Validation of a microwave-assisted micellar extraction method for the oxibendazole determination in soil samples

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Validación de un método de extracción micelar en microondas para la determinación de oxibendazole en muestras de suelo

Validació d'un mètode d'extracció micel·lar en microones per a la determinació d'oxibendazole en mostres de sòl

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RESUMEN

El oxibendazol es un fármaco veterinario utilizado como antihelmíntico para animales domésticos y de granja. Dicho fármaco puede introducirse en el medio bien directamente, a través de las heces o la orina, o bien indirectamente, cuando se utilizan residuos ganaderos como enmienda orgánica sobre suelos agrícolas. En este trabajo se ha desarrollado un método sencillo, rápido, fiable y sensible basado en el proceso de extracción micelar en microondas (MAME), usando Genapol X-080 (0.5%) como surfactante, para la cuantificación de oxibendazol en muestras de suelo. Dos métodos clásicos basados en agitación mecánica y por ultrasonidos seguidos de una extracción en fase sólida, fueron comparados con el proceso MAME. El oxibendazol fue determinado mediante cromatografía liquida de alta eficacia (HPLC) con detector de fluorescencia. En la optimización del proceso MAME se evaluaron cuatro parámetros: volumen de surfactante, agitación, potencia de irradiación y tiempo de extracción. Las condiciones óptimas fueron 20 ml de Genapol X-080 (0.5%), potencia de irradiación, 1000 W, tiempo de extracción, 2 minutos y agitación. En estas condiciones del proceso MAME se obtuvieron buenas recuperaciones (alrededor de 90%), significativamente superiores a las encontradas por el procedimiento clásico (por debajo de 55%). El método se aplicó a dos suelos de Madrid (centro de España). Los resultados confirmaron que las propiedades físico-químicas del suelo influyen decisivamente en la eficacia de la extracción.

Palabras clave: benzimidazoles, antihelmínticos, MAME, suelo, contaminantes emergentes.

SUMMARY

Oxibendazole is a veterinary drug used as an antihelmintic for companion and farm animals. This drug may enter into the environment directly, via excretion of faeces and urine by grazing animals, or indirectly, when manure of treated farm animals are used as soil organic amendment. In this work, a simple, fast, reliable and sensitive method based on microwave-assisted micellar extraction (MAME) procedure, using Genapol X-080 (0.5%) as surfactant, has been developed for the quantification of oxibendazole in soil samples. Two classical methods, based on mechanical shaking and ultrasonic assisted extraction followed by solid phase extraction, were compared with the MAME procedure. Oxibendazole was determined by high-performance liquid chromatography (HPLC) with fluorescence detector. In the optimization of the MAME procedure were evaluated four parameters: surfactant volume, stirring, irradiation power and extraction time. Optimum conditions were 20 ml of Genapol X-080 (0.5%), irradiation power of 1000 W, extraction time of 2 minutes and stirring. At these MAME conditions, good recoveries (around 90%) were obtained, significantly higher than those found by classical method (below 55%). Two different soils from Madrid (central Spain) were included in the study. Results confirmed the extraction effectiveness is strongly influenced by physico-chemical soil properties.

Keywords: benzimidazoles, antihelmintics, MAME, soil, emerging pollutant.

RESUM

L'oxibendazol és un fàrmac veterinari utilitzat com antihelmíntic per animals domèstics i de granja. Aquest fàrmac pot introduir-se en el medi bé directament, a través de la femta o l'orina, o bé indirectament, quan s'utilitzen residus ramaders com esmena orgànica sobre sòls agrícoles. En

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aquest treball s'ha desenvolupat un mètode senzill, ràpid, fiable i sensible basat en el procés d'extracció micel·lar en microones (MAME), usant Genapol X-080 (0.5%) com tensioactiu, per a la quantificació d'oxibendazol en mostres de sòl. Dos mètodes clàssics basats en agitació mecànica i ultrasons seguits d'una extracció en fase sòlida, van ser comparats amb el procés MAME. L'oxibendazol va ser determinat mitjançant cromatografia líquida d'alta eficàcia (HPLC) amb detector de fluorescència. A la optimització del procés MAME es van avaluar quatre paràmetres: volum de tensioactiu, agitació, potència d'irradiació i temps d'extracció. Les condicions òptimes van ser 20 ml de Genapol X-080 (0.5%), potència d'irradiació, 1000 W, temps d'extracció, 2 minuts i agitació. En aquestes condicions del procés MAME es van obtenir bones recuperacions (al voltant de 90%), significativament superiors a les que es troben amb el procediment clàssic (per sota de 55%). El mètode es va aplicar a dos sòls de Madrid (centre d'Espanya). Els resultats van confirmar que les propietats fisicoquímiques del sòl influeixen decisivament en l'eficàcia de l'extracció.

Paraules clau: benzimidazols, antihelmíntics, MAME, sòl, contaminants emergents.

INTRODUCTION

Veterinary medicines are widely used in treating cattle and can be released into the environment, either directly in faeces or urine or indirectly after the application of the manure as organic fertilizer. Numerous veterinary medicines such as hormones, antibiotics and antiparasitics have been detected in soil, surface water and groundwater [1-3]. As emerging contaminants, the faeces-borne drugs are suspected of causing adverse effects in both humans and wildlife. It is therefore necessary to develop analytical methods to determine drugs in environmental samples. In the past few years, several studies on the potential impact of veterinary medicines on the environment and on animal health have been conducted [4,5].

Benzimidazoles (mebendazole, fenbendazole, oxfendazole, oxibendazole, albendazole and triclabendazole) are one of the main groups of antihelmintics used clinically because of their broad spectrum of activity, wide safety margin and effectiveness against adults, larvae and eggs [6]. All benzimidazoles share the same central structure with 1,2-diaminobezene. Oxibendazole (Figure 1) is of particular interest since its effectiveness against certain gastrointestinal parasites that are resistant to other benzimidazoles [7]. After administration, benzimidazoles are absorbed into the bloodstream and transported to different parts of the body [6]. Then, benzimidazoles can be partially metabolized and excreted through faeces and urine. In the particular case of oxibendazole, the most persistent metabolite is the parent drug [8,9]. Gokbulut et al. [8] detected oxibendazole in horse faeces between 12 and 72 hours after administration. The peak concentration of dry faecal was detected approximately after 24 hours (0.53 mg/g). According to Danaher et al. [9], the withdrawal period for oxibendazole is 7 days in pig, indicating that it is one of the least persistent benzimidazole residues.

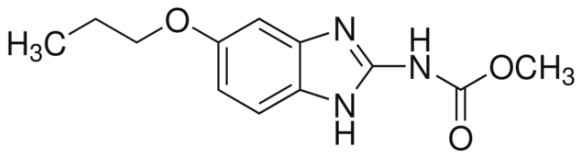

Figure 1. Chemical structure of oxibendazole.

It is necessary to develop new analytical methods to detect veterinary medicines at low concentrations and to estimate their environmental impact. In general, the extraction of veterinary medicines in soil samples is based on classical processes of shaking with organic solvents followed by an additional cleanup step through a solid phase extraction (SPE) column [3,4,10]. Soxhlet extraction is another method frequently used to extract organic pollutants from soil samples; the major disadvantages are large volumes of organic solvent usage (300-500 ml) and long extraction times (up to 24-48 hours) [11-12]. In microwave-assisted extraction (MAE), the soil is treated with an organic solvent and heated with microwave energy. MAE has been applied to extract different organic compounds from soil samples. The extraction efficiency is comparable to that of the classical techniques, such as soxhlet, ultrasonic assisted extraction or liquid-liquid extraction and other more recent ones such as SFE (supercritical fluid extraction) or PLE (pressurized liquid extraction) [11-14]. The use of micellar media combined with the microwave energy, called microwave-assisted micellar extraction (MAME), offers advantages such as low toxicity, low cost, and friendly to the environment [11,12,15]. MAME has been applied successfully to extract organic contaminants such as pesticides [12,16], PAHs [17], PCBs [18], and pharmaceuticals [19] from soil and sediment samples.

To our knowledge, there are not data related with determination of oxibendazole in soil samples. A MAME method for determination of two benzimidazole fungicides chemically similar to oxibendazole has been developed in sandy soil samples [12]. The aim of this study was to develop and validate a simple method for the extraction of oxibendazole in two soil samples with different characteristics using MAME technology followed by liquid chromatographic determination.

MATERIAL AND METHODS

Chemical

Oxibendazole (Dr. Ehrenstorfer, Augsburg, Germany) was used as the standard reference material (purity = 98.5%) (see Figure 1 for structure). A stock solution of oxibendazole at concentration of 125 µg/ml and prepared in methanol was stored at -18 ºC. Working standard solutions at 12.5 µg/ml was prepared from stock solution by adding acetonitrile. The non-ionic surfactant Genapol X-080 (Oligoethylene glycol monoalkyl ether) was obtained from Fluka (Sigma-Aldrich Chemie, Steinheim, Germany)**.** Acetonitrile and methanol were of HPLC grade and obtained from Sigma-Aldrich Chemie. Ammonium acetate (analytical grade) was from Panreac (Barcelona, Spain). Water was purified with a Milli-Q system (Millipore, Bedford, MA). C18 (500 mg, 6 ml) and Strata-X polymeric (500 mg, 6 ml) SPE cartridges from Phenomenex (Torrance, CA) were used for the cleanup procedure.

Table 1. Physico-chemical Soil Properties (0-30 cm) Alcalá de Henares (Soil A) and Aranjuez (Soil B).

parameter	Soil A	Soil B
pH	7.5	7.3
EC (dS/m)	0.20	0.7
Organic matter (%)	1.0	3.6
Total nitrogen (%)	0.1	0.2
Carbonates (%)	2.1	<2
Phosphorus (mg/kg)	19	450
Ca (mg/kg)	2807	4252
Mg (mg/kg)	100	318
Na (mg/kg)	9.8	174
K (mg/kg)	368	1199
Total sand (%)	44.7	45.8
Total silt (%)	37.6	28.7
Clay (%)	17.7	25.5
Texture class, (USDA 1994)	Loam	Clay sandy loam

Procedures

An extraction method of oxibendazole from soil samples is presented. Bulk soil samples were collected from the surface layer (0–30 cm depth) of two agricultural areas, Alcalá de Henares (soil A) and Aranjuez (soil B), both sites in central Spain (Madrid). Soil samples were air-dried, sieved (<2mm) and analyzed, according to Spanish official methodology for soil analysis [20]. In brief, electric conductivity (EC) and pH were measured in 1:2.5 soil-to-water ratio; organic matter and total nitrogen content were determined using the Walkley–Black and Kjeldahl methods, respectively; the percentage of carbonates was measured using a calcimeter; available phosphorus was evaluated using sodium bicarbonate at pH 8.5; available nutrients were extracted with NH_4Ac 0.1 N and assessed using atomic absorption spectrometry (AA 240 FS, Varian, Victoria, Australia); soil texture was analyzed using a Bouyoucos densimeter. Characteristics of the two studied soils are shown in Table 1. Soil A was used to optimize and validate the oxibendazole determination.

Spiking soil. Soil samples (1.0 g) were placed in the extraction vessels and spiked with oxibendazole working solution (12.5 µg/ml) to reach concentrations of 0.4 or 0.8 µg/g. Samples were shaken and stored in dark at room temperature for 24 hours.

Classical extractions. Two extraction methods, two SPE cartridges (C18 and Strata-X polymeric) and two solvents were evaluated. Soil samples (1.0 g) were placed in 50 ml centrifuge tubes, with 5 ml of methanol or acetonitrile. Samples were mechanically shaken for 30 minutes or shaken in ultrasonic water bath for 20 minutes. Then, the extracts were centrifuged at 13750*g* (15ºC, 15 min). After collecting the supernatant, a second extraction was carried out at the same conditions. The supernatants from both extractions were mixed and brought up 40 ml with Milli-Q water. The mixture was passed through the SPE cartridge previously conditioned with 5 ml of methanol or acetonitrile and followed by 5 ml of methanol-water or acetonitrile-water (30:70, v/v), according the solvent extraction used. After loading the extract, the cartridge was washed with 5 ml of the organic solvent-water (30:70, v/v) solution and dried under vacuum for 15 min. The analyte was eluted with 6 ml of methanol or acetonitrile and evaporated to dryness under a stream of nitrogen at 45ºC using a Liebisch evaporator (Labortechnik, Bielefeld, Germany). The residue was dissolved in 1 ml of methanol or acetonitrile and 10 µl was injected on the HPLC system according to the conditions described below. Three replicates were performed for each sample.

Table 2. *Summary of the tested conditions to optimize the MAME process.*

Surfactant volume (ml)	Stirring	Irradiation power (W)	Extraction time (min)
10	No	600	2
		800	2
		1000	2
20	No	600	2
		800	2
		1000	2
20	Yes	600	2
		800	2
		1000	2
20	Yes	600	4
		800	4
		1000	4
20	Yes	600	8
		800	8
		1000	8

MAME. Spiked soil samples (1.0 g) were mixed with 20 ml of Genapol X-080 (0.5%). Surfactant concentration was selected according to Halko et al. [12]. In that work MAME was applied for benomyl and carbendazim, two benzimidazole fungicides chemically very similar to oxibendazole. The vessels were introduced into a microwave Multiwave 3000 (Anton Paar, Graz, Austria) equipped with a rotor XF100-8 of eight vessels, and optional agitation during microwave heating. The microwave conditions were chosen according to the experiment to be performed. They are summarized in Table 2. After the MAME procedure, the vessels were allowed to cool at room temperature, transferred to 50 ml centrifuge tubes and centrifuged at 13750*g* for 10 min at 15 ºC with a Beckman (Fullerton, CA) centrifuge (J2-21). Finally, the supernatant was brought up to 20 ml, transferred to a glass vial and injected into the HPLC system (Milford, MA) equipped with the following components: a gradient pump (600 Controller) with a degasser, a 717 plus autosampler, a column heater, and a fluorescence detector model 2475Multi λ. An aliquot of 10 µl of the micellar extract was injected on a Phenomenex Luna C18 (2) column (150 x 4.6 mm i.d.; 5 µm particle size) with a Phenomenex C18 precolumn (4.0 x 3.0 mm i.d.; 5 µm particle size). The mobile phase consisted of acetonitrile - ammonium acetate 0.025 M, pH 6.6 (50:50, v/v) and was pumped at rate of 1 ml/min. The column temperature was maintained at 35 ºC. The fluorescence detector was set at an excitation wavelength of 280 nm and an emission wavelength of 320 nm. Samples of soil A and B without oxibendazole were analyzed. No interference was detected.

Statistics

The data analyses were performed using the statistical package Statgraphics Plus, release 5.0 (Manugistics, Maryland, USA).

RESULTS AND DISCUSSION

It is necessary to know the analyte characteristics before designing an extraction procedure. Information about the physical and chemical properties of benzimidazo-

les can be found in bibliographic database or by simple calculations, however there are available few data about chemical properties of oxibendazole. Properties such us octanol-water partition coefficients and pK_a values provide important information regarding solubility and ion exchange properties, and give an useful indication for liquid chromatography separation. The octanol-water partition coefficient and pK_a values were calculated by Danaher et al. [9]. Oxibendazole exists in a non-ionized form at pH values higher than 8.3 and has a K_{ow} in the range of 1.86-2.63. In general, mass transfer and matrix effects are the most influential factors in extraction of organic compounds from soil samples [21]. Regarding oxibendazole solubility, it is insoluble in water and has a limited solubility in methanol and acetonitrile (below 150 mg/l in methanol and 15 mg/l in acetonitrile according our previous lab experiences).

Oxibendazole determination. Optimization of the MAME.

A loam soil from Alcalá de Henares (soil A), was used in the optimization study (Table 1). Classical extraction methods were previously tested. The assayed methods included a first extraction step with methanol or acetonitrile under mechanical or ultrasonic shaking, followed by SPE using a C18 or a polymeric cartridge. Extraction recoveries are shown in Table 3. Total recoveries were less than 55%.

Table 3. Mean recoveries (%) of oxibendazole in soil samples using shaking and ultrasonic assisted extraction methods at a concentration of 0.8 µg/g (n=3).

Extraction method	Solvent	SPE cartridge	Recovery (%)
Mechanical shaking 20 min	Methanol	C18	43±3
		Strata polymeric	50±5
Ultrasonic 20 min	Methanol	C18	55±6
		Strata polymeric	46±4
	Acetonitrile	C18	47±5

In view of the poor recoveries acquired, a method based on MAME technology is presented to determine oxibendazole in soil samples. Genapol X-080 was added as surfactant; its concentration was selected according to Halko et al. [12]. The authors found recoveries close to 100% for benomyl and carbendazim, two benzimidazole fungicides chemically very similar to oxibendazole, in sandy soil samples. Four variables were considered in the MAME optimization process: surfactant volume, stirring, irradiation power and extraction time. Table 2 shows a summary of the tested conditions to optimize the MAME process. Firstly, the surfactant volume was evaluated. The volume must be sufficient to ensure that the entire sample is immersed, and the proportion of soil sample in the extraction solution should not exceed 30-34% (w/v) soil/solvent ration [11]. In the present study, soil (1 g) was mixed with 10 or 20 ml of Genapol X-080 (0.5%). The oxibendazole recovery at 0.4 µg/g was studied at three irradiation power levels (600, 800 and 1000 W) for two minutes. Results are shown in Figure 2.

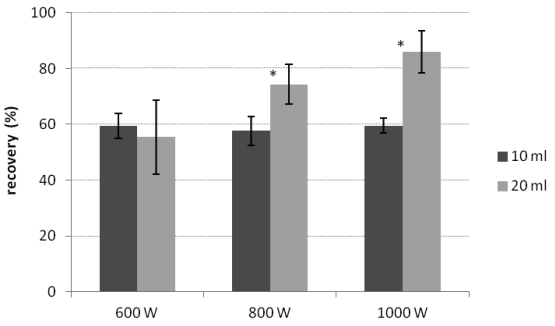

*Figure 2. Recoveries (%) for oxibendazole in soil samples (0.4 µg/g) obtained by MAME at three irradiation powers for two minutes using 10 and 20 ml of Genapol X-80 0.5%. * Significant differences at p<0.05 (n=6).*

At power of 600 W, the recovery values with surfactant volumes of 10 and 20 ml were similar and less than 60%. However, recoveries increased significantly ($p<0.05$) by raising the power at 800 and 1000 W in a surfactant volume of 20 ml. Twenty millilitres of Genapol X-080 (0.5%) were chosen as optimum. Secondly, the stirring influence was studied during the microwave extraction. The oxibendazole recovery at a theoretical concentration of 0.4 µg/g, was compared at three levels of irradiation power (600, 800 and 1000 W), for two minutes with or without stirring. Results are shown in Figure 3. The best recovery percentages (86.0-95.1%) ($p<0.05$) were obtained when soil samples were stirred during MAME process, which may be because of stirring improves the mass transfer. Therefore, sample stirring during MAME process was considered necessary.

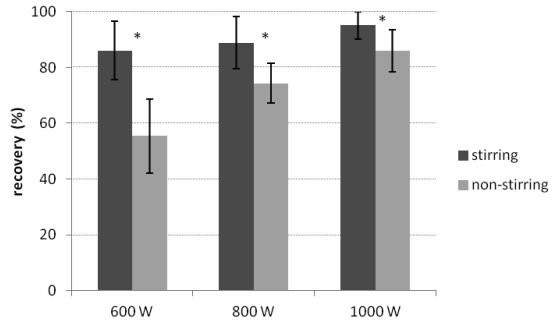

*Figure 3. Recoveries (%) for oxibendazole in soil samples (0.4 µg/g) obtained by MAME with 20 ml of Genapol X-80 0.5% at three irradiation powers for two minutes with different stirring conditions. * Significant differences at p<0.05 (n=6).*

Finally, irradiation power and extraction time were optimized by applying a 3^2 experimental design at three levels and two factors with four replicates. The irradiation levels were 600, 800 and 1000 W; extraction times were 2, 4 and 8 minutes. The experimental design allows to estimate the influence of two factors simultaneously on the oxibendazole recovery. Two concentration levels (0.4 and 0.8 µg/g) were studied. Figure 4 shows the response surface for oxibendazole at 0.4 µg/g (a) and at 0.8 µg/g (b). Analogues results were found for the two levels of concentration.

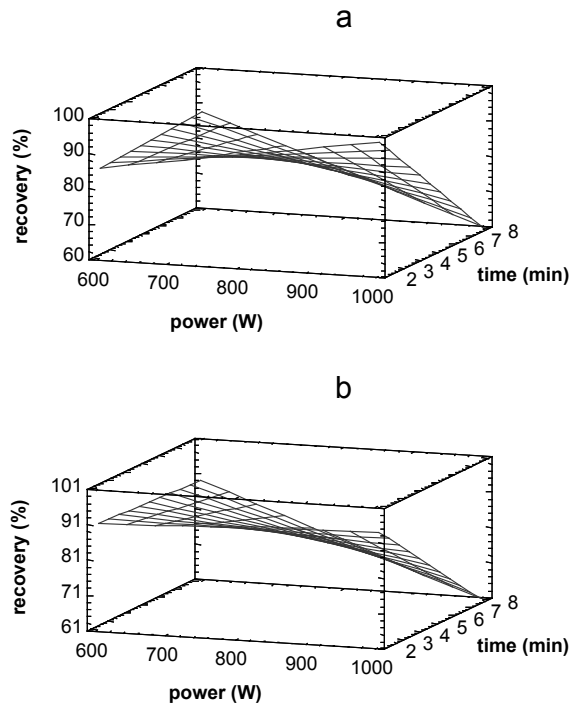

Figure 4. Response surfaces obtained from the experimental design for the effect of microwave power and radiation time on oxibendazole extraction from soil samples at concentrations of (a) 0.4 µg/g and (b) 0.8 µg/g.

The highest recoveries were obtained with high power and short time. Time was the most influential variable in the MAME extraction process. The statistical analysis confirmed that the optimum conditions were irradiation power of 1000 W for 2 minutes. At these conditions, the recoveries obtained with MAME methodology were around 90% (Figure 4), higher than found by classical procedures (below 55%, Table 3). Moreover, since oxibendazole extraction is carried out in only two steps (microwave heating and centrifugation), MAME resulted a faster method than the classical ones. The non-ionic surfactant Genapol X-080 used in the MAME procedure has been shown to be a suitable agent to extract oxibendazole in soil samples, being the optimum conditions 1 g of soil mixed with 20 ml of Genapol X-080 (0.5%), and subjected to 1000 W of irradiation power for two minutes with stirring.

The optimum conditions obtained for the MAME procedure were applied to extract oxibendazole in a clay sandy loam soil (soil B, Table 1) at two concentrations (0.8 and 2 µg/g). Mean recoveries were 72.4 and 71.0%, respectively. Although the recovery percentages for oxibendazole in soil B were satisfactory, in general, they were lower than found for soil A (above 90%). It may be due to the different soil characteristics. Soil B showed higher organic matter and clay content than soil A, thus, analyte–matrix interactions might increase and desorption and mass transfer decrease. These results highlight that the effectiveness of the oxibendazole extraction is strongly influenced by the physico-chemical soil properties. In this sense, soil A, a loam soil with 1% of organic matter showed recovery percentages above 90%, and soil B, a clay sandy loam with 3.6% of organic matter, showed recoveries above 70%. Similar result were found by Halko et al. [12] in a study about the determination of benzimidazole fungicides in three sandy soils using MAME procedure. The effectiveness of MAME extraction went down if the organic matter content in soil rose. In particular, benzimidazole recoveries of 55-98% were obtained in soils which presented organic matter percentages less than 4%, however, when the organic matter in soil increased to 12.5%, the recoveries decreased to 37-76%.

Analytical characteristics of the method

Linear regression was applied to obtain oxibendazole external calibration plot. The linearity was proven using seven calibration plots ranging from 0.02 to 2.2 µg/ml in Genapol X-080 at 0.5%. The calibration curve showed good linearity, with a coefficient R^2 of 0.999. The limit of detection (LOD) of oxibendazole in the HPLC system was calculated by injecting solutions containing progressively smaller amounts of oxibendazole and was found to be 0.05 ng [22]. In addition, the LOD in samples was calculated as 3 times the signal-to-noise ratio (LOD = 3 S/N) and was found to be 0.10 µg/g for soil samples (dry samples). The limit of quantification (LOQ = 10 S/N) was found to be 0.33 µg/g.

Table 4. Analytical precision and accuracy of oxibendazole determination in soil samples.

Parameter (%)	Concentration levels (µg/g)		
	0.4	0.8	2.0
Recovery	95.1	90.3	87.1
RSD intra-day (n=6)	5.2	7.7	2.9
RSD inter-day (n=5)	5.8	7.0	7.0

To evaluate the accuracy of the extraction and chromatography procedures, the recoveries from blank soil samples spiked with oxibendazole at three levels (0.4, 0.8, and 2.0 µg/g (n = 6)) were determined (intra-day analysis). A oxibendazole/acetonitrile solution at 12.5 µg/ml was used to spike the blank samples. The samples were in contact with the solution for 24 hours before the MAME process was performed. The mean recoveries were in the range 87.1 to 95.1% (Table 4), with RSD (relative standard deviation) lower than 8% (n=6). The inter-day precision was evaluated over five consecutive working days, determining the recovery at the same three levels. The inter-day precision was in the range 5.8-7.0%, expressed as RSD (%). The developed method showed low detection limit, good recoveries, and inter-day and intra-day precision RSD values lower than 8%; therefore, this extraction method resulted analytically satisfactory and it could be applicable to the oxibendazole determination in soil samples.

CONCLUSIONS

The combination of MAME methodology and HPLC with florescence detection has allowed to develop a simple, fast, reliable and sensitive analytical method to determine oxibendazole in soil samples, resulting an alternative to the classical extractions with organic solvents. The optimum microwave conditions obtained after the experimental design were 1000 W of irradiation power for two minutes with stirring. At these conditions, high extraction efficiencies (around 90%) were obtained for oxibendazole

at the two fortification levels evaluated. The effectiveness of the extraction was strongly influenced by the physico-chemical soil properties.

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