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Article

Simultaneous GC-NCI-MS Determination of Persistent Organic Pollutants and Current-Use Pesticides in Breast Milk Samples

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To preserve human health, persistent organic pollutants (POPs) and current-use pesticides (CUPs) should be monitored in fatty tissues, including breast milk. Therefore, this study aimed to optimize sample preparation conditions using a 3^2 factorial design for the determination of POPs and CUPs by gas chromatography coupled to mass spectrometry with negative chemical ionization (GC-NCI-MS). The method was validated for 57 POP and CUP compounds using hexane:acetone for extraction and clean-up by dispersive solid phase extraction (d-SPE) with Florisil®. The matrix effect was compensated by extracted analytical calibration. Method validation showed satisfactory results with limits of detection of 3 to 13 ng g^{-1} of fat. The method presented adequate accuracy (recoveries from 72 to 117%) and precision (relative standard deviation (RSD) ≤ 18%) and was applied to breast milk samples from Rio Grande do Sul State, southern Brazil, wherein all samples contained at least one compound. With principal component analysis, it was possible to associate the pesticides detected with the city of origin of the samples and the number of pregnancies of nursing mothers. Additionally, the analytical method was effective for the determination of trace levels of POPs and CUPs in breast milk and can be applied in biomonitoring studies.

Keywords: breast milk, POPs, pesticides, GC-NCI-MS, sample preparation

Introduction

Pesticides are intensively used in agriculture to eliminate pests and diseases and maintain the quality and durability of crops. Nonetheless, these compounds can be toxic to the environment and human health as they can be bioaccumulated and remain in the environment for generations, being transported to remote places, including the polar region. Pesticides and other contaminants coming from multiple external sources enter the human body, thereby posing a potential risk to human health.[1] The Stockholm Convention is a global treaty to protect human health and the environment from chemicals such as pesticides that have harmful impacts on human health or the environment. This convention aimed to implement actions that should be taken to eliminate and replace persistent organic pollutants (POPs), as well as monitoring the persistence in the environment. POP compounds are characterized by being lipophilic and can be accumulated especially in the adipose tissues of animals. In the human case, POPs can be accumulated in breast milk. This is a matrix with high fat content and the breastfeeding is a way of releasing the contaminants to the new organism. According to the Global Monitoring Program (GMP), created by the Stockholm Convention, breast milk can be used as a bioindicator in assessing the effectiveness of eliminating the production and the use of POPs.[2] Breast milk is the first option of infant feeding providing immunoprotection and supplying nutrients and bioactive compounds to the infant. Environmental and dietary factors potentially lead to excessive chemical exposure during neonatal life, including lactation. The World Health Organization (WHO) recommends a breastfeeding of up to two years with the first six months being exclusive breastfeeding. On the other hand, pollutants are known to be present in human milk.[3]

Most studies related to analyzing pesticides in breast milk are about persistent organochlorine pesticides.[4-18] Some applications focus more on other classes of pesticides (e.g., pyrethroids,[19-25] organophosphates[21-25] and carbamates[25]) and few studies have been developed for non-persistent pesticides covering several classes.[26-28] However,

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POPs are only a part of the chemicals with potential for environmental contamination. More recent pesticides, such as pyrethroids, can be included in this group that has emerged as an alternative to pesticides with greater toxic potential. Due to this dynamic, monitoring these residues is not necessarily restricted to prohibited pesticides, albeit current-use pesticides (CUPs) are also important.[21] Several works[19,29,30] have reported the bioaccumulation of POPs and CUPs (e.g., pyrethroids) in breast milk.Thus, developing analytical methods for the simultaneous multiclass determination of POPs and CUPs residues in breast milk is paramount to allow extensive monitoring of exposure to these compounds.

The QuEChERS (quick, easy, cheap, effective, rugged, and safe) method was developed initially by Anastassiades *et al*.[31] for the determination of pesticide residues in food and has been employed in various food matrices. Acetate[32] and citrate[33] QuEChERS methods were developed later and became world reference to the Association of Official Analytical Chemists (AOAC) and to the European Committee for Standardization, respectively. The QuEChERS method is probably the most successful development in sample preparation for the determination of organic compounds in food samples[34,35] and can be an adequate procedure for breast milk samples.

Gas chromatography coupled to mass spectrometry with negative chemical ionization (GC-NCI-MS) was selected in others works to enhance sensitivity in several determinations of pesticide residues.[36-38] The NCI mode is an ionization technique that is frequently used in GC-MS for analyzing electrophilic molecules with high selectivity and sensitivity in a very effective way.[39]

Given the above, this study aimed to develop, validate, and apply an effective multiresidue method for simultaneous determination of POPs and current-use pesticides in breast milk samples. A 3^2 factorial design was applied to develop the sample preparation step, evaluating different extraction solvents and sorbents for the clean-up step. Analysis was performed by GC-NCI-MS to achieve high sensibility. The method developed was applied in breast milk samples collected in Rio Grande do Sul State, southern Brazil, and principal component analysis (PCA) was applied to correlate detected pesticides with samples information.

Experimental

Chemicals and apparatus

Solvents *n*-hexane and formic acid of chromatographic grade were purchased from Sigma-Aldrich (St. Louis, USA) and acetone, acetonitrile and glacial acetic acid from J. T. Baker (Phillipsburg, USA). Certified standards with high purity from LGC Standards (Wesel, Germany) were used to prepare individual standard stock solutions in acetone. Bromophos ethyl and deuterated trifluralin-d_{14} were used as internal (IS) and surrogate (SS) standards, respectively. Anhydrous sodium acetate, anhydrous magnesium sulfate ($MgSO_4$) and sodium chloride analytical grade were acquired from J.T. Baker (Phillipsburg, USA). Sorbents primary secondary amine (PSA), octadecylsilane (C18) and aminopropyl (NH_2), with 40 μm of particle size, were from Agilent Technologies (Santa Clara, USA), Supel™ QuE Z-Sep+ was from Sigma-Aldrich (St. Louis, USA) and Florisil® 60-100 mesh was from J. T. Baker (Phillipsburg, USA). Florisil® was prepared by heating in muffle at 550 °C overnight and transferred to an oven at 130 °C for 5 h and immediately deactivated through the addition of 8% (m/v) ultrapurified water as described by Orso *et al*.[40] EMR-Lipid® and Polish® were from Agilent Technologies (Santa Clara, USA) as well the syringe filters of 13 mm and 0.2 μm of porosity. Ultrapurified water was obtained with a Direct-Q 3 UV system from Millipore, (Molsheim, France). Analytical balances AUW-220D and UX-420H from Shimadzu (Kyoto, Japan), vortex mixer Microtecnica model QL901 (Curitiba, Brazil), refrigerated centrifuge Novatecnica NT825 (Piracicaba, Brazil) and evaporator Biotage TurboVap® LV (Düsseldorf, Germany) were used.

Measurements were carried out on a gas chromatograph CP 3800 coupled to a triple quadrupole mass spectrometer MS1200 from Varian (Walnut Creek, USA). The GC-NCI-MS system was equipped with CP 8400 autosampler, electronic flow control, injector 1079 with programmable temperature vaporizing and data acquisition software MS Workstation 6.9.2.

Instrumentation conditions

Gas chromatographic separation was performed on the capillary column VF-5-MS (5% phenyl and 95% dimethylpolysiloxane) with 30 m × 0.25 mm internal diameter, 0.25 μm film thickness from Agilent Technologies (Santa Clara, USA). The oven temperature program was as follows: 80 °C, held for 1 min; 25 °C min^{-1} to 190 °C, held for 1 min; 5 °C min^{-1} to 280 °C. Total run time was 25 min with solvent delay of 5 min. Injection volume of 2 μL was used in a splitless mode with an initial temperature of 80 °C, increased at 200 °C min^{-1} to 300 °C and held for 13.2 min before return to initial temperature. Temperature of ion source was set at 230 °C, transfer line at 275 °C and MS/MS manifold at 40 °C. Chemical ionization was done

at 70 eV with methane (9 torr) as reagent gas. Carrier gas was helium 99.9999% purity (Air Liquide, Brazil) at 1.0 mL min^{-1}. Analyses were performed in a single MS mode with selected ion monitoring (SIM).

Chromatographic conditions were based on a previous study developed in our research group by Kolberg *et al.*[36] for the NCI mode. We decided for this ionization mode due to the higher signal/noise ratio obtained for the selected analytes. Different ionization source current (100, 150 and 200 mA) and the voltage of the electro multiplier (900, 1100 and 1200 V) were evaluated in order to select the best signal/noise ratio. The proposed method was validated evaluating the parameters: linearity, analytical curve, limits of detection and quantification, precision (as repeatability and intermediate precision), trueness (as recovery) and matrix effect according to SANTE.[41]

Sample preparation evaluation

Experimental planning and PCA analysis

In order to evaluate the influence of variables like extraction solvent (acetone:hexane, ethyl acetate and acetonitrile) and clean-up sorbents (Florisil®, C18 and EMR-Lipid®), a 3^2 factorial design was performed using random configuration generated by the software Statistica version 8.0,[42] resulting 9 different combinations evaluated in duplicate. The variables and the levels are presented in Table S1 (Supplementary Information (SI) section).

Considering the difficulty in obtaining blank samples, the factorial design tests were performed with 6 mL of reconstituted infant formula, that has a similar composition compared with breast milk, followed by extraction with 6 mL of solvent. Partition was obtained with the addition of 2.4 g $MgSO_4$ and 0.6 g NaCl. The clean-up step was performed with 600 mg of $MgSO_4$ and 200 mg of Florisil® or C18. For the EMR-Lipid® tests, 800 mg of this sorbent and 1.6 g of Polish® were used. A 2 mL aliquot of the cleaned extract was used for evaluation of coextractives removal and another for GC-NCI-MS analysis after filtration in 0.2 µm syringe filter. The principal component analysis (PCA) was performed correlating the results of the breast milk samples with the answers of the questionnaire. The PCA evaluation was done using Statistica 8.0 software[42] and the data were previously auto scaled. For each response, a PCA was generated for the evaluation of possible correlation of the samples results.

Evaluation of coextractives after the clean-up step

The clean-up efficiency of the extract was evaluated quantitatively through the amount of coextractives resulting from each sample preparation procedure. For comparison, 2 mL volume of the extracts was evaporated to dryness in the TurboVap® LV for 30 min at 60 °C before and after clean-up. The amount of coextractives present in each extract after the clean-up step was evaluated gravimetrical as described by Oshita and Jardim[43] in order to select the most suitable solvent and sorbent. The removal of coextractives was calculated according to the mass difference of coextractives before and after the clean-up as described by Sapozhnikova and Lehotay.[44]

Validation of the method for determination of POPs and CUPs in breast milk

Figure 1 presents the proposed method for the determination of POPs and CUPs in breast milk. The method validation was performed according to Araujo,[45] Zanella *et al.*[46] and SANTE,[41] as present in Table S2 (SI section).

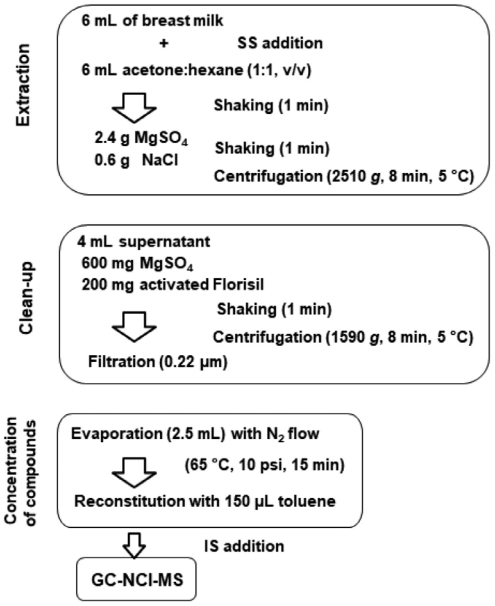

Figure 1. Sample preparation procedure for the determination of POPs and CUPs in breast milk.

Method applicability

Following the WHO[47] recommendation, sampling of breast milk was performed between 3 and 8 weeks after childbirth in the Rio Grande do Sul state, Brazil. Samples were stored in previously sterilized glass vials at –20 °C until analysis. Not all samples were from mothers in the first pregnancy. Women who accepted to participate in the study signed a free and informed consent form, which describes the objectives of the study and that participation, would be voluntary. With the term, a questionnaire was applied to the nursing mothers including questions about

the characteristics of the nursing mothers, such as age, if was the first gestation, as well as the type of feeding, locality where it resides, proximity to chemical industries or agricultural areas. In total, 20 nursing mothers donated breast milk and completed the questionnaire. To avoid a biased measurement of results, the most important questions were answered objectively, with numeric values or closed answers like yes/no. Data analysis was carried out in order to evaluate, through the frequency of the answers, the profile of the donors to correlate with the possible contamination of the breast milk samples. The study was approved by the Research Ethics Committee of Universidade Federal de Santa Maria, Brazil (CAAE: 06095212.8.0000.5346).

Results and Discussion

GC-NCI-MS conditions for the determination of POPs and pesticides in breast milk

All compounds were analyzed according to the conditions described in the "Instrumentation conditions" sub-section. Quantification was performed in SIM mode and the analytes were identified according to their retention time (t_R), quantification ion (target ion) and qualifier ions. The SIM parameters for each compound, including internal and surrogate standards, are presented in Table S3 (SI section).

Bromophos ethyl (internal standard) and trifluralin-d_{14} (surrogate) presented t_R values of 12.4 and 7.3 min, respectively, and the monitored ions were 79 + 169 + 256 and 319 + 350, respectively. The internal standard was used to check the stability of the GC-NCI-MS system but not for calibration. The surrogate standard was added before extraction and was used to check if the sample preparation and analysis were performed correctly. In order to verify the t_R of each analyte and its characteristic ions (SIM mode), 2 μL of each standard was injected at 500 and 250 μg L^{-1} for pesticides and polychlorinated biphenyls (PCBs), respectively, in the full scan mode. With these results, the monitored ions of each analyte were selected (Table S3, SI section). Preferentially the selected ions should have a high mass to minimize interferences in the detection system.

Optimization of chromatographic system

For the accomplishment of factorial design 3^2, the signal response (area) acquired for the representative compounds aldrin, azoxystrobin, bifenthrin, bioallethrin, bromophos methyl, cyfluthrin, cypermethrin, chlordane, chlorpyrifos ethyl, chlorpyrifos methyl, dieldrin, endosulfan alpha, endosulfan beta, endosulfan sulfate, endrin, esfenvalerate, fenvalerate, fipronil, HCH alpha, HCH beta, HCH gamma, heptachlor, heptachlor epoxide, HCB, mirex, oxyfluorfen, pendimethalin, permethrin, prothiofos, quintozene, tefluthrin, tetradifone, transfluthrin and trifluralin were evaluated. The interactions between the variables studied were verified by interactive plotting for each compound with a confidence level of 95%. When analyzing the experiment by analysis of variance (ANOVA), it was possible to observe that the *p*-value that indicates if the areas obtained in the described tests are statistically different. Figure S1 (SI section) presents the results for representative compounds of different chemical groups: cyfluthrin (pyrethroid), HCB (organochlorine), prothiophos (organophosphorus) and trifluralin (dinitroaniline).

As can be observed independently of the chemical group, the behavior was similar for all compounds and the best analytical response was obtained using a mixture of acetone and hexane for the extraction step, followed by a clean-up with Florisil®.

Evaluation of coextractives after the clean-up step

For the extract clean-up tests with EMR-Lipid® presented good performance when acetonitrile was used as solvent, although an exchange to acetonitrile would be required when others nonpolar solvents are used (Figure S2, SI section). In addition, when the extracts containing solvents different of acetonitrile were added directly to the EMR-Lipid® the resulting volume after the centrifugation step was insufficient for the Polish® step. Possibly this condition occurs due that the Lipid EMR® was developed for methods that use acetonitrile as the extraction solvent.

The gravimetric analysis was performed with the purpose of quantifying the presence of coextractives in the extracts before and after clean-up. For this evaluation, an aliquot of the extract was evaporated and another aliquot was subjected to the clean-up step without evaporation. Acetonitrile extracted smaller amount of coextractives from the matrix when compared with ethyl acetate and acetone:hexane. For this reason, acetonitrile has been widely used in methods for determination of pesticide residues in food and environmental samples.[48] The amounts of coextractives were evaluated by mass difference between evaporated extracts, obtained with and without clean-up, and it was possible to evaluate how much coextractives from matrix was removed by the sorbents using the factorial design tests.

The acetonitrile extraction and the respective clean-up sorbents provide less coextractives in the final extract. The use of EMR-Lipid® removed at least 90% of coextractives

from samples, proving the efficiency of this sorbent. For the extraction of POPs with lipophilic characteristics, solvent such as hexane is used to improve the extraction of these compounds, and it is used with less nonpolar solvents such as acetone to reduce coextractive extraction.[49] Methods for breast milk analysis require very low limits of detection. In our study, the extraction with acetone and hexane was chosen because was the one that presented the best analytical responses. In Figure S3, the total ion chromatograms obtained from a blank sample spiked at 250 μg L^{-1} extracted with a mixture of acetone:hexane, followed by three different sorbents for the clean-up step. The extraction procedure using acetone:hexane and Florisil® showed about twice the analytical response compared to EMR-Lipid® clean-up. In addition, for the clean-up with EMR-Lipid® it would be necessary to evaporate the extract to exchange the solvent to acetonitrile prior the clean-up step.

Optimized method

The bioaccumulation of hydrophobic compounds, due to their high lipophilicity, stability and resistance to degradation, occurs in the fatty portion of breast milk.[50,51] Considering that the fat percentage in breast milk change during lactation, the concentration of pesticides in breast milk is expressed in relation to the fat content. For this reason, the enrichment factor is one of the most important steps in the method of analyzing breast milk because, according to GMP recommendations, results should be presented is ng g^{-1} of fat.[52,53] Thus, the validated method (Figure 1) was the one that obtained the better analytical response, even though it was not the one that obtained the greatest removal of coextractives.

Validation results

Selectivity, linearity, analytical curve and matrix effect

The selectivity evaluation was performed by comparing the chromatograms of the blank sample, blank reagent and spiked blank sample. There are no interferers with the same quantification ions at the same t_R of the analytes evaluated, evidencing the method selectivity. Regarding the chromatographic separation, the method was not selective for six analytes, because they had the same quantification ions and the same t_R. The quantification was calculated as the sum of them, as was the case of the analytes HCH beta and HCH gamma, PCB 28 and 31, PCB 118 and 149.

The linearity for each compound, evaluated in triplicate injections of solutions prepared in spike blank samples, confirmed that the response was linear from the limit of quantification (LOQ) until 300 μg L^{-1}. The use of extracted analytical curve is an alternative for calibration where blank samples are spiked with different concentrations before the extraction step. Because the method has a stage of enrichment of the analytes, this type of calibration becomes interesting as it allows to compare the analytical curve and the samples in the same conditions. In addition, the matrix effect and recovery values can be compensated. The matrix effect is characterized by suppression or increment of the analytical signal and is considered to be low when < 20%, medium for 20-50% and high for > 50%.[41] As can be observed for most compounds, an increase in the signal occurs because the components from matrix can act as protectors and preferentially adsorb the active sites of the chromatographic system.[54,55] ME with significant values (> 20%) are very common in the analysis of complex samples such as food.[56,57] In this work, matrix matched calibration was used in order to compensate the matrix effect observed.

The adequacy of the analytical calibration curves was confirmed by the analysis of the variance for the fit of a least squares model for each analyte. For the exclusion of replicates, the maximum value of 22.2% of the data set, excluding the points with extreme residual value identified by the Grubbs test. For compounds that obtained a linearity of 5 to 300 μg L^{-1}, the maximum of excluded points were four. Analytical curves were considered adequate as long as they comprised at least seven levels and at least one replica of all concentrations. According to the results, propiconazole did not obtain a significant regression and kresoxim methyl and parathion ethyl, although they obtained a significant regression evaluated by the *F* test and determination coefficient (r^2) of 0.989 and 0.964, respectively, it was identified lack of adjustment of the model, because the residues are not distributed randomly along the analytical curve and these compounds were not included in the validation.

Limit of quantification (LOQ) and detection (LOD), trueness and precision

Method LOQ was established as the lowest spike level of the analyte with accuracy of 70-120% and precision ≤ 20%. The LOD was calculated dividing the LOQ by 3.33. It should be noted that the concentration factor of the extraction method of 16.7 times allowed the quantification of the compounds at ng g^{-1} levels. LOD and LOQ values are presented in Table S3 (SI section). LOQ values were considered adequate for monitoring pesticide residues in breast milk.

As presented in Table S3 (SI section), LOD and LOQ values were from 3 to 13, and 9 to 43 ng g^{-1} fat,

respectively. Limits of quantification reported in the literature regarding analyses of breast milk are variable since it depends on the instrumental technique used for analysis, the analytes selected and the sample preparation method. Nevertheless, LOD and LOQ values reached by this study were considered adequate for monitoring pesticide residues in breast milk and are comparable to the ones reported in literature and presented in Table S4 (SI section), especially considering that this study reported a multirresidue determination of several classes of POPs and CUPs at lower levels that the another multiresidue method.[28]

The trueness and precision parameters were evaluated by the analysis of four levels of fortification in six replicates, by calculation of recovery and relative standard deviation, respectively, and the results are presented in Table S3 (SI section). To obtain the concentration in ng g^{-1} fat, the average fat content (3.5 g *per* 100 mL) present in the 20 samples evaluated and the method concentration factor (16.7 times) were considered. Therefore, the levels 5, 10, 25 and 50 μg L^{-1} correspond to 9, 17, 43, and 86 ng g^{-1} of fat. Validation results showed in Table S3 (SI section) indicated that the proposed method presented adequate recovery (72 to 117%) with relative standard deviation (RSD) ≤ 18% for the 57 compounds studied under repeatability and intermediate precision conditions.

Method application to real samples

The proposed method was applied in 20 samples collected in different cities of Rio Grande do Sul state and the results found in breast milk samples are presented in Table 1. All samples showed contamination by at least one compound and 14 pesticides were detected in breast milk samples. The pesticide with the highest concentration was lambda-cyhalothrin, and it was detected in 40% of the samples in the range of 1.8 to 5.2 μg g^{-1}. This pesticide is frequently used in foliar application, as well for cereals, vegetables and fruits storage. The pesticides bifenthrin and transfluthrin, from the pyrethroids group, were also detected, emphasizing the importance of the development of multiresidue methods.

Dicofol, that is under review to be included in the list of POPs, was detected in a sample at 0.19 μg g^{-1} and endosulfan beta was detected in 55% of the samples. It should be noted that endosulfan became part of the POPs group by the Stockholm Convention in 2011 and in Brazil its use was allowed until 2013. In a previous study conducted by our laboratory,[5] HCB was present in 75% of the samples analyzed, followed by HCH gamma (40%), heptachlor (30%), HCH beta (25%), mirex (20%) and HCH alpha (10%). With respect to HCB, similar results were found in this present study once it was detected in 70% of the samples. Similar results were reported for samples from Belgium,[58] which analyzed 190 samples of breast milk and HCB and HCH beta were detected in 86 and 22% of the samples, respectively. In Croatia, the study developed by Klinčić[59] detected HCB and HCH in 100% of samples when analyzing 38 samples. In both studies the 1-chloro-4-[2,2-dichloro-1-(4-chlorophenyl)ethenyl]benzene (4,4'-DDE) that is a 4,4'-dichlorodiphenyltrichloroethane (DDT) degradation product was also detected in 100% of the samples.

In Brazil, studies carried out by Mesquita[60] with 50 samples showed following percentage of contamination:

Table 1. Compounds found in breast milk samples

Sample	Concentration / (μg g^{-1} fat)																			
	P30	P32	P36	P37	P38	P39	P41	P42	P44	P45	P54	P55	P56	P62	P66	P68	P88	P90	P92	P95
Bifenthrin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.20
Cyhalothrin lambda	3.19	1.79	n.d.	n.d.	n.d.	n.d.	5.21	n.d.	n.d.	5.18	n.d.	n.d.	4.93	n.d.	2.99	3.82	n.d.	n.d.	n.d.	3.38
Dicofol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.19	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Endosulfan beta	0.15	0.045	n.d.	0.13	n.d.	0.13	n.d.	n.d.	0.24	n.d.	n.d.	0.84	0.21	0.083	0.16	0.10	n.d.	n.d.	0.081	n.d.
Epoxiconazol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.18	0.37	n.d.	0.88	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.83	0.64	n.d.	1.0
HCH beta + gamma	0.037	0.040	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.055	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
HCB	0.18	0.14	0.13	n.d.	0.13	0.067	n.d.	n.d.	0.13	0.17	n.d.	0.12	0.13	0.13	0.12	0.14	n.d.	0.14	0.13	n.d.
Mirex	0.070	0.046	0.11	0.10	0.70	0.21	0.48	< 0.009	0.05	0.11	0.10	0.47	0.66	n.d.	0.11	0.18	0.069	0.21	0.048	0.088
PCB 52	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.15	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Pendimethalin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.21	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Transfluthrin	n.d.	0.046	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Tetradifon	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.17	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Trifluralin	0.087	0.084	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.10	n.d.	0.085	n.d.	n.d.	n.d.	n.d.	n.d.	0.089	n.d.	n.d.

n.d.: not detected; HCB: organochlorine; PCB: polychlorinated biphenyls.

4,4'-DDE 100%, 4,4'-DDT 90%, HCH beta 84%, 1,1-dichloro-2,2-bis(4-chlorophenyl)ethane (4,4'-DDD) 82%, endosulfan alpha 78%, chlordane gamma 74%, HCH alpha 56%, HCH gamma 32%, aldrin 54%, dieldrin 52%, endrin 20%, endosulfan beta 14%, methoxychlor 20% and mirex 38%. Palma *et al.*[61] presented that in all 62 breast milk samples analyzed, some pesticides were detected, being the compound 4,4'-DDE (0.32-12.03 μg g^{-1} fat), endosulfan beta (0.54-0.61 μg g^{-1} fat) and 4,4'-DDT (2.62-12.41 μg g^{-1} fat) detected in 100, 44 and 13% of the samples, respectively. The other pesticides analyzed were found below the LOQ (0.0013-0.108 μg mL^{-1}). Deltamethrin was detected in 37% of these samples, aldrin and endosulfan alpha, both in 32%, HCH alpha in 18%, trifluralin in 11% and lindane in 6%.

In our study, the DDT compound and its metabolites DDD and DDE were not detected in the analyzed samples. In this sense, results reported by Du *et al.*[62] indicated a clear declining trend in the total DDTs in breast milk in Western Australia during the last decades. Takazawa *et al.*[63] reported the same trend for background air in East Asia, reflecting the recent activities to eliminate production and use of DDTs under the Stockholm Convention. The same situation was pointed out by Wasser *et al.*[64] in a study conducted in Israel. They observed that POPs levels declined significantly since 1982.

As reported by the studies from Pedersen *et al.*[27] and Yildizdas *et al.*,[26] non-persistent pesticides from several classes were also found in our study. Pyrethroids (bifenthrin cyhalothrin lambda and transfluthrin) and herbicides of the dinitroanilines group (pendimethalin and trifluralin) were some of the CUPs present in the evaluated samples.

Although different analytical methods and number of samples used in the studies can be observed that POPs are still present in the environment, and can be accumulated in humans besides other pesticides that have their permitted use. Although the presence of pesticides was detected in all samples, breast milk should not be replaced, since it has properties essential for the development of the infant.

PCA of the correlation between samples information and POPs and CUPs results

A correlation between the results of the samples and the information with respect to the origin (city) and whether the mothers were primiparous or multiparous was observed. Table 2 shows the cumulative variance in each main component for the two elaborated PCA, one in relation to the city and another in relation to the number of gestations of the mothers.

Table 2. Results of cumulative variance obtained by PCA for the main components city and number of gestations of the nursing mother

Principal components (PC)	City of the nursing mothers		Number of gestations	
	Variance / %	Cumulative variance / %	Variance / %	Cumulative variance / %
1	25.7	25.7	20.1	20.1
2	21.3	47.0	18.8	38.9
3	13.4	60.4	12.4	51.3
4	9.5	69.9	10.3	61.6
5	8.5	78.4	8.5	70.1
6	6.8	85.2	7.1	77.2

Figure 2 shows the graph of the PCA weights demonstrating the groups with respect to the origin of the samples and the detected compounds. With 47% of the information accumulated by principal components PC1 and PC2, it was possible to observe the separation of three groups. With PC1, two groups were observed, one formed by the city Bagé and another formed by the cities Três Passos, Bossoroca, Cachoeira do Sul and Tiradentes do Sul. PC2 information generated the third group formed by Tupanciretã.

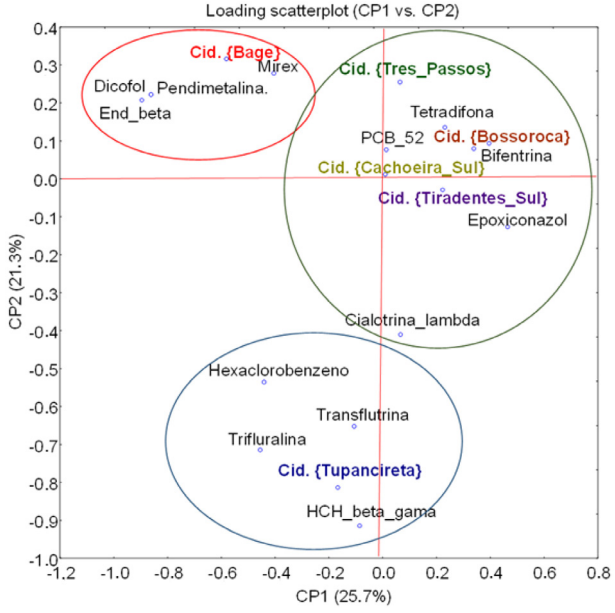

Figure 2. PCA weights demonstrating the groups with respect to the origin of the samples and the detected compounds.

Dicofol and pendimethalin were detected only in samples from Bagé, and the highest concentrations of the POPs endosulfan beta and mirex were also detected in the samples from this city, justifying the formation of this group. In the second group, formed by the set of cities, epoxiconazole was detected in samples of Tiradentes do Sul and Bossoroca. The highest concentrations of cyhalothrin

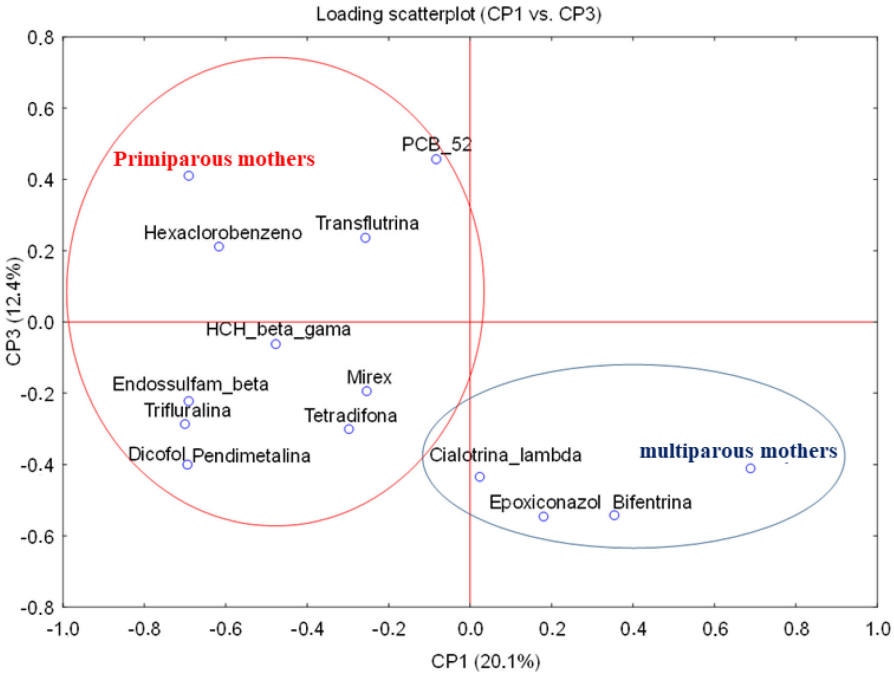

Figure 3. PCA weights of PC1 × PC3 demonstrating the groups regarding the number of gestations of the nursing mother and the contaminants present in the samples.

lambda were detected in the samples from Tiradentes do Sul and Cachoeira do Sul. Tetradifone and PCB52 were detected only in the samples from Tiradentes do Sul and bifenthrin in one sample from Bossoroca.

The group formed by Tupanciretã presented the transfluthrin, detected only in one sample of this city, trifluralin that was detected in 100% of the samples and the POPs HCB and HCH alpha + beta that were detected in higher concentrations than in the other cities. It can also be observed that a grouping occurred by geographic region, except for Cachoeira do Sul, belonging to the central region, which is in the group of the cities of the northwest of the state.

Regarding the number of gestations of the nursing mother, two groups were observed in Figure 3. When analyzing the weight chart with PC1 containing 20.1% of the data variance and PC3 with 12.4%, the groups were formed. The fact that the mother is primiparous is associated with the detection of more compounds and the presence of POPs. The compounds transfluthrin, tetradifone, trifluralin and pendimethalin, despite the use being authorized in Brazil, have an indication of more restricted use than the compounds cyhalothrin lambda, epoxiconazole and bifenthrin, which are related to the multiparous nurse samples. These results reinforce the importance of collecting samples from primiparous mothers for the accomplishment of monitoring of POPs in samples of breast milk, but the analyses of breast milk from multiparous nurse mothers also is important to follow the situation related with the exposition to current use pesticides.

Conclusions

The results obtained herein allowed us to conclude that the method used for sample preparation is advantageous compared to previous methods that have been used to analyze POPs in breast milk, since it is a simple and fast procedure with fewer analytical steps, which simplified the method and reduced error probability. Sample preparation was optimized according to the results of the factorial design 3^2 that allowed, by evaluating the interaction graph, to determine which factors significantly influence sample preparation. Moreover, the results of the matrix effect evidenced the need to use prepared blank curves of the matrix, and the extracted analytical curve compensates the recovery results besides maintaining the same sample preparation conditions. Infant formula for babies can be used as a blank as it is difficult to collect large volumes of breast milk samples and contamination-free samples, making it easier to develop method validation.

Therefore, the proposed method to determine POPs and CUPs in breast milk using GC-NCI-MS was effective because it met the validation parameters for chromatographic methods, presenting high selectivity and sensitivity and enabling adequate limits of detection to be reached to apply the method in biomonitoring studies. The method was applied in 20 samples collected in different regions of

Rio Grande do Sul State, Brazil, and demonstrated that in addition to POPs, other compounds are present in breast milk, evidencing the need to develop analytical methods that are increasingly more comprehensive. With the use of PCA, it was possible to obtain a correlation between the city of origin of the sample and the presence of POPs and CUPs in breast milk, as well between the primiparous or multiparous mothers.

Supplementary Information

Supplementary information of the proposed analytical method is available free of charge at http://jbcs.sbq.org.br as PDF file.

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Author Contributions

Mariela S. Vieira was responsible for conceptualization, data curation, formal analysis, methodology, software, validation, visualization, writing original draft; Giovana Ferronato for formal analysis, methodology, software, validation, writing original draft; Herliana D. F. Abreu for visualization, writing-review and editing; Osmar Damian Prestes for investigation, methodology, resources, supervision, writing-original draft; Martha B. Adaime for conceptualization, funding acquisition, writing-review and editing; Renato Zanella for conceptualization, funding acquisition, project administration, supervision, visualization, writing-review and editing.

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