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RESEARCH PAPER

Development of a method for the simultaneous determination of multi-class pesticides in earthworms by liquid chromatography coupled to tandem electrospray mass spectrometry

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Abstract

Agricultural intensification, and in particular the use of pesticides, leads over the years to a loss of biodiversity and a decline of ecosystem services in cultivated zones and agricultural landscapes. Among the animal communities involved in the functioning of agro-ecosystems, earthworms are ubiquitous and recognized as indicators of land uses and cultural practices. However, little data is available on the levels of pesticides in such organisms *in natura*, which would allow estimating their actual exposure and the potentially resulting impacts. Thus, the objective of this study was to develop a sensitive analytical methodology to detect and quantify 27 currently used pesticides in earthworms (*Allolobophora chlorotica*). A modified QuEChERS extraction was implemented on individual earthworms. This step was followed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The whole analytical method was validated on spiked earthworm blank samples, with regard to linearity (from 1 to 100 method limit of quantification, $r^2 > 0.95$), intra-day precision (relative standard deviation (RSD) < 15%), inter-day precision (RSD < 20%), recoveries (mainly in the range 70–110%), and limits of detection and of quantification (inferior to 5 ng/g for most of the pesticides). The developed method was successfully applied to determine the concentrations of pesticides in nine individuals collected *in natura*. Up to five of the selected pesticides have been detected in one individual.

Keywords Pesticide residues · Earthworm · LC-MS/MS · Ecotoxicology · Trace analysis · Bioaccumulation

Introduction

Pesticides are widely used in agricultural systems to increase crop yields and ensure crop protection against diseases and pests. During the application of pesticides, losses to the surroundings are unavoidable. This is due, among others, to wash off from treated foliage, spray drift, or seed treatments [1–3]. The soil is then the primary sink for these contaminants, which may affect the non-target soil living organisms and impact the ecosystems.

The use of sentinel species belonging to the soil macrofauna to assess soil contamination by pesticides and exposure of non-target wildlife could be relevant for ecotoxicological risk assessment of such contaminants. Among soil animals, earthworms represent a key component of soil ecosystem due to their biological, chemical, and physical actions in soils and as they represent the largest biomass [4–6]. They play a crucial role toward microbial activity and for soil functioning and fertility [7]. The pore network they create in soil modifies its structure, increasing aeration, drainage, and breakdown, and incorporation of organic matter thus enhancing its nutritional status [8]. They are also part of food webs, providing food for many vertebrates and invertebrate predators [9]. Earthworms are naturally in contact with the solid, aqueous, and gaseous soil phases and, as a result, are directly exposed to

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contaminants through soil ingestion or contact. This can induce a risk to their survival, behavior, productivity, and ability to perform their valuable functions in the soil ecosystem [10, 11]. Consequently, this can affect soil development and maintenance processes and further impact trophic webs and beneficial fauna populations in agro-ecosystems [12]. Moreover, since they constitute important prey for invertebrate predators such as gastropods and carabids as well as vertebrate species such as birds or terrestrial mammals, the levels of contaminants they contain could also be useful for addressing the risks associated with the biomagnification processes of pollutants.

Studies regarding pesticide quantification in soil macrofauna are relatively much less common than those for insects. In the literature, pesticide studies in earthworms generally deal with toxicity tests or assess bioavailability of chemicals to earthworms using laboratory experiments with different types of soils [6, 11, 13, 14]. Most of the papers on bioaccumulation assessment in earthworms focus on one or a few target compounds [15–18]. There is very little information on pesticide multi-residue analysis in earthworms whereas the organisms are in contact with pesticide mixtures in the natural environment. Pesticides may increase mortality rates of earthworms and have indirect adverse effects on reproduction, neurologic functions, and behavior especially at high concentrations [14]. It is important to notice that sublethal effects of pesticides have also been observed on earthworm growth and reproduction, underlying the importance to develop analytical methods that allow the quantification of low levels of contamination [11, 12].

Multi-residue analyses of currently used pesticides in wildlife are a challenging issue, due to the large variety of molecules used in the field having different physicochemical properties, the complexity of biological matrix, and the limited amount of biological material particularly in small species such as earthworms. Thus, analytical methods for quantifying pesticides in earthworms are scarce. Suitable sample preparation, including extraction and cleanup, has to be implemented to limit the presence of interfering matrix compounds during the analysis. Mainly two extraction techniques are employed: simple solid-liquid extraction by an organic solvent [17] which involves high quantities of solvent and is time-consuming, or ultrasound-assisted extraction [15, 16, 18] that reduces sample preparation time and required high solvent volumes. Purification is then necessary and is performed by solid-phase extraction (SPE) or dispersive SPE (dSPE). More recently, Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) extraction has been widely implemented for the analysis of a great variety of compounds in environmental matrices, including living organisms. The advantages of this method are a low solvent consumption together with short extraction and cleanup times [19]. The required selectivity and sensitivity are then achieved by the development and optimization of a separation by gaseous or liquid chromatography (GC or LC) coupled to a detection by mass spectrometry (MS) or tandem mass spectrometry (MS/MS).

Although it is needed for risk assessment and biological conservation purposes, to the best of our knowledge, there is not any existing available analytical multi-residue method for currently used pesticide analysis in earthworms. Thus, the present study describes the development and validation of a simple, rapid, robust, and sensitive analytical methodology based on a modified QuEChERS extraction followed by an accurate analysis using LC-MS/MS for the detection and quantification of 27 LC-amenable pesticides in earthworms. The developed method was successfully applied to earthworms collected *in natura* in a French area. The pesticides selected in this study were chosen on the basis of their relevance to the sampling site.

Materials and methods

Studied pesticides

The selected pesticides are herbicides, fungicides, and insecticides (Table 1) representative of agricultural practices (i.e., frequency and amount of pesticides, based on agricultural surveys) on the "Zone Atelier Plaine et Val de Sèvres" (ZAPVS), Villiers-en-Bois, France, where the earthworms have been collected.

Reagents and materials

Ultra-pure water has been produced by a MilliQ® gradient A10 water purification device equipped with an EDS-PAK cartridge and a 0.2-μm Millipak® 40 filter from Merck-Millipore (St. Quentin en Yvelines, France). Ammonium acetate and UPLC-MS grade acetonitrile (ACN) and methanol (MeOH) have been acquired from Biosolve Chimie (Dieuze, France). Dimethylsulfoxide (DMSO) (LC grade) and heptane (purity ≥ 90%) have been purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Acetic acid (purity ≥ 99%) has been obtained from Fluka.

Analytical standards (≥ 97.5% purity) of imidacloprid, pirimicarb, metconazole, epoxiconazole, thiamethoxam, thiacloprid, bifenthrin, deltamethrin, lambda-cyhalothrin, boscalid, fluoxastrobin, metrafenone, pendimethalin, napropamide, cloquintocet-mexyl, propiconazole, tau-fluvalinate, cycloxydim (purity 96.4%), and cypermethrin (purity 92.0%) have been purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). The internal standard (≥ 97.5% purity) imidacloprid-d4 has also been acquired from Sigma-Aldrich. The standard of pyroxsulam (purity 99.5%) has been purchased from Cluzeau Info Labo (Sainte Foy la Grande, France). The standards of metazachlor, dimethachlor, cyproconazole, prochloraz (purity 99%), diflufenican, and

Table 1 Mass spectrometric parameters for the multi-residue LC-MS/MS determination of 27 pesticides in earthworms (*HERB*, herbicide; *INSECT*, insecticide; *FUNG*, fungicide)

Compound	Class	Rt (min)	DP (V)	MRM1	CE (eV)	MRM 2	CE (eV)	Transition ratio
Thiamethoxam	INSECT	2.1	7	292 > 211	11	292 > 181	19	1.8
Imidacloprid	INSECT	2.6	11	256 > 175	15	256 > 209	17	1.2
Thiacloprid	INSECT	3.2	20	253 > 126	20	253 > 186	12	15.9
Pyroxsulam	HERB	3.9	16	435 > 195	26	435 > 124	46	7.5
Pirimicarb	INSECT	4.1	30	239 > 72	18	239 > 182	18	1.8
Metazachlor	HERB	4.4	15	278 > 134	22	278 > 210	10	1.4
Dimethachlor	HERB	4.4	20	256 > 224	15	256 > 148	25	1.9
Clomazone	HERB	4.5	23	240 > 125	18	240 > 89	46	18.9
Cyproconazole	FUNG	4.8	27	292 > 70	18	292 > 125	24	1.9
Boscalid	FUNG	5.0	30	343 > 307	20	343 > 140	20	2.8
Napropamide	HERB	5.2	21	272 > 129	16	272 > 171	18	0.8
Epoxiconazole	FUNG	5.2	30	330 > 121	23	330 > 123	20	5.4
Metconazole	FUNG	5.4	29	320 > 70	22	320 > 125	36	6.0
Fluoxastrobin	FUNG	5.5	27	459 > 427	18	459 > 188	36	2.9
Propiconazole	FUNG	5.5	37	342 > 159	34	342 > 69	22	2.5
Prochloraz	FUNG	5.7	27	376 > 308	11	376 > 70	23	2.4
Cycloxydim	HERB	5.8	23	326 > 280	16	326 > 180	22	0.8
Metrafenone	FUNG	5.8	19	409 > 209	14	409 > 227	16	1.6
Pyraclostrobin	FUNG	5.8	20	388 > 194	12	388 > 163	25	1.1
Diflufenican	HERB	5.8	30	395 > 266	28	395 > 246	32	3.8
Cloquintocet-mexyl	HERB	6.1	30	336 > 238	18	336 > 192	35	2.4
Pendimethalin	HERB	6.2	12	282 > 212	10	282 > 194	17	6,0
Lambda-Cyhalothrin	INSECT	6.5	6	467 > 225	12	467 > 141	46	3.3
Cypermethrin	INSECT	6.6	20	433 > 191	12	433 > 416	8	0.7
Deltamethrin	INSECT	6.7	10	523 > 281	14	523 > 506	8	0.9
Bifenthrin	INSECT	6.8	32	440 > 181	14	440 > 166	44	66.1
Tau-fluvalinate	INSECT	6.8	15	503 > 181	30	503 > 208	12	1.0

clomazone (97%) have been obtained from Riedel de Haën (Seelze, Germany). The standard of pyraclostrobin (99.9% purity) has been purchased from Fluka and phenacetin-ethoxy-1-^{13}C (98 atom % ^{13}C) used as injection standard has been acquired from Isotec (Ohio, USA).

Individual standard pesticide stock solutions (1000 mg/L) have been prepared in ACN and stored at − 20 °C. They remain stable over a period of at least 3 months. Working solutions have been obtained weekly by the appropriate mixture and dilution in ACN of the stock solutions. These solutions have been used for spiking blank earthworm samples to prepare the matrix-matched calibration standards.

The QuEChERS extract tubes have been supplied from Agilent Technologies (Massy, France). The citrate buffer (pH 5–5.5) contains 1 g of sodium citrate, 4 g of $MgSO_4$, 1 g of NaCl, and 0.5 g of disodium citrate sesquihydrate, whereas the acetate one (pH 4.8) is composed of 1.5 g sodium acetate and 6 g of $MgSO_4$. Several materials have been evaluated for dispersive solid-phase extraction (dSPE). Primary secondary amine (PSA which contains 150 mg of PSA and 900 mg of $MgSO_4$) and PSA/C_{18} (containing 900 mg of $MgSO_4$, 150 mg of PSA and 150 mg of C18) have been purchased from Macherey Nagel (Düren, Germany); Enhanced Matrix Removal Lipid (EMR lipid containing $MgSO_4$) has been obtained from Agilent Technologies.

Analysis by liquid chromatography tandem mass spectrometry

A liquid chromatographic system H-Class UPLC from Waters (St. Quentin en Yvelines, France) has been used for the separation of the target pesticides. The chromatographic column is a Kinetex Phenyl-Hexyl (100 × 2.1 mm; 2.6 μm) from Phenomenex (Le Pecq, France). The mobile phases are composed of (a) 0.01% acetic acid with 0.04 mmol/L ammonium acetate in ultra-pure water and (b) MeOH using a 0.4-mL/min flow rate. The oven temperature has been fixed at 60 °C and the injection volume has been set at 2 μL. The following

gradient has been applied: start at 5% (b), then rise to 90% in 7 min, then increase up to 100% (b) for 2 min. Next, the gradient has been decreased back to initial conditions and the column has been equilibrated for 3 min.

The chromatographic system is coupled to a Xevo TQ-S triple-quadrupole mass spectrometer (Waters) equipped with a StepWave ion guide. The multiple reaction monitoring (MRM) mode is performed for the MS/MS detection using an electrospray source in the positive ionization mode (ESI+). The optimum ESI-MS/MS settings have been obtained by direct infusion of each individual pesticide standard into the electrospray source. The declustering potential (DP) and collision energy (CE) leading to the highest signal intensities for each pesticide are displayed in Table 1. Two transitions have been followed for each analyte. The most intense MS/MS transition (MRM1) is used for the quantification and the second one (MRM2) for confirmation of each target compound. The retention time and the MRM ratios between both target ion transitions are also used as identification parameters. The optimized ionization source working parameters are as follows: capillary voltage 3200 V, desolvation temperature 450 °C, source offset 50 V, source temperature 150 °C, nitrogen desolvation and nebulizer gas flows of 900 and 150 L/h, respectively.

Earthworms

Earthworms of the species *Allolobophora chlorotica* have been collected from a fallow in Versailles, France (48°48′31″ N, 2°05′26″E), that had not been treated with pesticides for more than 20 years. These individuals have been used as blank matrix for method development. These pesticide-free samples have been first analyzed to confirm the absence of contamination with the targeted pesticide residues (see Electronic Supplementary Material (ESM) Fig. S1). These blank samples have been used for method development and validation and for matrix-matched calibration curves.

In the "Zone Atelier Plaine et Val de Sèvres" located in the West of France, the *A. chlorotica* individuals have been manually collected from the soil in different areas. This species of earthworms has been chosen because of its ubiquity in the different sampled areas, and more importantly because it lives close to the soil surface. It is thus potentially highly exposed to and impacted by pesticides [5].

Earthworms have been stored for 48 h in Petri dishes on a damp filter paper to void gut content and then frozen at − 80 °C until analysis.

The authors declare that the experiments on animals were conducted in accordance with local Ethical Committee laws and regulations.

QuEChERS extraction

The QuEChERS extraction combines two major steps: an initial salting-out liquid-liquid extraction followed by a dSPE cleanup. After homogenization, an aliquot (250-mg wet weight) of earthworm sample has been weighed in a 50-mL polypropylene centrifuge tube. For method development, the extract has been spiked with 125 μL of a mixture of the target pesticides (100 μg/L in ACN).

The earthworm extract has first been mixed with 6 mL of water and vortexed for 10 s. The water has first been added to the dry extract in order to weaken the interactions between the analytes and the matrix and to facilitate solvent accessibility in the matrix pores. Then, 5 mL of ACN (containing the internal standard imidacloprid-d4 at 100 μg/L) and 3 mL of heptane have been added to the tube which has been vortexed during 15 s. Next, the citrate buffering salt has been added and the tube has been straightaway vigorously manually shaken for 10 s, then vortexed for 1 min and finally shaken using a Geno/Grinder® (SPEX SamplePrep, Stanmore, UK) for 6 min at 1250 spm. Subsequently, the mixture has been centrifuged for 6 min at 5000 rpm, at room temperature. A 3-mL aliquot of the ACN extract layer has been transferred into the sample cleanup vial containing PSA/C18.

The extraction has been repeated once again with 5 mL of ACN. At the end of the extraction, 3 mL of the ACN layer has again been transferred to the dSPE tube. The cleanup vial has been subjected to vortexing (1 min) and centrifugation (5 min at 10000 rpm, at room temperature). Subsequently, 4 mL of the supernatant has been transferred to a 10-mL glass tube with 100 μL DMSO in order to prevent evaporation of the target analytes. Then, ACN has been evaporated under a gentle stream of nitrogen at room temperature. Finally, 50 μL of ^{13}C-phenacetin (200 ng/mL in MeOH) has been added to the residual DMSO. The remaining residue has been dissolved in 1 mL of MeOH/water (10:90, *v*/*v*) prior to LC-MS/MS analysis.

Method validation

Method performance and validation have been evaluated by assessing linearity, recovery, precision (as repeatability and within-lab reproducibility, RSD), matrix effect (ME), and method limits of detection (MLD) and of quantification (MLQ). Validation has been carried out using spiked blank extracts.

The specificity of the method has been determined using both chromatographic and mass criteria. Indeed, retention time, both characteristic MRM transitions, and MRM1/MRM2 ratio have been used for identification by comparing responses of analytical standards and samples, with a tolerance of 20%.

The MLD and MLQ have been estimated as the analyte concentration leading to a peak with a signal-to-noise (S/N) of 3 on the qualifier MRM2 transition, and a S/N of 10 on the quantifier MRM1 transition, respectively.

The linearity has been performed by preparing six different matrix-matched calibration curves with six levels of concentration in an analytical range between MLQ and 100 MLQ for each compound. Linearity of the calibration curves, obtained by plotting the peak area against the concentration of the corresponding standard, has been expressed by the square correlation coefficient (r^2).

Matrix effects (MEs) have been examined in parallel by comparing the signals of matrix-matched standards ($S_{\text{spiked after extraction}}$) with the signals of the standards in solvent (S_{solvent}). The MEs have been calculated at three levels of concentration (5 MLQ, 20 MLQ, 100 MLQ) with the following equation (Eq. 1). Values less than or greater than 0 correspond to matrix-induced signal suppression and enhancement, respectively.

$$\text{ME} = \left(\frac{S \text{ spiked after extraction}}{S \text{ solvent}} - 1\right) \times 100 \quad (1)$$

To calculate extraction recoveries of the whole process, blank samples have been spiked in triplicate at three concentrations (5 MLQ, 20 MLQ, 100 MLQ) and the signals of the samples spiked at the beginning of the protocol ($S_{\text{spiked before extraction}}$) have been compared to those of samples spiked after extraction ($S_{\text{spiked after extraction}}$) with the following equation (Eq. 2):

$$\text{Recovery } (\%) = \frac{S \text{ spiked before extraction}}{S \text{ spiked after extraction}} \times 100 \quad (2)$$

The intra-day precision of the method, expressed as the relative standard deviation RSD (%), has been determined by comparing the standard deviation of the recoveries of three replicate analyses of blank matrix spiked at three levels of concentration (5 MLQ, 20 MLQ, 100 MLQ) ran under the same conditions (same day with the same instrument and the same operator). Inter-day precision, as within-laboratory reproducibility, has been evaluated with the same levels but with analysis performed on 3 distinct days and by different operators in order to introduce some variations in the process. It is also expressed as the RSD of the series of measurements. Precision values below 20% have been targeted.

Quantification

The quantification has been done using matrix-matched calibration. In each batch, 6 calibration points ranging from MLQ to 100 MLQ have been performed as described in section "Method validation." The overall robustness of each analysis has been controlled through the checking of the peak area of the internal standard imidacloprid-d4. Moreover, ^{13}C phenacetin has been added in the vials just before injection to test possible variations in the injection volume. Finally, the instrumental performance has been controlled regularly by injecting quality control samples corresponding to mixtures of the analytical standards at 2 MLQs, 10 MLQs, and 50 MLQs.

Results and discussion

Chromatographic separation

Based on our previous works where we achieved the complete chromatographic separation of pesticides with a large range of log Kow, we have first tested the same column and aqueous phase and compared the results obtained with MeOH or ACN as organic phase [20]. The separation is more efficient with MeOH. Then, the column temperature and chromatographic gradient have been optimized in order to achieve the best separation, improve peak shapes, and avoid tailing peaks.

Optimization of sample preparation

Developing one single sample preparation procedure for all the targeted pesticides was challenging due to their different physicochemical properties. Moreover, the method has to be efficient enough to eliminate interfering compounds from the matrix and, at the same time, to minimize the loss of analytes and provide enough sensitivity to analyze the pesticides at trace levels. Indeed, matrix components remaining in the extract can cause ion suppression or enhancement of the analytes during the electrospray ionization. It is crucial to limit these interfering compounds that influence sensitivity and might lead to inaccurate results. Thus, our extraction protocol is based on the QuEChERS extraction in order to fulfill all these requirements. The extraction procedure is based on an initial salting-out extraction with a solvent (mainly ACN) followed by a dispersive solid-phase extraction (dSPE). The QuEChERS extraction has originally been developed to extract pesticides in fruits and vegetables [21]. Due to its ease of use, rapidity, effectiveness, and low solvent consumption, it is currently widely used to analyze a broad range of chemical residues in biotic matrices [19, 22, 23]. We have optimized each step of the QuEChERS extraction in order to obtain the best responses by LC-MS/MS for the majority of the target pesticides. Each experiment has been performed in triplicate. The results have been normalized: independently for each compound, the tested condition leading to the highest peak area has been attributed an answer of 100%; the other responses are expressed on this basis. The results were then compared in order to choose between the tested conditions.

Choice of the extraction buffer

To assist the transfer of the target compounds in the organic phase and to facilitate the phase separation between water and ACN, citrate or acetate salts are added in the first step of the extraction. The American AOAC Official Method 2007.01 and the European Standard Method EN 15662 involve the use of the acetate and citrate buffer, respectively. Both methods have been assessed using 10 mL of water and 10 mL of ACN for the extraction. The results are shown in Fig. 1. The signal intensities are higher with the citrate-based buffer for nearly all the compounds; therefore, this salt has been used for all subsequent development steps.

Ratio of the extraction solvents

In order to determine the ratio water/ACN leading to the more efficient extraction, different volumes have been evaluated. Three different volumes of water (3, 6, and 9 mL) have been tested with a constant volume of ACN (10 mL). The results are presented in Fig. 2. The extractions conducted with the ratios ACN/water 10/9 and 10/6 lead to the best results for 14 and 13 compounds, respectively, with equivalent extraction efficiencies for several pesticides. Thus, for both ratios, we have evaluated if we could enhance the extraction efficiency with 10 mL of ACN but using two successive extractions with 5 mL each (Fig. 2). The extraction efficiency is effectively enhanced, and the best results have been obtained with 6 mL of water and two successive extractions with 5 mL of ACN each.

Cleanup

In order to remove interfering matrix components while retaining the target pesticides, a cleanup step is generally necessary, and it is performed using dSPE sorbents. Different phases are available to selectively trap impurities and co-extractants from the matrix. In order to trap polar organic acids, pigments, sugars, and fatty acids, PSA is commonly used whereas for retaining non-polar compounds, C18 is generally employed. EMR lipid, a more recent phase selectively trapping lipidic compounds, has been evaluated too. We have tested these three phases and compared the results to those obtained without any purification step. The normalized signal areas of the analytes with and without the dSPE phases are presented in Fig. 3. The best results have been obtained with PSA/C18 as purification phase.

Matrix effects

The matrix could enhance or decrease ionization of pesticides in the mass spectrometer electrospray source. The MEs are reported in Table 2. The matrix components slightly suppress ionization of the MS signals for the majority of the target compounds, with a signal decrease lower than 50%. A higher suppression is observed for three compounds: the herbicide cycloxydim, and the insecticides bifenthrin and tau-fluvalinate. On the contrary, an increase is observed for the herbicide cloquintocet-mexyl, whatever the level of concentration. To compensate for matrix effects typically observed in living organisms, we have performed matrix-matched calibration and we have used the signal areas of the internal standard imidacloprid-d4 to control the extraction performance.

Method validation

Due to the efficiency of the sample preparation and the sensitivity of the LC-MS/MS detection, the developed method is very sensitive. Limits of detection are lower than 5 ng/g for 25 targeted compounds out of 27, and even lower than 1 ng/g for 21 compounds (Table 2). Limits of quantification are lower than 5 ng/g for 24 targeted pesticides and even lower than 1 ng/g for 17 pesticides. The pyrethroids lambda-cyhalothrin and cypermethrin exhibit the highest MLQs, with 50.8 and 32.7 ng/g, respectively. These data are in the same order of magnitude or

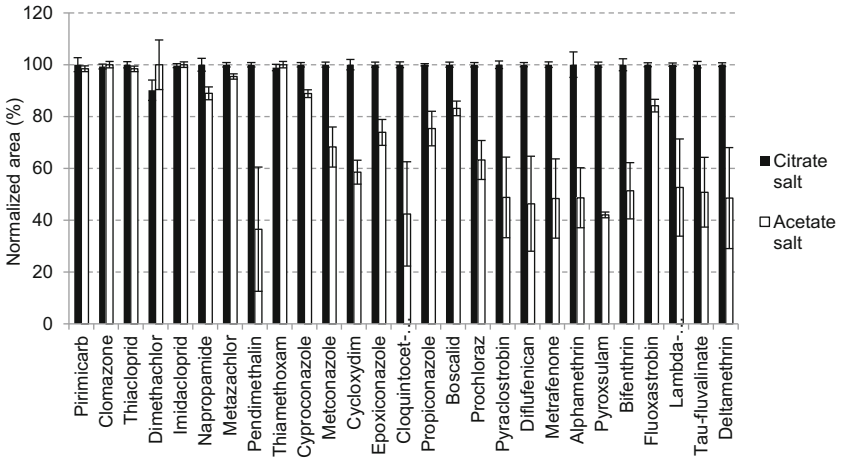

Fig. 1 Normalized responses obtained for earthworms extraction (10 mL water, 10 mL ACN, no dSPE cleanup) using both existing QuEChERS buffers. Targeted pesticides are spiked at 100 μg/L (replicate measurements, $n = 3$)

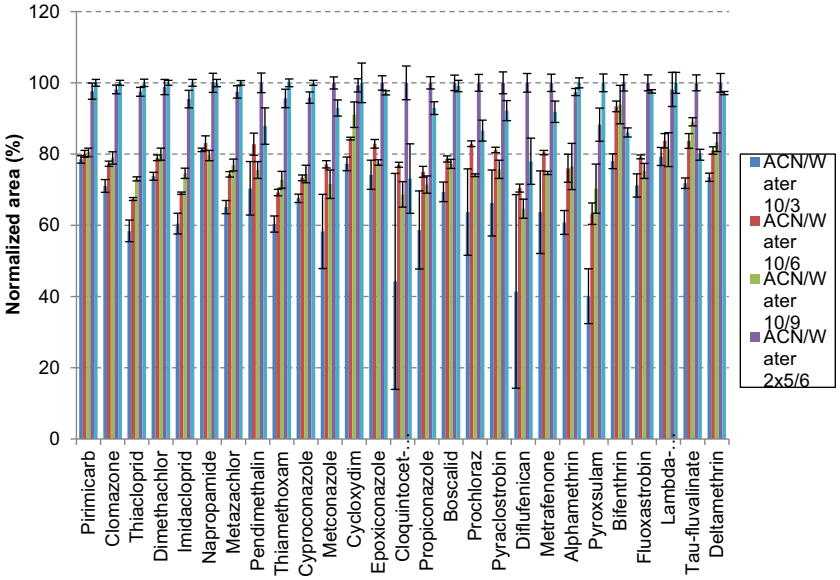

Fig. 2 Normalized responses obtained for earthworms extraction (citrate buffer, no dSPE cleanup) using different ratios acetonitrile/water. Targeted pesticides are spiked at 100 μg/L (replicate measurements, $n = 3$)

lower than in the few published studies where limits of detection or quantification of pesticides in earthworms are specified. Moreover, the limits achieved in our work have been obtained through a multi-residue method whereas published studies are mainly dealing with one or a few compounds. As example, Chang et al. (2016) worked with 1 g of earthworm and obtained limits of detection of 2 and 20 ng/g for bifenthrin and lambda-cyhalothrin, respectively [18]. In their study on alpha-cypermethrin, Diao et al. (2011) achieved a limit of detection of 50 ng/g [15].

The linearity of the method has been checked on 3 days, in the range MLQ–100 MLQ by considering the determination coefficient (r^2). The matrix-matched curves show good linearity (correlation coefficients greater than 0.95) for all target analytes over this range (Table 2).

Satisfactory recoveries of the whole protocol have been reached for all compounds, with most values comprised between 70 and 110%, except for pyroxsulam with around 30% (Table 2). This compound is essentially lost during the purification step where it stays bound to the PSA sorbent.

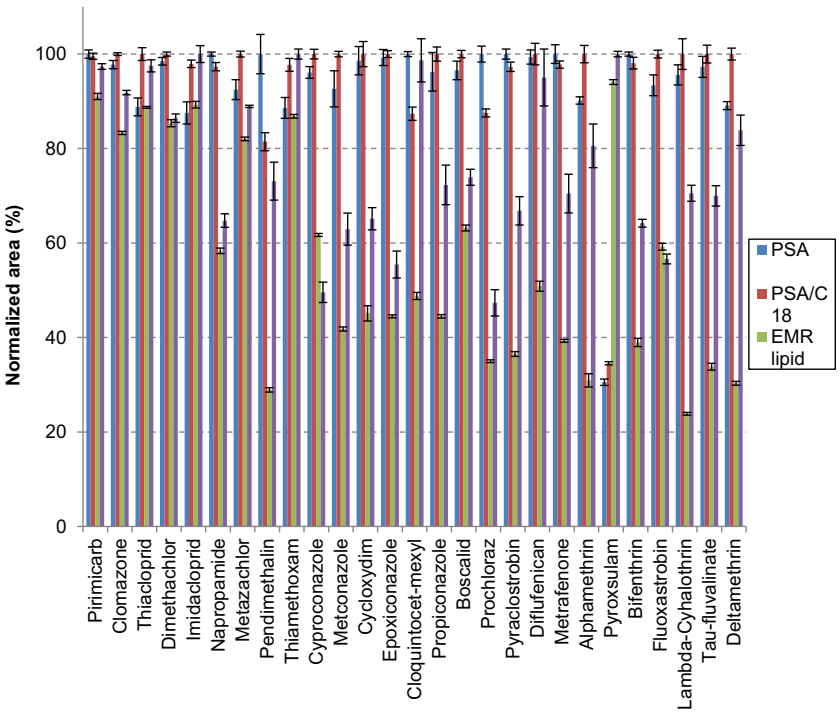

Fig. 3 Normalized responses obtained for earthworms extraction (citrate buffer, 6 mL water, 2 × 5 mL ACN) using different cleanup phases. Targeted pesticides are spiked at 100 μg/L (replicate measurements, $n = 3$)

Table 2 Validation parameters for the multi-residue LC-MS/MS method for the determination of 27 pesticides in earthworms

Compound	MLD	MLQ	ME (%)			Recovery (%)			Intra-day (%RSD)			Inter-day (%RSD)		
	ng/g	ng/g	5 LQ	20 LQ	100 LQ	5 LQ	20 LQ	100 LQ	5 LQ	20 LQ	100 LQ	5 LQ	20 LQ	100 LQ
Thiamethoxam	0.2	0.4	−37	−5	−14	103	114	113	5.8	5.9	1.3	13.7	10.9	9.0
Imidacloprid	0.2	0.4	47	33	−5	78	92	108	2.9	2.2	2.7	2.6	6.2	3.6
Thiacloprid	0.05	0.1	−5	5	−8	108	102	96	3.7	1.0	2.4	4.6	5.4	6.1
Pyroxsulam	0.2	0.5	−6	−1	−1	31	36	33	3.5	4.8	5.9	1.3	3.0	0.9
Pirimicarb	0.05	0.1	−9	−6	−9	101	105	106	4.5	3.1	1.9	10.4	2.9	2.1
Metazachlor	0.2	0.5	−25	−19	−22	102	102	106	3.4	2.3	1.6	4.9	2.8	1.8
Dimethachlor	0.2	0.5	−26	−15	−20	110	104	107	2.3	1.1	2.0	5.6	5.4	6.9
Clomazone	0.1	0.2	−20	−14	−18	99	101	107	3.3	2.1	1.0	3.4	3.3	2.4
Cyproconazole	0.5	1.4	−54	−51	−54	104	102	110	1.1	2.8	1.1	3.1	8.9	2.4
Boscalid	0.5	1.6	−38	−35	−39	97	99	108	1.8	2.5	0.4	5.6	2.5	2.3
Napropamide	0.2	0.5	−34	−30	−38	100	95	107	7.3	2.3	0.6	7.5	2.6	1.8
Epoxiconazole	0.2	0.4	−31	−34	−39	94	94	103	6.2	5.9	2.0	11.3	7.1	5.1
Metconazole	0.2	0.5	−41	−37	−47	92	93	105	13.4	5.5	2.2	10.0	6.9	7.4
Fluoxastrobin	0.4	1.5	−31	−23	−25	97	99	96	1.7	1.7	2.6	1.2	3.3	3.6
Propiconazole	0.3	0.7	−38	−36	−44	78	89	106	9.4	5.7	2.9	3.5	7.1	2.5
Prochloraz	0.2	0.4	−42	−34	−44	76	93	106	6.7	11.7	2.2	12.3	15.9	7.5
Cycloxydim	1.5	5.1	−66	−79	−56	72	70	74	6.2	13.7	4.2	4.5	3.2	17.2
Metrafenone	0.5	1.4	−33	−21	−32	81	88	105	12.5	4.2	2.2	11.9	11.2	5.3
Pyraclostrobin	0.1	0.3	−26	−16	−27	93	90	104	9.8	7.0	2.9	7.9	13.6	8.4
Diflufenican	0.2	0.4	18	24	38	77	84	109	7.4	7.1	2.1	5.8	17.1	2.4
Cloquintocet-mexyl	0.2	0.5	263	216	294	79	83	100	13.7	6.2	3.6	13.3	8.3	7.8
Pendimethalin	1.5	4.5	28	26	16	76	82	99	7.6	4.9	2.0	17.5	1.8	1.7
Lambda-Cyhalothrin	15.2	50.8	−12	−29	−21	84	84	97	9.1	6.2	6.2	9.3	11.1	1.3
Cypermethrin	9.8	32.7	−34	−46	−39	94	93	94	7.9	4.6	4.4	9.1	8.6	8.7
Deltamethrin	1.2	3.9	−26	−35	−32	84	82	98	10.2	5.4	8.0	11.8	14.7	6.6
Bifenthrin	0.2	0.4	−66	−65	−63	71	74	76	10.8	6.5	9.3	14.0	4.0	5.0
Tau-fluvalinate	1.3	4.2	−66	−67	−61	85	91	89	7.5	6.4	8.7	5.8	5.6	4.1

The repeatability (intra-day precision) measured for each target pesticide is presented in Table 2. Intra-day precisions are mainly lower than 10% for all targeted pesticides, at low, middle, and high concentrations. Occasionally, for some pesticides, the repeatability values exceed 10%, but remain below 15%. For inter-day precision, values are slightly higher but do not exceed 20%. Overall, it can be concluded that the QuEChERS procedure performed well for nearly all the targeted pesticide residues.

Application to samples collected from natural soils

The developed method has been applied to the analysis of nine earthworms *A. chlorotica* collected from different soils of the ZAPVS, France. The results are presented in Table 3. Among the analyzed earthworm, two samples did not contain any of the selected pesticides. The other analyzed earthworms contain at least one and up to five of the selected pesticides (ESM Fig. S2). Ten out of the 27 targeted pesticides have been detected at least once in the earthworms sampled. Among them, the most often detected pesticides are the insecticide imidacloprid and the herbicide diflufenican, both detected in four samples onto the nine analyzed. The highest concentrations of pesticides are 35.0 and 16.2 ng/g for imidacloprid and diflufenican, respectively. The triazole fungicides cyproconazole, epoxiconazole, and propiconazole have been detected in three, two, and two samples, respectively. These results confirm that the developed method is suitable to assess traces of currently used pesticides in earthworms and that these organisms can be exposed to several substances in their natural environment. This raises important ecotoxicological, agronomical, and biodiversity conservation issues and should be taken into account for the ecological risk assessment of pesticide use.

Table 3 Analysis of the targeted pesticides in earthworms by QuEChERS-LC-MS/MS (concentrations expressed in ng/g wet weight)

Compound	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9
Thiamethoxam	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD
Imidacloprid	*11.0*	< MLD	< MLD	*11.0*	< MLD	*35.0*	*11.3*	< MLD	< MLD
Thiacloprid	< MLD	< MLD	< MLD	< MLD	< MLD	*0.17*	*0.13*	< MLD	< MLD
Pyroxsulam	*< MLQ*	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD
Pirimicarb	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD
Metazachlor	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD
Dimethachlor	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD
Clomazone	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD
Cyproconazole	< MLD	*3.2*	< MLD	< MLD	*5.0*	*4.2*	< MLD	< MLD	< MLD
Boscalid	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	*2.3*
Napropamide	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD
Epoxiconazole	< MLD	*2.0*	< MLD	< MLD	*1.7*	< MLD	< MLD	< MLD	< MLD
Metconazole	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD
Fluoxastrobin	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD
Propiconazole	< MLD	< MLD	< MLD	< MLD	*< MLQ*	*3.5*	< MLD	< MLD	< MLD
Prochloraz	*1.2*	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD
Cycloxydim	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD
Metrafenone	< MLD	*2.5*	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD
Pyraclostrobin	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD
Diflufenican	*1.6*	< MLD	< MLD	*1.1*	*< MLQ*	*16.2*	< MLD	< MLD	< MLD
Cloquintocet-mexyl	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD
Pendimethalin	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD
Lambda-Cyhalothrin	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD
Cypermethrin	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD
Deltamethrin	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD
Bifenthrin	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD
Tau-fluvalinate	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD	< MLD

< MLQ: compound detected but with level inferior to the MLQ. < MLD: not detected

In italics, compounds that have been detected or quantified in the samples

Conclusion

The methodology developed and validated in this work enables the effective and sensitive multi-residue analysis of 27 currently used multi-class pesticides in earthworms *A. chlorotica***.** The methodology, based on a QuEChERS's extraction followed by a selective and sensitive analysis by LC-MS/MS, has been carried out from only 250 mg of the sample which is lower than the average weight of one individual. Nevertheless, the sensitivity and selectivity obtained are still in the low nanograms per gram range with high repeatability.

Although the method has been developed on earthworm species *Allolobophora chlorotica*, the protein, lipid, and carbohydrate contents of earthworms (that represent potential analytical interferents) vary relatively little from one species to another. It would be therefore quite possible to transpose this method to other species. In that case, the matrix effects and recoveries should be checked. Moreover, the matrix-matched calibration levels have to be performed with the corresponding earthworm species. Given the diversity of the pesticides analyzed (different chemical families and physicochemical properties), the scope of the method could be expanded to other pesticides from the same families, or to other families, provided the extraction recovery and matrix effects are evaluated.

The validated methodology has been successfully applied to the analysis of earthworms collected in natural soils from a French area. The present findings show the presence of ten targeted pesticides in the analyzed samples, with up to five pesticides in one earthworm. The neonicotinoid insecticide imidacloprid and the herbicide diflufenican are the most frequently detected pesticides, with measured concentrations up to 35 and 16 ng/g *ww*, respectively. As information on quantification of currently used pesticides in earthworms and terrestrial wildlife is scarce, this methodology can be used to improve knowledge about this organism as a sentinel species and exposure of terrestrial food webs to currently used pesticides.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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