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Article

Simultaneous Determination of Different Classes of Pesticides in Breast Milk by Solid-Phase Dispersion and GC/ECD

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Este estudo apresenta desenvolvimento, validação e aplicação de um método multirresíduo para determinação de nove agrotóxicos e um produto de degradação (α-endossulfam, β-endossulfam, α-HCH, γ-HCH, aldrim, p,p'-DDE, p,p'-DDT, cipermetrina, deltametrina e trifluralina) em amostra de leite humano por dispersão em fase sólida e cromatografia gasosa com detector de captura de elétrons (GC/ECD). O método é considerado simples e eficiente, combinando a extração e a purificação em uma única etapa e quantificação por adição padrão para eliminar efeito de matriz. Os limites de detecção e quantificação do método variaram entre 0,002-0,079 μg mL^{-1} e 0,013-0,108 μg mL^{-1}, respectivamente. O método proposto foi aplicado para a análise de 62 amostras de leite humano coletadas entre fevereiro e junho de 2010 em Lucas do Rio Verde-MT. p,p'-DDE (0,32-12,03 μg g^{-1} de gordura), p,p'-DDT (2,62-12,41 μg g^{-1} de gordura) e β-endossulfam (0,54-0,61 μg g^{-1} de gordura) foram quantificados em 29%, 5% e 3% das amostras analisadas, respectivamente. Aldrim foi encontrado abaixo do limite de quantificação do método em 7% das amostras. Embora esses compostos tenham sido encontrados nas amostras analisadas, as mães foram orientadas a continuar o aleitamento materno por ser considerado benéfico durante a infância.

This study presents the development, validation and application of a multiresidue analytical method intended to determine nine pesticides and a degradation product (α-endosulfan, β-endosulfan, α-HCH, γ-HCH, aldrin, p,p'-DDE, p,p'-DDT, cypermethrin, deltamethrin, and trifluralin) in breast milk samples by solid-phase dispersion and gas chromatography with electron capture detector (GC/ECD). The proposed method is considered simple and efficient, combining extraction and clean up in a single step and quantification performed by standard addition to avoid the matrix effect. The method limits of detection and quantification varied between 0.002 and 0.079 μg mL^{-1} and 0.013 and 0.108 μg mL^{-1}, respectively. The proposed method was applied to analyze 62 breast milk samples collected between February and June 2010 in Lucas do Rio Verde, Mato Grosso, Brazil. p,p'-DDE (0.32-12.03 μg g^{-1} of fat), p,p'-DDT (2.62-12.41 μg g^{-1} of fat) and β-endosulfan (0.54-0.61 μg g^{-1} of fat) were quantified in 29%, 5% and 3% of the samples, respectively. Aldrin was found below the method quantification limit in 7% of samples. Although these compounds were found in the analyzed samples, mothers were oriented to carry on feeding the infants as the breast milk is considered the optimum food during infancy.

Keywords: pesticides, solid-phase dispersion, gas chromatography, breast milk, matrix effect

Introduction

Brazil is one of the world's largest pesticide consumers, and the state of Mato Grosso stands out in the national scenario as one of the major agricultural producers and pesticide consumers. Lucas do Rio Verde, a city having 37,000 inhabitants located 285 km North from Cuiabá, the state capital, is considered one of the largest agricultural producers in Mato Grosso. In 2010, 410,000 hectares of soybean, corn and cotton were cultivated in the municipality, and around 5.1 million liters of pesticides, especially herbicides, insecticides and fungicides were sprayed over those fields.[1,2]

During and after their application, pesticides can spread through different environmental compartments and reach places far from the application areas.[3] As a result, people

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living in a city near or surrounded by large fields can be highly exposed to those compounds. That exposure can become more dangerous in case of accidents, such as the one that occurred in Lucas do Rio Verde in 2006, when an agricultural plane that was spraying a soybean crop in the urban area surroundings accidentally sprayed a desiccant over the city. The accident not only affected vegetables and ornamental, fruit and medicinal plants but also the inhabitants of the city, who reported symptoms such as vomiting, diarrhea and hives.[1]

Adverse effects of pesticides to human health have been described in the literature,[4] as well as exposition routes such as by drinking water, skin absorption, inhalation, and through food.[5] Residue of pesticides in breast milk is a concern since infants do not have a fully developed detoxification mechanism, their immune systems and other organs are immature and milk is appointed as the best sole nutrient source for them. Breast feeding is considered to be one of the major excretory pathways of xenobiotics from the mother body, which makes it the main source of pesticide residue transfer to newborns.[6] On the other hand, breast milk-fed newborns have higher immunity, normal growth, better digestive process and gastrointestinal system, enhanced mother-son bond, and better emotional, cognitive and nervous system development.[7]

Breast milk (3 to 5% fat) is an aqueous fluid whose characteristics allow it to partition quite well both hydrosoluble and liposoluble compounds.[8] Due to their highly lipophilicity and persistency and worldwide use in the past to control disease vector, organochlorine pesticides, mainly dichlorodiphenyltrichloroethane (DDT) and its metabolite dichlorodiphenyldichloroethylene (DDE) are the most frequently studied pesticides in breast milk in several countries.[9-24] Another class of pesticides currently used, pyrethroids, is also under study.[10,25]

Solid-phase, liquid-liquid, and sonication extraction techniques have been frequently used for extraction and clean-up of breast milk samples followed by analyte determination using gas chromatography with electron capture detector (GC/ECD) and mass selective detectors.[9-15,19-22,25] Most of published methods intend to determine isolated analyte or substances with similar physical and chemical properties. The challenging with this complex matrix is to obtain an efficient multi-residue method that is able to determine molecules with different properties, since humans are exposed to different classes of pesticides.

Considering a possible exposure of newborns in Lucas do Rio Verde to pesticides through breast milk, this work aimed to develop and validate a multi-residue analytical method in order to determine simultaneously nine pesticides and a degradation product belonging to three chemical groups: dinitroanilines (trifluralin), organochlorines (α-HCH, γ-HCH, aldrin, α-endosulfan, β-endosulfan, p,p'-DDE and p,p'-DDT) and pyrethroids (cypermethrin and deltamethrin) and to evaluate their presence in 62 breast milk samples collected in the aforementioned locality. Although trifluralin and the pyrethroids cypermethrin and deltamethrin are not persistent pesticides, they were included in this study considering their intensive use in the municipality of Lucas do Rio Verde and high octanol-water partition coefficient (Kow > 4.6) which indicates a potential for bioaccumulation.

Experimental

Reagent and chemicals

Acetone, *n*-hexane, dichloromethane, toluene, sodium sulfate and Celite®, residue analysis grade, were obtained from Tedia Brasil Ltda, Merck, Qhemis, and Mallinckrodt Chemicals. High purity standards were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) (p,p'-DDT 98.9%, p,p'-DDE 99.9% and aldrin 99.2%), Chem Service, Inc. (West Chester, PA, USA) (γ-HCH 99.5%), Pestanal® (Sigma-Aldrich Chemie Steinheim, Germany) (α-endosulfan 99.6%, β-endosulfan 99.9%, cypermethrin 95.1% and deltamethrin 99.9%), and Dr Ehrenstorfer GmbH (Augsburg, Germany) (heptachlor 99.5%, trifluralin 99.5% and α-HCH 97.5%). Stock standard solutions were prepared in toluene for chromatographic analysis, and in acetone when used to spike the control samples.

Sample preparation

A pool of pasteurized breast milk samples from the breast milk bank of Julio Müller Hospital, Cuiabá, Mato Grosso state, collected from donors living in the urban area and not directly exposed to the studied pesticides, was employed during development and validation method steps and also for analytes quantification in the analyzed samples. The absence of pesticides in this control sample was previously evaluated.

The samples were collected and kept following the instructions present in the technical norms of the BLH-BR Network for Breast Milk Banks/Fiocruz-IFF-BLH,[26] which the breast milk bank of the Julio Müller Hospital is registered at and integrated with.

Spiked samples were prepared by adding 500 μL of the standard mixture of analytes to 5 mL of control sample, followed by addition of 5 mL of acetone for deproteinization and homogenization during 5 min in a sonication bath. This procedure resulted in two spiked samples levels for each analyte as follow: α-endosulfan (0.015 and 0.103 μg mL^{-1}),

β-endosulfan (0.013 and 0.108 μg mL^{-1}), α-HCH (0.027 and 0.109 μg mL^{-1}), γ-HCH (0.028 and 0.111 μg mL^{-1}), aldrin (0.015 and 0.101 μg mL^{-1}), p,p'-DDT (0.107 and 0.153 μg mL^{-1}), p,p'-DDE (0.015 and 0.109 μg mL^{-1}), cypermethrin (0.104 and 0.157 μg mL^{-1}), deltamethrin (0.108 and 0.144 μg mL^{-1}), and trifluralin (0.015 and 0.107 μg mL^{-1}).

Extraction procedure

The extraction procedure consisted on combining the extraction and purification phases in a single step by using the solid-phase dispersion technique with Celite® and sonication extraction. Twelve different conditions were evaluated for selecting the solvents or mixtures of the solvents used in the extraction (Table 1). A control sample was analyzed for each condition.

Five milliliters of unfrozen breast milk control samples were transferred to an 80 mL centrifuge tube and spiked as mentioned. Samples spiked at the highest level (in duplicate) were used. Two grams of Celite®, previously activated in a muffle furnace at 600 °C for 12 h, and 10 mL of the solvents or their mixture (Table 1) were added to the mixture. The resulting mixture was agitated by sonication during 20 min followed by centrifugation (5 min, 2,000 rpm) for phase separation. The supernatant was transferred to a pear-shaped glass flask. The extraction procedure was repeated as indicated in Table 1. The extracts were then combined and concentrated in a rotary evaporator (35-40 °C) until near dryness, and the analytes were recovered in toluene (1.5 mL) and transferred to an autosampler vial containing 50 μL of the internal standard solution (heptachlor-around 10 μg mL^{-1}). The final extracts were kept under refrigeration (4 °C) for identification and quantification using GC-ECD.

The best extraction condition was selected based on the criteria recommended by Thier and Zeumer[27] and Sanco,[28] i.e., acceptable mean recoveries were those within the range 70-120% with an associated repeatability [coefficient of variation (CV)] ≤ 20%. In case of multi-residue method, recoveries outside this range may be accepted if demonstrating good precision.[28]

Analytes were identified by comparison with the retention times of the standards and were quantified by standard addition with standards solutions. Added concentrations ranged from the lowest to highest fortification level value, prepared in duplicate in the extract of control matrix without the analytes, in order to eliminate the matrix effect.

After optimization of the extraction procedure, the proposed method consisted on agitation by sonication during 20 min of 5 mL of unfrozen sample, 2 g of Celite®, and 10 mL of *n*-hexane:acetone (1:1, v/v), followed by centrifugation, and extraction separation. Extraction with 10 mL of *n*-hexane:acetone (1:1, v/v) was performed in duplicate, followed by two extractions with 10 mL of *n*-hexane:dichloromethane (4:1, v/v). Extracts were combined, concentrated until near dryness, and analytes were recovered in toluene (1.5 mL) and transferred to an autosampler vial containing 50 μL of the internal standard solution (heptachlor: around 10 μg mL^{-1}).

Equipment

Analytes were identified and quantified using gas chromatograph (HP 6890 series GC System) equipped

Table 1. Extraction conditions evaluated

Condition	Solvent or mixture of solvents	Condition	Solvent or mixture of solvents
1	dichloromethane:hexane (1:3, v/v) (1×) *n*-hexane:acetone (1:2, v/v) (1×)	7	dichloromethane (3×)
2[a]	*n*-hexane:acetone (1:1, v/v) (2×) *n*-hexane:dichloromethane (4:1, v/v) (2×)	8	*n*-hexane:acetone (1:1, v/v) (1×) dichloromethane:acetone (9:1, v/v) (1×) *n*-hexane (1×)
3	dichloromethane:hexane (1:3, v/v) (1×) *n*-hexane:acetone (1:2, v/v) (1×) *n*-hexane:acetonitrile (1:1, v/v) (1×)	9	*n*-hexane:acetone (1:1, v/v) (1×) *n*-hexane (1x) *n*-hexane:dichloromethane (7:3, v/v) (1×)
4	dichloromethane:hexane (1:3, v/v) (1×) *n*-hexane:acetone (1:2, v/v) (1×) methanol (1×)	10	*n*-hexane:acetone (1:1, v/v) (1×) *n*-hexane (1×) *n*-hexane:acetonitrile (1:1, v/v) (1×)
5	*n*-hexane:acetone (6:4, v/v) (3×)	11	*n*-hexane:acetone (1:1, v/v) (1×) dichloromethane:hexane (9:1, v/v) (1×) *n*-hexane:acetonitrila (1:1, v/v) (1×)
6	*n*-hexane:acetone (1:1, v/v) (3×)	12	dichloromethane:acetone (9:1, v/v) (3×)

[a]Selected condition for further validation.

with an electron capture detector and a 30 m long HP-5 chromatographic column with 0.25 μm of film thickness (5% phenylmethylsiloxane) and 250 μm of diameter. Ultrapure nitrogen 5.0 (99.999%, Linde Gás Brasil) was used as carrier and make-up gases at flow rate of 1 mL min^{-1} and 60 mL min^{-1}, respectively. Injector and detector temperatures were 250 °C and 280 °C, respectively. A volume of 1 μL was injected in the split mode (ratio 20:1). The column was set at 92 °C, ramped at 15 °C min^{-1} until 175 °C (13 min hold time) and heating at 10 °C min^{-1} until 280 °C (9 min). The chromatographic run time was 40 min. The chromatograms were recorded and processed using Chemstation version D.03.00.611 (Agilent Technologies Inc, California, USA).

Analytical method validation

The efficiency of the method was evaluated considering its accuracy (recovery percentage) and precision (coefficient of variation of the results obtained in the replicates, n = 5).

After selection of the best extraction conditions (essay 2 in Table 1), the recovery studies were carried out using the two spike levels for the pesticides. Those levels were selected considering the quantification limit of the chromatographic system (GC-ECD), 75 ng for trifluralin, aldrin, α-endosulfan, p,p'-DDE and β-endosulfan, 125 ng for γ-HCH, and 500 ng for p,p'-DDT, α-HCH, deltamethrin and cypermethrin.

The limit of detection (LOD) and limit of quantification (LOQ) of the method were calculated according to criteria established by Thier and Zeumer and Sanco.[27,28] The method LOQ is the lowest spike level of validation meeting the method performance acceptability criteria described, which are recovery between 70-120% with CV ≤ 20%. LOD is characterized by the smallest concentration of a compound in the analytical sample, for which the particular analytical method produces signal values which differ with 95% probability from those given at nil concentration in the analytical sample, and it was estimated from recovery experiment at the smallest fortification level using equations 1 to 5 as follow:

$$LOD = \frac{2 \cdot t_{f,95} \cdot \hat{\sigma}_{com}}{S} \quad (1)$$

The standard deviation ($\hat{\sigma}_{com}$) (equation 2) is computed from the standard deviation of the blank signal ($\hat{\sigma}_B$) (equation 3) and from the standard deviation $\hat{\sigma}_A$ (equation 4), estimated during the experiment with the lowest fortification level.

$$\hat{\sigma}_{com} = \sqrt{\frac{(m-1) \cdot \hat{\sigma}_A + (n-1) \cdot \hat{\sigma}_B}{m+n-2}} \quad (2)$$

$$\hat{\sigma}_B = \sqrt{\frac{\sum_{i=1}^{n} (B_i - \bar{B})^2}{n-1}} \quad (3)$$

where m is the number of analytical values (A_i) and n is the number of blank values (B_i). Degree of freedom (f) = m + n – 2.

$$\hat{\sigma}_A = \sqrt{\frac{\sum_{i=1}^{n} (A_i - \bar{A})^2}{m-1}} \quad (4)$$

where $\bar{B}$ and $\bar{A}$ are the mean blank concentration and mean analytical concentration obtained in the recovery study, respectively.

The sensitivity of the analytical method (S), which means the change in signal value per change of concentration, can be estimated from the mean analytical concentration value ($\bar{A}$) and from the lowest fortification level – concentration value (q) (equation 5).

$$S = \frac{\bar{A}}{q} \quad (5)$$

Stability study

Considering the elapsed time between the sample collection and the laboratory sample processing, a stability study of the pesticides in frozen samples was performed. Spiked control samples (n = 5) at the low level applied in this study were frozen for 12 days and the aforementioned analytes concentrations were determined after this time as previously described.

Fat content determination

Total milk lipid concentrations were determined by the crematocrit method, which allows the determination of the cream content and the calculation of both fat and energetic contents in milk. The analytical technique was carried out as described elsewhere.[29]

Method application

The validated method was applied to analyze 62 breast milk samples collected from breast-feeding mothers living in Lucas do Rio Verde, Mato Grosso state. The research protocol was approved by the Ethics Committee of Hospital Universitário Júlio Müller of UFMT in Cuiabá, Mato Grosso, Brazil under the number 511/CEP-HUJM/08. All women signed a Written Informed Consent Form

before they were enrolled in the study and responded a questionnaire about their ages and their children, parity, education, profession, contact or exposure to pesticides.

Mature milk samples were drawn during 21-56 days post-delivery, as recommended elsewhere,[30] from mothers with age ranging between 18 to 49 years old and with good health conditions. Samples were stored in glass flasks at –4 °C until analysis, which did not exceed more than 12 days.

Results

The multi-residue method was developed based on the selection of extraction solvents or their mixtures aiming to extract efficiently the selected pesticides, which present different physical and chemical properties (Table 2). The octanol-water partition coefficient (log K_{ow}) for these substances ranged from 3.69 to 6.91 and water solubility varied from 0.0002 to 8.52 mg L^{-1} indicating high lipophilicity and potential biomagnification.

Validation method was performed employing pasteurized breast milk. Although the pasteurization step procedure could possibly degrade some pesticide in the control sample or even cause the formation of new compounds, it did not interfere in the extraction efficiency. It is important to mention that the pasteurization of human milk did not show statistically significant changes in the concentration of sodium, potassium, calcium, phosphorus, magnesium, protein, fat, lactose, or in osmolarity.[32] The real analyzed samples did not suffer this procedure previously to analysis.

Celite® was selected as sorbent taking into account its capacity to retain fat from matrix. This material has been employed in studies which describe pesticide and organic compounds determination in several matrices.[33]

Method development and validation

Taking into account the complexity of the matrix, i.e., breast milk with high fat content, the matrix effect was assessed according to recommendations.[34] The matrix effect was determined by the relationship between the values of the angular coefficients of the analytical curves from standard solutions prepared in organic solvent (toluene) and in the breast milk matrix (control sample extract obtained from proposed method). Since values of this relationship (Table 3) were higher than those considered acceptable (< 10%),[34] the quantification was performed using the standard addition method, in which the analytical responses of the analytes were obtained with standard solutions prepared in the extract of control matrix.

To correct the end volume of the extract and the volume of injection, internal standard was employed, being added in the final extract. Heptachlor was selected as internal standard since it was the least detected organochlorine pesticide in environmental and food samples in Brazil[20-23] and recent studies only detected heptachlor epoxide.[35] In addition, it was well resolved from the studied pesticides and give excellent response in GC/ECD. Ratio of analytes

Table 2. Physical and chemical properties of the studied pesticides

Analyte	Use	Culture or pest	Formula	MM / (g mol^{-1})	$LD_{50\ rat}$ / (mg kg^{-1})	log K_{ow} (pH 7)	$t_{1/2}$ / days	Solubility H_2O / (mg L^{-1})	P_{vap} / mPa	K_H / (Pa m^3 mol^{-1})
α-endosulfan	I	grains, fruits, vegetables, cotton	$C_9H_6Cl_6O_3S$	406.93	38	4.75	50	0.32	0.83	1.48
β-endosulfan	I	grains, fruits, vegetables, cotton	$C_9H_6Cl_6O_3S$	406.93	38	4.75	50	0.32	0.83	1.48
α-HCH	I	fruits, vegetables, animal facilities	$C_6H_6Cl_6$	290.82	177	3.82	175	2.0	5.99	3.58×10^{-04a}
γ-HCH	I	seeds, soil, trees, wood	$C_6H_6Cl_6$	290.82	163	3.50	980	8.52	4.4	1.483×10^{-06}
Aldrin	I	cotton, corn	$C_{12}H_8Cl_6$	364.91	39	6.5	28	0.027	3	1.72×10^{01}
p,p'-DDT	I	malaria control	$C_{14}H_9Cl_5$	354.49	113	6.91	6200	0.006	0.025	8.43×10^{-01}
Cypermethrin	I	cotton, coffee, bean, corn, soybean	$C_{22}H_{19}Cl_2NO_3$	416.3	287	5.3	60	0.009	0.00023	2.00×10^{-02}
Deltamethrin	I	cotton, coffee, bean, corn, soybean	$C_{22}H_{19}Br_2NO_3$	505.2	87	4.6	13	0.0002	0.0000124	3.10×10^{-02}
Trifluralin	H	cotton, rice, sugar cane, corn	$C_{13}H_{16}F_3N_3O_4$	335.28	> 5000	5.27	181	0.221	9.5	10.2

MM: molecular mass; LD_{50}: lethal dose; log K_{ow}: octanol-water partition coefficient; $t_{1/2}$: half-life in soil; P_{vap}: vapor pressure (25 °C); K_H: Henry's law constant (25 °C); [a](20 °C); I: insecticide; H: herbicide; (PPDB, 2014).[31]

Table 3. Retention time and matrix effect on the quantification of pesticides by GC-ECD

Analyte	Rt / min	Angular coefficient (AC)		ACSC/ACMC
		Solvent curve (SC)	Matrix curve (MC)	
Trifluralin	12.74	0.89×10^{-02}	0.050×10^{-02}	18
α-HCH	13.57	2.9×10^{-02}	0.17×10^{-02}	17
γ-HCH	15.25	2.0×10^{-02}	0.17×10^{-02}	12
Aldrin	23.03	2.7×10^{-02}	0.13×10^{-02}	21
α-endosulfan	26.29	2.0×10^{-02}	0.080×10^{-02}	25
p,p'-DDE	27.20	0.17×10^{-02}	0.020×10^{-04}	850
β-endosulfan	28.00	5.7×10^{-01}	0.080×10^{-02}	715
p,p'-DDT	28.35	0.61×10^{-02}	0.12×10^{-02}	5
Cypermethrin	33.67	0.020×10^{-02}	0.010×10^{-03}	20
Deltamethrin	36.17	0.020×10^{-02}	0.020×10^{-03}	10

Rt: retention time; ACSC: angular coefficient-solvent curve; ACMC: matrix curve.

area and internal standard area and ratio of analytes concentration and internal standard concentration were calculated and employed for quantification.

Among the twelve evaluated extraction conditions, condition 2 (Table 1) was the one that resulted in the most satisfactory results for all analytes, with recoveries ranging between 67 to 120% (Figure 1). For this condition, average recovery and relative standard deviation (RSD) for the low spiked level (67-120%, ≤ 20%) and for the high spiked level (70-75%, ≤ 9%), respectively were considered satisfactory for the recovery experiments (Table 4).

The detection and quantification limits of the method (Table 4) were calculated based on the recovery results obtained with the lowest spiking level and on the control samples.[27,28] These values are useful for a first assessing of presence of pesticides in breast milk. Following the Comission Directive 2006/141/EC,[36] infant formulae and follow-on-formulae shall not contain residues of individual pesticides at levels exceeding 0.01 mg kg^{-1} of the product. The selectivity of the method was evaluated by the absence of interfering peaks at the same retention times of the analytes (Figure 2). With the exception of p,p'-DDE, no interfering peak was identified in the control sample. The values of such areas in the control samples were considered in p,p'-DDE concentrations calculation.

In Table 5, methods for analysis of pesticides in human milk described in the literature are listed showing concisely extraction procedures and instrumentation used as well as limits of quantification and detection. The great majority used liquid-liquid extraction followed by cleanup and gas chromatography with electron capture detector to determine mostly organochlorinated pesticides.

The results of the recovery experiment of the spiked control breast milk samples (lowest spiking level) kept frozen (–4 °C) (Table 6) show that it is possible to maintain samples at the spiked sample level studied frozen for 12 days without any modification of the concentration of the analytes.

Method application: pesticides in breast milk

Characteristics of the samples

The proposed method was applied to analyze 62 samples from breast-feeding mothers living in Lucas do Rio Verde, MT, Brazil, who took part in a survey carried out from February to June 2010 in which they answered a

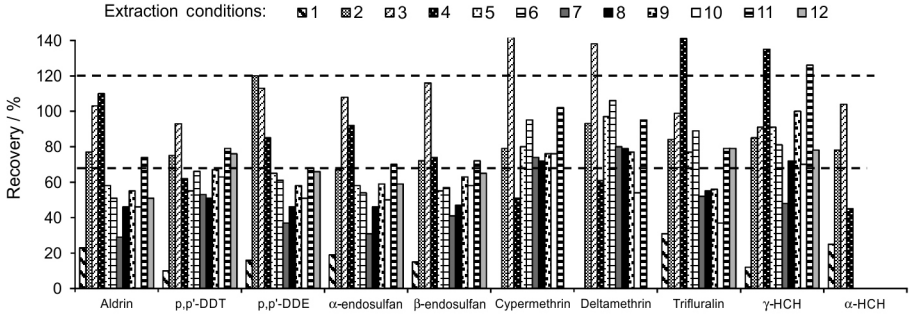

Figure 1. Average recovery results for the pesticides using studied extraction conditions and spiked sample fortified at the highest level.

Table 4. Accuracy (recovery), precision (coefficient of variation), and detection and quantification limits of the proposed method

Analyte	Low level			High level			LOD / (μg mL^{-1})	LOQ / (μg mL^{-1})
	Amount / (μg mL^{-1})	Average[a] / % (Interval)	CV / %	Amount / (μg mL^{-1})	Average[a] / % (Interval)	CV / %		
Trifluralin	0.015	84 (67-111)	20	0.107	70 (66-73)	2	0.015	0.015
α-HCH	0.027	78 (63-97)	14	0.109	72 (69-77)	2	0.027	0.027
γ-HCH	0.028	85 (67-105)	16	0.111	73 (68-80)	4	0.022	0.028
Aldrin	0.015	77 (74-79)	2	0.101	70 (66-78)	4	0.002	0.015
α-endosulfan	0.015	67 (65-73)	3	0.103	71 (66-84)	6	0.003	0.015
p,p'-DDE	0.015	120 (112-132)	13	0.109	75 (70-82)	4	0.005	0.015
β-endosulfan	0.013	72 (64-83)	6	0.108	75 (67-92)	9	0.006	0.013
p,p'-DDT	0.107	75 (65-92)	11	0.153	71 (63-88)	8	0.079	0.107
Cypermethrin	0.104	79 (74-84)	3	0.157	71 (59-88)	9	0.019	0.104
Deltamethrin	0.108	93 (81-111)	12	0.144	71 (67-80)	4	0.057	0.108

[a]n = 5; CV: coefficient of variation; LOD: method's limit of detection; LOQ: method's limit of quantification.

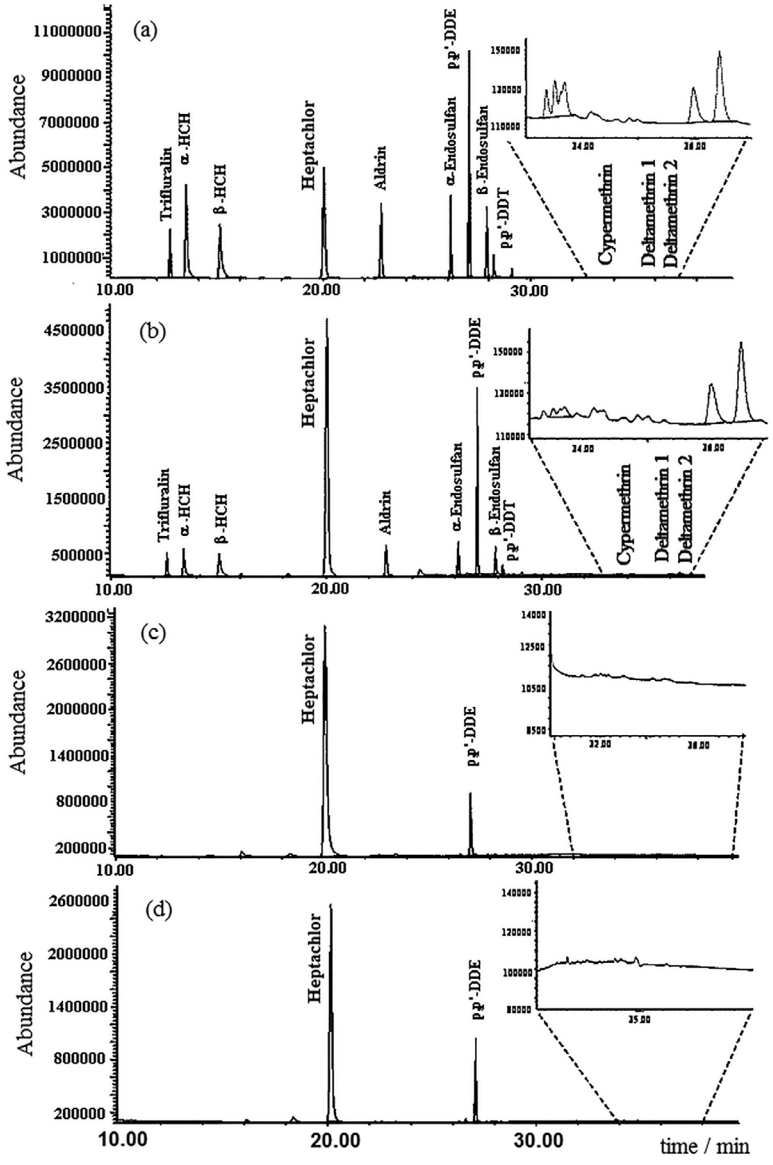

Figure 2. GC-ECD chromatograms: (a) control sample spiked at the highest spike level; (b) control sample spiked at the lowest spike level; (c) control sample extracted using the proposed method; (d) example of analyzed sample.

Table 5. Comparison between the methods described in the literature and the method proposed for determining pesticides in breast milk

Analyte	Analytical procedure			Recovery / %	CV / %	LOD/LOQ	Site	Reference
	Extraction	Clean-up	Instrument					
α-endosulfan, β-endosulfan, γ-HCH, α-HCH, aldrin, p,p'-DDT, p,p'-DDE, cypermethrin, deltamethrin, trifluralin	SPD (Celite®; Hex, acetone, DCM)	–	GC/ECD	67-120	3-20	0.003-0.079 μg mL^{-1}	Lucas do Rio Verde-MT, Brazil	This study
α-endosulfan, β-endosulfan, β-HCH, γ-HCH, aldrin, dieldrin, HCB, p,p'-DDT, p,p'-DDE	LLE (Hex, acetone)	SPE (Florisil, Hex, DCM)	GC/ECD	53-109	–	0.01-0.04 mg kg^{-1}	Indonesia	9
p,p'-DDT, p,p'-DDE, p,p'-DDD, o,p'-DDT, o,p'-DDE, o,p'-DDD, cyfluthrin, deltamethrin, cypermethrin, permethrin	OC: LLE (Hex) Pyrethroid: LLE (pH 4, ACN)	OC: H_2SO_4 Pyrethroid: GPC (silica gel columns with Hex, ethyl ether)	GC/ECD	–	–	OC: 0.042-0.049μg mL^{-1} Pyrethroid: 0.181-0.576 μg mL^{-1}	South Africa	10
HCB, β-HCH, γ-HCH, Σ-HCH, dieldrin, o,p'-DDE, p,p'-DDT, p,p'-DDE, p,p'-DDD, o,p'-DDT, Σ-DDT	LLE (Hex, ACN, EtOH)	SPE (Florisil, Na_2SO_4; DCM, Hex)	GC/ECD	80-93	–	1 ng g^{-1} of fat	Tunisia	11
DDE, DDD, α-HCH, β-HCH, γ-HCH, HCB	LLE (Chloroform, MeOH, Hex)	H_2SO_4	HRGC/ECD	–	–	0.98-1.64 ng g^{-1} of fat	Croatia	12
DDTs, HCHs, HCBs, CHLs	LLE (Diethyl ether, Hex)	–	GC/ECD	92-101	–	–	India	13
p,p'-DDT, p,p'-DDE, p,p'-DDD, o,p'-DDT, dicofol	LLE (Hex, EtOH, diethyl ether, DCM)	–	GC/MS	84-94	–	0.01-0.20 ng g^{-1} of fat	China, Korea and Japan	14
4,4'-DDE, 4,4'-DDD, 4,4'-DDT, ΣDDT, 2,4'-DDE, 2,4'-DDD, 2,4-DDT	LLE (Hex)	H_2SO_4	GC/ECD	–	–	–	Mozambique, South Africa	15
α-HCH, β-HCH, γ-HCH, δ-HCH, o,p'-DDE, p,p'-DDE, o,p'-DDD, p,p'-DDD, o,p'-DDT, p,p'-DDT	SPE (MeOH, Hex)	–	GC/ECD	80-93	0.56-9.86	0.0004 mg kg^{-1} of milk	Cidade dos Meninos, Duque de Caxias-RJ, Brazil	19
α-HCH, β-HCH, γ-HCH, HCB, aldrin, γ-chlordane, α-endosulfan, dieldrin, trans-nonachlor, β-endosulfan, p,p'-DDT, p,p'-DDE, p,p'-DDD, methoxychlor, mirex	SPE (C_{18}, Hex, ActOH, MeOH, ACN)	SPE (Florisil; Hex, DCM, petroleum ether, ActOH, acetone)	GC/ECD	72-110	2.2-5.8	0.0030-0.1720 ng mL^{-1}	Rio de Janeiro and Grande Rio-RJ, Brazil	20
HCB, o,p'-DDT, o,p'-DDE, o,p'-DDD, p,p'-DDE, p,p'-DDD, p,p'-DDT, dieldrin, endrin, *cis*-heptaclorepoxi, α-chlordane, γ-chlordane, oxychlordane, trans-nonachlor, Σ Parlar (toxaphene), α-HCH, β-HCH, γ-HCH, Σ endosulfan	LLE (Na_2SO_4, Hex, ActOH)	GPC (Bio-Beads S-X3 with Hex and ActOH)	GC/ECD	–	–	0.001 μg g^{-1} of fat	São Paulo-SP and Belo Horizonte-MG, Brazil	21
p,p'-DDT, p,p'-DDE, p,p'-DDD	SPE C_{18} - ActOH, acetone, MeOH	SPE (C_{18} - DCM, ActOH, petroleum ether, acetone, Hex	GC/ECD	82-103	2.2-3.1	0.0040-0.0340 ng mL^{-1}	Madeira River Basin-AM, Brazil	22
Bifenthrin, λ-cyhalothrin, permethrin, cyfluthrin, cypermethrin, es/fenvalerate, deltamethrin, tetramethrin, phenothrin, resmethrin	Hex:DCM (2:1) SPD (Florisil, ActOH-DCM(2:1), C_{18}, ACN	–	GC/MS	48-91	3-20	8.3-3600 pg g^{-1} of fat	Mozambique, South Africa	25

SPD: solid-phase dispersion; SPE: solid-phase extraction; LLE: liquid-liquid extraction; GPC: gel permeation chromatography; –: not shown; DCM: dichloromethane; Hex: hexane; ACN: acetonitrile; MeOH: methanol; ActOH: ethyl acetate; EtOH: ethanol; OC: organochlorine compounds; GC: gas chromatography; ECD: electron capture detector; MS: mass spectrometer; LOD: method's limit of detection; LOQ: method's limit of quantification.

Table 6. Stability study of the pesticides in frozen breast milk samples (12 days)

Analyte	Spiked level / (μg mL^{-1})	Average[a] / % (Interval)	CV / %	Analyte	Spiked level / (μg mL^{-1})	Average[a] / % (Interval)	CV / %
Trifluralin	0.015	98 (93-97)	2	p,p'-DDE	0.015	121 (109-127)	7
α-HCH	0.027	114 (103-116)	5	β-endosulfan	0.013	111 (104-110)	3
γ-HCH	0.028	113 (106-115)	4	p,p'-DDT	0.107	97 (64-121)	25
Aldrin	0.015	111 (100-116)	6	Cypermethrin	0.104	129 (107-136)	12
α-endosulfan	0.015	117 (106-123)	8	Deltamethrin	0.108	105 (94-119)	9

[a]n = 5; SL: spiked level; CV: coefficient of variation.

questionnaire. The following results are a description of the data collected during the study.

Among the breast-feeding mothers, 64% were between 20 and 29 years old, the average age being 26 years (RSD = 6). Their level of education demonstrates that 100% of them are literate, having at least started basic education.

Their professional information show that 21% of them have worked in fields. Only one breast-feeding mother (1.6%) declared to work with pesticide, as an agronomist responsible for a grain warehouse. Some of them (6.5%) worked in the rural area, but not in direct contact with pesticides. Regarding their living place, 43.5% said they have lived in the rural area for some time. Regarding pregnancy and childbirth, 71% were multiparous and 29% were primiparous, with three childbirths in average.

When asked about the use of domestic pesticides, 50% reported to use some sort of product. Pyrethroids were the most mentioned, either in the form of spray or tablets for electric dispenser. Among the breast-feeding mothers who said they use pesticides at home, 33% used them once or twice a week, whereas 15% used them daily. With respect to fumigation provided by specialized companies, 36% said they have used that service, 53% of them in the last six months. Around 81% of the breast-feeding mothers have been living in Lucas do Rio Verde for up to 10 years, of which 26% have lived there for just 1 year.

Detection and quantification of pesticide residues in breast milk samples

Fifty-five percent of the samples had some of the studied pesticides. In 16% of the samples more than one pesticide was detected (Table 7). The frequency of detection per pesticide is shown in Table 8. Considering the fact that organochlorine compounds preferably accumulate in adipose tissues, Table 9 presents the levels of the quantified analytes in μg mL^{-1} of milk and in μg g^{-1} of fat. The average fat content of such samples is also presented. The identification of the analytes in one of the analyzed sample, compared with a spiked control sample, is shown in Figure 2.

Table 7. Number of analytes detected in breast milk samples (n = 62) from breast-feeding mothers living in Lucas do Rio Verde, Mato Grosso state

Number of analytes detected in the sample	n	Frequency of detection / %
0	28	45
1	24	39
2	6	10
3	4	6
TOTAL	62	100

Table 8. Frequency of pesticides detection in breast milk samples (n = 62) from breast-feeding mothers living in Lucas do Rio Verde, Mato Grosso state

Analyte	Total of samples with pesticides	Detected (> LOD and < LOQ)	Quantified (> LOQ)	Frequency of detection / %
p,p'-DDE	33	15	18	53
Aldrin	7	7	0	11
p,p'-DDT	5	2	3	8
β-endosulfan	3	1	2	5

LOD: method's limit of detection; LOQ: method's limit of quantification.

p,p'-DDE was found in 53% of the samples while p,p'-DDT was detected only in 8% (Table 8). Only three of the analytes β-endosulfan, p,p'-DDT and p,p'-DDE were quantified in 19 samples (31%). p,p'-DDT was quantified in samples where p,p'-DDE was also quantified and the ratio between those two compounds confirms a non-recent exposure to DDT.

Discussion

Among the studied pesticides, those whose use is still authorized in Brazil, α-endosulfan, β-endosulfan, cypermethrin, deltamethrin and trifluralin are commonly applied in soybean, corn, cotton, rice, sorghum, and bean fields in Lucas do Rio Verde.[2] The other analytes, i.e., aldrin, p,p'-DDT, α- and γ-HCH were widely used in the past. All these substances are stable and lipophilic (Table 2),

Table 9. Pesticide residues in breast milk taken from breast-feeding mother living in Lucas do Rio Verde, Mato Grosso state, in μg mL^{-1} of milk and μg g^{-1} of fat

Analyte	Range[a] / (μg mL^{-1})	Average fat content / %	Range[a] / (μg g^{-1})	Median	3rd quartile / (μg g^{-1})
β-endosulfan	0.016-0.020	3.1	0.54-0.61	< LOD	< LOD
p,p'-DDT	0.170-0.397	4.1	2.62-12.41	< LOD	< LOD
p,p'-DDE	0.021-0.543	3.8	0.32-12.03	< LOQ	1.01

[a]Range of concentrations among quantified samples.

which can facilitate their accumulation in the mother's fat and further elimination through breast milk during the lactation period.

The analysis of complex matrices requires additional care in controlling interferences. The matrix effect is caused by the presence of matrix constituents that can result in an increase or decrease in the analytical response. Due to the high lipid content of human milk that can be co-extracted by extracting solvents, the study of matrix effect was carried out showing the need to use matrix matched standard solutions to overcome this effect.

The proposed method is an alternative for the simultaneous determination of different classes of pesticides (organochlorines, pyrethroids and dinitroanilines). A diversity of organic solvents and solvent mixtures were necessary to extract the pesticides with different properties from the matrix. Extraction with hexane:acetone (1:1, v/v) and hexane:dichlorometane (4:1, v/v) (condition 2, Table 1) was selected to carry out the validation method. It showed good results regarding accuracy and precision, and the detection and quantification limits were comparable to those presented in the literature (Table 5). Furthermore, the method is simple, rapid, does not require sophisticated equipment, and uses a low quantity of organic solvent. By using solid-phase dispersion with Celite®, it was possible to extract the pesticides and purify extracts in a single step.

Among the p,p'-DDT metabolites, p,p'-DDE is the most frequently detected among the general population, in view of its low metabolization rate and the fact that food is the primary source of contamination with it. That prevalence of p,p'-DDE over p,p'-DDT suggests a prior exposure to DDT, as the average period needed for the metabolization of p,p'-DDT to p,p'-DDE is twelve months.[23]

Three samples presented quantifiable concentrations of p,p'-DDT of the same order of magnitude as p,p'-DDE and much higher than the ones from the other mothers. These concentrations are outliers. These three mothers had in common the fact that they either lived near agricultural area or work in the rural area.

Excluded these outliers, DDE levels ranged from 0.32 to 6.78 μg g^{-1} of fat, and were similar to the ones determined in other regions of Brazil, such as in Porto Alegre in 1987/88[34] and Rio de Janeiro in 2000;[20] and higher than the ones determined in São Paulo and Belo Horizonte in 2001.[21] These are studies carried out in very urbanized areas in which the mothers were exposed environmentally and not occupationally. Our results were also of the same order of magnitude as the one carried out in the Madeira River area, Amazonas in 2001/2002.[22] This studied population lives alongside the Madeira River, was not occupationally exposed and the diet assessment indicated that the most frequently consumed food was fish. Thus, the authors concluded that the found levels were associated to fish consumption. Other studies carried out in Brazil before the ban of DDT use in agriculture in 1985,[18,23] detected much higher concentrations than the ones developed after the ban. DDT use for health campaign in Brazil was forbidden only in 1997 and was used for malaria mosquito control in endemic areas until this year.

In comparison with data provided by international studies, the levels found herein are above those of p,p'-DDE and below those of α-HCH and γ-HCH found in Croatia,[12] and below those of α-HCH and γ-HCH and above those of p,p'-DDE and p,p'-DDT found in China.[17]

Although higher than some other results reported in Brazil and worldwide, this difference was not enough to discourage breast-feeding, so all the mothers that took part in this research received a document containing the results of the analyses and an express recommendation to continue breastfeeding, since it provides important benefits for the health of both the baby and mother.

Conclusion

The developed multi-residue method was demonstrated to be efficient for the determination of different classes of pesticides by combining the analyte extraction and extract purification into a single step. The method was used to evaluate the presence of analytes in breast milk samples taken from women living in Lucas do Rio Verde, Mato Grosso state, a region characterized by large pesticide consumption.

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