

Vortex-assisted matrix solid phase dispersion method for the determination of multiclass pesticides in earthworm

Método de dispersão da matriz em fase sólida assistida por vórtex para a determinação de pesticidas multiclasse em minhocas

Renata Rodrigues de Moura^{1,2,3*}, Sergiane Souza Caldas², Caroline Lopes Feijo Fernandes^{1,3}, Júlia Oliveira Penteado^{1,3}, Ana Luiza Muccillo-Baisch^{1,3}, Ednei Gilberto Primel², Flavio Manoel Rodrigues Da Silva Júnior^{1,3}

¹Universidade Federal do Rio Grande (FURG), Programa de Pós-Graduação em Ciências da Saúde, Faculdade de Medicina, Campus da Saúde, Rua Visconde de Paranaguá, 102, Rio Grande-RS, Brasil, cep 96203-900.

²Universidade Federal do Rio Grande (FURG), Escola de Química e Alimentos, Laboratório de Análise de Compostos Orgânicos e Metais, Campus Carreiros, Av. Itália Km 8, Rio Grande-RS, Brasil, cep 96203-900.

³Universidade Federal do Rio Grande, Instituto de Ciências Biológicas, Laboratório de Ensaios Farmacológicos e Toxicológicos, Campus Carreiros, Av. Itália km 8, Rio Grande, Rio Grande do Sul, Brasil Brasil, cep 97900-000.

*rodriguesdmoura@hotmail.com

Abstract

The pesticides contamination may be result in accumulation and consequent adverse effects on representative organisms of ecosystem services such as earthworms. The use of earthworms to evaluate soil contamination by pesticides is of great relevance because this organism main macro-decomposer of the soil. However, there are few analytical methods that quantify different pesticides classes in earthworms. Thus, the aim of this study was to develop and validate a multiclass method based on vortex-assisted matrix solid-phase dispersion (VA-MSPD) and gas chromatography-mass spectrometry (GC-MS) to determine residues of the pesticides aldrin, atrazine, DDT, dieldrin, difenoconazole, endrin, heptachlor, metribuzin, mirex, S-metolachlor, tebuconazole in earthworm *Eisenia andrei*. The best results were obtained with 0.5 g sample, 0.5 g C18 as sorbent, 5 mL acetonitrile as extraction solvent. Analytical recoveries ranged between 70 and 118% with RSD $\leq$ 20% for repeatability and 60 to 140% with RSD $\leq$ 20% for intermediate precision. Limit of quantification (LOQ) values ranged from 0.05 to 0.5 mg kg⁻¹. VA-MSPD showed to be easy and fast to use, employing little mass of sample and sorbent, small volume of solvent, high extraction efficiency, cleaning and extraction in a single step. The method was also successfully validated and applied to soil samples.

Keywords: Multiclass method, pesticides, soil invertebrates, VA-MSPD.

Resumo

A contaminação por agrotóxicos pode resultar em acúmulo e efeitos adversos em organismos representativos dos serviços ecossistêmicos, como as minhocas. O emprego de minhocas para avaliar a contaminação do solo por agrotóxicos é de grande relevância, pois este organismo é o principal macrodecompositor do solo. Entretanto, existem poucos métodos analíticos que quantificam diferentes classes de pesticidas em minhocas. Portanto, o objetivo deste estudo foi desenvolver e validar um método multiclasse baseado na dispersão da matriz em fase sólida assistida por vórtex (VA-MSPD) empregando cromatografia gasosa com espectrometria de massa (GC-MS) para determinação dos pesticidas aldrin, atrazina, DDT, dieldrin, difenoconazol, endrin, heptacloro, metribuzin, mirex, S-metolacolor, tebuconazol em minhoca *Eisenia andrei*. Os melhores resultados foram obtidos com 0,5 g amostra, 0,5 g C18 como adsorvente e 5 mL de acetoneitrila como solvente extrator. As recuperações analíticas variaram entre 70-118% (RSD $\leq$ 20%) para repetibilidade e 60-140% (RSD $\leq$ 20%) para precisão intermediária. Os limites de quantificação (LOQ) variaram de 0,05-0,5 mg kg⁻¹. VA-MSPD mostrou-se fácil e rápido de usar, empregando pouca massa de amostra e adsorvente, pequeno volume

de solvente, alta eficiência de extração, limpeza e extração em uma única etapa. O método também foi validado e aplicado em amostras de solo.

Palavras-chave: Método multiclasse, pesticidas, invertebrados, solo, VA-MSPD.

Introduction

Most of pesticides are deliberately applied to the soil or sprayed on crop fields. There is evidence that soils, sediments, surface and underground bodies of agroecosystems are contaminated with varying levels of pesticide residues (ppt - ppm) [1]. According to physicochemical properties, pesticides that have a high octanol-water partition coefficient (K_{ow}), long half-life in soil (DT50), low water solubility (S) and vapor pressure (P), tend to persist in the soil, circulating in ecosystems and can be accumulated by many living organisms and even migrate through food chains [2,3]. The deleterious effects caused by pesticides are often difficult to detect on non-target organisms, since many of these effects tend to manifest only after prolonged exposure [4]. Among edaphic organisms, earthworms are important members of the soil ecosystem, contributing to the improvement of soil biophysical conditions, such as soil structure and immobilization of nutrients. In addition, the earthworms perform a crucial role in the dynamics of the microbial community, contributing to soil fertility, reflecting in the growth and development of plants [5,6].

Earthworms are naturally in contact with the solid, aqueous and gas phases from the soil and are therefore directly exposed to contaminants via contact or soil ingestion [7]. Experimental studies show that the exposure of earthworms to soil contaminated by pesticides has serious effects on the health of this macro-decomposer. Among these effects are irritation, swelling, changes in enzyme and locomotor activities, growth inhibition and high mortality caused by organochlorine pesticides [8–12]. When exposed to soils contaminated by triazines the earthworms showed a reduction in the synthesis of adenosine triphosphate (ATP); and triazoles have been shown to be cumulative in earthworms [12,13]. Consequently, these effects on earthworms can generate an environmental imbalance, since they are one of the main terrestrial macro-decomposers, and due to its role in the food chain.

Most studies carried out on earthworms investigate the toxicological effects caused by their exposure to soil contaminated by pesticides [8–12]. Usually assessing the effect of a pesticide, or the same class and / or chemical group, as well as possible synergistic interactions caused in earthworms by the association between pesticides and metals [14]. Despite the importance of this macro-decomposer in development processes and maintenance of the soil, there are few studies that carry out the determination of pesticide residues in earthworms [7,15].

In the Brazilian scenario, the low number of studies related to detection in environmental matrices is incompatible with the high consumption of pesticides in the country [16,17]. The scarcity of data regarding the quantification of pesticide residues ends up limiting the understanding of the behavior of these contaminants in the soil and in non-target organisms, contrasting scientific data and the environment [18]. This is because these data are extremely important for carrying out complex environmental studies, such as the assessment of ecological risks [19,20].

Accelerated solvent extraction (ASE) [15] and modified QuEChERS extraction [7] have been used in the sample preparation technique for pesticides in earthworm. To the best of our knowledge, matrix solid phase extraction (MSPD) have not been used for this purpose yet. MSPD was proposed by Barker in 1989 to extraction of drugs from biological tissues [21], and the technique is based on the dispersion of the sample with the aid of a solid support that acts as an abrasive, promoting the rupture of the physical structure of the sample, which suffers a dispersion on the surface of the support material, forming a new phase which provides the isolation of the matrix analytes [2,21,22]. In the VA-MSPD, the cartridge packaging step was replaced by a vortex-assisted extraction reducing the extraction step time and the variations resulting from this process [2].

In this context, unprecedentedly, this study proposed the use of the vortex-assisted matrix solid-phase dispersion (VA-MSPD), aiming at the development and validation of a multiclass method to determination of residues of the pesticides aldrin, atrazine, DDT, dieldrin, difenoconazole, endrin, heptachlor, metribuzin, mirex, S-metolachlor, tebuconazole in earthworms (*Eisenia andrei*). The

method was also tested in the soil matrix in order to obtain a versatile method, aiming at application in earthworm samples and samples from their habitat (soil). The pesticides under investigation were chosen based in their tendency to remain in the soil (e.g.: high K_{ow} and low vapor pressure), their presence in Brazilian soils, high toxicity and persistence in the environment, besides the pesticide marketing history in Brazil [19,23].

Experimental

Chemicals

Aldrin, atrazine, atrazine-d5, DDT, dieldrin, difenoconazole, endrin, heptachlor, metribuzin, mirex, S-metolachlor, tebuconazole analytical standards (purity >99%) were purchased from Sigma Aldrich (São Paulo, Brazil). Acetonitrile, acetone and ethyl acetate of chromatographic grade were provided by Mallinckrodt (Phillipsburg, NJ, USA). Octadecyl (C18) 0.050 mm was acquired from Phenomenex (Torrance, CA), sea sand with particle diameter of 0.088–0.105 mm, this sand was previously characterized by dos Santos (2019)[24].

Standard stock solutions of 1000 mg L^{-1} of the target compounds were prepared in acetonitrile and all solutions were stored at -18°C . The standard stock solutions were used for preparing the pesticide working solutions of 100 mg L^{-1} . Working solutions were used to prepare analytical curves and sample spiking [2].

Apparatus and chromatographic conditions

Analyzes were performed using a Shimadzu gas chromatograph, model QP2010 Plus GC–MS, composed of auto-sampler (AOC-20i) and quadrupole mass spectrometer detector. The GC separation was carried out by ZB-5MSi capillary column (30 m x 0.25 mm x 0.25 μm) (Phenomenex). The control of the analytical instrument and data treatment were done through the software GC–MS solution version 2.7[®] (Shimadzu, USA). Helium (99.999% purity) was the carrier gas. Injection volume was 2 μL in *splitless* mode [24,25]. Each compound was monitored in full scan mode in the range m/z 50–500 in order to optimize the MS parameters. MS conditions were fixed for each compound, in an attempt to select a precursor ion with higher m/z ratio (greater selectivity) and abundance (highest sensitivity). Ionization mode by electron-impact (EI) and 70 eV of ionization energy was used [2,24,25]. The separation temperature program was optimized by using the pesticide standard solution in a concentration of 1.0 mg L^{-1} .

The injector temperature was 300°C . The temperature of the interface, in the MS system, was set at 280°C and the source of ionization at 300°C . The optimized GC oven temperature program was 70°C (held for 1 min), followed by a $19^\circ \text{C min}^{-1}$ ramp to 160°C , then by a $25^\circ \text{C min}^{-1}$ ramp to 190°C , by a $65^\circ \text{C min}^{-1}$ to 250°C , and a final ramp of $30^\circ \text{C min}^{-1}$ to 300°C (held for 5 min). A solvent delay time of 7.0 min was set to prevent instrument damage.

A perfluorotributylamine (PFTBA) solution was used to calibrate the MS. The mass spectrometer (MS) system was defined in selective ion monitoring (SIM) mode and each compound was quantified based on the peak area using three qualifier ions (Table 1). European SANTE (2019) recommendations for the identification and confirmation of pesticides were followed [26].

Table 1. Retention time, GC-EI-MS conditions, octanol-water partition coefficient (K_{ow}) and vapor pressure.

Pesticides	Rt (min)	Monitored ions (m/z)	Segment	Time window (min)	K_{ow}^b	Vapor pressure (mmHg) ^b
Atrazine-d5	8.58	205 ^a , 220, 178	1	7.0 - 9.5	2.6	3×10^{-7}
Atrazine	8.58	200 ^a , 215, 173	1		2.61	3×10^{-7}
Metribuzin	8.86	198 ^a , 103, 144	1		1.65	4.35×10^{-7}
Heptachlor	8.98	272 ^a , 100, 237	1		6.10	4×10^{-4}
S-Metolachlor	9.16	162 ^a , 133, 238	1		3.13	3.14×10^{-5}
Aldrin	9.20	263 ^a , 293, 257	1	9.51 - 10.5	6.50	1.2×10^{-4}
Dieldrin	9.78	263 ^a , 108, 277	2		5.40	5.89×10^{-6}
Endrin	9.92	263 ^a , 79, 245	2		5.40	5.89×10^{-6}
DDT	10.17	235 ^a , 237, 165	2		6.91	1.6×10^{-7}
TPP	10.25	326 ^a , 233, 215	2		4.59	2×10^{-6}
Tebuconazole	10.27	125 ^a , 250, 252	2	10.51 - 14.0	3.7	1.3×10^{-8}
Mirex	11.05	272 ^a , 237, 274	3		6.89	8×10^{-7}
Difenoconazole	12.91	265 ^a , 323, 267	3		4.36	2.5×10^{-10}

^aions used for quantification. ^bMacBean C, ed; e-Pesticide Manual. 15th ed., ver. 5.1, Alton, UK (2008-2010)

Earthworm and soil samples

For the method validation, earthworm (*Eisenia andrei*) and tropical artificial soil were cultivated and prepared, respectively, in the Laboratory of Pharmacological and Toxicological Tests - LEFT of the Federal University of Rio Grande - FURG. The earthworm used has an average of 40.43% protein, 9.87% lipids and 9.11% carbohydrates [27]. The artificial tropical soil is composed of 70% fine sand, 20% kaolin clay, 10% coconut fiber powder [28,29].

For the method applicability, earthworm and soil samples were collected in the countryside of Rio Grande, Rio Grande do Sul, Brazil (32°00'15.5"S 52°07'50.6"W), where agricultural activities are diverse and intense.

All samples were lyophilized (Bench-top freeze dryer, Model: L101, Biobras) and crushed for later use in the development, method validation and application.

Optimization of the Vortex-assisted matrix solid-phase dispersion (VA-MSPD) procedure

The optimized parameters in the VA-MSPD procedure in earthworms (*Eisenia andrei*) were: extraction solvent type, sorbent type, sorbent proportion to sample mass, maceration time and vortex agitation time.

The extraction solvent, sorbent type, and proportion of sorbent in relation to the sample mass were evaluate in a one-variable-at-time way. After some extraction conditions were fixed, an experimental design was constructed based on a 2² factorial design, experimental error was carried out three central points, a total of 7 experiments. Maceration time (1, 5 and 9 min) and vortex agitation time (1, 3 and 5 min) were evaluated at three levels (Table 2).

Table 2. Full factorial planning matrix $2^2 + 3$ central points with coded values and real values (in parentheses).

Assay	Maceration time (min)	Vortex agitation time (min)
1	-1 (1)	-1 (1)
2	-1 (1)	1 (5)
3	1 (9)	-1 (1)
4	1 (9)	1 (5)
5	0 (5)	0 (3)
6	0 (5)	0 (3)
7	0 (5)	0 (3)

Recovery assays were performed in triplicate and the extracts were injected three times each ($n=9$) [26,30]. Atrazine-d5 was added before extraction, as surrogate standard (SS) at $50 \mu\text{g kg}^{-1}$ and triphenylphosphate (TPP) after the extraction as internal standard at $100 \mu\text{g kg}^{-1}$ [24,26].

The initial conditions for the extraction of pesticides from earthworm were performed by using 0.5 g earthworm samples spiked with pesticides at 0.5 mg kg^{-1} level. After solvent evaporation (approximately 30 min), the sample was blended with 1 g of sorbent in a ceramic pestle with a mortar to obtain a homogeneous mixture that was transferred to a 15 mL polypropylene tube. Afterward, 5 mL of extraction solvent was added to the tube. The suspension was vortex stirred for 1 min and centrifuged for 10 min at 10000 rpm. Then, 2 μL of the extract was injected into the chromatographic system [2].

Statistical analysis

The experimental design and statistical analysis of the experimental results was carried out with the Statistica 5.0 Portable software (2007; StatSoft Inc., Tulsa, USA). The tests were made in triplicate and assessed by Tukey's test with a 95% confidence level.

Method validation

The validation parameters evaluated were limit of detection (LOD), limit of quantification (LOQ), linearity, accuracy, repeatability (intraday precision), intermediate precision (interday precision), matrix effect (ME) and robustness as recommended in the document by SANTE (2019) in compliance with INMETRO (2020) [26,30].

For each analyte, the LOD and LOQ of the method were obtained by measuring the ratio between the signal and the baseline, 3 and 10 times, respectively [2,26,30]. Different analyte concentrations diluted with earthworm extracts VA-MSPD were injected to determine the LOD and LOQ, these values were confirmed experimentally [2].

To evaluate the linearity of the method, matrix matched calibration was performed at the concentration level of the LOQ of each compound to a concentration equivalent to 16-fold LOQ value for earthworms and 12-fold LOQ value for soil. Pesticides standard solution dilutions were performed with blank extract from the matrix extracted using the optimized VA-MSPD method [2,24]. Three replicates were injected in triplicate ($n=9$) at 6 concentration levels of the calibration standards for the earthworm matrix [24] and 5 concentration levels for the soil matrix [2]. An external calibration was

also performed at the same concentrations by diluting the standard pesticide solution in acetonitrile [2,26,30].

The method accuracy was assessed through recovery tests for each peak level studied, in compliance with INMETRO (2020) and SANTE (2019). At the beginning of the process, 0.5 g samples blank earthworm or soil samples were spiked by adding a known volume of standard solution containing a mixture of pesticides [2]. The spike levels were concentration equivalent to the LOQ, 2-fold LOQ and 4-fold LOQ for earthworms and equivalent to the LOQ, 2-fold LOQ for soil. Triplicate extractions were performed for each spike level and injected three times ($n=9$). The method precision was expressed as relative standard deviation (RSD) assessed with respect to the repeatability and intermediate precision [26,30].

The evaluation of the ME was calculated by overlap the slopes of the matrix matched calibration and the calibration solutions prepared in solvent [24,31]. The robustness was evaluated through recovery of VA-MSPD in different matrices (earthworms and soil) [26,30].

Applicability

The validated method applicability was performed with earthworm and soil samples collected in the interior of the city of Rio Grande, RS, Brazil. The samples were previously frozen ($-18\text{ }^{\circ}\text{C}$), lyophilized, crushed and homogenized before analysis. The analyzes were performed in triplicate of sample and injection ($n = 9$).

Results and Discussion

VA-MSPD

One-factor-at-a-time parameters

The first variable evaluated was the extraction solvent. The polarity of the solvent is the key factor in the VA-MSPD, because, together with the sorbent, it determines the extraction efficiency and the purity of the final extracts [2].

To evaluate the influence of the extraction solvent, 5 mL acetonitrile, acetone and ethyl acetate was used. The solvent that showed satisfactory recoveries for most analytes was acetonitrile, between 70 - 120% range and RSD <20%, as recommended by SANTE (2019) and INMETRO (2020) (Figure 1). Acetonitrile, acetone and ethyl acetate have $\log K_{ow}$ values of -0.34, 0.11 and 0.28, respectively. According Moldoveanu and David (2013), solvents with much lower $\log K_{ow}$ are capable of eluting strongly retained compounds, and also claim that acetonitrile can elute / extract from the sorbent C18 compounds with significantly higher $\log K_{ow}$ values, as is the case of the compounds studied in this work [32]. Acetonitrile is preferred for use with biological samples because it helps to precipitate highly polar proteins, its selectivity and its intermediate polarity provides extraction of a smaller amount of lipophilic interferences and the extraction of a wide range of pesticides with different polarities; how is the case of the studied analytes [33–36]. Acetonitrile have showed high extraction efficiency in MSPD for the extraction of pesticides in other matrices, such as fish and crab hepatopancreas [2], eggs [2], pollen [37], tea [38], tobacco [39] and foraging honeybees [40].

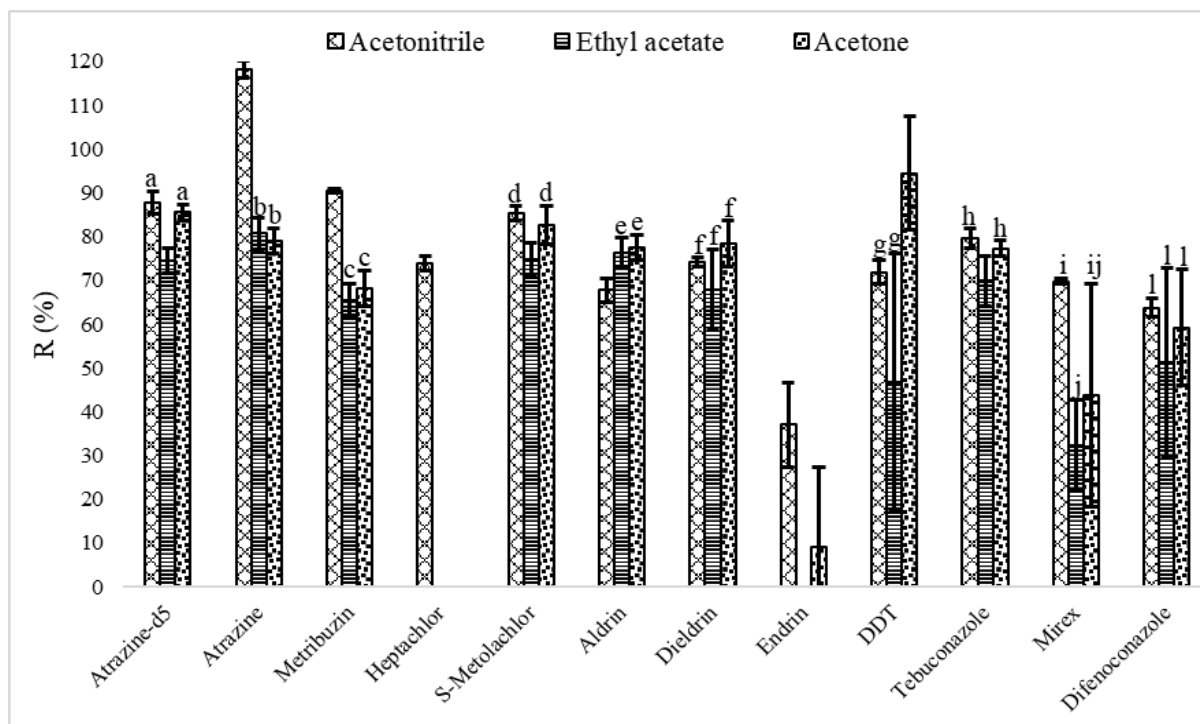


Figure 1. Effect of the type of extraction solvent on the recoveries of the pesticides (conditions of extraction: 0.5 g sample spiked with $50 \mu\text{g L}^{-1}$, 1.0 g C18, 5 mL extraction solvent, 5 min macerating, 1 min vortex, 10 min at 10000 rpm). Error bars indicate RSD (%) ($n=9$); same letters between the averages of each compound, are not significantly different according to the Tukey test (significance level of 5%).

After choosing acetonitrile as extraction solvent, the sorbents C18 and sand were investigated. Experiments without sorbent were also carried out to investigate if the composition of the earthworms could be acting as a sorbent, since they ingest soil, that usually are rich in silicates. The sorbent that showed the best recoveries for most analytes was C18 (Figure 2). The best result can be attributed to C18 due to its lipophilic and hydrophobic characteristics. In step homogenization of the sample with the C18 (lipophilic phase bound), weak intermolecular forces act that promote the effective interruption of the sample and the interaction of the analytes with C18 through the sorption process. Thus, as C18 has a hydrophobic character and the analytes studied in this work have behavior that ranges from moderately polar to apolar, consequently the interactive adsorption forces are stronger than absorption and the analytes tend to be adsorbed on C18 [41–43].

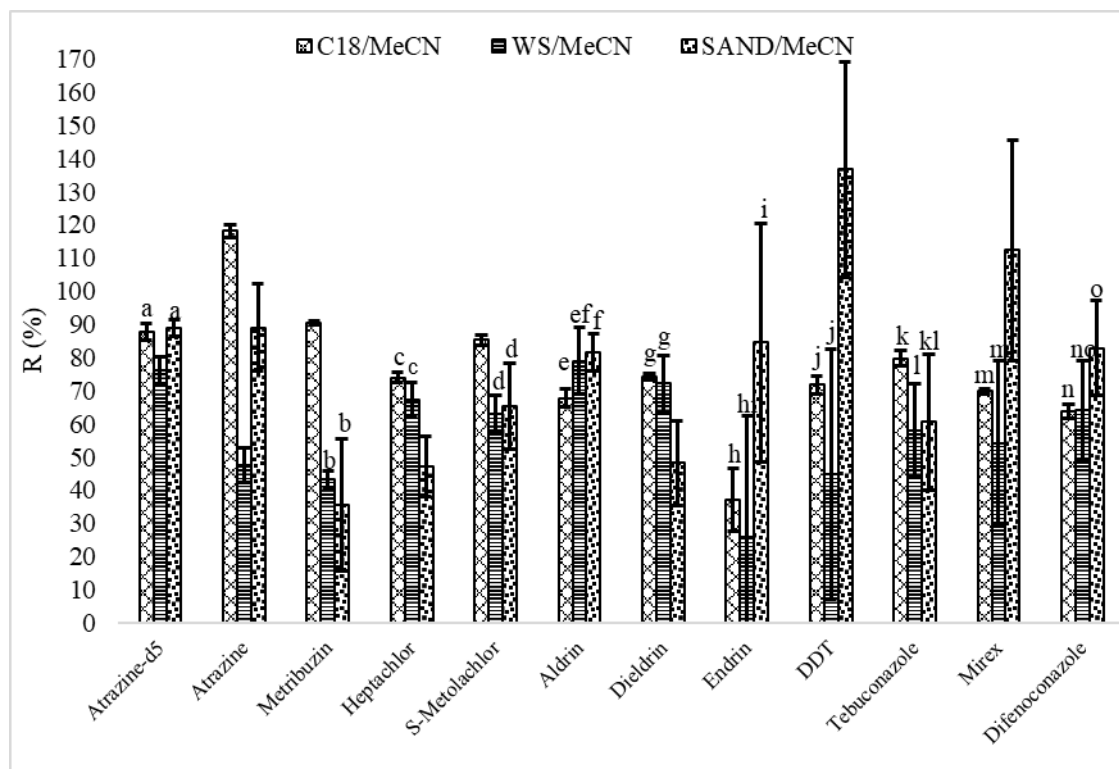


Figure 2. Effect of sorbents on pesticide recoveries (conditions of extraction: 0.5 g sample spiked with $50 \mu\text{g L}^{-1}$, 1.0 g sorbent, 5 mL acetonitrile, 5 min macerating, 1 min vortex, 10 min at 10000 rpm). Error bars indicate RSD (%) ($n=9$); same letters between the averages of each compound, are not significantly different according to the Tukey test (significance level of 5%). C18/MeCN: C18 with acetonitrile; WS/MeCN: without sorbent, only sample and acetonitrile; SAND/ MeCN: sand with acetonitrile.

For experiments carried out with sea sand and without sorbent, recoveries outside 70-120% can be attributed to the use of inert solids, such as sand, in the homogenization step. These sorbents perform abrasion and disruption of the sample, but do not have the same efficiency as the bonded phase sorbents [42].

Commonly, the proportion is one part of sample to four parts of sorbent [33,41]. However, according to the literature, the higher the $\log K_{ow}$, the better recoveries are achieved by decreasing the proportion of sorbent in relation to the sample mass [44]. To improve recoveries of pesticides aldrin, endrin, mirex and difenoconazole the proportions 1:1 (sample: C18), 1:2 and 1:4 were tested in triplicate (Figure 3). The 1:1 sample:sorbent proportion showed recoveries of 70 to 120%, with an $\text{RSDr} \leq 13\%$, for all compounds with significant differences from 1:2 and 1:4 (sample:C18) when a statistical test was carried out (t -test, $P > 0.05$). This result is in accordance with the literature, since the organochlorines aldrin, endrin, mirex and the triazole difenoconazole have a high $\log K_{ow}$ value [44].

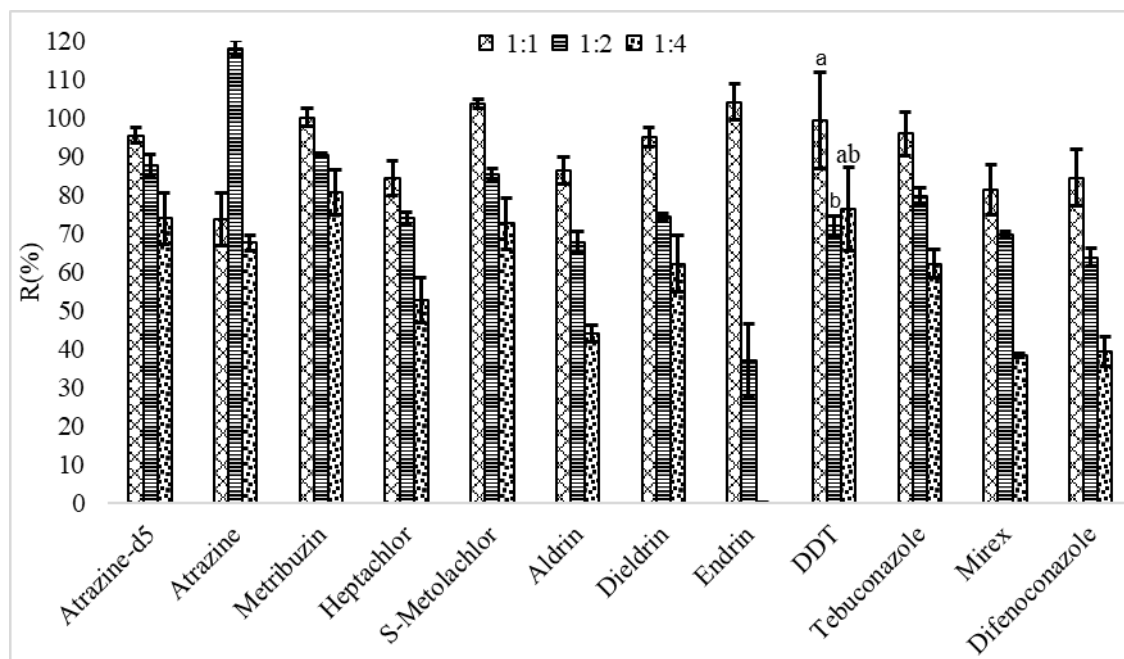


Figure 3. Effect of sorbent on pesticide recoveries using 0.5 g of sample to 0.5 (ratio 1:1), 1.0 (ratio 1:2) and 2.0 (ratio 1:4) g of C18, respectively (conditions of extraction: 0.5 g sample spiked with 50 $\mu\text{g L}^{-1}$, 5 mL acetonitrile, 5 min macerating, 1 min vortex, 10 min at 10000 rpm). Error bars indicate RSD (%) ($n=9$); same letters between the averages of each compound, are not significantly different according to the Tukey test (significance level of 5%).

2² factorial design

The 2² factorial design with 3 central points carried out to evaluate the influence of the maceration and vortex agitation times on the extraction efficiency showed no significant difference among them (Figure 4). The most commonly applied maceration time from 5 to 10 min [33]. Nevertheless, aiming at having faster method, the shortest maceration times (1 minute) and vortex agitation (1 minute) represented by assay 1 were chosen since there was no significant difference between them. Other maceration times in MSPD for biological and environmental samples are reported, such as 5 min fish and crab hepatopancreas [2], 2 min for tobacco [39] and 1.5 min for eggs [34], soil and river sediment [45]. However, this method showed maceration time even less than the cited works. In relation to time by the vortex agitation, the best result obtained is consistent with that reported in the literature to vortex-assisted MSPD, such as the determination of pesticide residues in fish and crab by gas chromatography with mass spectrometry [2].

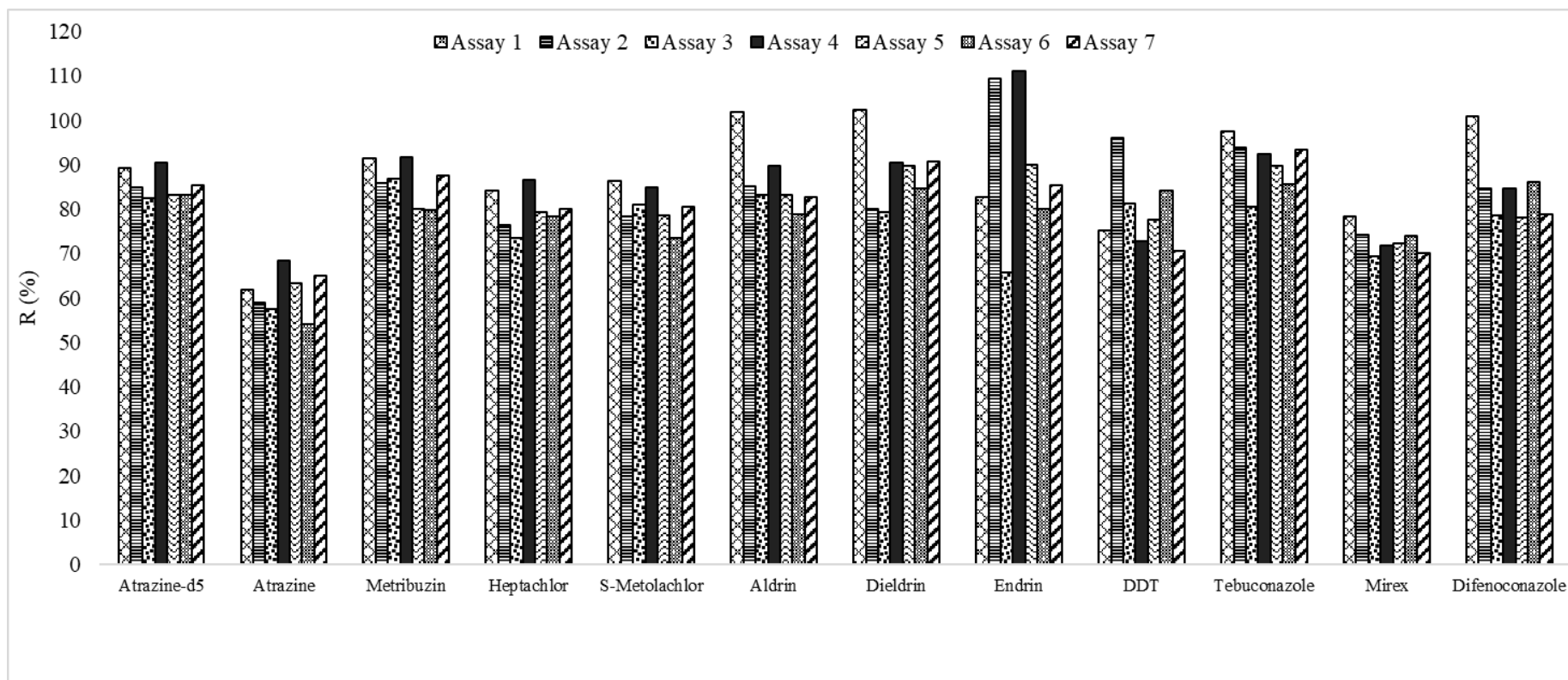


Figure 4. Recovery assays using different maceration and vortexing times (conditions of extraction: 0.5 g sample spiked with 50 $\mu\text{g L}^{-1}$, 0.5 g C18, 5 mL acetonitrile). Assay 1: 1 min macerating, 1 min vortex; assay 2: 1 min macerating, 5 min vortex; assay 3: 9 min macerating, 1 min vortex; assay 4: 9 min macerating, 5 min vortex; assays 5 to 7: 5 min macerating, 3 min vortex.

Method validation

Since the factors affecting the MSPD procedure have been optimized, the method was validated to earthworm and soil matrices.

Linearity and detection and quantification Limits

Adequate linearity was obtained in the concentration range from the LOQ to 16-fold LOQ to earthworm and LOQ to 12-fold LOQ to soil, with determination coefficient (R^2) higher than 0.99 complying with SANTE's recommendations [26] (Table 3).

LOQ values for these compounds varied from 0.05 to 0.5 mg kg⁻¹ for earthworm and soil matrices. It is unfeasible to compare the limits obtained in the work with other the same application because it is the first time the VA-MSPD is applied for earthworm; and in relation to the soil, it is found in the literature application of VA-MSPD in soil, but there is not for the pesticides approached in this work [33]. Andrade et. al. (2014) evaluate the bioavailability of DDT, DDD, DDE and dieldrin in soil contaminated by bioassays earthworm (*Lumbricus terrestris*) [15]. Earthworm samples were extracted by accelerated solvent extraction (ASE) with acetone and hexane, the extract was dried and reconstituted in hexane followed by successive liquid-liquid extractions with acetonitrile. The LOQ for each of the analytes was 0.8 mg kg⁻¹ based on a 2 g sample of either soil or earthworms. When comparing this study with the present work, we developed a method that analyzes multiclass of pesticide residues, with lower LOQ values, a miniaturized extraction procedure, so it uses less solvent volume, less steps consequently it is faster, cheaper and reduces analyst exposure to toxic solvents.

Table 3. LOQ and analytical curve parameters in earthworm and soil matrices.

Pesticides	Earthworm			Soil		
	LOQ (µg/kg)	Linearity (µg /kg)	R^2	LOQ (µg /kg)	Linearity (µg /kg)	R^2
Atrazine-d5	50	50- 800	0.9994	50	50-600	0.9929
Atrazine	250	250-4,000	0.9991	250	250-3,000	0.9941
Metribuzin	250	250-4,000	0.9972	250	250-3,000	0.9956
Heptachlor	250	250-4,000	0.9987	250	250-3,000	0.9950
S-Metolachlor	100	100-1,600	0.9991	100	100-1,200	0.9966
Aldrin	100	100-1,600	0.9994	100	100-1,200	0.9947
Dieldrin	500	500-8,000	0.9990	500	500-6,000	0.9947
Endrin	250	250-4,000	0.9991	250	250-3,000	0.9956
DDT	100	100-1,600	0.9972	100	100-1,200	0.9981
Tebuconazole	50	50-800	0.9992	50	50- 600	0.9987
Mirex	250	250-4,000	0.9994	250	250-3,000	0.9964
Difenoconazole	100	100-1,600	0.9992	100	100-1,200	0.9928

Accuracy and precision

Recoveries varied from 70 to 118% for repeatability with $RSD \leq 20\%$ were obtained for all compounds referring to the earthworm matrix and recoveries in the varied from 60 to 123% with $RSD \leq 16\%$ for all compounds to the soil matrix (Table 4) complying with INMETRO and SANTE's recommendations [26,30]

The recoveries obtained for the earthworm matrix when studying the intermediate precision, most compounds showed values within the acceptable interval, varying from 60 to 140 with $RSD \leq$

20%. Therefore, all compounds showed values within from 60 and 140%, in accordance with the SANTE validation guide (2019) and INMETRO (2020) [26,30].

Table 4. Recovery values ($n=9$) and relative standard deviation (RSD) obtained with the MPSD method at three concentration levels in earthworm and two three concentration levels in soil matrices.

Pesticides	Earthworm						Soil	
	Repeatability			Intermediate Accuracy			Repeatability	
	1 LOQ	2 LOQ	4 LOQ	1 LOQ	2 LOQ	4 LOQ	1 LOQ	2 LOQ
	R (%) ±RSD	R (%) ±RSD	R (%) ±RSD	R (%) ±RSD	R (%) ±RSD	R (%) ±RSD	R (%) ±RSD	R (%) ±RSD
Atrazine-d5	70 ± 11	92 ± 6	77 ± 8	99 ± 14	99 ± 14	68 ± 9	93 ± 7	102 ± 3
Atrazine	83 ± 4	108 ± 8	79 ± 2	136 ± 8	115 ± 15	66 ± 4	76 ± 15	89 ± 7
Metribuzin	77 ± 7	95 ± 10	73 ± 1	134 ± 7	113 ± 16	67 ± 1	104 ± 5	96 ± 2
Heptachlor	76 ± 5	109 ± 7	77 ± 4	67 ± 6	75 ± 9	108 ± 7	63 ± 2	61 ± 14
S-Metolachlor	86 ± 11	100 ± 9	75 ± 5	93 ± 5	99 ± 4	70 ± 16	110 ± 7	75 ± 16
Aldrin	78 ± 12	89 ± 10	71 ± 5	65 ± 10	81 ± 20	79 ± 10	64 ± 9	67 ± 6
Dieldrin	80 ± 5	111 ± 3	86 ± 5	123 ± 7	106 ± 20	85 ± 12	80 ± 2	77 ± 2
Endrin	76 ± 20	96 ± 15	89 ± 4	100 ± 9	86 ± 12	94 ± 13	68 ± 10	63 ± 4
DDT	78 ± 20	118 ± 6	84 ± 14	77 ± 12	88 ± 12	62 ± 10	82 ± 15	70 ± 11
Tebuconazole	92 ± 6	110 ± 9	103 ± 2	70 ± 3	81 ± 8	70 ± 16	123 ± 8	91 ± 3
Mirex	66 ± 12	84 ± 4	73 ± 2	64 ± 9	70 ± 12	101 ± 6	64 ± 4	60 ± 2
Difenoconazole	93 ± 7	96 ± 8	80 ± 8	108 ± 5	111 ± 4	82 ± 10	100 ± 5	68 ± 5

Matrix Effect

The matrix effect (ME) may be influenced by the analyte, matrix, sample preparation, instrumentation and operational conditions; therefore, variable and unpredictable. The ME is attributed to co-extractives that may be present in the sample, these ME can be observed as an enrichment or suppression in the response as compared with those produced by solutions of the analyte in solvents [24,46].

According to the literature the ME is considered low for a range of signal suppression / enrichment of $-20\% < ME\% < +20\%$, medium for $-50\% < ME\% < -20\%$ or $+20\% > ME\% > +50\%$ and high to $ME\% < -50\%$ or $ME\% > +50\%$ [47]. In this context, most of the analytes showed medium to high ME, except for the analytes endrin, DDT and difenoconazole, which showed signal enrichment below 20% to earthworm. In soil samples, the ME was low to medium for all analytes, except metribuzin, which showed signal enrichment above 50% (Figure 5). In these cases, it is recommended that the analytical curve be constructed on the matrix extract.

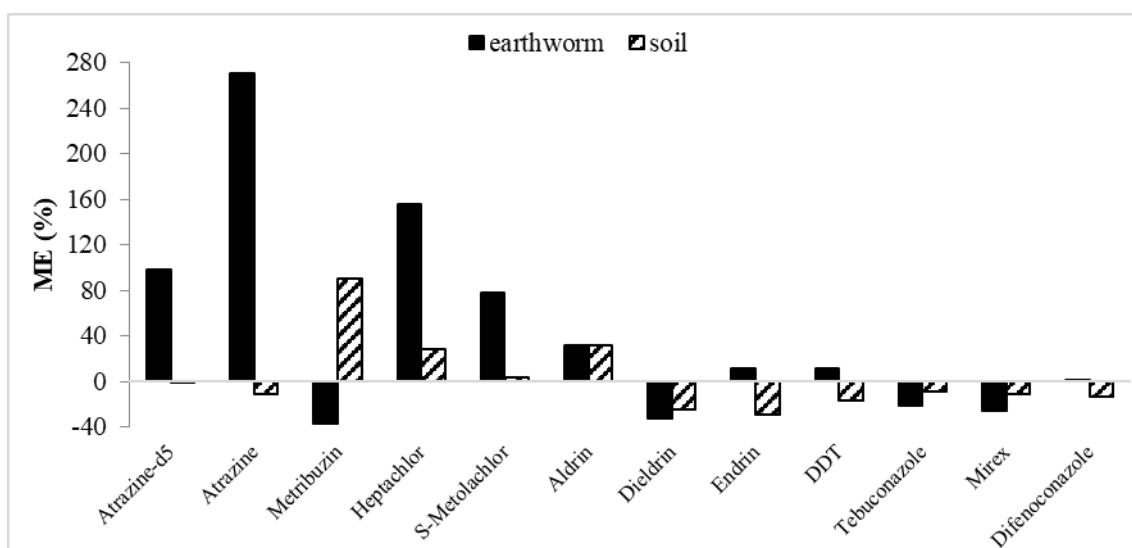


Figure 5. Result of the Matrix Effect from the overlapping of the curves in the solvent and in the earthworm extract (black bars) and in the soil extract (striped bars).

Robustness

The robustness of the method was assessed through recovery tests on earthworm and soil samples, in triplicate the samples were spiked at the 4LOQ level. Recoveries were between 68 and 99% with $RSD \leq 19\%$ to earthworm and 79 to 101% with $RSD \leq 12\%$ to soil (Table 5) [30] therefore, the developed method proved to be robust and able to be used in different matrices such as biological matrix (earthworm) and soil.

Table 5. Recovery values ($n=9$) and relative standard deviation (RSD) obtained with the MPSPD method at 4 LOQ concentration level in earthworm and soil samples.

Pesticides	Earthworm	Soil
	4 LOQ	
	R (%) ±RSD	R (%) ±RSD
Atrazine-d5	90 ± 1	80 ± 4
Atrazine	86 ± 13	80 ± 2
Metribuzin	99 ± 1	85 ± 2
Heptachlor	72 ± 19	94 ± 5
S-Metolachlor	68 ± 6	85 ± 10
Aldrin	76 ± 1	79 ± 6
Dieldrin	95 ± 3	101 ± 8
Endrin	81 ± 2	91 ± 2
DDT	91 ± 14	87 ± 12
Tebuconazole	96 ± 1	91 ± 7
Mirex	96 ± 1	99 ± 11
Difenoconazole	79 ± 1	84 ± 7

Applicability

The quantification was performed using the matrix matched calibration at five concentrations, to compensate for the matrix effect in each matrix (cultivated earthworm and artificial soil). The applicability of the method was performed with earthworm and soil samples collected in the interior of Rio Grande, RS, Brazil.

In the earthworm matrix, the triazinone metribuzin and the organochlorines heptachlor, dieldrin, endrin, DDT and mirex were detected in concentrations lower than their respective LOQs. In the soil matrix, pesticides from the organochlorines group, aldrin, dieldrin, endrin, DDT and mirex, were also detected in concentrations lower than their respective LOQs. Although the LOQs presented are relatively high close to other more sensitive techniques, studies with soils from other Brazilian regions have already detected high concentrations of pesticides (up to 1243.68 mg kg⁻¹), with emphasis on organochlorines [16].

Metribuzim belongs to the most commercialized pesticide class in Brazil, the herbicides [23]; and in relation to intervention reference values (RGV's) for soils, Brazilian legislation and the European Union do not stipulate a limit value for metribuzin [20,48]. In Brazil, Polese et. al. 2002 investigated the presence of metribuzin in 22 soil samples, the analyzes gave below the LOD value for all samples [49].

The use of organochlorines in Brazil was banned in the 1960s due to high toxicity, lipophilicity, bioaccumulative, estrogenic, carcinogenic and resistant to the degradation process of the environment. However, the presence of these compounds in the environment can still be detected [50,51]. Regarding the organochlorines detected in this work, in Brazil there are reports of other studies that investigated these pesticides in soil [16], however only DDT (397 mg kg⁻¹) [52] was found in a concentration higher than RGV's by Brazilian and European legislation.

Conclusion

The VA-MSPD multiclass method allowed the simultaneous determination of organochlorines, triazoles, triazines and triazinones pesticides for the first time in earthworm matrix. The advantages of the proposed method are speed and ease of use, associated with a small sample mass and solvent volume, resulting in high extraction efficiency and the possibility of cleaning and extraction in a single step. VA-MSPD is an alternative to extraction that does not require specific and sophisticated equipment, while reducing the exposure time of the analyst.

The method presented satisfactory accuracy and precision. When applied to different matrices (earthworm and soil) the VA-MSPD multiclass method proved to be robust, also presenting as a differential the possibility of multiclass pesticides residues analysis in the biota (earthworm) and in the habitat (soil) in which the earthworm was collected. Therefore, the developed method is an efficient tool for investigating the distribution of pesticides in contaminated soils.

Conflict of Interest

The authors declare that there is no conflict of interest

References

- [1] Z. Vryzas, Pesticide fate in soil-sediment-water environment in relation to contamination preventing actions. *Curr. Opin. Environ. Sci. Heal.*, 4 (2018) 5–9.
- [2] S. S. Caldas; C. M. Bolzan; E. J. De Menezes; A. L.V. Escarrone; C. M. G. Martins; A. Bianchini; E. G. Primel, A vortex-assisted MSPD method for the extraction of pesticide residues from fish liver and crab hepatopancreas with determination by GC-MS. *Talanta*, 112 (2013) 63–68.
- [3] J. Ma; L. Pan bo; X. Yang; X. Liu; S. Tao; L. Zhao; X. Qin; Z. Sun; H. Hou; Y. Zhou, DDT, DDD, and DDE in soil of Xiangfen County, China: Residues, sources, spatial distribution, and health risks. *Chemosphere*, 163 (2016) 578–583.
- [4] V. I. Lushchak; T. M. Matviishyn; V. V. Husak; J. M. Storey; K. B. Storey, Pesticide toxicity: A mechanistic approach. *EXCLI J.*, 17 (2018) 1101–1136.
- [5] P. Lavelle, Faunal Activities and Soil Processes: Adaptive Strategies That Determine Ecosystem Function., *Adv. Ecol. Res.*, 27 (1997) 93–132.
- [6] M. Blouin; M. E. Hodson.; E. A. Delgado; G. Baker; L. Brussaard; K. R. Butt; J. Dai; L. Dendooven; G. Peres; J. E. Tondoh, A review of earthworm impact on soil function and ecosystem services. *Eur. J. Soil Sci.*, 64 (2013) 61–182.
- [7] G. Daniele; F. Lafay; C. Pelosi; C. Fritsch; E. Vulliet, Development of a method for the simultaneous determination of multi-class pesticides in earthworms by liquid chromatography coupled to tandem electrospray mass spectrometry. *Anal. Bioanal. Chem.*, 410 (2018) 5009–5018.
- [8] R. K. Hans; R. C. Gupta; M. U. Beg, Toxicity assessment of four insecticides to earthworm, *Pheretima posthuma*. *Bull. Environ. Contam. Toxicol.*, 45 (1990) 358–364.
- [9] D. Xu; Y. Wen; K. Wang, Effect of chiral differences of metolachlor and its (S)-isomer on their toxicity to earthworms., *Ecotoxicol. Environ. Saf.*, 73 (2010) 1925–1931.
- [10] Y. Shi; Q. Zhang; D. Huang; X. Zheng; Y. Shi, Survival, growth, detoxifying and antioxidative responses of earthworms (*Eisenia fetida*) exposed to soils with industrial DDT contamination. *Pestic. Biochem. Physiol.*, 128 (2016) 22–29.
- [11] V. D. Dani; A. J. Simpson; M. J. Simpson, Analysis of earthworm sublethal toxic responses to atrazine exposure using ¹H nuclear magnetic resonance (NMR)-based metabolomics. *Environ. Toxicol. Chem.*, 37 (2018) 473–480.
- [12] N. Cui; H. Xu; S. Yao; Y. He; H. Zhang; Y. Yu, Chiral triazole fungicide tebuconazole: enantioselective bioaccumulation, bioactivity, acute toxicity, and dissipation in soils. *Environ. Sci. Pollut. Res.* 25 (2018) 25468–25475.
- [13] M. Svobodová; K. Šmídová; M. Hvězdová; J. Hofman, Uptake kinetics of pesticides chlorpyrifos and tebuconazole in the earthworm *Eisenia andrei* in two different soils. *Environ. Pollut.*, 236 (2018) 236, 257–264.
- [14] H. Uwizeyimana; M. Wang; W. Chen; K. Khan, The eco-toxic effects of pesticide and heavy metal mixtures towards earthworms in soil. *Environ. Toxicol. Pharmacol.*, 55 (2017) 20–29.
- [15] N. A. Andrade; T. Centofanti; L. L. McConnell; C. J. Hapeman; A. Torrents; A. Nguyen; W. N. Beyer; R. L. Chaney; J. M. Novak; M. O. Anderson; K. B. Cantrell, Utilizing thin-film solid-phase extraction to assess the effect of organic carbon amendments on the bioavailability of DDT and dieldrin to earthworms. *Environ. Pollut.*, 185 (2014) 307–313.
- [16] C. L. F. Fernandes; L. M. Volcão; P. F. Ramires; R. R. Moura; F. M. R. Silva-Junior, Distribution of pesticides in agricultural and urban soils of Brazil: A critical Review. *Environ. Sci. Process. Impacts.*, 22 (2020) 256–270.
- [17] A. F. Albuquerque; J. S. Ribeiro; F. Kummrow; A. J. A. Nogueira; C. C. Montagner; G. A. Umbuzeiro, Pesticides in Brazilian freshwaters: A critical review. *Environ. Sci. Process. Impacts.*, 18 (2016) 779–87.
- [18] B. I. Escher; H. M. Stapleton; E. L. Schymanski, Tracking complex mixtures of chemicals in our changing environment. *Science*, 367 (2020) 388–392.
- [19] C. L. F. Fernandes; L. M. Volcão; R. R. Moura; P. F. Ramires; F. M. R. Silva-Júnior, Which pesticides are contaminating a brazilian soils? *Res. Soc. Dev.*, 9 (2020) e114932569.

- [20] Brasil Resolução CONAMA nº 420, de 28 de dezembro de 2009. CONAMA - Cons. Nacional de Meio Ambiente (2009). <http://www2.mma.gov.br/port/conama/legiabre.cfm?codlegi=620>.(accessed on 10 April 2020).
- [21] S. A. Barker; A. R. Long; C. R. Short, Isolation of drug residues from tissues by solid phase dispersion. J. Chromatogr. A., 475 (1989) 353–361.
- [22] R. M. Rebelo; E. D. Caldas, Environmental Risk Assessment of Aquatic Systems Affected By Pesticide Use. Quim. Nova., 37 (2014) 1199–1208.
- [23] IBAMA Boletim Anual de Produção, Importação, Exportação e Vendas de Agrotóxicos no Brasil, Boletim(2019).<https://www.ibama.gov.br/relatorios/quimicos-e-biologicos/relatorios-de-comercializacao-de-agrotoxicos> (accessed on 15 June 2020).
- [24] E. O. Santos; J. O. Gonzales; J. C. Ores; L. C. Marube; S. S. Caldas; E. B. Furlong; E. G. Primel, Sand as a solid support in ultrasound-assisted MSPD: A simple, green and low-cost method for multiresidue pesticide determination in fruits and vegetables. Food Chem., 297 (2019) 124926.
- [25] K. L. Soares; M. B. R. Cerqueira; S. S. Caldas; E. G. Primel. Evaluation of alternative environmentally friendly matrix solid phase dispersion solid supports for the simultaneous extraction of 15 pesticides of different chemical classes from drinking water treatment sludge. Chemosphere., 182 (2017) 547–554.
- [26] SANTE, Analytical quality control and method validation procedures for pesticide residues analysis in food and feed [sante/12682 /2019](https://ec.europa.eu/food/sites/food/files/plant/docs/pesticides_mrl_guidelines_wrkdoc_2019-12682.pdf), (2019). https://ec.europa.eu/food/sites/food/files/plant/docs/pesticides_mrl_guidelines_wrkdoc_2019-12682.pdf (accessed on 20 June 2020).
- [27] M. P. Bag; S. C. Mahapatra; P. S. Rao; D. Chakrabarty; H. Pal, Nutritive potential of earthworm (*Eisenia foetida*) meal in the diet for Nile tilapia (*Oreochromis niloticus*) fingerlings. Int. Res. J. Pharm. Appl. Sci., 2 (2012) 117–123.
- [28] ABNT Ecotoxicologia terrestre — Toxicidade aguda — Método de ensaio com minhocas (Lumbricidae). ABNT NBR 15537 (2014).
- [29] M. Garcia; J. Römbke; M. T. Brito; A. Scheffczyk, Effects of three pesticides on the avoidance behavior of earthworms in laboratory tests performed under temperate and tropical conditions. Environ. Pollut., 153 (2008) 450–456.
- [30] INMETRO, ORIENTAÇÃO SOBRE VALIDAÇÃO DE MÉTODOS ANALÍTICOS DOQ-CGCRE-008.(2020).http://www.inmetro.gov.br/Sidoq/Arquivos/CGCRE/DOQ/DOQ-CGCRE-8_05.pdf. (accessed on 10 April 2020).
- [31] C. F. Poole, Matrix-induced response enhancement in pesticide residue analysis by gas chromatography. J. Chromatogr. A., 1158 (2007) 241–250.
- [32] S. Moldoveanu; V. David. " Mobile Phases and Their Properties " in Essentials in Modern HPLC Separations, Elsevier, 2013.
- [33] D. Wianowska; M. Gil, New insights into the application of MSPD in various fields of analytical chemistry. TrAC - Trends Anal. Chem., 112 (2019) 29–51.
- [34] M. R. R. Souza; C. O. Moreira; T. G. Lima; A. Aquino; H. S. Dórea, Validation of a matrix solid phase dispersion (MSPD) technique for determination of pesticides in lyophilized eggs of the chicken *Gallus gallus domesticus*. Microchem. J., 110 (2013) 395–401.
- [35] R. Weng; S. Lou; X. Pang; Y. Song; X. Su; Z. Xiao; J. Qiu, Multi-residue analysis of 126 pesticides in chicken muscle by ultra-high-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry. Food Chem., 309 (2019) 125503.
- [36] A. A. El Megid; M. E. Abd Al Fatah; A. El Asely; Y. El Senosi; M. M. A. Moustafa; M. A. O. Dawood, Impact of pyrethroids and organochlorine pesticides residue on IGF-1 and CYP1A genes expression and muscle protein patterns of cultured *Mugil capito*. Ecotoxicol. Environ. Saf., 188 (2020) 109876.
- [37] C. Torres-Perea; D. Muñoz-Rodríguez; S. Medina-Peralta; C. Carrera-Figueiras; Y. B. Moguel-Ordóñez, Recovery evaluation of organophosphorus pesticides from bee pollen by matrix solid-phase dispersion extraction using sorbents based on silica and titania. IOP Conf. Ser. Mater. Sci. Eng., 45 (2013) 01.

- [38] Y. Cao; H. Tang; D. Chen; L. Li, A novel method based on MSPD for simultaneous determination of 16 pesticide residues in tea by LC-MS/MS. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.*, 998 (2015) 72–79.
- [39] B. Lozowicka; E. Rutkowska; I. Hrynko, Simultaneous determination of 223 pesticides in tobacco by GC with simultaneous electron capture and nitrogen-phosphorous detection and mass spectrometric confirmation. *Open Chem.*, 13 (2015) 1137–1149.
- [40] A. I. García-Valcárcel; E. Molero; J. L. Tadeo; M. D. Hernando, Determination of selected environmental contaminants in foraging honeybees. *Talanta* 148 (2016) 1–6.
- [41] S. A. Barker, Matrix solid phase dispersion (MSPD). *J. Biochem. Biophys. Methods.*, 70 (2007) 151–162.
- [42] A. L. Capriotti; C. Cavaliere; P. Giansanti; R. Gubbiotti; R. Samperi; A. Laganà. Recent developments in matrix solid-phase dispersion extraction. *J. Chromatogr. A* 1217 (2010) 2521–2532.
- [43] M. Kemmerich; M. Demarco; G. Bernardi; O. D. B. Prestes; M. Adaime; R. Zanella, Balls-in-tube matrix solid phase dispersion (BiT-MSPD): An innovative and simplified technique for multiresidue determination of pesticides in fruit samples. *J. Chromatogr., A* 1612 (2020).
- [44] M. Li; Q. Sun; Y. Li; M. Lv; L. Lin; Y. Wu; M. Ashfaq; C. Yu, Simultaneous analysis of 45 pharmaceuticals and personal care products in sludge by matrix solid-phase dispersion and liquid chromatography tandem mass spectrometry. *Anal. Bioanal. Chem.*, 408 (2016) 4953–4964.
- [45] P. Zhao; J. Zhao; S. Lei; X. Guo; L. Zhao, Simultaneous enantiomeric analysis of eight pesticides in soils and river sediments by chiral liquid chromatography-tandem mass spectrometry. *Chemosphere.*, 204 (2018) 210–219.
- [46] B. Lozowicka; G. Ilyasova; P. Kaczynski; M. Jankowska; E. Rutkowska; I. Hrynko; O. Mojsak; J. Szabunk, Multi-residue methods for the determination of over four hundred pesticides in solid and liquid high sucrose content matrices by tandem mass spectrometry coupled with gas and liquid chromatograph. *Talanta.*, 151 (2016) 51–61.
- [47] A. Economou; H. Botitsi; S. Antoniou; D. Tsiipi, Determination of multi-class pesticides in wines by solid-phase extraction and liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A.*, 1216 (2009) 5856–5867.
- [48] Z. Li; A. Jennings, Worldwide regulations of standard values of pesticides for human health risk control: A review. *Int. J. Environ. Res. Public Health.*, 14 (2017) E826.
- [49] L. Polese; E. F. G. C. Dores; E. F. G. Jardim; S. Navickiene; M. L. Ribeiro, Determination of herbicides residues in soil by small scale extraction. *Eclet. Quim.*, 2 (2002) 00.
- [50] M. Tankiewicz; J. Fenik; M. Biziuk, Solventless and solvent-minimized sample preparation techniques for determining currently used pesticides in water samples: A review. *Talanta.*, 86 (2011) 8–22.
- [51] A. Samsidar; S. Siddiquee; S. M. Shaarani, A review of extraction, analytical and advanced methods for determination of pesticides in environment and foodstuffs. *Trends Food Sci. Technol.*, 71 (2018) 11.
- [52] O.M. Brilhante; R. Franco, Exposure pathways to HCH and DDT in Cidade dos Meninos and its surrounding districts of Amapa, Figueiras and Pilar, metropolitan regions of Rio de Janeiro, Brazil. *Int. J. Environ. Health Res.*, 3 (2006) 205-17.