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Accurate determination of selected pesticides in soya beans by liquid chromatography coupled to isotope dilution mass spectrometry

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ABSTRACT

A sensitive, accurate and simple liquid chromatography coupled with mass spectrometry method for the determination of 10 selected pesticides in soya beans has been developed and validated. The method is intended for use during the characterization of selected pesticides in a reference material. In this process, high accuracy and appropriate uncertainty levels associated to the analytical measurements are of utmost importance. The analytical procedure is based on sample extraction by the use of a modified QuEChERS (quick, easy, cheap, effective, rugged, safe) extraction and subsequent clean-up of the extract with C_{18}, PSA and Florisil. Analytes were separated on a C_{18} column using gradient elution with water–methanol/2.5 mM ammonium acetate mobile phase, and finally identified and quantified by triple quadrupole mass spectrometry in the multiple reaction monitoring mode (MRM). Reliable and accurate quantification of the analytes was achieved by means of stable isotope-labelled analogues employed as internal standards (IS) and calibration with pure substance solutions containing both, the isotopically labelled and native compounds. Exceptions were made for thiodicarb and malaoxon where the isotopically labelled congeners were not commercially available at the time of analysis. For the quantification of those compounds methomyl-$^{13}C_2{}^{15}N$ and malathion-D10 were used respectively. The method was validated according to the general principles covered by DG SANCO guidelines. However, validation criteria were set more stringently. Mean recoveries were in the range of 86–103% with RSDs lower than 8.1%. Repeatability and intermediate precision were in the range of 3.9–7.6% and 1.9–8.7% respectively. LODs were theoretically estimated and experimentally confirmed to be in the range 0.001–0.005 mg kg^{-1} in the matrix, while LOQs established as the lowest spiking mass fractionation level were in the range 0.01–0.05 mg kg^{-1}. The method reliably identifies and quantifies the selected pesticides in soya beans at appropriate uncertainty levels, making it suitable for the characterization of candidate reference materials.

1. Introduction

Pesticides comprise a large number of compounds, with extremely diverse physico-chemical properties. They are applied to crops at various stages of cultivation to provide protection against pests and during post-harvest, to prolong storage life and preserve quality. The use of pesticides may however generate residues which involve a risk for both the environment and human health. In order to guarantee food and feed safety and to regulate international trade, the European Union has established maximum residue limits (MRLs) to minimize the presence of pesticide residue levels in different food stuffs [1].

Suitable analytical methodology for monitoring purposes is required to avoid human exposure to pesticide residues through food. In Europe, official control and commercial laboratories involved in the pesticides residues field need to deal with large amounts of analyses as well as fulfilling the requirements for assuring the quality of their results, especially in relation to accreditation according to ISO 17025 [2]. To cope with thousands of possible individual pesticide-matrix combinations, multi-residue methods are needed. Nevertheless, these methods require full validation. Aware of the related difficulties, the EU Directorate General for Health and Consumers (SANCO) produced guidelines for method validation and quality control procedures for pesticide residues in food and feed, which have been recently revised [3]. For each individual pesticide residue,

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the acceptability criteria set for the validation parameters includes a method repeatability threshold of 20% and recovery in the range 70–120%. Although these conditions are acceptable for the application of routine analyses in control laboratories, more stringent accuracy criteria are needed for measurement procedures used to assign values to a certified reference material in order to be suitable for quality control purposes.

The guidance document on pesticide residue analytical methods, published by the Organisation for Economic Co-operation and Development (OECD) [4], establishes that food commodities are classified in groups according to their mayor composition. Full validation is needed for a representative commodity and extension of the scope to other matrices from the same group requires demonstrating that the method performance for the new matrix is not affected. In this study soya beans are selected as representative matrix for a high oil content commodity in line with the classification for vegetables, fruits and cereals proposed by SANCO.

Accurate determination of pesticides in fatty food matrices, such as soya beans, remains a challenge due to the complexity of the matrix and low mass fraction levels to be dealt with. Chromatographic techniques, such as gas chromatography (GC) or liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS), are nowadays commonly applied techniques for routine pesticide determination as they provide satisfactory selectivity and sensitivity [5–7]. LC is preferred over GC in the case of either thermolabile or newly developed pesticides, usually having high polarity and low volatility [8–10]. Regardless of the chromatographic technique chosen, a clean-up step is usually required after extraction, particularly when dealing with complex matrices in order to eliminate matrix effects caused by co-extracted compounds. The matrix effects observed are generally suppression or enhancement of the detector response due to co-eluting matrix constituents [11], interferences in the determination of the analyte of interest, or even shortening of the lifetime of the analytical equipment. These effects can seriously affect quantification at trace levels and concern both, GC and LC, coupled to MS detection. In addition to the removal of matrix constituents by specific dedicated clean-up protocols and chromatographic separations, other strategies could be simultaneously applied in order to reduce matrix effects to an acceptable level. The most applied approaches are external matrix matched calibration, calibration by standard addition, using analogue internal standard or stable isotopically labelled standards [12,13]. The latter appears to be the most robust and efficient approach [11]. Isotope dilution mass spectrometry (IDMS) is based on the addition of isotopically labelled analogues of the analytes to the sample, which are considered the ideal internal standards as they are expected to show identical behaviour to the target analyte in sample pretreatment (provided isotopic equilibrium can be reached before extraction), chromatographic separation as well as in the compound ionization.

The predominant sample treatment applied for pesticide determination in fatty vegetables is based on liquid extraction with organic solvent, followed by clean-up with solid phase extraction (SPE), gel permeation chromatography (GPC) or low-temperature fat precipitation [14,6,15]. Other procedures include microwave-assisted extraction (MAE), supercritical fluid extraction (SFE), matrix solid phase dispersion (MSPD) or solid phase micro-extraction, although they have been applied with limited success [16]. Moreover the so called QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) has been increasingly used in the last years due to its simplicity, minimum number of sample processing steps and effectiveness for cleaning-up complex samples [17–19]. The technique was originally introduced for the extraction of pesticides from food matrices and has also been applied for other compounds, such as mycotoxins, plant toxins and veterinary drugs [20–22]. It comprises first an extraction of pesticides from the matrix with acetonitrile, then a salting out step which involves a phase separation of aqueous and organic phases, and a subsequent clean-up of the extract by dispersive SPE (d-SPE).

This study includes the development and validation of a method for pesticide determination in soya beans by LC-MS/MS, with the primary focus to reach a suitable level of measurement uncertainty. Rather than an extensive list, a restricted range of pesticides was targeted including methomyl, imidacloprid, carbendazim, malaoxon, thiodicarb, malathion, iprodione, tebuconazole, diazinon and chlorpyrifos. The pesticides were chosen by paying attention to a number of aspects such as the physicochemical properties, representation of different pesticide families, the analytical challenges related to their analysis, their frequency of use or the commercial availability of the isotopically labelled analogous. In order to improve the cleanliness of the sample extracts the QuEChERS method was adapted for the selected pesticides and matrix. In addition, accurate quantification of all considered compounds was achieved due to the application of IDMS and calibration with pure substance solutions containing both, the isotopically labelled and native compounds.

2. Experimental

2.1. Reagents and materials

Calibration substances of methomyl (99.5%), imidacloprid (99.0%), malaoxon (98.0%), thiodicarb (99.0%) were obtained from Dr. Ehrenstorfer (Augsburg, Germany). Carbendazim (98.0%), malathion (97.2%), iprodione (99.3%), tebuconazole (99.6%), diazinon (98.3%) and chlorpyrifos (99.5%) were purchased from Pestanal©, Sigma-Aldrich Co St. Louis (Mo, USA).

Isotopically labelled compounds used as internal standards were purchased either as neat crystals when available, or in solution. Carbendazim-d_4 (benzimidazole 4,5,6,7-d_4, 99.3 at% D) and iprodione-d_5 (3,5-dichlorophenyl-2,4,6-d_3; hydantoin-5,5-d_2, 98 at% D) were obtained from CDN Isotopes, Quebec, Canada. Methomyl-$^{13}C_2{}^{15}N$ (acetohydroxamate-$^{13}C_2$; ^{15}N, 99.0 at% C and 98 at% N, 100 ng μL^{-1} in acetone) was purchased from Cambridge Isotopes laboratories, Inc., Andover, MA, USA. Malation-d_{10} (diethyl D10, liquid, 99.0%), diazinon-d_{10} (diethyl D10, liquid, 97.5%), chlorpyrifos-d_{10} (diethyl D10, neat crystal, 98.0%), tebuconazole-d_6 (ethylene D4, methylene D2, 100 ng μL^{-1} in acetone, 96.5%) were obtained from Dr. Ehrenstorfer (Augsburg, Germany).

Acetonitrile (MeCN) and methanol (MeOH) of LC-MS grade were purchased from Merck KGaA (Darmstadt, Germany), and deionised water ($>18\ M\Omega\ cm$) was obtained from a Milli-Q system (Millipore, Brussels, Belgium). Formic acid (98%), ammonium formate and acetate ($\geq 99\%$) as well as NaCl were from Fluka, Sigma-Aldrich NV/SA (Bornem, Belgium), acetic acid ($\geq 99\%$) and $MgSO_4$ were from Sigma-Aldrich NV/SA (Bornem, Belgium) and primary–secondary amine (PSA), C18 as well as LC-Florisil® sorbents (100 g, bulk) were provided by Supelco (Bellefonte, PA, USA).

2.2. Preparation of calibration solutions

Individual stock solutions of both native and isotopically labelled pesticides were prepared gravimetrically at the mass fraction of approximately 1 mg g^{-1} in MeCN, with the exception of carbendazim, a compound much less soluble, which was prepared at the mass fraction of approximately 0.1 mg g^{-1} in MeOH. The solutions were kept in capped amber vials at $-20\ ^{\circ}C$ until use. Two intermediate solutions, one containing a mixture for the native and another a mixture of the isotopically labelled compounds, were prepared at mass fraction levels in the range

of approximately 4–20 mg kg^{-1} depending on the pesticide. The dilution of the stock solutions was performed gravimetrically in MeCN containing 0.1% of formic acid. The intermediate calibration solutions were once again diluted gravimetrically with H_2O and MeCN (final proportion of 50:50, v/v) to obtain the working calibration solutions used for quantification purposes.

2.3. Sample treatment procedure

Soya beans were purchased of-the-shelf from a local grocery specialised in organic products. For the study a portion of the material was milled and fortified with selected pesticides. In summary, the milling consisted on a previous freezing approximately 900 g of soya beans with liquid N_2 and milling them into a Palla vibrating mill (KHD Humboldt Wedag, Köln, Germany) during 8 min at −180 °C. The resulting powder was distributed in glass bottles and stored at −20 °C. The top particle size of the soya powder as determined by laser diffraction was 500 μm.

The extraction procedure applied introduces variations (mostly in the clean-up step) to the so called modified QuEChERS method, optimized by Mastovska et al. for the extraction of pesticides from cereal grains, and applied also in fatty matrices such as flaxseeds, peanuts and doughs [23,24]. After homogenization of the sample, a 3 g portion was weighed into a 50 mL PTFE centrifuge tube. Fifteen mg of the IS mixture solution (IS intermediate stock solution) was subsequently added. The tube was vigorously shaken, first by means of a vortex during 2 min and then by using a wrist action shaker (IKA Labortechnik, Staufen, Germany) at 300 rpm (maximum speed) during 10 min. The bottle was allowed to stand open at room temperature and protected from light during 1.5 h for equilibration. Subsequently, 10 mL of deionised water and 15 mL of MeCN were added. The tube was then vigorously shaken manually during 1 min to fully disperse the sample in the solvent and placed in a wrist action shaker for 1 h at 300 rpm to allow swelling of the matrix and extraction of the analytes. 4 g of $MgSO_4$ and 1 g of NaCl were added to each tube, which was vigorously shaken by hand during 1 min avoiding powder agglomeration. The tubes were again shaken for 15 min, to obtain a homogeneous mixture, and centrifuged during 5 min at 4000 rpm. After centrifugation, the upper organic phase showed a gelatinous consistency and therefore a quick shaking by hand of the tube and a second centrifugation step was necessary. The clean-up procedure consisted of a d-SPE as follows: 1 mL of the MeCN extract was transferred to a 4 mL vial containing 150 mg of $MgSO_4$, 150 mg PSA, 50 mg C_{18} and 150 mg of Florisil, which was vortexed for 1 min and centrifuged at 4000 rpm for 5 min. Finally, 300 μL was placed into an autosampler vial and diluted with 300 μL of H_2O containing 0.1% (v/v) formic acid, and injected into the LC-MS/MS.

An additional pre-concentration of the extract was necessary for the quantification of iprodione at low mass fraction levels (10 fold times). For this purpose, a higher volume of the MeCN extract (3 mL) was cleaned by transferring it to a 4 mL vial containing 150 mg of $MgSO_4$, 300 mg PSA, 100 mg C_{18} and 300 mg of Florisil, and vortexed for 1 min. 2 mL of the extract was placed in a clean vial and evaporated under a gentle stream of nitrogen at 40 °C nearly to dryness. 100 μL of MeCN and 100 μL H_2O containing 0.1% (v/v) respectively were added and the vial was vortexed for 1 min prior to injection into the LC.

2.4. LC-MS/MS analysis

The chromatographic analyses were performed with an Agilent HP-1200 series rapid resolution HPLC (Agilent Technologies, Waldbronn, Germany) equipped with a binary pump, online degasser, thermostated autosampler, and thermostated column compartment. Separations were carried out using a Kinetex-C_{18} of 150 mm × 4.6 mm id, 2.6 μm particle size analytical column with an in-line filter of 0.5 μm porosity × 0.1 mm id (both from Phenomenex, Bester B.V., Amstelveen, The Netherlands). The mobile phases, A and B, were Milli-Q-water and methanol respectively. Under final conditions, both phases were modified with 2.5 mM ammonium acetate. The optimized gradient programme started with 30% of B, followed by a linear gradient up to 80% B in 4.5 min, which was then kept constant for 3 min. Subsequently, the mobile phase was increased linearly up to 95% of B in 1.5 min and held for 7.5 min. Initial conditions were reached in 0.5 min with 7 additional min for equilibration time before the next injection. The mobile phase flow rate was set at 0.4 mL min^{-1} and the temperature of the column was kept at 30 °C. The injection volume for calibration solutions and sample extracts was 10 μL.

The HPLC was coupled to a triple quadrupole mass spectrometer 3200 QTrap (Applied Biosystems, Inc., Foster City, CA, USA) operated in the triple quadrupole configuration, with Q1 and Q2 set to unit resolution. The instrument control and the data processing were done by software Analyst 1.5 (Applied Biosystems). Pesticides were recorded in the multiple reaction monitoring (MRM) mode using scheduled windows, with two transitions per compound, for both identification and quantification purposes. $[M+H^+]$ served as precursor ion in all cases, and the most intense transition was used to quantify the response of each species in standards and sample extracts. To optimize the MS/MS conditions for each analyte, experiments were carried out by direct infusion of individual standards solutions of 1 μg mL^{-1} of each pesticide (except for iprodione, which was infused at 5 μg mL^{-1}) in acetonitrile–ultrapure water 1:1 v/v, containing 0.1% formic acid. Infusion was carried out using a syringe pump (Harvard Apparatus, Kent, UK) at a flow rate of 10 μL min^{-1}. Table 1 shows the optimized compound-specific mass parametric settings.

Instrument specific parameters were scheduled MRMs with detection window of 100 s, a target time of 0.5 s, which allowed the acquisition of at least 15 data points per peak, and a pause between mass ranges of 5 ms. The electrospray ion source (Turbolonspray) was operated in the positive mode. The interface heater was set to 120 °C, the probe temperature at 600 °C and a capillary voltage of 3 kV was applied. Nitrogen was used as curtain and nebulizer gas (GS1 and GS2) at 20, 40 and 50 psi, respectively, as well as collision gas (CAD) at 9 psi.

2.5. Identification and quantification

Identification and confirmation of pesticides were based on the criteria specified in Commission Decision 2002/657/EC [3]:

(a) The retention time of the analyte in the extract should correspond to that of the calibration standard with a tolerance of ± 0.2 min.
(b) The presence of three identification points (precursor ion and two fragments, see Table 1).
(c) The relative ion intensities had to comply with the permitted tolerances (± 30%).

Quantification was based on peak area and was performed using the internal standard (IS). For each IS/analyte pair, the relative response factor was determined with calibration solutions containing isotopically labelled and native compounds. As IS, eight isotopically labelled pesticides were used. For thiodicarb and malaoxon, with no isotopically labelled congener available, methomyl-$^{13}C_2{}^{15}N$ and malathion-D10 were used respectively, as they are structurally close related to each other.

2.6. Method validation

The validation of the method was performed in accordance with ISO/IEC 17025 [2] and taking into consideration DG SANCO

Table 1
MRM transitions and compound-related MS parameters.

Pesticide	RT (min)	Ion transition (*m/z*)	Declustering potential (V)	Entrance potential (V)	Collision energy (V)	Collision cell entrance potential (V)	Collision cell exit potential (V)	Ion ratio (%RSD)
Methomyl-$^{13}C_2{}^{15}N$	9.1	**166.1 > 91.1**	16.1	2.4	8.8	10.6	2.5	
		166.1 > 108.9	16.1	2.4	8.8	12.0	2.7	
Methomyl	9.1	**163.0 > 88.0**	21.4	2.3	9.8	11.8	2.4	55.1 (1.8)
		163.0 > 106.0	21.4	2.3	9.8	12.4	2.3	
Imidacloprid-D4	10	**260.2 > 213.1**	27.4	4.1	15.3	20.9	2.9	
		260.2 > 179.2	27.4	4.1	15.3	22.2	2.3	
Imidacloprid	10	**256.1 > 209.0**	34.0	3.4	13.1	20.4	3.8	86.6 (6.1)
		256.1 > 175.1	34.0	3.4	13.1	23.5	2.3	
Carbendazim-D4	11	**196.1 > 164.2**	31.2	4.2	11.1	23.8	5.1	
		196.1 > 136.1	31.2	4.2	11.1	40.6	2.1	
Carbendazim	11	**192.1 > 160.0**	38.7	4.7	10.6	24.8	2.5	19.0 (6.6)
		192.1 > 132.0	38.7	4.7	10.6	41.6	2.1	
Malaoxon	11.5	**315.1 > 127.0**	33.3	3.7	13.9	15.4	2.4	130.2 (2.4)
		315.1 > 99.01	33.3	3.7	13.9	35.0	2.4	
Thiodicarb	12	**355.0 > 88.1**	24.2	4.0	15.0	23.5	2.3	50.4 (3.0)
		355.0 > 108.0	24.2	4.0	15.0	19.6	2.5	
Malathion-D10	13.9	**341.2 > 132.2**	19.5	4.0	13.8	16.9	3.0	
		341.2 > 290.2	19.5	4.0	13.8	9.7	4.7	
Malathion	13.9	**331.0 > 127.2**	26.1	3.7	13.9	16.4	2.5	41.7 (5.0)
		331.0 > 285.2	26.1	3.7	13.9	9.6	4.0	
Irpodione-D5	14.8	**335.1 > 250.1**	33.7	4.2	18.9	19.3	7.8	
		335.1 > 293.0	33.7	4.2	18.9	14.1	9.3	
Irpodione	14.8	**330.1 > 245.1**	35.5	4.2	12.9	19.6	4.6	6.8 (12.0)
		330.1 > 174.0	35.5	4.2	12.9	43.9	3.0	
Tebuconazole-D6	15.2	**314.3 > 72.1**	50.9	3.7	14.3	44.7	2.5	
		314.3 > 125.0	50.9	3.7	14.3	48.3	2.8	
Tebuconazole	15.2	**308.2 > 70.0**	44.8	4.1	15.9	43.1	2.2	14.1 (10.0)
		308.2 > 125.0	44.8	4.1	15.9	51.4	2.0	
Diazinon-D10	15.5	**315.3 > 170.3**	32.7	5.1	14.1	32.2	2.2	
		315.3 > 154.3	32.7	5.1	14.1	27.2	2.3	
Diazinon	15.5	**305.1 > 169.1**	47.7	4.7	15.0	27.0	2.4	61.6 (6.4)
		305.1 > 153.1	47.7	4.7	15.0	26.7	2.3	
Chlorpyrifos-D10	17.1	**362.0 > 99.0**	35.6	4.6	17.7	46.1	2.4	
		362.0 > 201.0	35.6	4.6	17.7	23.7	2.9	
Chlorpyrifos	17.1	**351.9 > 199.9**	38.4	3.8	15.1	29.0	3.0	148.5 (3.2)
		351.9 > 97.0	33.4	3.8	15.1	53.5	2.2	

Transition in bold: MRM used for quantification.

guidelines [3] in relation to selectivity, linearity, recovery, repeatability, within laboratory reproducibility, detection and quantification limits (LOD and LOQ), stability of the extracts, robustness and measurement uncertainty. Experiments were carried out on 5 days and recovery studies were carried out at three different mass fraction levels.

The selectivity of the method was assessed by analysing blank soya samples and checking for the absence of interfering peaks in the retention time segment of each target analyte. Analytes were distinguished from possible co-eluting matrix interferences by comparing the relative intensity of the ratio between quantifying and qualifying MRM transition obtained in the sample to that corresponding to pure calibration standards (see Section 2.5).

On each measurement day, calibration was performed by injecting the set of calibration solutions in triplicate, and in a random order. Calibration curves were constructed by plotting the peak area ratio (native/isotopically labelled) versus the mass fraction ratios (native/isotopically labelled), obtained from standard solutions at five different target mass fraction levels for each pesticide in solvent (H_2O:MeCN 50:50, v/v, containing 0.1% formic acid), in the range 0.03–1.27 mg kg^{-1} for methomyl, thiodicarb and tebuconazole, 0.012–0.064 mg kg^{-1} for imidacloprid and chlorpyrifos, 0.006–0.254 mg kg^{-1} for carbendazim, and 0.001–0.025 mg kg^{-1} for malathion, malaoxon, iprodione and diazinon. These values, which were selected taking into account both the method sensitivity and different MRL for each pesticide, correspond to pesticide mass fraction levels of 0.025–1 mg kg^{-1} for methomyl, thiodicarb and tebuconazole; 0.013–0.5 mg kg^{-1} for imidacloprid and chlorpyrifos; 0.05–2 mg kg^{-1} for carbendazim; 0.005–0.2 mg kg^{-1} for malathion, malaoxon, iprodione and diazinon in the final extracts, after extraction of 3 g sample with 15 mL MeCN (11.8 g) and subsequently diluted 50:50 (v/v) with H_2O, prior to injection into the LC-MS/MS. The linearity of each calibration curve within the selected working range was firstly evaluated by visual inspection of the plotted data (area ratio and mass fraction ratio of analytes and isotopically labelled standards), and then by the residual plots and calculation of the correlation coefficient (r).

In order to assess the LOD and LOQ of the method, on each of the 5 days of the validation three matrix blanks were spiked at a level corresponding to the lowest point of the calibration curve for each compound. The samples were processed as described above and injected into the LC-MS/MS in triplicate. The LOD and LOQ were estimated as 3 and 10 times respectively the standard deviation of the signal expressed in mass fraction units. In addition, the LOD and LOQ values from the calibration data were estimated and compared with those previously obtained. According to SANCO guidelines [3], lowest spike mass fraction levels used in the recovery experiments were considered as the validated LOQs in this study.

For the determination of the method repeatability and within-laboratory intermediate precision, five blank samples were spiked at a mass fraction level corresponding to the middle point of the calibration curve for each compound (0.1 mg kg^{-1} for methomyl, thiodicarb and tebuconazole; 0.05 mg kg^{-1} for imidacloprid, chlorpyrifos and carbendazim; 0.02 mg kg^{-1} for malathion,

malaoxon, iprodione and diazinon), processed and injected in triplicate. The same procedure was repeated on four additional days. From the results obtained, repeatability and within-laboratory intermediate precision were calculated using the single-factor analysis of variance (ANOVA) as described by Van der Veen et al. [25].

Due to the lack of an appropriate CRM, the recovery was assessed by spiking experiments at three different mass fraction levels on five samples each. The recovery values were estimated by comparing both the measured and the theoretical mass fractions. In the case of related compounds (thiodicarb-methomyl and malathion-malaoxon [1]), the sum of the spiked mass fractions for each compound was considered as the theoretical mass fraction, and the measured mass fraction was the sum of the measured mass fractions obtained for each related compound.

The investigation of method robustness focused on potentially critical factors identified during the method development and affecting the sample preparation procedure. The solvents used for extraction and shaking times are optimized elsewhere [23,24]. Conservative values were selected for this method and therefore small variations of these parameters are not expected to influence the results. Instead the clean-up of the sample treatment procedure was the focus of this investigation as the step requires a previous weighing and mixing of C_{18}, Florisil and PSA into a vial. A fractional factorial experimental design (2^{3-1} plus one central point in triplicate) was applied, where the amount of the three sorbent substances was varied $\pm 5\%$ (mass) of the nominal value (50 mg C18, 150 mg Florisil and 150 mg PSA). For each replicate analysis, a sample (3 g) of soya was spiked with the pesticides at mass fraction level similar to those used for precision studies and extracted according to the above mentioned procedure. The homogenized extract was split in order to perform the clean-up corresponding to each of the points of the experimental design. The resulting extracts were injected in triplicate in a random sequence. Design of the experiments and evaluation of the data were done using Statgraphics 5.0. [26].

The stability of the extracts was assessed by injecting spiked samples ($n=5$) at the day of preparation, and then reanalysed after 2 weeks. During that period, the extracts were kept in closed (capped) vials and stored at $+4\ ^{\circ}C$ in darkness.

3. Results and discussion

3.1. Optimization of LC-MS/MS conditions

Once the MS parameters were optimized for each pesticide (see Section 2.4), chromatographic conditions were investigated in order to achieve separation of all analytes and to maximize both sensitivity and precision of the analytical method. These studies were carried out by injecting standard solutions into the system. Different C_{18} analytical columns were tested using different aqueous-organic mobile phase gradients, with either MeCN or MeOH. All eluents contained 0.1% (v/v) of formic acid and were evaluated at different flow rates. In addition, columns with small particle size ($<2\ \mu m$) were checked as an attempt to improve the separation efficiency [27]. However in the latter case the capability for data acquisition of the detector was not sufficient for a proper definition of the chromatographic peaks. Since poorly defined peaks could affect negatively the reproducibility of the method, small particle size columns were not considered for further experiments. A better response and peak shape were obtained when comparing aqueous-MeOH versus aqueous-MeCN as mobile phases. Good separation of all the compounds was also achieved with the latter mobile phase, with the exception of diazinon and tebuconazole, for which complete separation in a reasonable time was only possible using a Kinetex C_{18}, 2.6 μm, $150 \times 4.6\ mm^2$ (Phenomenex) column. The overlapping of the chromatographic signals of these two compounds is per se not a problem for their identification and quantification since they can be easily distinguished in the MS. However, a complete chromatographic separation of these two compounds was favoured to avoid any potential ion suppression or any other ionization effect. Therefore the Kinetex column and a MeOH-aqueous gradient were selected for the following method optimization steps.

Flow rates were studied in the range 0.2–0.6 mL min^{-1} with the aim of minimizing the analysis time. Although the capacity of the turbo ionspray source allowed operating flow rates up to 0.8–1.0 mL min^{-1}, attention was paid to this parameter, as some authors observed a particular decrease in sensitivity when increasing the flow rate, probably associated with dilution effects or instability of the spray [28]. For most of the target compounds an increase in the analytical signal was proportional to the decrease of the flow rate, influenced by higher sampling efficiency of the MS equipment at lower flow rates. However the sensitivity of the method was not effectively affected since the background signal behaves in a similar manner. In the case of chlorpyrifos and especially for iprodione and tebuconazole, a strong enhancement on the signal was observed at low flow rate. In those cases the increase was not proportional to the flow rate, possibly due to an easier ionization of the compounds or a higher stability of the spray at lower flow rates. This study was repeated after mobile phase composition and ionization source parameter optimization, and similarly to the rest of pesticides, a lower analytical signal was obtained for chlorpyrifos, iprodione and tebuconazole at increased flow rates. As a compromise the flow rate of the method was set to 0.4 mL min^{-1}.

The ionic strength and pH of the mobile phase are parameters that can affect both the ionization efficiency and the chromatographic separation [29,30]. Hence the influence of mobile phase additives, when added to both aqueous and organic solvents, was investigated with regards to the signal intensity for the selected pesticides. In a preliminary study the following commonly used modifiers in LC-MS, with acidic or saline character, were tested using the same H_2O-MeCN gradient (see Section 2.4): 5 mM ammonium formate, 0.1% formic acid, sum of these two, 5 mM ammonium acetate, 0.1% acetic acid, and sum of these two. The modification of pH in the mobile phase uniquely affected the retention time of carbendazim, a compound with alkaline property. The benzimidazole compound was eluted first when an acidic modifier was used and third (after imidacloprid and before malaoxon) when no modifier or either formate/acetate were employed, while it was eluted in the second place (after methomyl and before imidacloprid) when a combination of acidic and basic modifiers was added simultaneously to the mobile phase (Fig. 1).

Fig. 2 shows how the different tested modifiers of the mobile phase affect the analytical signal of each compound. As can be seen, the presence of any of the tested additives affected negatively the response for carbendazim, especially when acetic acid or ammonium acetate was employed. However this effect was not seen as crucial due to both, the high sensitivity obtained for carbendazim using ESI-MS and the higher allowed MRL when compared with other target pesticides. The addition of acetic acid, ammonium acetate or a mixture of both also produced a very strong decrease of the signal for the rest of analytes, with the exception of tebuconazole, with no effect, and iprodione, for which the addition of acetic acid produced a strong increase in the signal ($\sim$10 times). When formic acid was added to the mobile phases a high enhancement in the mass spectrometer response was obtained for iprodione ($\sim$7 times), for diazinon ($\sim$2 times) and for tebuconazole ($\sim$2 times), however, a decrease in the signal was observed for imidacloprid, malathion, malaoxon and thiodicarb.

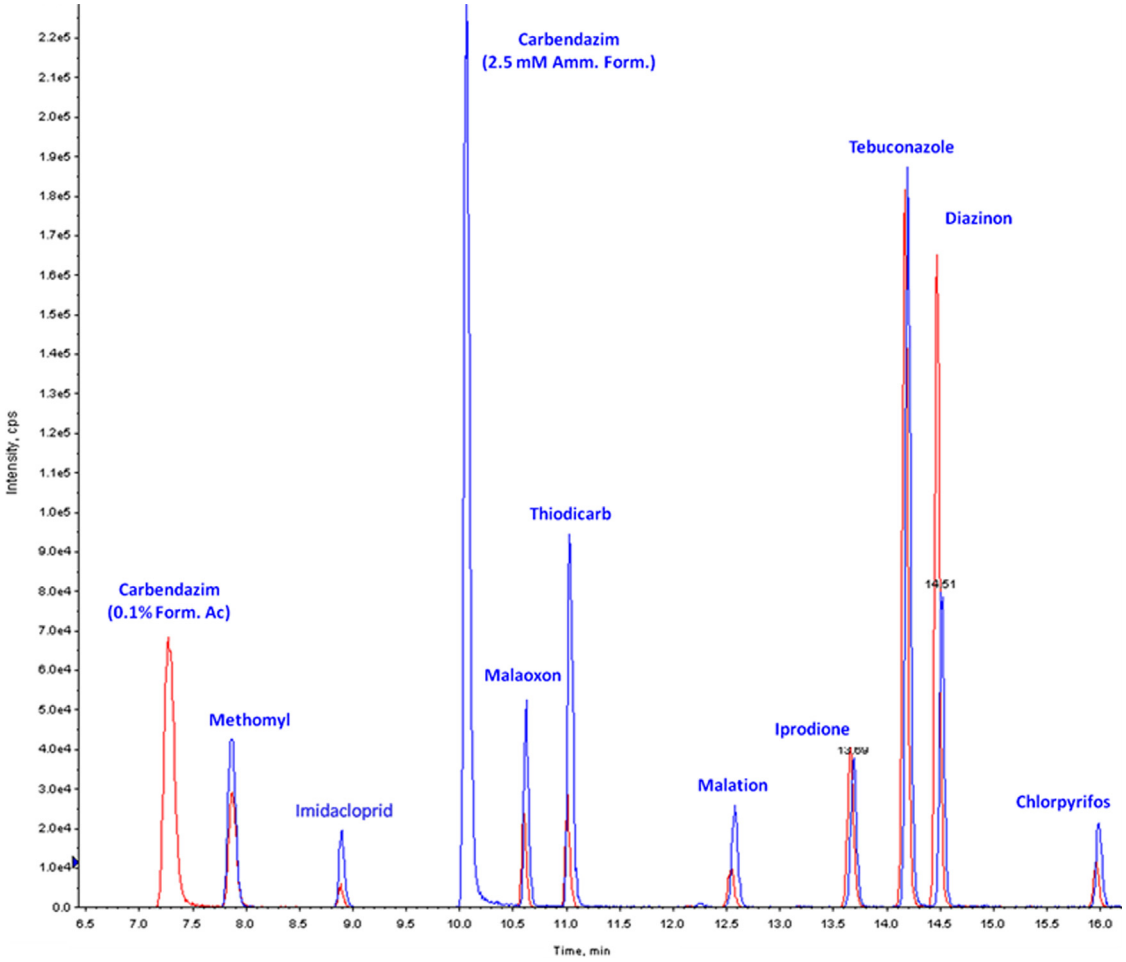

Fig. 1. Combined HPLC-MS chromatogram from a standard mixture of pesticides at 0.1 mg kg^{-1} for methomyl, thiodicarb and Tebuconazole; 0.05 mg kg^{-1} for Imidacloprid, Malaoxon, chlorpyrifos and carbendazim; 0.02 mg kg^{-1} for malathion, iprodione and diazinon MRL (carbendazim 0.2 × MRL and iprodione 2 × MRL). Red: 0.1% formic acid and blue: 2.5 mM ammonium formate. Other conditions as described in the experimental section. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

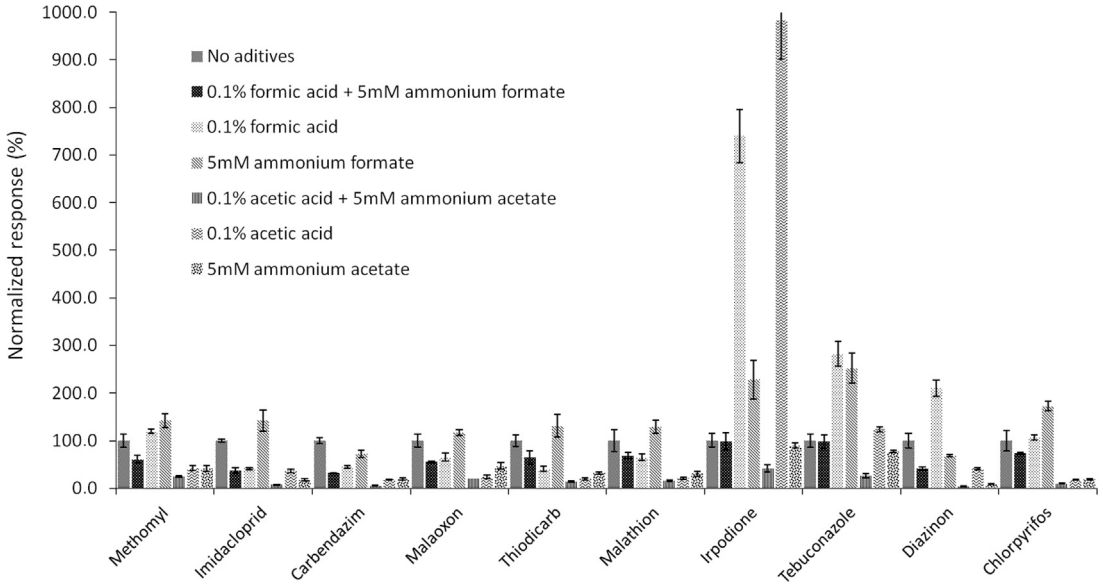

Fig. 2. Influence of modifiers added to the mobile phase (H_2O–MeOH) on the analytical response.

The addition of ammonium formate produced an increase in the signal of iprodione, tebuconazole and chlorpyrifos of about 2 fold, and slightly for imidacloprid, malathion and methomyl. According to these results it was decided to choose ammonium formate to continue with the optimization of the method as a compromise for the group of pesticides under study. In order to optimize the

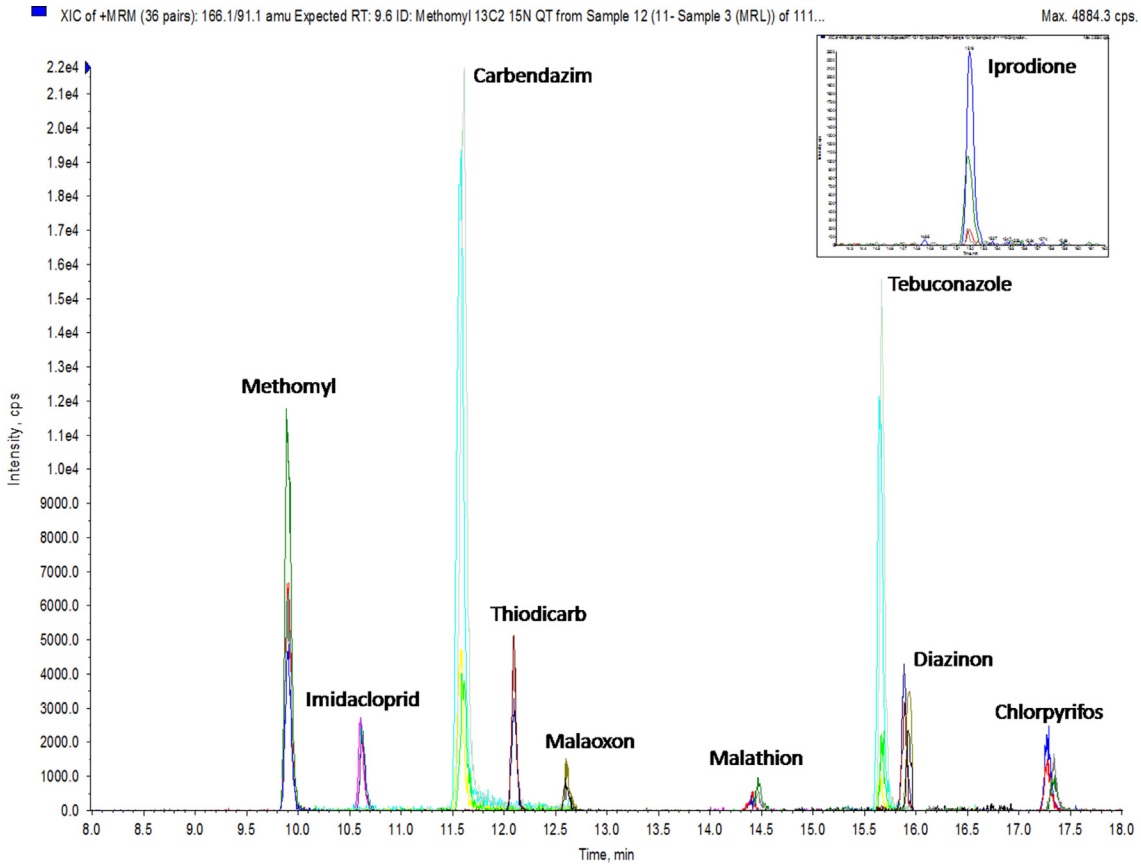

Fig. 3. Chromatogram of soya sample spiked with selected pesticides (0.1 mg kg^{-1} for methomyl, thiodicarb and tebuconazole; 0.05 mg kg^{-1} for imidacloprid, malaoxon, chlorpyrifos and carbendazim; 0.02 mg kg^{-1} for malathion, iprodione and diazinon). at MRL concentration levels.

Table 2
Working range and external calibration equation[a]. Results are shown for 1 day and are representative for all validation days ($n=5$).

Pesticide	MRL in soya beans [mg kg^{-1}]	Mass fraction linear range		Calibration equation[a]	$S_{R,c}$	r	Lack of fit P-value ($\alpha=0.05$)
		Matrix [mg kg^{-1}]	Standard [mg kg^{-1}]				
Methomyl	0.1	0.018–1	0.003–0.127	$Y=0.114+1.921X$	0.072	0.9991	0.9342
Imidacloprid	0.05	0.011–0.5	0.002–0.064	$Y=-0.02+0.749X$	0.133	0.9987	0.5300
Carbendazim	0.2	0.015–0.4	0.001–0.051	$Y=0.039+1.065X$	0.034	0.9999	0.9110
Malaoxon	0.02	0.009–0.2	0.001–0.025	$Y=0.499+2.701X$	0.155	0.9945	0.9951
Thiodicarb	0.1	0.014–1	0.003–0.127	$Y=0.015+0.816X$	0.040	0.9999	0.9987
Malathion	0.02	0.016–0.2	0.001–0.025	$Y=0.249+2.640X$	0.417	0.9991	0.8245
Iprodione	0.01	0.010–0.1	0.013–0.254	$Y=0.162+1.929X$	0.074	0.9998	0.8055
Tebuconazole	0.15	0.021–1	0.003–0.127	$Y=0.083+0.974X$	0.080	0.9998	0.8134
Diazinon	0.02	0.004–0.2	0.001–0.025	$Y=-0.016+0.817X$	0.073	0.9997	0.8824
Chlorpyrifos	0.05	0.018–0.5	0.002–0.064	$Y=0.057+0.437X$	0.125	0.9966	0.4994

$S_{R,c}$: regression standard deviation.
r: correlation coefficient.

[a] Obtained from triplicate injection of the standard solutions. Y: area ratio analyte/I.S.; X: concentration ratio analyte/I.S.

concentration of ammonium formate in the mobile phases, standard solutions of each pesticide were injected in triplicate using increasing concentrations of the modifier (0.1, 2.5, 5 and 10 mM). An optimum was found at 2.5 mM. Higher concentrations did not produce any significant improvement on the signal of any of the analytes, and on the other hand responses for iprodione and chlorpyrifos were slightly decreased.

Once the chromatographic method was set up, variables affecting the ESI source were studied and optimum values were selected (Section 2.4). Using these conditions, scheduled MRM was applied for quantification and confirmation transitions with detection windows of 100 s. Bearing in mind that peak widths were approximately 8–20 s, a target scan time of 0.5 s was selected to achieve adequate peak definition. With these settings quantitative results and compound confirmation were reproducible.

3.2. Extraction and clean up procedure

As already indicated, sample extraction and clean-up were carried out by a modified QuEChERS procedure [23,24]. To this method a number of adaptations were introduced. The sample intake was reduced from 5 to 3 g of sample to allow a complete swelling of the sample and proper mixing with the solvents. As an attempt to obtain cleaner extracts and minimize matrix effect,

different sorbent materials for the d-SPE were assessed. Florisil, was earlier selected by Ngyen et al. [31] for the cleaning of soybean oil extracts by d-SPE previous to the analysis of pesticides, and for the cleaning of extracts of other fatty vegetables by SPE [6]. It was now tested in combination with C_{18} and PSA, in order to obtain very clean extracts and minimize matrix effect. The specific properties and extraction capabilities of each sorbent material were applied simultaneously to the soya beans sample obtaining clear sample extracts, and decreasing the ion suppression for tebuconazole, diazinon and chlorpyrifos, when comparing the same procedure but using C18 and PSA as the only sorbents in the cleaning cocktail. The mix of sorbent materials and the procedure finally selected is specified in Section 2.3.

Potential matrix effects were tested as described by Matuszewski et al. [32], by comparing the analytical signal (peak area) obtained from a standard solution prepared in solvent (H_2O–MeCN 50:50, v/v) with those obtained for blank matrix extracts spiked with standard solution, both at the final mass fractions corresponding to the middle point of the external standard calibration. In general low or negligible matrix effects were observed. Some ionization suppression was detected for diazinon (16%). For thiodicarb, imidacloprid and carbendazim the ionization suppression was at a level of 10–12%. An ionization enhancement effect for iprodione (10%) was observed. For the rest of the compounds signal suppression below 10% was obtained (from 3 to 7%), and therefore matrix effect was considered not significant. According to these results the extracts clean-up process can be considered satisfactory.

The application of matrix matched calibrations for quantification resulted in recovery rates in many cases far worse than the ambitious accuracy targets a priori established for this method. Uniquely imidacloprid showed a recovery above 90%. Recoveries ranging between 60 and 75% were obtained for chlorpyrifos, tebuconazole and carbendazim, while for the rest of the compounds it ranged between 80 and 90%. In the case of thiodicarb and methomyl, very low (40%) and very high (135%) recoveries were obtained respectively. Considering the matrix effect discussed above (low or negligible), these results (recoveries $< 90\%$) suggested either incomplete extraction of analytes from the matrix or some analyte losses during the sample preparation (reasons for methomyl high recoveries are given later in this section).

As alternative approach to overcome the difficulties linked to the matrix matched calibration (no correction of sample preparation bias), the calibration was carried out with solvent-based solutions of the analytes, to which stable isotope-labelled analogues were added as internal standards. The application of isotope dilution mass spectrometry offers the advantage of compensation for both, signal suppression or enhancement caused by residual matrix compounds and the loss of analyte during sample processing.

In the case of thiodicarb and methomyl similar recoveries as for the matrix matched calibration were obtained (i.e. 40% for thiodicarb and 135% for methomyl). These results are in agreement with those found by other authors, describing the partial degradation of thiodicarb to methomyl during the sample preparation [8,33]. For confirmation purposes, a standard solution of thiodicarb and a soya sample spiked with thiodicarb, both at the MRL mass fraction level, were processed according to the sample preparation procedure (Section 2.3) and injected in the LC-MS system. This experiment showed that degradation of thiodicarb only occurs in the spiked soya and not in pure solvent. The effect is therefore influenced by the sample matrix and it is not provoked by the sample treatment procedure. During the analysis this degradation is however not necessarily a problem, since according to the residue definition [1], the results should be expressed as the sum of the two related compounds (thiodicarb and methomyl). Results for malathion and malaoxon were also expressed as sum of both as they are a related pair of compounds as well [1].

3.3. Method validation

3.3.1. Specificity and selectivity

The specificity of the method was evaluated by the analysis of 5 different blank soya bean samples. No interfering peaks from endogenous compounds were found at the retention times of the target analytes.

Pesticides were identified in the samples according to their retention time, and distinguished from any possible coeluting matrix interference by comparing the relative intensity of the ratio between quantification and qualifying MRM transition obtained pure calibration standards (Table 1), with that corresponding to sample. In all cases the MRM ratios in samples were well within the tolerance range for relative ion intensities as indicated in the current European guidelines [3]. No significant interferences from the soya bean matrix were detected. For illustration, Fig. 3 shows a chromatogram obtained for a spiked soya bean sample.

3.3.2. Linearity and working range

The working range is usually defined by the objective of the analysis. Since the purpose of the method is to analyse soya beans containing the selected pesticides close to the MRL [1], the working range was established accordingly. Calibration curves were obtained as described in Section 2.6. The statistical parameters calculated from least-square regression are presented in Table 2. The correlation coefficients (r) were higher than 0.99, and the P-values for the lack-of-fit test ($\alpha = 0.05$) higher than 0.05, which confirm the absence of curvature and the linearity of the analyte responses over the tested range. In the case of iprodione the curvature was visually observed at the highest mass fraction level during three out of the five validation days, indicating that this compound is only quantifiable in a narrower linear range. This effect could probably be caused by the saturation of the electrospray ionization [34] at high iprodione mass fraction. For this compound the working range was adapted accordingly.

3.3.3. Performance characteristics

LODs and LOQs of each analyte were evaluated by daily spiking three blank soya samples at the lowest level of the working range for each pesticide. The limits were theoretically estimated as $3S_0$ and $10S_0$ respectively, where S_0 corresponds to the standard deviation of the measured mass fraction at the lowest level of the calibration curve. LODs were confirmed experimentally, and the lowest spike mass fraction levels used in the recovery experiments were considered as validated LOQs in this study [3]. As shown in Table 3, LOQs for all pesticides were below the respective MRLs. The precision of the method, estimated through the repeatability and within-laboratory intermediate precision is

Table 3
LODs, LOQs and contributions to the uncertainty budget of the proposed method.

Pesticide	LOD [mg kg^{-1}]	Estimated LOQ [mg kg^{-1}]	Validated LOQ [mg kg^{-1}]	rep RSD (%)	ip RSD (%)	Truenes RSD (%)	U (%)
Methomyl	0.005	0.018	0.05	5.6	7.6	4.9	9.1
Thiodicarb	0.004	0.014	0.05				
Imidacloprid	0.003	0.011	0.025	5.1	2.9	5.7	7.4
Carbendazim	0.004	0.015	0.02	7.4	1.9	6.3	8.1
Malathion	0.005	0.016	0.01	5.9	8.7	5.6	10.4
Malaoxon	0.003	0.009	0.01				
Iprodione	0.003	0.010	0.01	7.2	1.8	7.2	9.0
Tebuconazole	0.006	0.021	0.05	3.9	2.2	3.3	4.5
Diazinon	0.001	0.004	0.01	5.4	1.3	5.0	6.3
Chlorpyrifos	0.005	0.018	0.025	7.6	1.9	5.2	6.9

Table 4
Mean recoveries (%) and RSD (%, $n=5$) obtained for the selected pesticides spiked at different levels in milled soya beans.

Pesticide	Spiked [mg kg^{-1}]	Recovery (%)	RSD (%)	Spiked [mg kg^{-1}]	Recovery (%)	RSD (%)	Spiked [mg kg^{-1}]	Recovery (%)	RSD (%)
Methomyl	0.05	103	4.2	0.10	94	1.4	0.20	101	3.2
Thiodicarb									
Imidacloprid	0.03	97	3.8	0.05	100	5.4	0.10	97	4.5
Carbendazim	0.02	101	4.4	0.04	98	4.5	0.08	97	5.3
Malathion	0.01	96	5.4	0.02	97	1.7	0.04	88	2.6
Malaoxon				0.02			0.04		
Iprodione	0.01	90	8.1	0.02	92	6.7	0.04	86	6.5
Tebuconazol	0.05	99	4.1	0.10	98	4.9	0.20	96	2.8
Diazinon	0.01	94	5.7	0.02	93	4.7	0.04	91	2.8
Chlorpyrifos	0.03	95	4.3	0.05	93	4.1	0.10	97	4.5

shown in Table 3. All repeatability and intermediate precision values expressed as RSD were below than 10%.

Table 4 summarizes the results obtained from the recovery studies carried out at three mass fraction levels of each individual pesticide. Values obtained for all pesticides were higher than 90%, with the exception of malathion plus malaoxon and iprodione at the highest mass fraction level, for which recoveries of 88% and 86% were obtained respectively, with RSD ranging from 1.4% to 8.1%. These results show the good accuracy of the proposed methodology, and therefore its suitability for the characterization of a candidate reference material.

3.3.4. Robustness

Robustness, defined as the capacity of an analytical method to remain unaffected by small but deliberate variations in method parameters, provides an indication of its reliability during normal use. As described in Section 2.6, the amount of three sorbent substances was varied $\pm 5\%$ of the nominal value (50 mg C_{18}, 150 mg Florisil and 150 mg PSA), and the effect of these variations were studied by a fractional factorial experimental design (2^{3-1} plus one central point) in triplicate. The lack-of-fit test showed that the models are adequate for the observed data at the 95% confidence level in all the cases ($P \geq 0.05$). For all the pesticides tested, the effects of the experimental factors were not significant ($P \geq 0.05$) in the selected experimental range, with the exception of chlorpyrifos, for which the amount of PSA seems to have a negative influence on the response ($P=0.035$). Thus we can conclude that the method is generally robust, although special care should be taken when weighing the PSA in order to avoid any influence in the recovery for chlorpyrifos.

3.3.5. Stability of extracts

The stability of the extracts was tested over a period of 2 weeks, and the extracts showed no signs of degradation. Between the injections, the extracts were kept at $+4\,^{\circ}C$ in the dark. It is however recommended to inject the extracts as soon as possible after preparation.

3.3.6. Measurement uncertainty

Measurement uncertainty estimation was based on the bottom-up approach together with validation data and the use of formal error propagation principles [35]. The main contributors to the total measurement uncertainty were the method repeatability, the intermediate precision, and the trueness (as estimated by the recovery experiment). The expanded uncertainty is calculated by combining the individual uncertainty contributions and applying a coverage factor of $k=2$ (level of confidence of approximately 95%), according to the formula

$$U = k \cdot \sqrt{\frac{RSD_{rep}^2}{n_1} + \frac{RSD_{ip}^2}{n_2} + \frac{RSD_{true}^2}{n_3}}$$

where U is combined expanded uncertainty; RSD_{rep} is relative standard deviation of repeatability; n_1 is number of independent samples used for analysis (25 for the repeatability study); RSD_{ip} is relative standard deviation of intermediate precision; n_2 is number of days (5 for the intermediate precision study); RSD_{true} is relative standard deviation of the estimation of trueness by the recovery experiment, i.e. identical to the RSD of the recovery study taking into account five measurements made for each mass fraction level; n_3 is number of mass fractions levels for recovery estimation (3 in this validation study). The uncertainty budget estimated for each analyte is presented in Table 3.

The guidelines for method validation and quality control procedures for pesticide analysis in food and feed [3] require an intermediate precision below 20%, and establish that a combined expanded uncertainty lower than 25% is expected for single-residue methods, particularly if isotopic labelled internal standards are used. As can be seen from Table 3, these requirements are well met in the method presented here, thus demonstrating the suitability for characterization of a candidate certified reference material for which more stringent requirements apply to make them fit for value assignment purposes.

4. Conclusions

In this study a simple and accurate LC-MS/MS method for the determination of 10 selected pesticide analytes in soya beans has been developed and validated. A clean-up step using Florisil in conjunction with C_{18} and PSA proved to be efficient in minimizing matrix effects, and the application of isotope dilution mass spectrometry allowed an accurate quantification. Full validation was carried out according to the ISO/IEC 17025 standard and to DG SANCO guidelines, showing excellent recoveries (86–103%) and precision ($U < 10.4\%$). The results confirm the suitability of the method to be applied for the assessment of homogeneity and stability as well as during characterization studies necessary in the development of a certified reference material.

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