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Optimization and Validation of an Extraction Method for Endosulfan Lactone on a Solid Substrate

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Abstract: Endosulfan lactone is a metabolite obtained from the biological oxidation of the insecticide endosulfan by action of the microorganisms present in the soil. This metabolite is more toxic and persistent than the parent compound. Therefore, it is extremely important to be able to determine the presence of this metabolite in the soil. However, accessible methods for extraction of endosulfan lactone in soil were not found in published literature. For this reason, the aim of this study was to evaluate two conventional methods of liquid–solid extraction for the determination of endosulfan lactone in solid substrate using two solvents (ethyl acetate and acetonitrile) and HPLC UV-VIS. The acetonitrile and rotary agitation extraction method was the one with the highest efficiency (97%), optimized using a factorial 3² response surface design, and validated in terms of linearity and precision. The linearity shown was $r > 0.999$ in a wide spike level (0.15–100 mg kg⁻¹), with the detection limit (DL) of 0.045 mg kg⁻¹ and quantification limit (QL) of 0.15 mg kg⁻¹. The extraction of endosulfan lactone in solid substrate using acetonitrile was more efficient than that used with ethyl acetate, so this method could be used to extract and quantify endosulfan lactone in agricultural soil.

Keywords: endosulfan lactone; HPLC UV-VIS; optimization; response surface; solvent extraction; ultrasound

1. Introduction

Organochlorine (OC) insecticides are synthetic broad-spectrum chemical compounds used in agriculture. Their chemical structure confers a high physical and chemical stability that allows them to be non-volatile, insoluble in water, highly soluble in fats, and with slow biodegradability, thus favoring their bioaccumulation in living beings and persistence in the environment due to their average life of 5 to 30 years [1].

Endosulfan (C₉H₆Cl₆O₃S) is a synthetic OC insecticide widely used in agricultural crops [2]. However, currently, the concern over its use and accumulation has been increasing worldwide because of its residual nature, high level of toxicity, persistence, long distance transport, and bioaccumulation in fatty tissues [3]. The main problem of the use of these substances is that, even though in some countries around the world they are banned, they are still used illegally on crops [3]. Further, they are part of the Persistent Organic Compounds (POCs) [4].

This insecticide, through biological oxidation and the oxidative enzymatic processes of the soil, is transformed into endosulfan sulphate, which is later degraded to more polar and

toxic compounds as endosulfan diol ($C_9H_8Cl_6O_2$), endosulfan ether ($C_9H_6Cl_6O$), and endosulfan lactone ($C_9H_4Cl_6O_2$) [3,5], the latter having greater toxicity and bioaccumulation in living beings [6] with an average lethal concentration of 0.004 mg endosulfan lactone/kg for *Eisenia fetida* (California red worm), indicating that the compound is extremely toxic [7]. Thus, it is important to highlight that the monitoring of these compounds, as well as that of persistent and non-persistent insecticides in soil samples, is crucial to protect the lives of humans and other living organisms [8]. However, accessible methods for extraction of endosulfan lactone in soil and solid substrate were not found in published literature [5,9].

Although it is true that there are already sophisticated and expensive methodologies for the extraction and quantification of insecticides in sediments [10] and soil [11–13], the process for insecticide metabolites is still poorly managed. Some of them need very sophisticated special equipment at a high cost and high energy consumption such as using the QuEChERS method [14], plus they require a procedure of purification before being detected by any chromatographic technique [15] and generate even more toxic products than the initial ones.

For this reason, it is necessary to look for more accessible and versatile alternative techniques such as solvent extraction [16] and ultrasound assisted techniques [5,17,18] where the extraction time is short and simple, low-cost equipment is used and the extraction efficiencies reach up to 96% in the soil, creating a more practical procedure [8,16]. Solid-phase extraction is the most commonly used method due to its simplicity, rapidity, and its ability to treat a large volume of samples with high recovery [19].

Furthermore, if a screening design is used, the most important factors that may have an effect on one or more responses of interest can be identified. This will reduce the number of factors to be investigated in further experimentation, in order to eliminate unimportant factors before investing time and money [20]. The screening design has a number of valuable features: it helps to improve the quality control process by determining the upper and lower control limits of a certain variable. Process can be refined by identifying the influencing factors in a less expensive way [21]. Minimizing the number of experiments while maximizing information is the ultimate goal [20,21].

Therefore, the main aim of this study was to evaluate two conventional methods of liquid–solid extraction for the determination of endosulfan lactone in solid substrate, by using a multifactorial ANOVA used a design of screening experiments. Further, the best method was optimized and validated.

For general knowledge, this is the first study which establishes and validates an extraction method for endosulfan lactone in an organic material such as a solid substrate.

2. Materials and Methods

2.1. Solvents and Analytical Standards

Ethyl acetate and acetonitrile were HPLC grade (Fermont, Monterrey, NL, Mexico). Endosulfan lactone (CAS Number 3868-61-9) was used as an analytical standard with purity >99%, provided by Sigma-Aldrich (St. Louis, MO, USA). Primary stock solution of the analyte was prepared in acetonitrile at a concentration of 1000 mg L⁻¹. For the construction of the calibration curve of endosulfan lactone, solutions of 0.15–1.05 mg kg⁻¹ were used; for the validation of the method higher concentrations were included: 0.1–10 mg kg⁻¹ [22] and 10–100 mg kg⁻¹ with a correlation coefficient (R^2) greater than 98%.

2.2. Raw Material

The solid substrate used was composed of 85% peat moss and 15% rabbit manure [23] that was previously dried, ground, and sieved to a particle size of 0.2 and 0.5 mm.

Rabbit manure was collected from a farm located in Tuxtla Gutierrez, Mexico (latitude 16°45'11" N and longitude 93° 06'56" W). The peat moss was a commercial product obtained from Promix Canadian Sphagnum from Quebec, Canada (latitude 52°0'00" N and longitude 72°00'00" W).

The agricultural soil samples used were collected from a superficial layer (0–5 cm) [24] from two places, the greenhouse of the Technological Institute of Tuxtla Gutierrez, Chiapas, Mexico (AS₁) (latitude 16°46′00″ N and longitude 93°05′00″ W), and from Nuevo Mexico, Chiapas, México (AS₂) (latitude 16°46′81″ N and longitude 93°43′81″ W), where endosulfan was used to eliminate screwworm from the corn crop more than 6 years ago.

2.3. Equipment

The following equipment was used: a Cole-Parmer sonicator (40 kHz, 700 kW, Vernon Hills, IL, USA), a Thermo Scientific MaxQ 2000 rotatory shaker (Waltham, MA, USA), an Eppendorf centrifuge 510 R (Hamburg, Germany) and a Flexar liquid chromatograph with autosampler and UV-VIS detector (Perkin Elmer) with a ODS ZORBAX chromatographic column (250 mm × 4.6 mm × 5 µm) (Agilent, Santa Clara, CA, USA).

2.4. Solid Substrate Parameters

The parameters determined to identify the quality of the solid substrate and agricultural soils (AS₁ and AS₂) were the amount of organic matter and carbon to nitrogen (C/N) ratio, based on the methodology described by Walkley and Black [25] and NOM-021-REC/NAT-2000 [24], respectively.

2.5. Analytical Method

2.5.1. Extraction Procedure with Acetonitrile

The method of Li et al. [16] was followed with modifications. Briefly, solid substrate was settled in a 250 mL Erlenmeyer flask where endosulfan lactone and acetonitrile were added, then mixed on a rotatory shaker at 175 rpm, for its subsequent centrifugation at 4000 rpm for 15 min at 20 °C. The supernatant was decanted and left to concentrate on a hood for 24 h up to 1 mL. The concentrate was filtered and analyzed by HPLC UV-VIS. Factors studied are shown in Table 1, keeping constant the amount of endosulfan lactone (1 mg kg⁻¹); results were analyzed by multifactorial ANOVA ($p < 0.05$) by Tukey HSD method in triplicate.

Table 1. Factors studied for acetonitrile extraction.

Solid Substrate (g)	Rotary Agitation Time (min)	Acetonitrile (mL)	Particle Size (mm)
5	60	50	0.2
10	90	75	0.5
15	120	100	—

2.5.2. Extraction Procedure with Ethyl Acetate

The method reported by Tiwari and Guha [5] was followed with minor modifications. Briefly, solid substrate was placed in 50 mL Falcon tube and ethyl acetate was added, along with endosulfan lactone. This mixture was sonicated and centrifuged at 4000 rpm for 15 min and the supernatant was separated to concentrate to 1 mL in a hood then was filtered and analyzed by HPLC UV-VIS. Factors studied are shown in Table 2, keeping constant the amount of endosulfan lactone (1 mg kg⁻¹); results were analyzed by multifactorial ANOVA ($p < 0.05$) by Tukey HSD method in triplicate.

Table 2. Factors studied for ethyl acetate extraction.

Solid Substrate (g)	Sonicated Time (min)	Ethyl Acetate (mL)	Particle Size (mm)
5	10	30	0.2
10	20		
15	30	67.5	0.5

2.5.3. HPLC UV-VIS Conditions

The HPLC UV-VIS analysis was performed on a Flexar chromatograph with acetonitrile-water (80:20, *v/v*) as mobile phase, constant flow of 1 mL min⁻¹, 217 nm as wavelength of maximum absorption and with an injection volume of 20 µL. The run time was 10 min and the retention time of endosulfan lactone was 5.3 min [16].

2.5.4. Experimental Design

The factors studied are found in Tables 1 and 2, with the endosulfan lactone extraction efficiency (%) as the response variable for both methodologies, following a completely randomized screening experiment design, using a multifactorial ANOVA ($p < 0.05$), giving a total of 72 experimental units. Factors that result in higher extraction efficiency are taken into consideration for the optimization and validation process.

2.5.5. Optimization Process

The better extraction procedure was optimized using a full factorial design with three replications and $p < 0.05$. To optimize, a factorial 3² response surface design was used, results were analyzed in statistical software Stat graphics Centurion XVI[®], manufactured in The Plains, Virginia, USA.

2.6. Validation Method

The validation method was performed according to recommendations in the procedure of SANTE/11813/2017 [26]. The obtained optimal conditions to extract endosulfan lactone were used to validate the extraction method. The validation was done using five parameters: the quantification limit (QL), which is the smallest concentration that an analyte can be quantitatively determined to be for a level of confidence of 95% to trace levels (<100 mg kg⁻¹); the detection limit (DL), which is the smallest concentration that can be measured by the analytical equipment; spike level, which is determined based on concentrations that helped build the calibration curve; variation coefficient (<20%) and precision (δ), which is the proximity between magnitude values obtained by replicated measurements, usually denoted by the standard deviation [27]. Precision is evaluated by repeatability, which is the standard deviation obtained analyzing a single sample several times in a short period of time without changing measurement equipment, reagents, or analyst; and the reproducibility, which is the standard deviation obtained analyzing several times the sample on different days, varying conditions such as equipment, reagents, or analyst [28], for this case the analyst changed without significant statistical difference in measurements.

Finally, the method established in this study was adopted to determine endosulfan lactone concentration in agricultural soil (AS₁ and AS₂) where endosulfan was added as an insecticide. The analysis was conducted in triplicate using the same experimental conditions.

3. Results

The chromatographic analysis of endosulfan lactone showed in Figure 1. The retention time was 5.3 min.

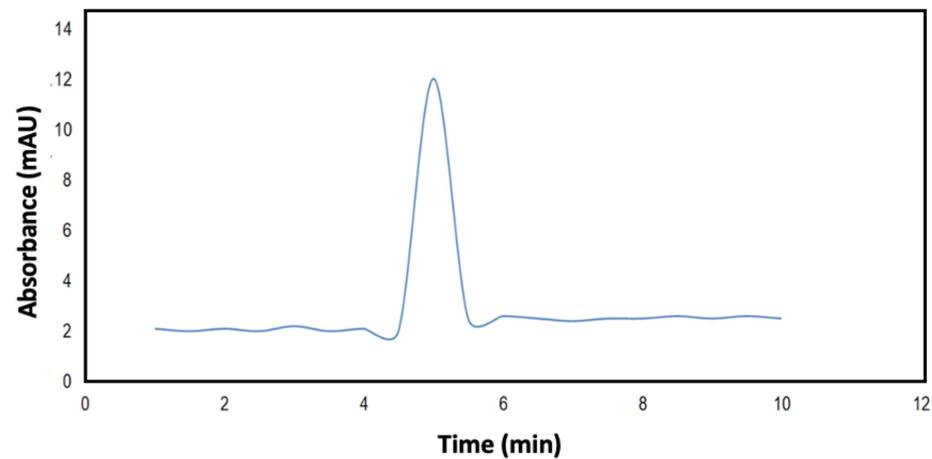


Figure 1. Chromatographic analysis of endosulfan lactone in solid substrate.

3.1. Solvent Extraction

According to the statistical analysis of the acetonitrile method results, quantity of substrate, volume of acetonitrile, particle size, and rotary agitation time had a statistically significant effect on the response variable ($p < 0.05$). The conditions where the best extraction efficiency (50–60%) was reached were 5 g of substrate (Figure 2A), 75–100 mL of acetonitrile (Figure 2B), 0.2 mm particle size (Figure 2C), and 120 min of rotary agitation (Figure 2D).

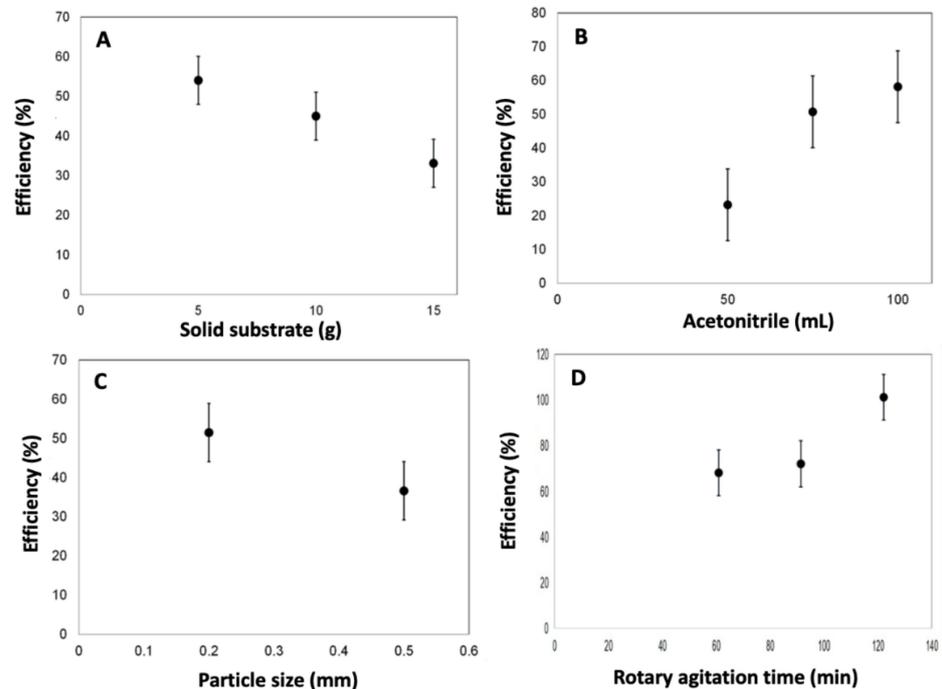


Figure 2. Analysis of means of (A) substrate (g), (B) acetonitrile (mL), (C) particle size (mm), and (D) rotary agitation time (min) in the method of extraction with acetonitrile, scanned with 95% of Tukey HSD.

The method of extraction using ethyl acetate generated an efficiency of 35 to 40% and any factor studied affected on the response variable (Figure 3A–D). Based on the results of both methodologies (acetonitrile and ethyl acetate), the acetonitrile method was used to optimize the response variable, since this obtains higher efficiency extraction.

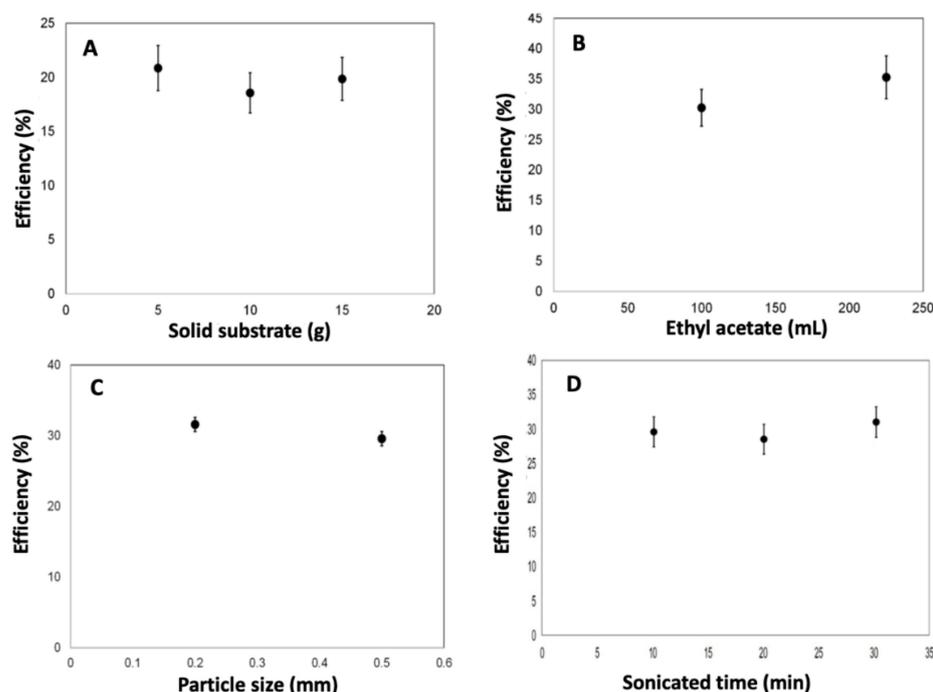


Figure 3. Analysis of means of (A) substrate (g), (B) ethyl acetate (mL), (C) particle size (mm), and (D) sonicated time (min) in the method of extraction with ethyl acetate, scanned with 95% of Tukey HSD.

3.2. Optimization Process

A factorial 3^2 response surface design was used, where the assessed factors were the amount of substrate (5 g, 10 g, and 15 g), the amount of acetonitrile (50 mL, 75 mL, and 100 mL) on the same response variable, since these showed little discrepancy between them. So, both variables were repeated in the optimization process to obtain more accurate data. Conversely, the particle size (0.2 mm) and agitation time (120 min) were keeping constant because of these parameters had a remarkable difference and generated an higher extraction efficiency

Optimal conditions obtained from the analysis in the statistical software were 12 g of substrate, 75 mL of acetonitrile and 77% of extraction efficiency, as can be seen in the response surface chart (Figure 4A) and Pareto chart (Figure 4B); acetonitrile volume has a positive influence on extraction efficiency and solvent volume has significant effect between 50 and 75–100 mL. Furthermore, the negative influence was observed with substrate quantity.

In the Pareto chart (Figure 4B), it is seen that the amount of acetonitrile has a positive influence on the extraction efficiency of the compound of interest contrary to what occurred with the amount of substrate. The Pareto chart provides a graphical representation of the factors influencing the response variable (extraction efficiency), in which the most influential factors are grouped at the top of the list. Factor bar, which overpasses graphically the significance line (red line), exert a statistically significant influence on the result.

The theoretical optimum provided by the Stat Graphics program was replicated in order to obtain the real optimum treatment with 12 g of substrate, 75 mL of acetonitrile, 120 min of rotary agitation, and 1 mg kg⁻¹ of endosulfan lactone with an extraction efficiency of 97%. This efficiency obtained in this study competes with the results of the very sophisticated special equipment of high cost and high energy techniques [14].

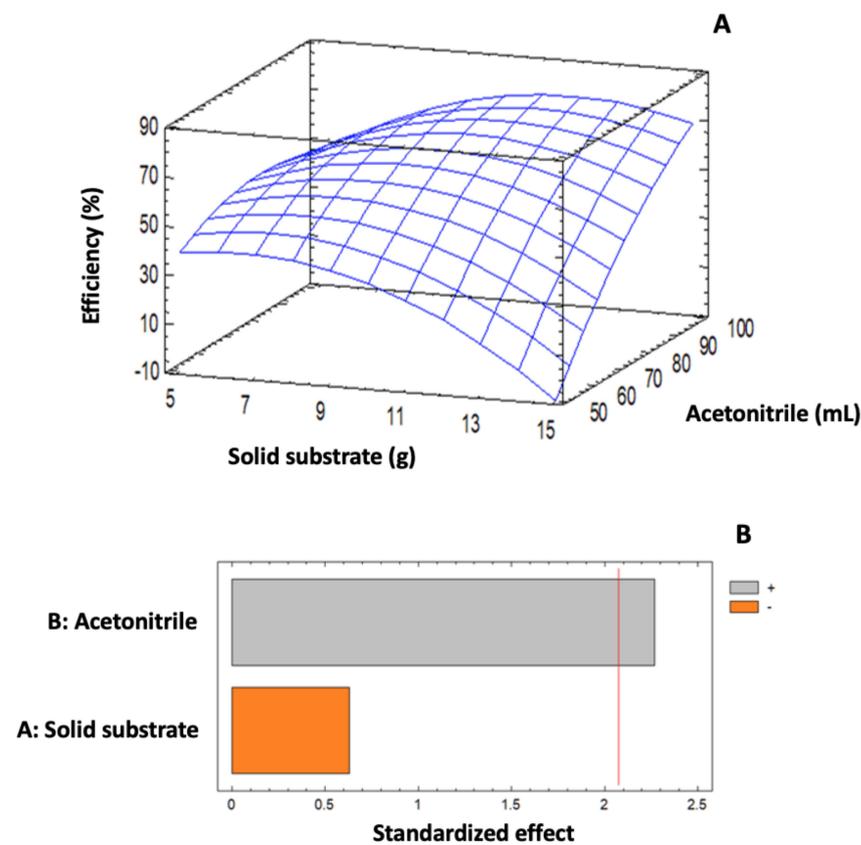


Figure 4. Graphs of response surface (A) surface estimated response, (B) Pareto chart standardized for efficiency (%) where with greater quantity of acetonitrile and least amount of substrate more extraction efficiency was obtained.

3.3. Validation Process

The proposed methodology was validated to ensure the reliability of the method in routine analysis applications, testing on a solid substrate simulating the organic matter and carbon–nitrogen (C/N) conditions of a real agricultural soil (Table 3).

Table 3. Parameters of solid substrate and agricultural soil ($p < 0.05$).

Parameter	Solid Substrate	AS ₁	AS ₂
Organic matter (%)	7.3 ± 0.34	7.6 ± 0.15	7.2 ± 0.28
C/N	14.92	13.51	14.05

The optimum conditions for the extraction of endosulfan lactone in solid substrate using a solid–liquid extraction methodology with acetonitrile were: 12 g of substrate, 75 mL of acetonitrile at 120 min of rotary agitation; the validation results shown in Table 4. It can be observed that the correlation coefficient (R^2) values were higher than 0.999, revealing excellent linearity for all the concentrations studied. Based on Currie [29], the QL is 3.33 times the DL, so, for this case QL is = 0.15 mg kg⁻¹ and DL is = 0.045 mg kg⁻¹. The extraction efficiency was the result of the optimization, given by the statistical program Stat graphics. The precision given by repeatability and reproducibility were the standard deviations of the measurements made with 13 repetitions for better statistical certainty, since for repeatability they were measured by the same equipment, the same day and the same analyst. For reproducibility it was carried out on three different days and by a different analyst.

Table 4. Validation data for the solid–liquid extraction method with acetonitrile, of endosulfan lactone in solid substrate.

Insecticide	Endosulfan Lactone
Linearity	$y = 16,025x$ $R^2 = 0.999$
Spike level (mg kg^{-1})	0.15–100
Recovery or efficiency (%), ($n = 27$)	97
DL (mg kg^{-1})	0.045
QL (mg kg^{-1})	0.15
Repeatability RSDr (%), ($n = 13$)	6.68
Reproducibility RSDr (%), ($n = 3$)	5.15
Variation coefficient (%)	13.77

The validated acetonitrile extraction method was used in agricultural soil (AS₁ and AS₂) exposed six years ago to endosulfan and endosulfan lactone found in a concentration of 0.65 mg kg^{-1} and 0.90 mg kg^{-1} , respectively. This demonstrates that this method can be used to quantify an endosulfan derivate toxic compound.

4. Discussion

The process of extraction with acetonitrile was more efficient than that used with ethyl acetate due to the physical characteristics of these two solvents, i.e., the dielectric constant is higher in acetonitrile to ethyl acetate (37.5 and 6.0, respectively) [30], which makes it a more polar solvent, as endosulfan lactone have a higher affinity for acetonitrile than for ethyl acetate. Both solvents are polar aprotic, i.e., they cannot form hydrogen bonds and contain no acidic hydrogen in their structure, causing them to interact with the endosulfan lactone moiety and forming electrostatic interactions which are stronger with the acetonitrile than ethyl acetate. This effect is called the effect of solvation, which takes place in two phases: (1) the molecules of the solute (endosulfan lactone) are surrounded by the molecules of the solvent (acetonitrile); (2) particles of solute are separated and swept away in the solution, so that, in the process of extraction, once the supernatant is obtained [31] the solvent is evaporated and concentrated, which leaves more endosulfan lactone for subsequent quantification in HPLC UV-VIS.

On the other hand, the quality of the agricultural soils (Table 3) analyzed in this research presented adequate values according to reporting by Vázquez-Villegas [32], which indicate that the soil quality favors greater bacterial proliferation and consequently the decompositions and mineralization [33] of different compounds as endosulfan into more toxic metabolites such as endosulfan lactone, since significant concentrations of this metabolite were found in these soils, indicating so despite the passage of time remaining in high concentration in both agricultural soils.

5. Conclusions

The extraction with acetonitrile and agitation was more efficient than the extraction with ethyl acetate and ultrasound for endosulfan lactone in solid substrate. The rotary agitation time for acetonitrile extraction had slight variations in the efficiency of extraction, so that at 120 min agitation, better extraction efficiency is obtained. This method could be used in the future to evaluate the concentration of endosulfan lactone in different materials with a high content of organic matter. It is suggested that this method be reproduced to extract insecticide metabolites with similar structures in agricultural soil.

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F.A.G.-M., and J.A.M.-M.; Project administration, R.M.-G. and J.J.V.-M.; Resources, P.T.V.-V., R.M.-G., A.C.-S., and J.J.V.-M.; Software, M.C.L.-H., V.M.R.-V., F.A.G.-M., and J.A.M.-M.; Supervision, P.T.V.-V. and R.M.-G.; Validation, M.C.L.-H., V.M.R.-V., F.A.G.-M., and J.A.M.-M.; Writing—original draft, P.T.V.-V. and R.M.-G.; Writing—review and editing, P.T.V.-V., R.M.-G., A.C.-S., and J.J.V.-M. All authors have read and agreed to the published version of the manuscript.

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