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Monitoring Pesticide Residues in Anuran Liver Tissue: A Proposal for the Sample Preparation Method

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In this work, a miniaturized version of the QuEChERS (quick, easy, cheap, effective, rugged, and safe) method was optimized, validated, and applied for extracting eight pesticide residues in anuran liver tissue samples. The extracts were quantified by high-performance liquid chromatography with diode array detection, and the detected residues in liver tissue samples were identified by tandem mass spectrometry-liquid chromatography. The optimized extraction method entailed the scale reduction of the QuEChERS method twenty times using 500 mg of liver tissue sample, 200 mg Mg_2SO_4, 50 mg NaCl, 1.5 mL of acetonitrile, and a mixture of 25 mg C_{18} + 25 mg PSA (primary-secondary amine) as dispersive solid-phase extraction sorbent. The method was validated using liver tissue samples spiked at two levels of pesticide concentrations according to the SANTE/11312/2021 document. Recoveries ranged from 91-110% with a relative standard deviation < 20%. The method robustness assessed by the Steiner and Youden test resulted in recovery rate variations of less than 2% for all pesticides after deliberated changes in seven variables. The validated method was applied to 72 liver tissue samples from two anuran species, registering residues of some pesticides in 31 samples. The proposed method proved to be efficient, precise, and robust for determining pesticide residues in anuran liver tissues.

Keywords: QuEChERS, HPLC, extraction, *L. macrosternum*, *Scinax x-signatus*

Introduction

Over the last 40 years, Brazil has continuously expanded its agricultural frontiers, advancing through different biomes, and reflecting an exponential increase in agricultural production.[1,2] However, high productivity rates are associated with the intense use of pesticides in crops, making Brazil the largest consumer of pesticides in the world since 2008.[3] One of the most recent agricultural frontiers is in the Baixo Jaguaribe, Ceará region, whose biome is the Caatinga, and which has been taken over by fruit growing and, more recently, by soybean and cotton cultivation.[4,5] With the intense use of pesticides in the region, environmental contamination problems have been reported, putting human health and non-target organisms at risk.[5,6]

Anurans are among the vertebrate groups most threatened by the impacts of agricultural intensification.[7,8] These amphibians, popularly known as toads, frogs, and tree frogs, inhabit terrestrial and aquatic environments and are sensitive to water quality and the availability of microhabitats. Habitat loss and the presence of toxic chemical residues are two of the major causes of the global decline in their populations.[9,10] Several reports[10-13] associate pesticides with reproductive dysfunction and abnormal development in anurans through interference with the endocrine system. Contamination of anurans by pesticides can be assessed by determining pesticide residues in their tissues. The liver is the largest organ in anurans and has the highest potential for retaining residues of pesticides absorbed through food.[13-15]

The most challenging analytical step in determining pesticide residues in anuran tissues is sample preparation due to the small mass of liver tissue and the need for efficient and selective isolation of pesticide residues from

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the anuran tissue matrix.[16-18] Few anuran tissue sample preparation methods are reported in the literature and present scarce information on the efficiency, precision, and robustness in the extraction of pesticide residues.[16-23]

QuEChERS (quick, easy, cheap, effective, rugged, and safe) is a sample preparation method that has been efficiently and robustly applied to the extraction of pesticide residues from different types of matrices, from highly aqueous to highly greasy, without substantially modifying the procedure proposed in 2003.[24-28] Furthermore, the QuEChERS method is not only straightforward and cost-effective but also employs readily available materials and equipment. Such a remarkable simplicity enables its execution in any analytical laboratory, even by less experienced analysts. The original QuEChERS method uses a sample mass of 10 g, requiring adaptation for smaller sample sizes, such as anuran liver tissues.[23] The miniaturization of the QuEChERS method makes it possible to adapt it to the available sample size, reducing the volume of solvents, reagent consumption, costs, and environmental impact compared to the original method.[22,29,30]

In this study, a miniaturized QuEChERS method was established for the extraction of atrazine, endosulfan (α- and β-), chlorpyrifos, and cypermethrin (α-, β-, θ-, and ζ-) residues (chemical structures in Figure S1, presented in the Supplementary Information (SI) section) from liver tissue of anurans, using high-performance liquid chromatography-diode array detection (HPLC-DAD). The method for determining residues of the eight pesticides was optimized, validated, and applied to liver tissue samples from two anuran species, *Leptodactylus macrosternum* and *Scinax x-signatus*, collected in the city of Tabuleiro do Norte, located in the Baixo Jaguaribe region, Brazil. These two species of anurans have high abundance, wide distribution, and low dispersal capacity, with terrestrial and arboreal habits.[31,32] The presence of pesticide residues in liver tissue samples was confirmed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).

Experimental

Chemicals

The standards for atrazine (98.0%), endosulfan (98.0%, sum of α-endosulfan and β-endosulfan), chlorpyrifos (98.0%) and cypermethrin (> 90%, sum of isomers) were purchased from Sigma-Aldrich (São Paulo, Brazil). Methanol and acetonitrile, chromatographic grade, were purchased from JT Baker (Xalostoc, Mexico) and Tedia® (Rio de Janeiro, Brazil). Ethyl acetate, PA ACS grade, was purchased from Synth (Diadema, Brazil). Ultrapure water was obtained from a MegaPurity system at 18.3 MΩ cm (Billerica, MA, USA). Spherical C_{18} silica (40-75 µm, 70 Å pore diameter, 480 m^2 g^{-1}) and PSA (primary-secondary amine, with 8.4% C and 3.3% N, 50 µm particle size) were purchased from Sigma-Aldrich (Darmstadt, Germany). Anhydrous $MgSO_4$ and NaCl, PA ACS grade, were obtained from ACS Científica (Sumaré, Brazil). The mobile phase solvents were individually filtered through Millex® PTFE (polytetrafluoroethylene) filters with 0.22 µm pore diameter.

Sampling and processing of liver tissue samples

The collection of anurans was authorized by the Biodiversity Information System of the Brazilian Institute of the Environment and Natural Resources (SisBio-IBAMA) (approval No. 58724-1). The Animal Ethics Committee of the University of São Paulo evaluated and approved the technical procedures (CEUA-USP number 4387250118). Seventy-two specimens were collected manually through nighttime active searching from 6:00 pm to 10:00 pm and transported in air-filled plastic bags to a nearby field laboratory set up for this purpose.[31] The collection period occurred because the two species studied are active at night. The frogs were then humanely euthanized with an intracardiac injection of lidocaine hydrochloride (30 mg kg^{-1}).[33,34] Immediately after euthanasia, the animals were dissected and separated liver tissue samples, weighed, and frozen at –20 °C for later analysis.

Optimization of mini-QuEChERS extraction

Pesticide-free samples of chicken liver tissue were simulacra of anuran liver tissue samples during the optimization of the extraction method. The original QuEChERS was chosen for sample preparation. Nevertheless, in this study, we present the reduction of the conventional approach scale by a factor of 20 to align with the available size of anuran liver tissue samples. The extracting solvent and cleanup sorbents in the extraction of atrazine, endosulfan, chlorpyrifos (α- and β-), and cypermethrin (α-, β-, θ-, and ζ-) were evaluated. These pesticides were selected because they are widely applied in the agricultural areas of the Baixo Jaguaribe region, have high absorption by the anuran tissues, and have proven to interfere with their reproduction and development.[35]

Briefly, for the mini-QuEChERS extraction of pesticides from liver tissue samples, 0.5000 g of chicken liver tissue was weighed and fortified with the mixture of studied pesticides, leaving it to rest for 10 min. Soon after, 1.5 mL of extracting solvent (acetonitrile, ethyl acetate, acetonitrile:ethyl acetate

(9:1, v/v), acetonitrile:water (9:1, v/v) or methanol) was added and vortexed for 60 s. Subsequently, 50 mg of NaCl and 200 mg of $MgSO_4$ were added and vortexed for 60 s. The samples were centrifuged at 3000 rpm for 5 min, drawing 1.50 mL of the supernatant. For the cleanup step of the extract, 30 mg of adsorbents (octadecylsilane, PSA, or PSA + octadecylsilane) were added to the supernatant, vortexed for 30 s and centrifuged at 3000 rpm for 5 min. A 1.00 mL aliquot of the purified extract was analyzed in the HPLC-DAD system.

Method validation according to the SANTE/11312/2021 document

The mini-QuECHERS-HPLC-DAD method was validated according to the SANTE/11312/2021 document from the European Community.[36] The selectivity was obtained by comparing the chromatograms of an extract of the liver tissue fortified with a pesticide mixture and an extract of a blank sample. The analytical curves for each pesticide were prepared in acetonitrile, in sample extract (after mini-QuEChERS extraction), and in the sample before extraction. Pesticide concentration ranges used in analytical curves were established from the limit of quantification (LOQ) to 100 × LOQ. The linearity was obtained by the correlation coefficient (r) between pesticide concentrations and their peak areas. LOQ values for each pesticide corresponded to the lowest concentration of the compound, which meets the method performance criteria (80-120% for recovery and relative standard deviation (RSD) < 20% for precision, estimated by the analytical curve prepared in the extract). The lowest concentration was obtained by analysis of pesticide standard solutions in decreasing concentrations until the registration of the minor chromatographic signal, repetitive and distinguishable of the baseline noise, in the pesticide retention times. The matrix effect for each pesticide on the proposed method was estimated from the angular coefficients of the analytical curves prepared in solvent and in the sample matrix, according to equation 1:

$$ME(\%) = \frac{(S_m - S_s)}{S_s} \times 100 \quad (1)$$

where ME (%) is the percentage of matrix effect; S_m is the angular coefficient of the analytical curve prepared in the sample matrix; S_s is the angular coefficient of the analytical curve prepared in acetonitrile.

The matrix effect expresses the percentage of suppression or gain in the analytical signal due to the interactions of pesticides with components of the anuran hepatic tissues. The precision and accuracy of the method were determined from mini-QuEChERS extractions of pesticides in liver tissue samples of anurans performed at two levels of concentration (3 × LOQ and 10 × LOQ) in six replicates. Precision measurements were evaluated in two dimensions: (*i*) repeatability was calculated with six extractions for each level of fortification of liver tissue samples performed under the same conditions on the same day; and (*ii*) intra-lab reproducibility was calculated with six extractions of fortified tissue samples at each concentration level performed within three days by three different analysts. The accuracy of the mini-QuEChERS method was expressed by the values of relative standard deviations (RSD) and the accuracy determined by the pesticide recovery rates of the fortified liver tissue samples, according to equation 2:

$$R(\%) = \frac{C_{extracted} - C_{initial}}{C_{spiked}} \times 100 \quad (2)$$

where R (%) is the percentage of pesticide recovery; $C_{extracted}$ is the concentration of analyte after the optimized mini-QuEChERS extraction; $C_{initial}$ is the concentration of analyte in the real sample; C_{spiked} is the concentration of a known amount of pesticide standard spiked into the real sample.

The robustness was studied by a fractional factorial design according to the Youden and Steiner test,[37] evaluating seven variables in two levels, the optimized and deliberately varied ones. Contact time between extractor and sample, agitation time, mass of salts, mobile phase solvent supplier, mobile phase flow, mobile phase composition, and oven temperature were the studied factors. The experimental matrix is in Table 1.

The effect of each variable in the optimized method was calculated by the difference between the mean pesticide recoveries at the optimized level and the mean recoveries at the varied level (equation 3).

$$E_{(var)} = \bar{R}_{optimized} - \bar{R}_{varied} \quad (3)$$

where $E_{(var)}$ is the effect of a variable; $R_{optimized}$ is the mean pesticide recovery in the optimized level of the variable; R_{varied} is the mean pesticide recovery in the deliberately varied level of the variable.

Determination of pesticide residues in liver tissues of two anuran species

After the validation study, the proposed method was applied to determine residues of the studied pesticides in 72 samples of liver tissue from two anuran species,

Table 1. Experiment matrix for evaluating the robustness of the mini-QuEChERS-HPLC-DAD method using the Youden and Steiner test[37]

Variable	Experiment								Level	
	1	2	3	4	5	6	7	8	Optimized	Varied
Contact time / min	A[a]	A	A	A	a[b]	a	a	a	10	12
Stirring time / s	B	B	b	b	B	B	b	b	60	90
Mass of NaCl/$MgSO_4$ / (mg mg^{-1})	C	c	C	c	C	c	C	c	50/200	60/240
Mobile phase solvent / supplier	D	D	d	d	d	d	D	D	JT Baker®	Tedia®
Mobile phase flow rate / (mL min^{-1})	E	e	E	e	e	E	e	E	1.0	0.8
Mobile phase organic component / %	F	f	f	F	F	f	f	F	80	82
Oven temperature / °C	G	g	g	G	g	G	G	g	30	35

[a]Capital letters: optimized levels; [b]lowercase letters: varied levels.

Leptodactylus macrosternum and *Scinax x-signatus*, collected in Tabuleiro do Norte, a municipality located in the region of lower Jaguaribe River, Ceará, Brazil. Samples of the same species that presented masses lower than the optimized sample mass of the mini-QuEChERS method were grouped into pools. All extracts that showed a peak in the retention times of a studied pesticide in HPLC-DAD were also analyzed by LC-MS/MS to confirm the peak identity.

HPLC-DAD and LC-MS/MS analyses

The samples were analyzed on a Waters® model Alliance HPLC system (Milford, MA, USA) equipped with a model e2695 quaternary pump, autosampler, column oven, and model 2998 PDA photodiode array detector. Data acquisition was controlled by Empower3 software. The chromatographic conditions used were: XSelect HSS T3 C_{18} Waters® column (150 mm × 4.6 mm, 3.5 µm particle size), oven temperature 30 °C, mobile phase MeOH:water (80:20, v/v) with a flow rate of 1.0 mL min^{-1}, 10 µL injection, and UV detection at 220 nm.

Samples with peaks in the retention times of pesticides detected in the HPLC-DAD chromatograms were also analyzed by LC-MS/MS to confirm the identity of the suspected peaks. The LC-MS/MS system consisted of a Waters Alliance liquid chromatograph coupled to a MicroMass Quattro microTM API mass spectrometer (Waters, Milford, MA, USA) with an electrospray ionization (ESI) interface. MS analysis conditions were injection volume of 10 µL, desolvation temperature of 350 °C, source voltage of 3.0 kV, source current of 100 µA, and capillary voltage of 5 V in positive ion mode. Analysis, data acquisition, and management were controlled by the MassLynx 4.0 software package. Specific multiple reaction monitoring (MRM) transitions for pesticide quantification and qualification were obtained after infusing 1.0 µg mL^{-1} of individual pesticide standards directly into the ESI interface, and the values obtained are in Table S1 (SI section).

Results and Discussion

Optimization of mini-QuEChERS extraction

The QuEChERS method was studied for extracting pesticide residues from anuran liver tissue samples due to its adaptability and robustness to different types of solid samples, from hydrophilic to high fat. Anuran liver tissue has a high lipid content, only lesser than adipose tissue, and has been the organ with the highest potential detection of pesticide residues.[7,38,39] Due to the limited availability of anuran liver tissue samples, the original QuEChERS method was scaled down by 20 times (sample mass, partitioning salts, cleanup sorbents, eluent volume). The sample mass was reduced to 500 mg, partitioning salts to 200 mg of $MgSO_4$ and 50 mg of NaCl, and the solvent volume to 1.5 mL. The extractor solvent and the cleanup dSPE (dispersive solid phase extraction) sorbent were optimized in the miniaturized QuEChERS method using a free-pesticide chicken liver tissue as a substitute sample. The optimum extractor solvent was evaluated by fixing the PSA:C_{18} mixture (25 mg:25 mg) as a cleanup sorbent. Figure 1 shows the pesticide recoveries according to the extractor solvents. Acetonitrile (ACN) was the only extractor solvent with recoveries for all compounds within 80 to 120%. Acetonitrile is considered an optimum extractor solvent for pesticide residues of different polarities and allows for smaller lipophilic co-extractives (waxes, fats, and pigments) from the sample.[40] This condition was confirmed by the extraction of cypermethrins, which presented rates greater than 100% with methanol and ethyl acetate due to the influence of lipophilic extractives from the liver tissue matrix. However, atrazine, the most hydrophilic pesticide from the mixture, showed 110% recovery with acetonitrile. The reduction of this matrix effect was evaluated by testing polar sorbents in the cleanup step. Figure 2 shows the pesticide recoveries according to the dSPE sorbent tested, using acetonitrile as the extractor solvent.

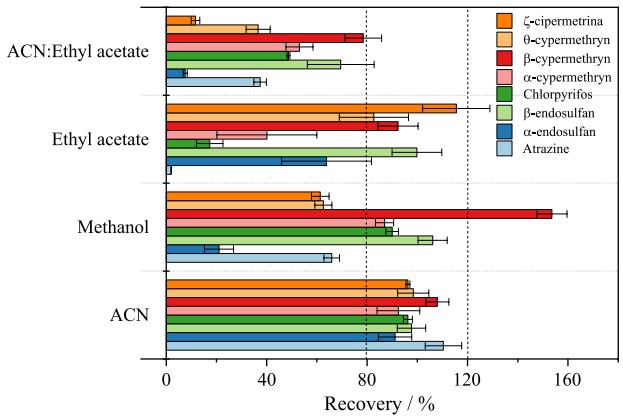

Figure 1. Pesticide recoveries from spiked liver tissue samples obtained by mini-QuEChERS using different extractor solvents and the PSA:C_{18} (1:1, m/m) as dSPE sorbent.

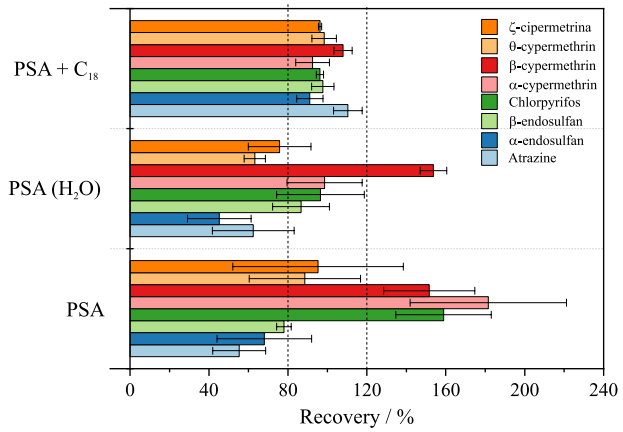

Figure 2. Pesticide recoveries from spiked liver tissue samples obtained by the mini-QuEChERS using some dSPE sorbents and acetonitrile as extractor solvent.

As observed in Figure 2, PSA as a cleanup sorbent reduces the effect of matrix extractives for extracting the most polar pesticides from the studied mixture, reducing the recovery rates of atrazine and endosulfans. Additionally, PSA was inefficient for matrix hydrophobic extractives, with a pronounced synergistic effect for extracting some cypermethrin isomers, with recoveries greater than 150%. The combination of a hydrophobic, octadecylsilane (C_{18}), and hydrophilic sorbents, PSA, in the cleanup step, was efficient for removing nonpolar and polar components from the liver tissue matrix without loss of pesticide recoveries. Thus, 25 mg of each sorbent was used for the cleanup step of the proposed method. The optimized method consisted of a liver tissue sample mass of 500 mg, 200 mg of $MgSO_4$ and 50 mg of NaCl as partitioning salts, 1.50 mL of acetonitrile as the extractor solvent, and 25 mg of C_{18} and 25 mg of PSA as dSPE sorbent.

Urban and Lesueur[41] found that the mixture C_{18} + PSA + $MgSO_4$ was an efficient cleanup sorbent for the extract from food samples without significant loss of the polar or apolar analytes. The results were comparable with specific sorbents for lipid removal, such as EMR-lipid (enhanced matrix removal-lipid), but with lower cost, better commercial availability of PSA and C_{18} sorbents, and a little reduction of analyte recovery. Ly *et al.*[42] also observed that the PSA and C_{18} mixture operated as a mixed mode in the cleanup of green tea leaf extracts after QuEChERS extraction of a few hundred pesticides. The authors observed that the sorbents allowed cleaner extracts without recovery loss of polar and apolar pesticide molecules.

Method validation for determining pesticides in anuran liver tissue samples

The proposed method for determining residues of the eight pesticides in anuran liver tissues was validated following the European Community guidance document for the method validation for analysis of pesticide residues in food and feed (document SANTE/11312/2021).[36] The method parameters subjected to validation were selectivity, linearity, range, matrix effect, limit of quantification, accuracy, precision (repeatability and intra-lab reproducibility), and robustness.

Selectivity

Despite the efficiency of the mini-QuEChERS method in extracting pesticides from anuran liver tissue samples, sample preparation is not selective enough to prevent the coextraction of matrix components. Thus, the combination with an analytical separation technique aims to ensure adequate selectivity of the analytes for unambiguous identification and quantification of pesticide residues in sample extracts. HPLC was the technique chosen for the analysis of the extracted compounds. The method selectivity was obtained by mini-QuEChERS extraction of pesticides followed by the HPLC-DAD separation of extracted compounds. The selectivity was measured by comparing the chromatograms of a blank extract and a spiked liver tissue extract with 125 μg kg^{-1} of pesticide standard mixture, Figure 3. The chromatogram of the blank sample resulted in a few matrix components extracted by the method, reaffirming the efficiency in the dSPE cleanup step using C_{18} and PSA sorbents. The chromatographic separation of pesticides using the XSelect HSS T3 C_{18} Waters® column and mobile phase MeOH:H_2O (80:20, v/v) allowed the elution of eight peaks in the chromatogram, referring to the endosulfan isomers (α- and β-) and cypermethrin (α-, β-, θ- and ζ-) with relatively good resolution between each other, as shown in Figure 3. Furthermore, the pesticide peaks did not show interference from extractives from the

liver tissue matrix. So, the mini-QuEChERS-HPLC-DAD method was selective for the residues studied in anuran liver tissue.

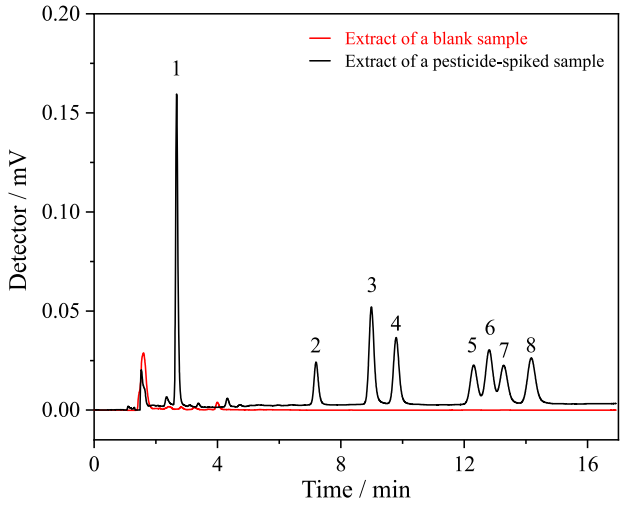

Figure 3. Chromatograms of a blank and a pesticide-spiked anuran liver tissue samples. Mobile phase: MeOH:H_2O (80:20, v/v), flow rate at 1.0 mL min^{-1}, detection UV at 220 nm, XSelect C_{18} HSS T3 column. Identification of peaks: (1) atrazine; (2) β-endosulfan; (3) chlorpyrifos; (4) α-endosulfan; (5) α-cypermethrin; (6) β-cypermethrin; (7) θ-cypermethrin; (8) ζ-cypermethrin.

Limit of quantification (LOQ)

The regulatory agencies did not tabulate maximum pesticide residue limits (MRL) in anuran tissues since the frogs are non-target organisms. However, liver tissues from animals targeted by veterinary medicines typically present MRLs of mg kg^{-1}.[43] Furthermore, concentrations of pesticide residues in anuran liver tissues have already been detected in the order of 1500 μg kg^{-1}. Therefore, the analytical method must be capable of determining pesticide residues in liver tissues at these concentration levels. The LOQ was defined by injecting decreasing concentrations of the pesticide-spiked sample extracts. The LOQ was the lowest concentration capable of producing a repetitive signal for the compounds in the HPLC-DAD chromatogram with a RSD of less than 20% and a recovery rate between 80-120%.[36] The experimentally measured LOQ values were 10.0 μg kg^{-1} for atrazine, 30 μg kg^{-1} for chlorpyrifos, 55 μg kg^{-1} for cypermethrins and α-endosulfan, and 75 μg kg^{-1} for β-endosulfan. The LOQ found are higher than for other pesticides in anuran tissues reported by other studies in the literature[16-18,23] that employed techniques such as GC-MS/MS (gas chromatography coupled with tandem mass spectrometry) or LC-MS/MS (liquid chromatography coupled with tandem mass spectrometry). While the proposed method exhibits lower detectability, it is crucial to highlight that the miniaturization of sample preparation led to a reduction in the generation of post-analysis organic residues, approximately 10% compared to residues generated by other methods documented in the literature.[16-21,23] In addition to mitigating the environmental impact of pesticide analysis, the proposal also aligns with contemporary efforts toward more sustainable laboratory practices. Moreover, HPLC-DAD remains more accessible to most chemical analysis laboratories worldwide, facilitating broader application of the proposed method. Still in this work, to confirm the identity of the suspected peaks of anuran liver tissue samples, the extracts obtained by the mini-QuEChERS method were also analyzed by LC-MS/MS, whose LOQ were lower than 1.0 μg kg^{-1} for pesticides, remaining at the same level as those reported in the literature 0.50 to 4.20 μg kg^{-1}.[16-21]

Range and linearity

The response linearity of the proposed method as a function of compound concentrations was evaluated by constructing analytical curves for each pesticide. The range considered was from the LOQ, the lowest concentration of pesticides quantifiable by the method, to a concentration of 50 × LOQ for all pesticides. Thus, the ranges were 10 to 500 μg kg^{-1} for atrazine, 55 to 2750 μg kg^{-1} for α-endosulfan and cypermethrins, 30 to 1500 μg kg^{-1} for chlorpyrifos, and 75 to 3750 μg kg^{-1} for β-endosulfan. Analytical curves using three calibration methods were prepared for all pesticides in their respective concentration ranges. As shown in Figure S2 and Table S2 (SI section), all analytical curves presented linear responses for pesticides in the range of concentrations evaluated, with correlation coefficients (r) between 0.9961 and 0.9998. Document SANTE/11312/2021 establishes as an acceptance criterion for analytical methods, correlation coefficients > 0.99.[36] Therefore, in the proposed method, the measured signal responds linearly to pesticide concentrations in anuran liver tissue samples within the concentration ranges. The most sensitive pesticide by the proposed method was atrazine, and the least was β-endosulfan (Table S2, SI section).

Matrix effect

As can be seen in Figure S2 (SI section), the analytical curves presented different straight-line slopes for the same pesticides according to the calibration method. This condition is qualitative evidence of the matrix effect in determining pesticide residues in the anuran liver tissue. Thus, the percentage of matrix effect (% ME) of the proposed method was quantified from equation 1, considering the angular coefficients of the analytical curves prepared in the matrix and acetonitrile, as recommended by the document SANTE/11312/2021.[36] According to the validation guidelines, if the method presents a % ME

greater than 20% either due to suppression or gain of sign for any pesticide, it is necessary to use the matrix-matched analytical curve to compensate for the matrix effect to quantify the compounds. According to Figure 4, most pesticides showed a signal suppression effect due to the liver tissue matrix. However, endosulfan isomers showed percentages of matrix effect higher than 20%. So, all pesticide residues were quantified using the matrix-matched calibration.

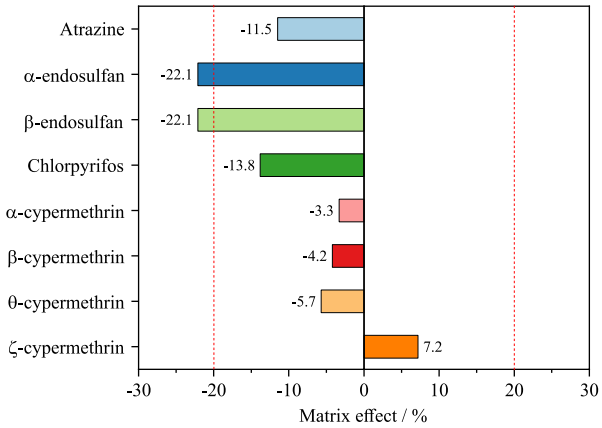

Figure 4. Percentages of the matrix effect in determining pesticides from anuran liver tissue samples using the mini-QuEChERS-HPLC-DAD method.

Accuracy and precision

The optimized mini-QuEChERS extraction was applied for the pesticides from liver tissue samples spiked at two pesticide concentration levels 3 × and 10 × LOQ, evaluating their accuracy and precision. The extractions were carried out over three days by three different analysts evaluating the precision in the levels of repeatability and intra-lab reproducibility of the method, as recommended by document SANTE/11312/2021.[36] Repeatability was measured based on the RSD of six extractions performed under the same conditions, and the intra-lab reproducibility of the method was determined by the RSD of 18 extractions performed by three analysts on three different days. The values obtained are in Table 2, with RSD values ranging from 0.4 to 19.3%. The most dispersion of results was for atrazine at the lowest fortification level, with an RSD of 10.9 to 19.3%. This result may be associated with the elution of atrazine (shorter retention time) in an initial region of the chromatogram, which presents more baseline instability with the elution of more polar extractives from the liver tissue matrix. For the other pesticides, the RSD values ranged from 0.4 to 7.9%, both for measuring repeatability and intra-lab reproducibility. The total precision for measuring the concentration of each pesticide in liver tissue samples was calculated by equation 4. The results in Table 2 indicated that the method presents good precision since the acceptance criteria established by document SANTE/11312/2021 is RSD < 20%.[36]

$$s_{total} = \sqrt{\left(\frac{s_{rep}^2}{n + s_{intra}^2} \right)} \quad (4)$$

where s_{total} is the total precision of the method, s_{rep} is the absolute standard deviation for repeatability, s_{intra} is the absolute standard deviation for intra-lab reproducibility, and n is the number of replicates for repeatability measurements.

The method accuracy was evaluated through the recovery rates of pesticides from spiked liver tissue samples. The average recovery rates of pesticides extracted in six replicates at the two fortification levels are in Figure 5. The recoveries ranged from 91 to 110% for all pesticides, meeting the acceptance criteria for an accurate method of determining pesticide residues in complex samples, from 70 to 120%.[36] Therefore, the method showed good accuracy even with the change of analysts and the

Table 2. Precision of the method for determining pesticide residues in anuran liver tissue based on mini-QuEChERS extraction with quantification by HPLC-DAD (n = 6)

Pesticide	Repeatability (RDS) / %		Intra-lab reproducibility (RDS) / %		Total precision (RDS) / %	
	3 × LOQ	10 × LOQ	3 × LOQ	10 × LOQ	3 × LOQ	10 × LOQ
Atrazine	19.3	0.6	10.9	3.1	15.0	3.1
α-Endosulfan	0.4	0.8	4.9	2.4	4.9	2.4
β-Endosulfan	2.9	0.6	2.4	1.1	2.7	1.1
Chlorpyrifos	2.1	0.7	1.6	0.7	1.8	0.8
α-Cypermethrin	1.9	2.7	7.9	3.1	7.9	3.3
β-Cypermethrin	1.4	1.0	1.3	1.6	1.4	1.6
θ-Cypermethrin	3.1	1.8	4.0	1.9	4.2	2.0
ζ-Cypermethrin	3.3	2.5	3.6	2.1	3.9	2.3

RSD: relative standard deviation; n: number of replicates; LOQ: limit of quantification.

day for pesticide extractions, proving robust to changes in optimized conditions, Figure 5.

A few methods have been related to extracting and determining pesticide residues in liver tissue or other anuran tissues. However, we did not identify validation studies proving the efficiency, precision, and robustness in determining pesticide residues in anuran tissues. In Table 3, we compare some reported methods with those proposed in this work for extracting pesticide residues in anuran tissue samples. The proposed miniaturized QuEChERS method is more economical, environmentally friendly, and efficient than those reported. The limit of quantification for the mini-QuEChERS-HPLC-DAD method can be improved using the mini-QuEChERS extraction before a more sensitive analytical technique, such as LC-MS/MS or GC-MS/MS.

Robustness by Youden and Steiner test

The method robustness for determining pesticide residues in liver tissue samples was evaluated using a 2^{7-4} fractional factorial design proposed by Youden and Steiner.[37] Seven variables of analytical technique (column oven temperature, mobile phase composition, mobile phase flow rate, mobile phase solvent supplier) and sample preparation (mass of salts, stirring time, and contact time) were evaluated at two levels (optimized and slightly varied). The response of assays was the pesticide recoveries from liver tissue samples. The effects of each variable on determining pesticides in liver tissues using the mini-QuEChERS-HPLC-DAD method were calculated using equation 3. Figure 6 shows the Pareto chart with the studied variables on pesticide recoveries from liver tissue

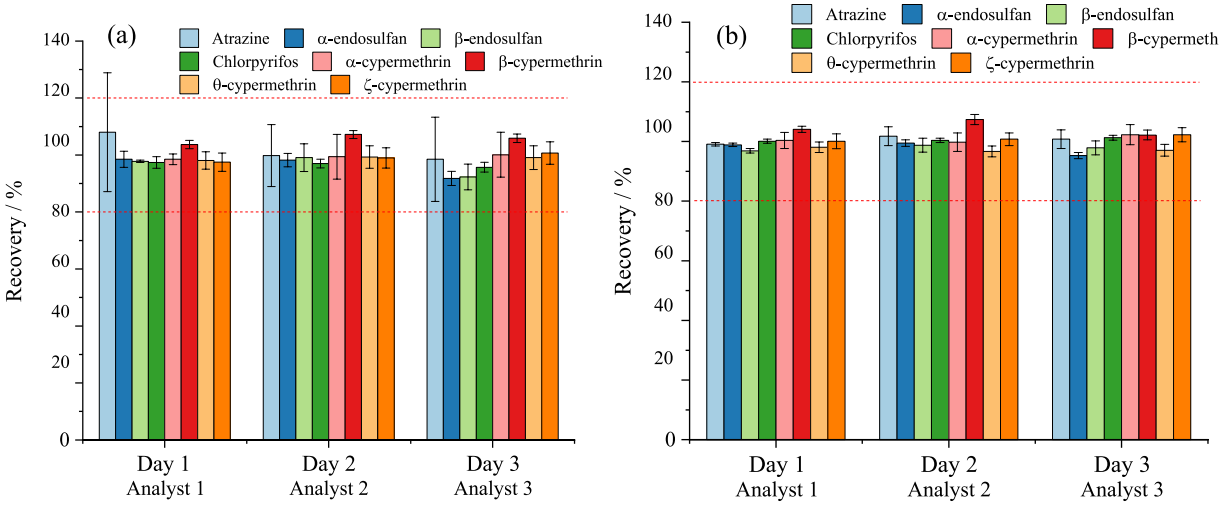

Figure 5. Recovery rates of pesticides in spiked anuran liver tissue samples with (a) 3 × LOQ and (b) 10 × LOQ obtained by the optimized mini-QuEChERS extraction with quantification by HPLC-DAD (n = 6).

Table 3. Comparison of the method characteristics for determining pesticide residues in anuran tissues

Pesticide	Extraction method	Analytical technique	Recovery / %	LOQ / (μg kg^{-1})	Solvent (volume / mL)	Reference
Clothianidin and metabolites	QuEChERS + solid phase extraction	LC-MS/MS	7-71	0.02-0.12	acetonitrile (20), hexane (3) methanol (0.2)	16
98 pesticides	pressurized liquid extraction	GC-MS/MS	75-120	0.50-4.20	dichloromethane (20)	17,18
Imidacloprid, atrazine, triadimefon, triadimenol, fipronil, and pendimethalin	solid-liquid extraction	LC-MS/MS	–	1.9-7.8[a]	methanol (10), distilled water (10), methyl *tert*-butyl ether (3)	19-21
Dichloro-diphenyl-trichloroethane (DDT) and deltamethrin	automated hot-Soxhlet extraction	GC-μECD	80	0.50	acetone + hexane (70), dichloromethane (30), *n*-decane (0.1)	23
Atrazine, endosulfan (α and β), chlorpyrifos, cypermethrin (α, β, θ, and ζ)	mini-QuEChERS	HPLC-DAD	91-110	10-75	acetonitrile (1.5)	this work

[a]LOD: limit of detection. LOQ: limit of quantification; GC-μECD: gas chromatography-microelectron capture detection; LC-MS/MS: liquid chromatography coupled with tandem mass spectrometry; GC-MS/MS: gas chromatography coupled with tandem mass spectrometry; HPLC-DAD: high-performance liquid chromatography-diode array detection.

samples obtained by the Steiner and Youden test. The most variation in response was +1.8% for atrazine when stirring time was 30 s higher than the optimized value. All other effects on pesticide recoveries were less than 1% after slight changes in the optimized method conditions. The sum of peak areas for the α-, β-, and θ-cypermethrin isomers was considered to calculate their recoveries since the overlap of peaks occurred after deliberated changes in the separation optimized conditions. The sum of the signals of these isomers also responds linearly to their concentrations. These results suggest that the proposed method is robust for determining pesticide residues in liver tissue samples, even if the optimized conditions cannot be applied in the mini-QuEChERS extraction or HPLC-DAD separation. None of the methods reported in the literature showed robustness studies that would enable a comparison with the mini-QuEChERS-HPLC-DAD.[17,18]

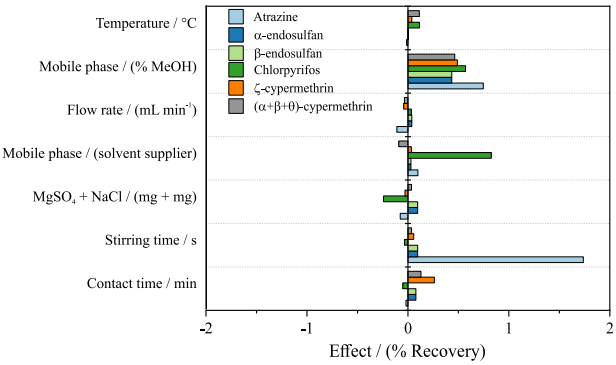

Figure 6. Effect of some variables in the robustness of the proposed method for determining pesticide residues in anuran liver tissue.

Application of the validated method to anuran liver tissue samples

The optimized and validated method was applied to liver tissue samples from two anuran species: *Scinax x-signatus* and *Leptodactylus macrosternum*. The species are common in semi-arid environments, abundant in South America, well-adapted to disturbed areas, and inhabiting or reproducing in all components of ecosystems (land, water, vegetation).[44,45] Seventy-two liver tissue samples from the collected species were subjected to the mini-QuEChERS-HPLC-DAD method to determine the eight studied pesticide residues. All samples with chromatographic peaks in the pesticide retention times were subjected to LC-MS/MS analysis to confirm the peak identity. The results are in Table S3 (SI section). Twenty-eight samples recorded the presence of endosulfan residues, four with chlorpyrifos residues, and seven with cypermethrin residues. The most contaminated samples were from *L. macrosternum* and two liver tissue samples from *S. x-signatus* with endosulfan residues. These results confirm that anuran liver tissue has the potential to retain pesticide residues, as indicated in other studies.[7,13]

Conclusions

In this work, a simple, efficient, and miniaturized sample preparation method based on the original QuEChERS combined with HPLC was developed for determining atrazine, chlorpyrifos, endosulfan (α- and β-), and cypermethrin (α-, β-, θ-, and ζ-) in anuran liver tissue samples. The original QuEChERS method was miniaturized, downscaling the sample size according to the availability of anuran liver tissue samples. Method conditions were optimized for 500 mg of sample, 1.5 mL of acetonitrile as extractor solvent, 200 mg of $MgSO_4$ + 50 mg of NaCl, and a mixture of 25 mg of C_{18} and 25 mg of PSA as cleanup adsorbent. The proposed method was validated regarding the main analytical parameters, following the procedures and acceptance criteria recommended by the European Community validation guidelines, SANTE/11312/2021 document. The validated method showed recovery rates between 91-110% for all eight pesticides, accuracy, and robustness for its purpose. The proposed method was applied to determine pesticides in liver tissue samples from two anuran species, detecting endosulfan, chlorpyrifos, and cypermethrin residues in more than 40% of the analyzed samples, with their presence confirmed by LC-MS/MS. In this way, the developed method proved suitable for extracting pesticide residues in anuran liver tissues, contributing to the need for monitoring non-target organisms for pesticide application with the expansion of monocultures in Brazil.

Supplementary Information

Supplementary information (chemical structures of studied pesticides, analytical parameters of the proposed method, pesticide residues in liver tissue samples) is available free of charge at http://jbcs.sbq.org.br as PDF file.

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Author Contributions

Igor M. Lima was responsible for the data curation, formal analysis, investigation, methodology and validation; Allyson L. R. Santos for

data curation, formal analysis, investigation, methodology, validation, writing review and editing; Andressa T. Vieira for data curation, formal analysis, investigation, methodology and validation; Patrícia M. Gondim for funding acquisition, investigation and writing (original draft, review and editing); Paulo Cascon for funding acquisition, resources, writing review and editing; Anizio M. Faria for funding acquisition, project administration, resources, supervision and writing (original draft, review and editing).

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