modified QuEChERS extraction and liquid chromatography–tandem mass spectrometry [19], also in 2008, Takatori et al. described a multiresidue method for the analysis of 99 pesticides in vegetables, fruits and cereals using liquid chromatography/tandem mass spectrometry [12], and Lee et al. developed a new methodology for the determination of 47 pesticides in cooked polished rice by LC–MS/MS [20]. In 2010, Niell

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et al. [21] analyzed nine multiclass herbicides in polished rice using an acetate buffered modification of QuEChERS but without the PSA clean-up. The PSA was avoided to increase the recovery of these herbicides, as they are acidic, they could be retained during the clean-up step. Mastovska et al. [22] have also studied several modifications to the original published QuEChERS for the analysis of pesticides in cereal grains. The authors used 25 mL of 1.5:1 of water:MeCN for the extraction of 5 g of milled rice, and the clean-up step was carried out using 150 mg of PSA and 50 mg of C-18 for lipid absorption. Another modification introduced in this study was the replacement of the traditional hand shaking from the original QuEChERS to an hour of orbital shaker to ensure adequate sample swelling. Also in 2010 Tsochatzis et al. [23] described a methodology for the analysis of nine pesticides commonly used in rice using alumina for the matrix-solid phase extraction. Mou et al. [24] described the determination of 15 phenylurea herbicides with HPLC, fluorescence detection, UV decomposition and post column derivatization in rice. The use of fluorescence detection improves the selectivity in complicated matrices allowing the determination of these compounds below the MRL.

Although in the last years the number of studies on pesticides in rice has been growing, still there is little information for the multiresidue analysis of pesticides, especially pre and post emergence herbicides in polished rice by LC–MS/MS. The aim of this work was to develop a multiresidue methodology amenable for the detection of pesticide residues commonly used in rice crops. Therefore, we sought to evaluate different sample preparations based on the QuEChERS method combined with LC-QqQ/MS for the determination of pesticide residues in polished rice.

2. Experimental

In this paper four sample preparation procedures were compared for three different sample sizes; (5 g; 7.5 g and 10 g): (a) original QuEChERS method [5] (method 1); (b) citrate buffered QuEChERS method [25] (method 2); (c) citrate buffered QuEChERS without a clean-up with PSA and C-18 (method 3); and (d) acetate buffered QuEChERS without the PSA clean-up [21] (method 4).

The PSA contains primary and secondary amino groups that remove acidic compounds from the extract, thus as many of the pesticides commonly used in rice cultivation are acidic, in the two latter methods no PSA was added.

The limits of detection (LODs), quantification (LOQs), percentage of recoveries and matrix effect were compared for the different experiments following the DG SANCO/10684/2009 of the European Quality Control Guidelines [26].

2.1. Materials and reagents

(a) Acetonitrile (MeCN) and water: HPLC-grade acetonitrile was purchased from J.T. Baker (Deventer, The Netherlands). A Milli-Q Plus ultra-pure water system from Millipore (Milford, MA, USA) was used throughout the study to obtain the HPLC-grade water used during the analyses.
(b) $MgSO_4$ and sodium acetate (NaOAc): certified anhydrous $MgSO_4$ and ACS grade anhydrous NaOAc, were obtained from Panreac (Barcelona, Spain) and Riedel-de-Haën (Selze, Germany), respectively. The $MgSO_4$ was baked for 5 h at 500 °C in a muffle furnace to remove phthalates and residual water.
(c) Sodium chloride (NaCl) and sodium citrate dehydrate were purchased from and J.T. Baker (Deventer, The Netherlands) while sodium citrate dibasic sesquihydrate was obtained by Sigma–Aldrich (St. Louis, MO, USA).
(d) Acids and bases: glacial acetic acid (HAc) was obtained from Merck and formic acid (98% purity) was obtained from Fluka (Steinheim, Germany). Solutions were prepared as needed. (e) SPE sorbents: primary secondary amine (PSA) sorbent and C-18, 40 μm particle size were obtained from Supelco (Bellefonte, PA, USA) and Varian (Palo alto, CA, USA), respectively.

2.2. Pesticide standards

Pesticide reference standards were obtained from Dr. Ehrenstorfer (Augsburg, Germany) and Riedel-de-Haën (Selze, Germany) and were stored at −30 °C.

Stock solutions of 1000–2000 mg L^{-1} of the individual standards were prepared in various solvents, 4 mix solutions of the pesticides were prepared from the stock solutions and the working standard pesticide solutions were prepared daily by appropriate diluting the 4 mix solution with mobile phase and stored at −18 °C until use. A triphenyl phosphate solution in MeCN was added to samples and/or standards to serve as the surrogate standard in all the experiments.

2.3. Instrumental and chromatographic conditions

Pesticide residue determinations were performed in an Agilent 1200 HPLC system with a binary pump, equipped with a reverse-phase C-8 analytical column of 150 mm × 4.6 mm and 5 μm particle size (Agilent Zorbax Eclipse XDB). Two different experiments were used in the positive mode and in negative mode. The mobile phases, A and B, were aqueous 0.1% formic acid and MeCN, respectively. The gradient program for the positive mode started with 20% of B, constant for 3 min, followed by a linear gradient up to 100% B in 30 min, then constant for 3 min. After this 33 min run time, 12 min of post-time followed using the initial 20% of B. For the negative mode the gradient program started with 50% of B constant for 3 min, followed by a linear gradient up to 100% B in 6 min, then constant for 3 min. After this 12 min run time, 5 min of post-time using the initial 50% B.

The flow rate was constant, 0.6 mL min^{-1} during the whole process for both methods and 10 μL of sample was injected in every case.

For the mass spectrometric analysis, an Agilent 6410 TripleQuad LC/MS system was applied. The ESI source was operated in positive and negative ionization modes and its parameters were as follows: gas temperature, 300 °C; gas flow, 9 L min^{-1}; nebulizer gas, 40 psi and capillary voltage, ±4000 V. Nitrogen was served as the nebulizer and collision gas. For the analysis in the positive mode, two segments with a ±1 min overlapping range around the borders were constructed. The start time of the first and second segments in the positive mode was 0 and 18.2 min, respectively whereas in the negative mode only one segment was used.

Agilent Mass Hunter Data Acquisition; Qualitative Analysis and Quantitative Analysis software was used for method development and data acquisition.

The screening of the real samples and the determination of co-extractants were performed in an high-performance liquid chromatography (HPLC) system (consisting of vacuum degasser, autosampler, and a binary pump) (Agilent series 1100, Agilent Technologies, Santa Clara, CA) equipped with a reversed-phase XDB-C18 analytical column of 4.6 mm × 50 mm and 1.8 μm particle size (Agilent Technologies, Santa Clara, CA). An amount of 20 μL of the sample extract was injected in each run. Mobile phases A and B were water/MeCN (95:5) (v/v) with 0.1% formic acid and MeCN/water (95:5) (v/v) with 0.1% formic acid. The chromatographic method held the initial mobile phase composition (10% B) constant for 1 min, followed by a linear gradient to 100% B up to 12 min, and kept for 5 min at 100% B. The flow rate used was 0.6 mL min^{-1}. The HPLC system was connected to a time-of-flight mass spectrometer Agilent MSD TOF (Agilent Technologies, Santa Clara, CA) equipped with an electrospray interface operating in the

Table 1
Comparative data of the four sample preparation methods compared.

	Method 1	Method 2	Method 3	Method 4
Extraction solvent	15 mL MeCN	15 mL MeCN.	15 mL MeCN.	15 mL MeCN with 1% HAc
Salts used for the salting-out	4 g $MgSO_4$, 1 g NaCl	4 g $MgSO_4$, 1 g NaCl, 1 g citrate dehydrate, 0.5 g sesquihydrate	4 g $MgSO_4$, 1 g NaCl, 1 g citrate dehydrate, 0.5 g sesquihydrate	7 g $MgSO_4$, 1.8 g $NaOAc{\cdot}3H_2O$
Clean-up	300 mg $MgSO_4$, 100 mg PSA	750 mg $MgSO_4$, 150 mg PSA, 150 mg C-18	1 g $MgSO_4$	1 g $MgSO_4$

positive mode, using the following operation parameters: capillary voltage, 4000 V; nebulizer pressure, 40 psi; drying gas flow rate, 9 L min^{-1}; gas temperature, 325 °C; skimmer voltage, 60 V; octapole dc 1, 37.5 V; octapole rf, 250 V; fragmentor voltage (in-source CID fragmentation) 190, 210, and 230 V. LC–MS accurate mass spectra were recorded across the range of 50–1000 *m/z*.

Accurate mass measurements of each peak from the total ion chromatograms were obtained using an automated calibrant delivery system to provide the correction of the masses. The instrument performed the internal mass calibration automatically, using a dual-nebulizer electrospray source with an automated calibrant delivery system, which introduces the flow from the outlet of the chromatograph together with a low flow (approximately 10 μL min^{-1}) of a calibrating solution which contains the internal reference masses purine ($C_5H_4N_4$ at *m/z* 121.050873) and HP-0921 ([hexakis-(1H,1H,3H-tetrafluoropentoxy)-phosphazene] ($C_{18}H_{18}O_6N_3P_3F_{24}$) at *m/z* 922.009798). The instrument provided a typical resolution of 9700 ± 500 (*m/z* 922).

The full-scan data recorded was processed with Applied Biosystems/MDS Sciex Analyst QS software (Frankfurt, Germany) with accurate mass application-specific additions from Agilent MSD TOF software and with Agilent Mass Hunter software (version B.01.03 Build 1.3.157.0 Patch 2) [27].

2.4. Sample preparation

A rice sample from a supermarket was used as blank matrix for all the fortification experiments and also for the matrix effect study. This sample was analyzed using the same methods as the fortified samples, no pesticide was found above the LOD of the methods tested.

The samples were dried for 24 h in a desiccator before being milled in a cereal grain mill purchased from SAMAP (Andolsheim, France).

For the recovery studies, a representative portion of a homogenized rice milled sample was weighed and transferred to a glass mortar, where it was fortified homogeneously with a standard solution in acetone to reach 10 μg kg^{-1} and 300 μg kg^{-1} of the studied pesticides, respectively. The mixture was then gently blended in the mortar for 30 min, to assess the homogeneity of the sample. The sample was allowed to stand at room temperature overnight, until analysis. Then, five extractions of 5, 7.5 and 10 g portions from the spiked rice were processed using the procedures described in Section 2.5.

As water content in rice is low, the addition of water before pesticide extraction was needed. A ratio of 1:1 water/MeCN was used for each of the amounts of sample used.

Eighteen real samples from different countries were analyzed using the acetate buffered QuEChERS method without PSA clean-up.

2.5. Analytical determination

The four methods presented similar procedures which were based on the Original QuEChERS method. This methodology consisted on the hydratation of *X* mL of milliQ water to *X* g of milled rice (i.e. for 7.5 g of sample 7.5 mL water were added) to form rice slurry for 1 h. Subsequently 15 mL of extraction solvent and 200 μL of 25 μg mL^{-1} triphenyl phosphate (TPP) standard in MeCN were added as a quality control during the entire procedure. Then a mixture of different salts was added and the extract was shaken vigorously for 4 min, followed by a centrifugation step for 5 min at 3700 rpm (1225 × *g*). A 5 mL aliquot was removed to a 15 mL PTFE centrifuge tube containing $MgSO_4$ with or without different sorbents for the clean-up step, depending on the method used. The extract was shaken in a vortex intensively for 20 s and centrifuged again at 3700 rpm (1225 × *g*) for 5 min. 1 mL of the extract was dried under a stream of nitrogen, then redissolved in MeCN and filtered through a 0.45 μm PTFE filter for LC-QqQ/MS analysis.

The main differences on the four methods tested were the type of salts and solvent used during the extraction and the adsorbents used for the clean-up. These differences are listed in Table 1. Moreover for methods 2 and 3, a pH adjustment of the extract after the clean-up was performed by adding 10 μL of a 5% formic acid solution in MeCN per mL extract.

The amount of sample *X* was 5, 7.5 and 10 g for each of the methods tested, and the pH of the extracts was measured in each step of the four different procedures.

2.6. Method performance

The following parameters were evaluated during the comparison of the methods: accuracy (% recovery), precision (% RSD), limit of detection (LOD), limit of quantification (LOQ) and matrix effect using LC-QqQ/MS and LC-TOF/MS.

The LOD values of each analyte were estimated on the injection of matrix matched standard solution at 10 μg kg^{-1} for the four tested methods. The calculation was based on the detection signal being three times over the average of background noise assayed, the LOQ values were estimated at 5* LOD.

The accuracy and precision of the methods were evaluated via recovery experiments with fortified samples at two fortification levels: 10 μg kg^{-1} and 300 μg kg^{-1} at five replicates for each level.

The linearity of the analytical procedures was tested with matrix-matched calibrations prepared by adding the standards to rice extracted with the four different methodologies in the range 5–500 μg L^{-1} for the three sample amounts (5, 7.5 and 10 g).

Additionally as it is well known that co-extractants depend not only on the type of matrix but also on the extraction method, the matrix background was compared for the three masses used (5, 7.5 and 10 g) and for the 4 methods at 300 μg kg^{-1} using LC-TOF/MS.

3. Results and discussion

3.1. Target compound selection

The 16 herbicides were selected as the most often used for rice production. According to the regulations of the European Union some of these herbicides are forbidden such as propanil, bromacil and imazapyr, others are pending like bispyribac sodium and others

Table 2
Instrument acquisition data used for the analysis of the selected pesticides by LC-QqQ/MS.

Pesticide	Mode of action	tR (min)	ESI mode	Quantitation MRM1	Confirmation MRM2	Fragmentor (V)	CE 1 (V)	CE 2 (V)
Azimsulfuron	Herbicide	17.3/**7.5**	+/−	447.1/178.1	425.0/182.1	120/90	10	15
				423.0/214.1	**423.0/135.1**	**150**	**10**	**27**
Azoxystrobin	Fungicide	21.1	+	404.0/372.0	404.0/344.0	120	10	20
Bensulfuron methyl	Herbicide	18.7/**8.3**	+/−	411.1/182.0	411.1/149.0	150	20	15
				409.1/195.8	**409.1/154.1**	**120**	**40**	**40**
Bentazone	Herbicide	7.9	–	239.1/197.0	239.1/132.0	120	15	20
Bispyribac sodium	Herbicide	19.2	+	453.1/297.1	453.1/179.1	150	15	20
Bromacil	Herbicide	14.2/6.2	+/−	261.0/205.0	261.0/188.0	90	10	20
				261.2/205.0	**261.2/188.0**	**150**	**15**	**15**
Carbaryl	Growth regulator/insecticide	17.2	+	202.0/145.0	202.0/127.0	140	10	20
Carbendazim	Fungicide	3.4	+	192.0/160.0	192.0/132.0	150	15	20
Carbofuran	Insecticide	16.6	+	222.0/123.0	222.0/165.0	90	20	10
Clomazone	Herbicide	19.4	+	240.1/125.0	240.1/89.0	150	20	60
Cyhalofop butyl	Herbicide	10.4	+	357.8/256.0	357.8/302.0	150/165	40	12
Diflubenzuron	Insecticide	22.1	+	311.0/158.0	311.0/141.0	120	10	20
Dimethoate	Acaricide/insecticide	11.2	+	230.0/199.0	230.0/171.0	90	5	10
Edifenphos	Fungicide	23.3	+	311.0/173.0	311.0/283.1	120	5	10
Epoxiconazole	Fungicide	21.0	+	330.1/123.1	330.1/121.1	120	10	15
Ethiofencarb	Insecticide	17.8	+	226.0/107.0	226.0/164.0	60	15	5
Fluroxypyr	Herbicide	14.5	+	255.0/181.0	255.0/209.0	120	20	15
Flutolanil	Fungicide	23.0	+	324.1/262.1	324.1/242.1	150	15	20
Imazapic	Herbicide	9.6	+	276.1/163.2	276.1/145.0	150	30	40
Imazapyr	Herbicide	6.5	+	262.1/149.1	262.1/217.0	150	30	30
Imazaquin	Herbicide	14.3	+	312.1/199.0	312.1/153.2	90/150	30	50
Imazosulfuron	Herbicide	18.6/8.2	+/−	413.1/156	413.1/153.0	150	20	5
				410.9/229.9	**410.9/153.9**	**120**	**15**	**20**
Imidacloprid	Insecticide	10.3	+	256.0/175.0	256.0/209.0	90	15	15
Iprodione	Fungicide	22.7	+	330.0/245.0	330.0/101.0	90	15	20
Kresoxim methyl	Fungicide	24.2	+	336.2/246.2	336.2/229.2	150	15	20
Malathion	Acaricide/insecticide	23.0	+	331.0/99.0	331.0/127.0	90	20	10
Metsulfuron methyl	Herbicide	15.7	+	382.1/167.1	382.1/141.1	150	15	15
Molinate	Herbicide	21.4	+	188.2/126.1	188.2/55.1	80	10	20
Oxydemeton methyl	Insecticide	4.1	+	246.9/125.0	246.9/169.0	80	20	10
Picoxystrobin	Fungicide	24.5	+	368.1/205.2	368.1/145.1	80	5	20
Propanil	Herbicide	9.1	-	218.0/161.8	216.0/159.9	120	10	15
Propiconazole	Fungicide	23.1	+	342.1/159.1	342.1/69.3	120	20	15
Pyrazosulfuron ethyl	Herbicide	20.3	+	415.1/182.1	415.1/139.1	120/90	25	50
Pyridaphenthion	Acaricide/insecticide	21.7	+	341.0/189.0	341.0/205.0	120	20	20
Tebuconazole	Fungicide	21.7	+	308.0/70.0	308.0/125.0	90	20	20
Tebufenozide	Insecticide	23.7	+	353.2/133.1	353.2/296.9	150	15	5
Thiacloprid	Insecticide	13.31	+	253.0/126	253.0/186	120	20	10
Thiamethoxam	Insecticide	7.6	+	292.0/211.0	292.0/181.0	90	10	20
Thiophanate ethyl	Fungicide	18.9	+	371.1/151.1	371.1/325.1	90	20	10
TPP	Surrogate St.	24.6	+	327.0/77.2	327.0/152.2	120	35	30
Triadimefon	Fungicide	21.6	+	294.2/197.1	294.2/225.0	150	10	10
Triadimenol	Fungicide	19.5 and 19.9	+	296.2/70.2	296.2/227.0	60	10	5
Tricyclazole	Fungicide	11.5	+	190.1/163.0	190.1/136.0	150	20	25

tR, retention time; CE, collision energy. Numbers in bold are the parameters used for the analysis of pesticides in the negative mode.

like pyrazosulfuron ethyl are still not considered in the legislation, nevertheless these herbicides are commonly applied in rice crops in exporting countries such as Argentina and Uruguay [28].

For the additional pesticides three different criteria were followed: (1) pesticides that have established MRLs by The Codex Alimentarius Commission [2], (2) pesticides most often used for rice production and (3) positive findings according to the residue data in www.pesticides-online.com database in the period 2000–2010 [29]. The comprehensive list covers 42 pesticides with different modes of action: herbicides, fungicides and insecticides from different chemical natures such as imidazolinones, phenoxyacetic acids, sulfonylureas, strobilurins, carbamates, organothiophosphates, conazoles and others.

3.2. Optimization of MS/MS conditions

The MS parameters were optimized with the objectives of: (i) obtaining the protonated molecule and (ii) selecting those transitions with higher molecular mass in order to avoid the disruptive effects of the matrix, as far as possible.

The optimization of the precursor ion and product ions was carried out by the injection of 1 μL of the individual pesticide standard solution directly into the mass spectrometer into a constant flow of MeCN/water (1:1) with a flow rate of 0.2 mL min^{-1}. Different fragmentor voltages (60, 90, 120 and 150 V) were applied and once the optimal fragmentor voltage was found, different collision energies (5, 10, 15 and 20 V) were investigated. In particular cases, like azimsulfuron and propanil different precursor ions were needed for an optimum optimization of the analyte. The two most intense transitions were chosen for merging and creating the method. The most intense transition was used as a quantifier while the other was used as a qualifier peak for the confirmatory analysis. These optimization parameters are included in Table 2. For cyhalofop butyl and kresoxim methyl only two transitions were obtained over all of the conditions studied, and one of them was not intense enough for the recovery tests thus making it impossible to obtain an adequate identification, therefore at 10 μg kg^{-1}, cyhalofop butyl and kresoxim methyl were not detected. However at 300 μg kg^{-1} it could be correctly detected and quantified.

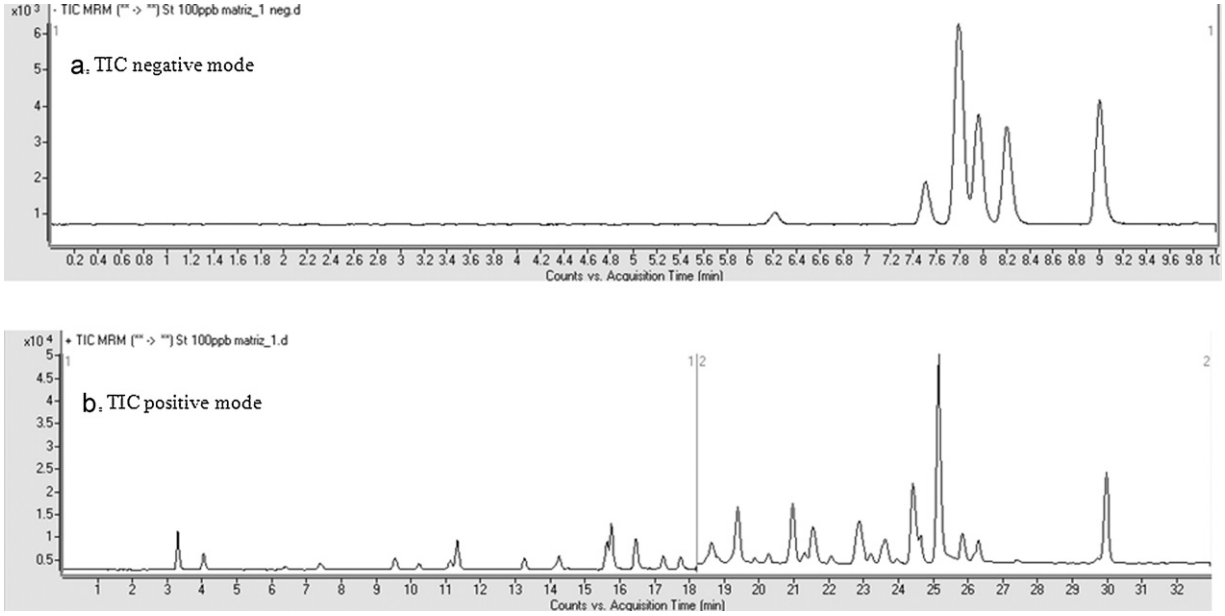

Fig. 1. (a) Total ion chromatogram at 300 μg kg^{-1} in polished rice for the 40 multi class pesticides in the positive ionization mode. (b) Total ion chromatogram for the 6 pesticides included in the negative ionization mode at the same concentration level.

For tebuconazole and triadimenol only low quantifier masses (m/z < 80) were detected, which turns to be a disadvantage as the selectivity is reduced [8].

Most of the pesticides under study were optimized in the positive form but as it is described in the literature bentazone and propanil were not possible to optimize them in the positive mode, so a method in negative mode was created for the analysis of these compounds [30,31].

Azimsulfuron, imazosulfuron and bensulfuron methyl were analyzed in both modes, negative and positive mode. However they were quantified using the positive mode because the sensitivity of the fragments was higher.

Fig. 1a and b shows the total ion chromatogram (TIC) at 300 μg kg^{-1} in rice extract for the 40 pesticides in the positive mode and the 5 pesticides included in the negative mode, respectively.

3.3. Selection of the amount of sample used

According to the literature, the selected amount of sample for pesticide residue analysis in cereals with different QuEChERS based methods is between 5 and 10 g [18,19,21,22,32,33].

Rice can be considered a difficult matrix due to its chemical composition, so high numbers of compound are co-extracted during the extraction procedures. For this reason, in this work 3 different amounts of sample for each of the 4 studied methods were compared so as to determine which the best conditions of analysis for milled rice are in terms of pesticide recoveries and matrix effects.

Table 3 shows the distribution of the pesticides (in percentage) with recoveries in the range 70–120% and RSD below 20% for each of the studied methods at 10 μg kg^{-1}. As can be seen from Table 3, the determination of pesticides using 7.5 g of sample presented the best performance followed by the use of 5 g of sample. The recoveries using 5 g are in general lower (but in many cases still acceptable) for the four methodologies. However for certain important rice pesticides like epoxiconazole in methods 3 and 4 or propanil in methods 2 and 3 the recoveries using 5 g does not meet the acceptable requirements. When sampling 10 g of milled rice the recovery rates are lower than the ones obtained with 5 or 7.5 g of sample and also, as it is explained in Section 3.4.4.2, the amount of co-extracted compounds from the matrix is bigger. For this reason, 7.5 g of milled rice is the amount of choice for the analysis of real samples. It is the best compromise between recoveries and the amount of co-extracted compounds.

3.4. Comparison of the methods

3.4.1. Accuracy and precision

For the recovery study, a spiked sample was prepared and the recoveries examined at 10 and 300 μg kg^{-1} spiking levels. The 4 different methods were performed five times at each spiking level. The data evaluation was carried out by comparing the peak intensities of the spiked samples to those obtained by matrix-matched standard calibration. The distribution of the recoveries and relative standard deviations are shown in Table 4.

The recoveries results show that method 4 is capable of determine 36 of the selected pesticides satisfactorily. The other 6 pesticides presented difficulties with this method; nevertheless these pesticides can be analyzed by at least one of the other three proposed methods with recoveries and RSD in the acceptance range of the DG SANCO Guidelines. Concerning the other three methods (methods 1–3) 33, 31 and 31 pesticides presented good recoveries and RSDs, respectively.

The acidic imidazolinone herbicides (imazapic, imazapyr, imazaquin, pK_a ~ 1.9–3.8) [34], widely used in rice crops [35] and

Table 3
Distribution of the pesticides (in percentage) with recoveries in the range 70–120% and RSDs below 20% for each of the studied methods.

Method number	Method	10 g (%)	7.5 g (%)	5 g (%)
1	Original QuEChERS	60	85	76
2	Citrate buffered QuEChERS	61	78	53
3	Citrate buffered QuEChERS without clean-up	61	78	58
4	Acetate buffered QuEChERS without clean-up	49	93	79

Table 4
Mean recoveries and RSD obtained for the selected pesticides spiked in milled polished rice, for the 4 methods assayed by LC-QqQ/MS.

Pesticide name	Method 1		Method 2		Method 3		Method 4	
	10 μg kg^{-1}	300 μg kg^{-1}	10 μg kg^{-1}	300 μg kg^{-1}	10 μg kg^{-1}	300 μg kg^{-1}	10 μg kg^{-1}	300 μg kg^{-1}
Azimsulfuron	87.1(3)	101.5(2)	108.6(13)	76.4(9)	108.6(19)	95.1(10)	103.3(12)	91.0(4)
Azoxystrobin	112.6(6)	99.6(2)	108.7(14)	90.6(4)	102.3(13)	93.3(7)	94.5(4)	89.5(1)
Bensulfuron methyl	82.7(10)	101.5(2)	97.1(15)	76.0(11)	113.4(14)	94.7(11)	105.9(14)	84.5(3)
Bentazone	85.7(8)	99.3(1)	82.9(5)	78.0(4)	92.0(6)	82.9(6)	75.2(7)	76.4(3)
Bispyribac sodium	51.1(37)	99.8(6)	81.6(22)	80.5(20)	62.2(14)	87.6(6)	84.1(27)	68.1(3)
Bromacil	108.3(12)	98.6(3)	110.8(11)	94.3(5)	112.8(10)	96.7(10)	96.0(5)	88.1(1)
Carbaryl	106.5(7)	102.6(14)	114.7(5)	97.3(7)	95.1(14)	99.4(8)	108.2(14)	90.1(2)
Carbendazim	97.5(7)	99.1(3)	114.1(8)	85.3(4)	81.9(8)	85.6(10)	87.6(4)	80.8(1)
Carbofuran	128.8(6)	95.6(7)	127.7(11)	94.0(3)	98.7(17)	90.4(10)	117.8(12)	85.1(3)
Clomazone	104.3(5)	102.7(7)	110.7(11)	86.6(6)	104.7(11)	77.8(14)	92.6(7)	78.1(6)
Cyhalofop butyl	NA	92.0(14)	NA	89.8(14)	NA	84.2(14)	NA	80.7(11)
Diflubenzuron	118.6(10)	105.5(6)	94.4(8)	91.0(6)	61.1(24)	59.0(5)	83.7(13)	75.6(7)
Dimethoate	102.6(7)	97.6(3)	96.5(9)	88.8(4)	106.1(20)	90.5(10)	86.4(13)	86.3(2)
Edifenphos	122.9(6)	110.7(8)	85.6(16)	84.5(6)	48.0(24)	63.1(8)	75.4(15)	68.1(4)
Epoxiconazole	101.3(9)	101.4(2)	102.1(18)	85.9(5)	67.3(10)	65.9(11)	74.3(12)	71.1(8)
Ethiofencarb	162.7(5)	98.9(3)	97.8(9)	89.6(5)	92.5(9)	88.9(10)	88.4(3)	79.0(5)
Fluroxypyr	98.6(4)	100.1(2)	38.3(34)	29.4(19)	118.1(20)	77.3(10)	77.6(20)	48.2(7)
Flutolanil	114.7(9)	101.7(2)	105.7(10)	96.4(3)	89.6(10)	82.5(7)	94.3(4)	86.1(1)
Imazapic	12.2(37)	99.4(0)	42.2(11)	31.4(14)	82.2(13)	88.0(3)	66.1(8)	50.1(2)
Imazapyr	6.7(42)	99.0(1)	33.1(22)	24.4(14)	74.6(12)	85.4(3)	29.4(7)	29.0(5)
Imazaquin	22.6(20)	99.7(1)	42.6(15)	33.6(15)	79.9(12)	78.9(10)	60.2(12)	58.0(2)
Imazosulfuron	78.3(14)	101.5(2)	96.4(16)	70.5(13)	93.7(10)	85.2(11)	90.9(18)	82.5(5)
Imidacloprid	96.7(8)	100.2(1)	108.6(6)	89.4(8)	91.4(12)	90.9(11)	120.7(5)	87.7(3)
Iprodione	89.6(7)	97.5(5)	99.3(15)	84.0(4)	73.3(19)	81.0(7)	66.2(18)	68.7(7)
Kresoxim methyl	NA	105.7(5)	NA	82.5(8)	NA	71.2(6)	NA	75.2(4)
Malathion	116.8(5)	100.8(6)	107.7(15)	90.1(2)	70.3(5)	80.0(7)	91.7(4)	83.9(2)
Metsulfuron methyl	70.0(9)	100.3(3)	87.7(12)	69.5(11)	115.2(15)	91.1(8)	100.8(9)	84.7(7)
Molinate	87.2(17)	123.6(18)	50.8(16)	51.6(4)	64.0(5)	68.0(5)	79.9(20)	66.0(20)
Oxydemeton methyl	105.9(9)	101.3(2)	108.9(15)	88.9(4)	94.5(15)	98.9(8)	90.4(6)	86.9(4)
Picoxystrobin	117.4(5)	107.4(2)	89.5(16)	84.6(5)	48.8(9)	69.5(7)	74.7(4)	78.8(3)
Propanil	100.7(9)	101.1(1)	88.5(9)	94.9(5)	77.4(6)	82.2(1.3)	86.3(5)	84.3(2)
Propiconazole	104.6(3)	104.0(2)	122.8(7)	80.6(9)	60.2(28)	63.8(11)	71.9(12)	58.9(4)
Pyrazosulfuron ethyl	113.8(11)	101.5(2)	106.9(15)	78.1(7)	94.0(16)	91.0(8)	95.1(11)	85.7(5)
Pyridaphenthion	85.0(8)	100.6(3)	86.7(13)	76.9(13)	69.3(7)	83.2(12)	103.7(12)	80.7(4)
Tebuconazole	117.7(6)	101.3(5)	102.1(8)	79.3(4)	59.8(6)	66.7(18)	91.0(14)	69.2(9)
Tebufenozide	87.8(9)	105.5(3)	98.0(12)	91.9(4)	53.8(12)	74.0(6)	75.9(8)	78.2(2)
Thiacloprid	112.6(2)	101.4(1)	122.6(11)	92.3(4)	90.6(10)	91.6(11)	120.8(14)	90.4(2)
Thiamethoxam	101.6(11)	98.5(3)	118.4(16)	87.8(4)	101.7(24)	90.6(11)	96.5(11)	90.1(3)
Thiophanate ethyl	118.7(17)	117.9(18)	58.1(19)	84.2(10)	104.0(16)	103.1(10)	105.4(13)	110.7(3)
Triadimefon	103.4(18)	101.6(4)	88.6(9)	85.0(2)	74.5(6)	73.9(11)	82.6(13)	79.7(8)
Triadimenol	117.5(8)	99.8(4)	99.1(8)	87.7(4)	87.7(12)	87.3(8)	87.2(10)	85.0(4)
Tricyclazole	101.3(3)	101.6(2)	111.3(3)	88.2(3)	112.1(8)	89.4(10)	101.2(3)	82.9(2)

Methods: (1) Original QuEChERS; (2) citrate buffered QuEChERS; (3) citrate buffered QuEChERS without clean-up; (4) acetate buffered QuEChERS without clean-up.
NA: not analyzed.

fluroxypyr, were expected to give higher recoveries when no PSA was added (methods 3 and 4) than those methods with PSA clean-up (methods 1 and 2) due to the capability of PSA to retain acidic compounds. Nevertheless, this assumption was not corroborated experimentally: Table 4 shows that method 3 presented good recoveries but method 4 did not meet the acceptable requirements for both fortification levels. Moreover, matrix effects are not relevant for these compounds (101.3, 98.9 and 106.6 for imazapyr, imazaquin and imazapic, respectively) and also the signal to noise ratio for these pesticides at 10 μg kg^{-1} is in the range 6–60, so a possible explanation could be the differences on the pH during the extraction methods; while in the final extract obtained with method 3 the pH is 4.1 due to the addition of 5% formic acid solution in MeCN, the pH obtained with method 4 is 6.2.

Concerning method 1 the recoveries of these four herbicides were good, the reason of these could be probably that the amount of PSA (100 mg), competing with the co-extractants from rice (organic and fatty acids) is relatively low, while in method 2 the amount of PSA (150 mg) interacts with the acid functionalities of these herbicides thus the recoveries were below 70% as it was expected.

In summary, based on the recovery results both the original QuEChERS (method 1) and the acetate buffered QuEChERS (method 4) presented the best performance for the analysis of the selected pesticides, except for imidazolinone herbicides where the citrate buffered QuEChERS without the clean-up with PSA and C-18 (method 3) gave the best results, so the analysis of pesticides in polished rice will depend on the scope of each laboratory.

In the present work method 4 was chosen not only because of its capability to determine most of the pesticides commonly used in rice, but also it eliminates the clean-up step, thus it appears to have the flexibility to include other acidic pesticides within the range of substances tested. Moreover as described in Section 3.4.4.2 the amount of co-extracted compounds seems to be smaller compared with method 1.

3.4.2. LOD and LOQ

The limits of detection (LODs) were estimated on the injection of matrix-matched standard solution at 10 μg kg^{-1} for the four methods, giving a signal-to-noise ratio of 3 and when a good fit of the spectra is obtained. In general, there are not important differences between the four methods; the average values are about, 4 μg kg^{-1} which is enough to meet the Regulation (EC) No. 299/2008 [36].

It should be pointed out that the LODs were as low as 0.07 μg kg^{-1} in the case of metsulfuron methyl with method 4 and 0.14 μg kg^{-1} for azimsulfuron with method 3.

Only cyhalofop butyl and kresoxim methyl showed LODs higher than 10 μg kg^{-1} maybe due to their low response under electrospray conditions, nevertheless they were detected and quantified properly at 300 μg kg^{-1}.

The LOQs were estimated at 5* LOD and were in the range 0.5–50 μg kg^{-1}.

3.4.3. Linearity

The quantification was performed for each pesticide from the average of two 5-point calibration curves for each method at the three different sample amounts by using LC-QqQ/MS.

The matrix-matched calibration standard concentrations were 5, 10, 50, 100 and 500 μg L^{-1}. The linearity for all pesticides was satisfactory with a correlation coefficient ≥0.998.

3.4.4. Matrix effects

To obtain a better understanding of matrix effects in quantitative analysis and to evaluate the use of matrix-matched standards, LC-QqQ/MS studies were carried out. LC-TOF/MS in full scan mode was also used to study the co-extractive interferences produced during the extraction of this commodity.

3.4.4.1. Matrix effects by LC-QqQ/MS. Matrix effect was evaluated by comparing the response of each pesticide obtained from a standard solution in solvent and that from a spiked sample and the corresponding slope in matrix/slope in solvent ratio was calculated and expressed as percentage, for each of the studied pesticides. Almost all the compounds presented signal suppression or enhancement, therefore the quantification was performed using matrix-matched standard calibration, which is in agreement with the recommendation in DG SANCO Guidelines [26].

Method 3 presented lower slopes compared with the other three methods for most of the pesticides and therefore as it is represented in Fig. 2 signal suppression was observed for almost 90% of the pesticides under study. In addition to this, method 4 presented the lowest percentage of pesticides without important (≥ ± 25%) matrix effect. In methods 1 and 2 signal enhancement and suppression was found depending on the pesticide. An example of the matrix effects presented for pyrazosulfuron ethyl and molinate is shown in Fig. 3. Regarding pyrazosulfuron ethyl no matrix effect was observed for method 2 (2%). Methods 1 and 4 presented around 20% of signal suppression whereas 44% of signal suppression was observed in method 3. Molinate showed signal suppression (34, 44, 26 and 47%) for methods 1–4, respectively, these examples confirm that matrix effect on pesticides in polished rice depends not only on the analytes but also on the method used.

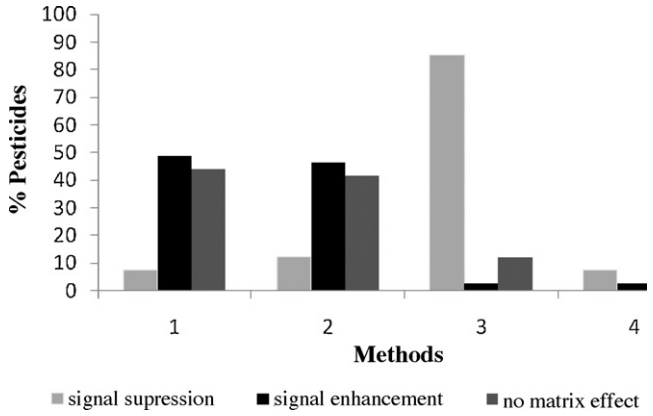

Fig. 2. Distribution of pesticides in percentage presenting different matrix effects for each one of the studied methods.

3.4.4.2. Study of co-extracted matrix components by LC-TOF/MS. The amount of co-extractants obtained after the extraction process selected for pesticide determination is a relevant parameter in routine laboratories not only because it can affect the performance of the method but also for the maintenance of the analytical equipment. The complexity of certain matrices leads to a decrease in the lifetime of chromatographic columns and can even cause problems in the ionization and detection systems of the analytical instrument. It is therefore necessary to select a methodology that allows the analysis of the largest number of analytes but not disregarding this factor.

One possible approach in quantitative analysis is to improve the pretreatment of the sample, but in multiresidue analysis it is not always straightforward, thus a reasonable solution is to reduce the amount of matrix components that are introduced into the analytical system.

In this work we evaluated the background of the matrix obtained with the four different extraction procedures at 300 μg kg^{-1} and for the three different amounts of sample, by injecting in LC-TOF/MS in full scan mode. In general, it was observed that the higher the amount of sample used, the higher the amount of co-extractive compounds present in the final extract for the four assayed methods. However this relation is not linear, either because the increase on the amount of sample and solubility is not always a linear pro-

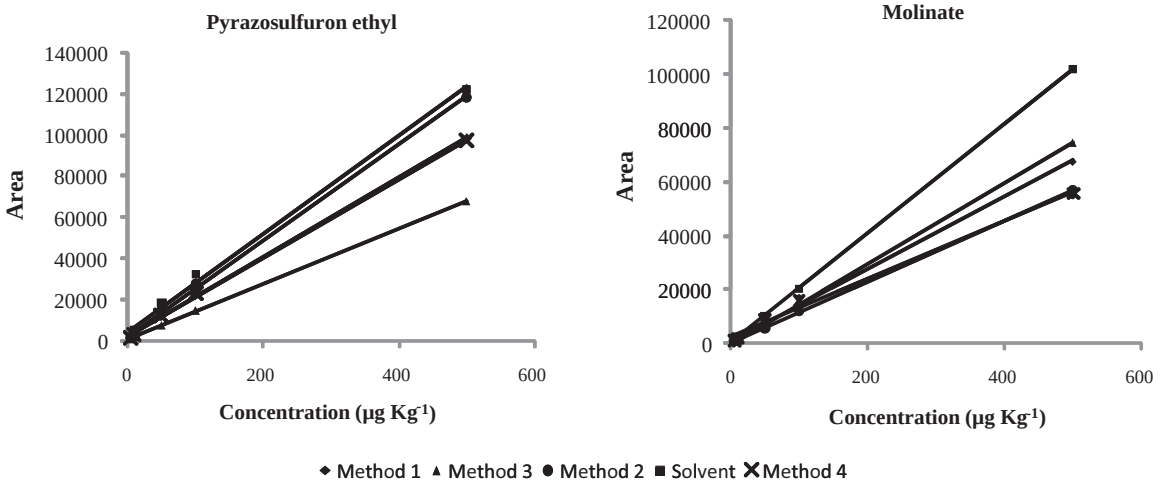

Fig. 3. Matrix effect by LC-QqQ/MS. Calibration curves of the different methods. (a) Pyrazosulfuron ethyl and (b) molinate.

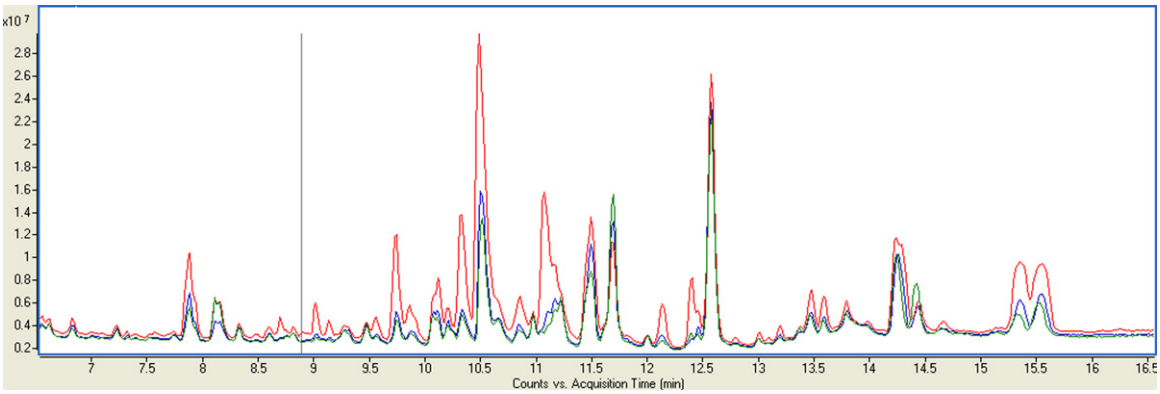

Fig. 4. Matrix effect by LC-TOF/MS. Comparison of the total ion chromatograms of a 300 μg kg^{-1} spiked sample obtained using buffered acetate QuEChERS method without PSA clean-up using three different amounts of sample: 5, 7.5 and 10 g. Red line 10 g, blue line 7.5 g, green line 5 g. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

cess or even to thresholds of competence between the interferences and the analytes during the ionization process.

When comparing one of the methods for the different amounts of sample (5, 7.5 and 10 g of sample) which means a concentration of 0.33; 0.5 and 0.66 g of sample/mL of extract, different results were obtained depending on the amount of sample used. Fig. 4 shows the TIC obtained with acetate buffered QuEChERS without the clean-up step (method 4) for the three amounts of sample. As it is shown in Fig. 4, the TICs corresponding to 5 and 7.5 g are similar still there is a slightly higher amount of co-extractants for 7.5 g of rice. The TIC obtained with the extraction of 10 g of sample presented a clearly higher amount of co-extractants; nevertheless there is not linear relationship between the amount of sample and the co-extractants for the three amounts of sample assayed. As it was discussed before the reason could be that higher amounts of sample produce higher amount of ions that can saturate the detector producing responses that are not linear and therefore not expected results.

As can be seen in Fig. 5 for 7.5 g of polished rice, cleaner extracts were obtained for citrate buffered QuEChERS (method 2) and acetate buffered QuEChERS without PSA clean-up (method 4) while original QuEChERS (method 1) give dirtier extracts. The TIC obtained when performing method 3 varies along the chromatogram time, until 8 min presents the highest background but at the end of the chromatogram the amount of co-extractants is similar to the rest of the other methods.

As methods 1 and 2 include a clean-up step, fewer amount of co-extractants were expected in their TICs. This was observed for method 2 but not for method 1; also method 4, where no clean-up was included, provided cleaner extracts than method 1. One reasonable explanation of this behaviour is the differences on the pH during the different extraction procedures. While the pH of the final extract in method 1 was 8.4 the pH of method 4 was 6.3, thus the higher pH of method 1 may have a strong influence on the co-extraction of matrix components and therefore higher background noise.

Concerning the differences between methods 1 and 2 despite the fact that the pH after the clean-up step is similar for both methodologies (around pH 8), the clean-up of method 2 involves C-18 sorbent which interacts with lipophilic compounds, sugars and other matrix components, this could be the reason of the differences in their TICs.

A comparison between citrate buffered QuEChERS with and without clean-up was performed for the 3 amounts of sample. The results showed that in general the chromatogram is dirtier when using the citrate method without the purification step for the three amounts of sample studied, which is logical since the adsorbents used (PSA and C-18) retain components of the matrix that can interfere in the analysis, giving cleaner extracts. Curiously from the min 11 of the chromatogram obtained with method 2 is much dirtier than the one obtained without the addition of PSA and C-18 (method 3) giving much more co-extractants. This fact should be due to differences on the pH during the extraction (pH 8 for method 2 vs. pH 6.4 for method 3). As in the case discussed before (Fig. 5) higher pH provided dirtiest extracts thus it seems that some components of the matrix are

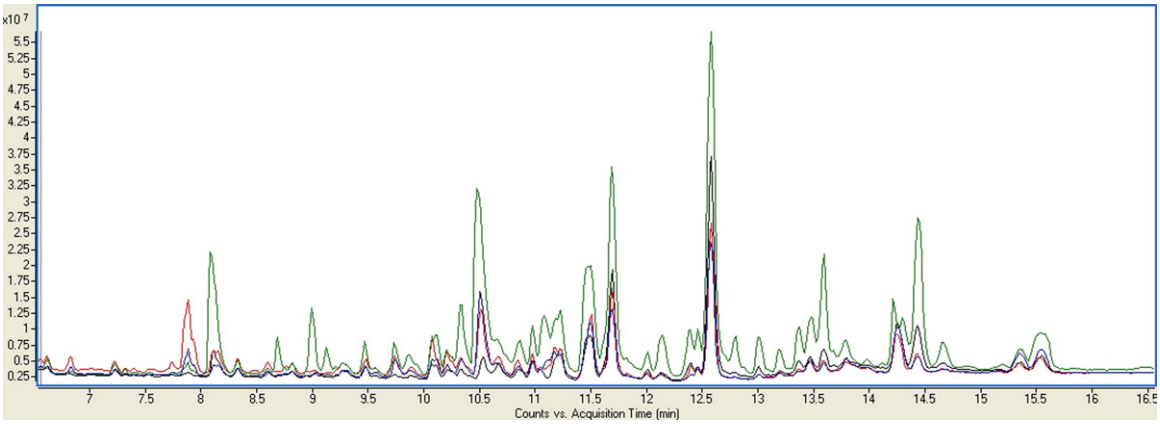

Fig. 5. Matrix effect by LC-TOF/MS. Comparison of the total ion chromatograms of a 300 μg kg^{-1} spiked sample obtained with the 4 methods tested using 7.5 g of milled polished rice. Green line: original QuEChERS; red line: citrate buffered QuEChERS without clean-up; blue line: citrate buffered QuEChERS; black line: acetate buffered QuEChERS without clean-up. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

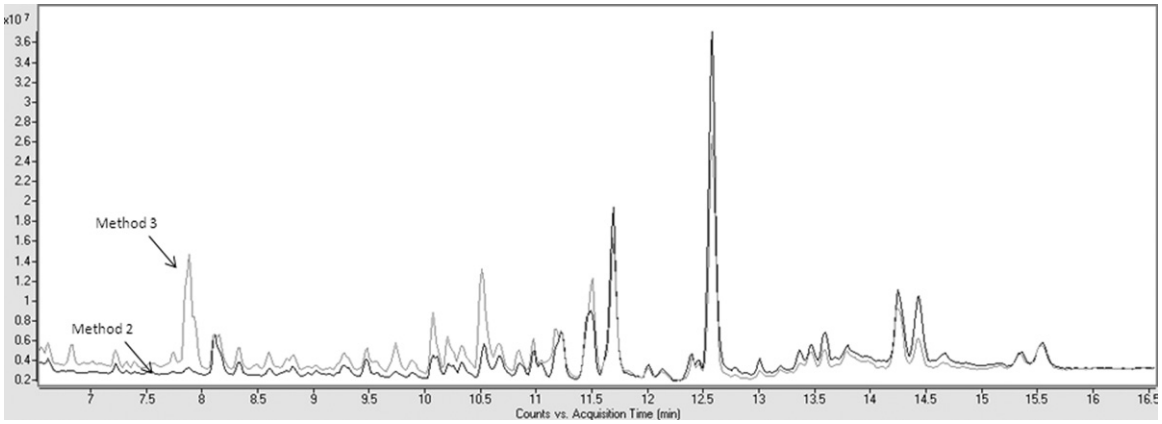

Fig. 6. Matrix effect by LC-TOF/MS. Comparison of the total ion chromatograms of a 300 μg kg^{-1} spiked sample obtained using both citrate buffered QuEChERS and citrate buffered QuEChERS without PSA and C-18 clean-up using 7.5 g of sample.

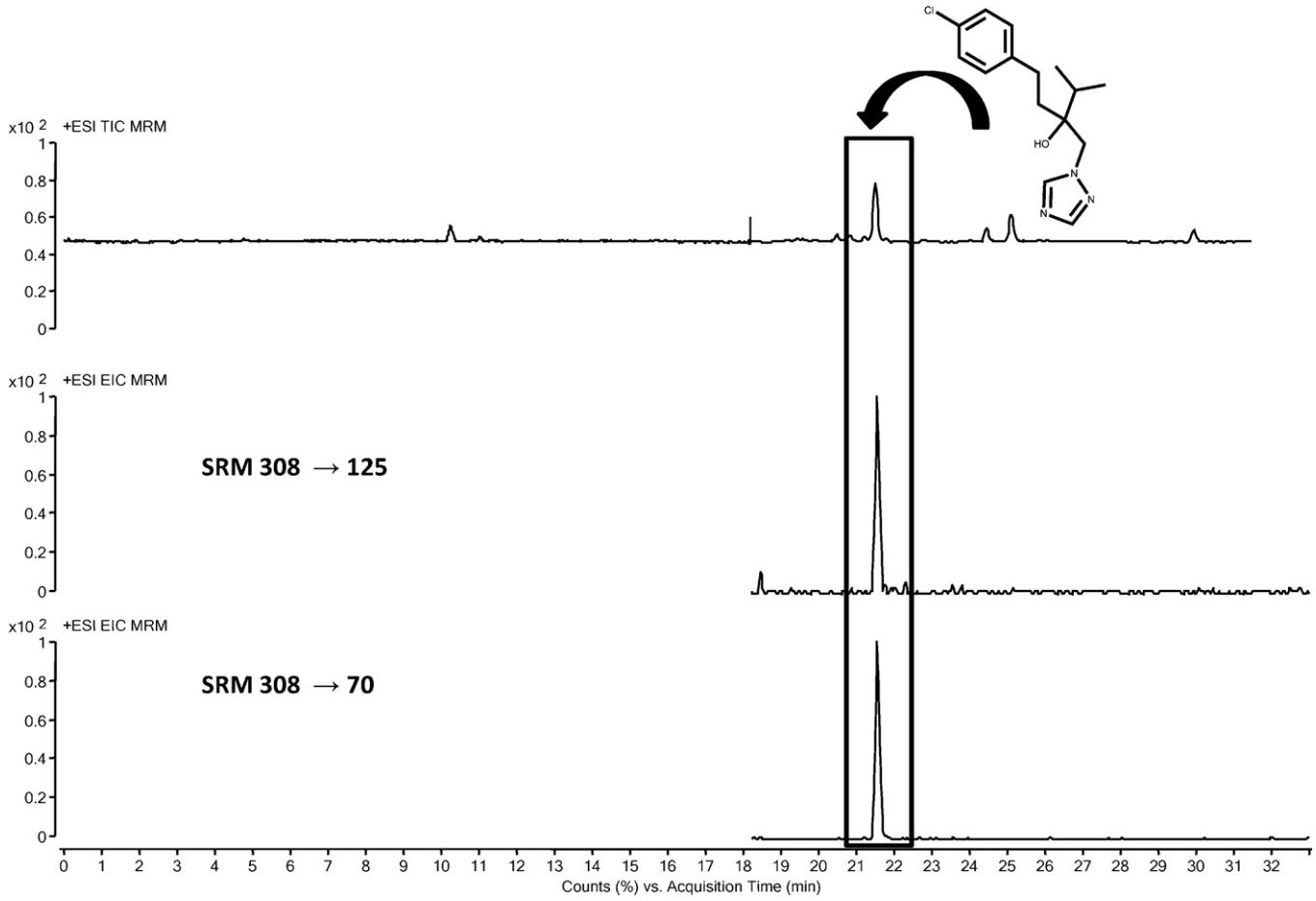

Fig. 7. Total ion chromatogram and SRM chromatograms of 15.8 μg kg^{-1} of tebuconazole found on a commercial sample of polished rice.

extracted when pH is around 8 but not when the pH is lower (see Fig. 6).

4. Application to real samples

The screening of 18 commercially available samples was performed by LC-TOF/MS followed by LC-QqQ/MS analysis for the quantification of positive samples. The samples were extracted with acetate buffered QuEChERS method without the PSA clean-up (method 4). Two of the samples showed a concentration of tebuconazole of 3.0 and 15.8 μg kg^{-1}, and one sample presents 3 μg kg^{-1} of propiconazole. No pesticides were observed in the other samples. The TIC and SRM chromatograms of a positive sample where tebuconazole was detected are shown in Fig. 7.

5. Conclusions

Four different procedures based on the QuEChERS method were compared for the determination of 42 pesticides including herbicides, fungicides and insecticides widely used in rice crops.

Most of the pesticides have shown recoveries in the range 70–120% and precision (RSD < 20%), meeting EU guidelines method performance criteria, even at 10 $\mu g\,kg^{-1}$.

Method 4 was chosen for validation and subsequent analysis of the real samples because it gives good results for the principal pesticides used in rice crops and also because it is a very simple and fast methodology without the clean-up step. Moreover regarding the co-extraction of matrix components, this method provides cleaner chromatograms and also matrix effect is not very pronounced.The usefulness of this method was proved in the analysis of 18 real samples demonstrating its suitability for routine analysis.

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