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Simultaneous analysis of aflatoxins B1, B2, G1, G2, M1 and ochratoxin A in breast milk by high-performance liquid chromatography/fluorescence after liquid–liquid extraction with low temperature purification (LLE–LTP)

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ABSTRACT

The aims of this study were to optimize and validate a methodology for the simultaneous analysis of aflatoxins B1, B2, G1, G2, M1 (AFB1, AFB2, AFG1, AFG2, AFM1) and ochratoxin A (OTA) in breast milk, and to analyze these mycotoxins in samples obtained from human milk banks in the Federal District, Brazil. The optimized analytical method was based on liquid–liquid extraction with low temperature purification (3.25 mL of acidified acetonitrile + 0.75 mL of ethyl acetate), followed by analysis by high-performance liquid chromatography with fluorescence detector (HPLC/FLD) and a photochemical post-column reactor. Limits of quantification (LOQ) ranged from 0.005 to 0.03 ng/mL, recoveries from 73 to 99.5%, and relative standard deviations (RSD) from 1.8 to 17.3%. The LLE–LTP extraction method was shown to be simple and cost-effective, since no columns were needed for clean-up. Only 2 of the 224 breast milk samples analyzed were positive for the mycotoxins, both samples containing AFB2 at the LOQ level (0.005 ng/mL). The identity of the mycotoxin detected was confirmed by liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). This result indicates that infants who are fed with breast milk from the milk banks are not at risk from aflatoxin and ochratoxin exposure.

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1. Introduction

Breast milk is considered the best source of nutrients for infants, providing a unique blend of nutritional and non-nutritional benefits [1]. However, toxic chemicals that nursing mothers are exposed to through the diet, such as mycotoxins, may be carried over to their milk [2].

Mycotoxins are secondary metabolites produced by a range of fungi [3] that contaminate various agricultural commodities, either before harvest or under post-harvest conditions [4]. *Aspergillus flavus* and *Aspergillus parasiticus* are the main producers of aflatoxins B1, B2, G1, G2 (AFB1, AFB2, AFG1, AFG2) [5], mycotoxins that are hepatotoxic and carcinogenic to humans [6]. *A. flavus* is commonly associated with aflatoxin (AFs) production in maize, peanuts and nuts, while *A. parasiticus* with peanuts [7]. Aflatoxin M1 (AFM1) is a hydroxylated metabolite excreted in milk of humans and animals exposed to AFB1 [8,9]. Ochratoxin A (OTA) is naturally produced by several species of *Aspergillus* and *Penicillium* [5], and has been shown to be nephrotoxic and a possible human carcinogen [6]. OTA is found in a wide range of cereals, beer, wine, cocoa, coffee, dried fruit and spices [10].

Several authors have reported the presence of mycotoxins in breast milk around the world. AFB1, AFB2 and their metabolites (AFM1, AFM2), as well as aflatoxicol, were found in samples from Ghana [11]. AFM1 was detected in samples from Australia, Thailand, Egypt, Italy, Turkey and Iran [12–19], and OTA was reported in breast milk samples from Italy, Turkey, Chile and Slovakia [14,15,20–22]. In Brazil, the incidence of AFM1 and OTA was reported to be low [23], and the presence of the other aflatoxins considered in this study has not yet been investigated in breast milk.

Milk is a complex matrix, and mycotoxins may bind to casein proteins, which makes their extraction a difficult procedure [24]. Methods normally used to analyze mycotoxins in milk are based on extraction with organic solvents, clean-up with solid phase extraction (SPE) or immunoaffinity columns, followed by concentration steps [13,20,25]. The LLE–LTP method is based on liquid–liquid extraction followed by a low temperature clean up step in which the organic phase is separated by freezing [26]. Detection and quantification of mycotoxins are performed mainly by liquid chromatography with either a fluorescence detector or mass spectrometry [15,25,27].

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Several countries have established regulatory limits to control the presence of AFM1 in milk and dairy products, but there are no guidelines for breast milk. In Brazil the limit for AFM1 in bulk milk is 0.5 μg/kg [28], the same limit established by the *Codex Alimentarius* [29].

This study aimed to optimize and validate a methodology for the simultaneous analysis of AFB1, AFB2, AFG1, AFG2, AFM1 and OTA in breast milk, and to investigate the presence of these mycotoxins in breast milk samples collected in the Federal District of Brazil. To the best of our knowledge, no single method that simultaneously analyzed these mycotoxins in breast milk using HPLC/FLD has been published in the literature.

2. Materials and methods

2.1. Chemicals and reagents

Acetonitrile, methanol and formic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA), ethyl acetate from Merck KGaA (Darmstadt, Germany), toluene from Mallinckrodt Baker (Phillipsburg, USA), acetic acid from J.T. Baker (Phillipsburg, USA), and anhydrous sodium sulphate from Vetec (Rio de Janeiro, Brazil). All solvents used were HPLC grade. Ultra-pure water was produced by a Milli-Q system (Millipore Corporation, USA). The syringe filters utilized were Millex^{TM} from Millipore (USA).

Aflatoxin standards were purchased from Sigma–Aldrich (St. Louis, MO, USA), and ochratoxin A from Biopure (USA). AFB1, AFB2, AFG1, AFG2 stock solutions were prepared in toluene-acetonitrile (9:1), AFM1 in acetonitrile and OTA in toluene-acetic acid (99:1). Working solutions were prepared and concentrations determined according to AOAC International [30]. A mixed working standard solution was prepared by diluting individual stock solutions to obtain a solution at concentrations of 50 ng/mL for AFM1, AFB1, AFG2 and OTA, 150 ng/mL for G1, and 12.5 ng/mL for B2. All standard solutions were stored at −20 °C and kept out of direct light.

All glassware used was soaked in diluted acid solution (110 mL/L sulfuric acid) for several hours to remove possible active adsorption sites. The glassware was then thoroughly rinsed with distilled water to remove all traces of acid [31]. After the analyses, all materials were decontaminated with sodium hypochlorite solution.

A pool of breast milk samples obtained from different donors was analyzed and none of the mycotoxins of interest was detected. This pool was considered blank, and used to optimize and validate the analytical method.

2.2. Extraction procedures

Three different extraction procedures were tested during method development: (1) solid-phase extraction (SPE) with C-18 cartridges [13]; (2) liquid–liquid extraction (LLE) [20], and (3) liquid–liquid extraction with low temperature purification (LLE–LTP), adapted from Goulart et al. [32]. In the SPE method, a 10 mL breast milk sample diluted in water (1:1) was passed through the C-18 cartridges (Merck, 3 mL), previously activated with acetonitrile and water, at a flow rate of 3.5 mL/min. The cartridges were washed (basic and acid solutions of acetonitrile), the mycotoxins eluted with acidified acetonitrile, and extracted twice with dichloromethane (2 mL). Extracts were evaporated and dissolved in methanol for analysis.

In the LLE method, 1 mL of breast milk was extracted with chloroform (2.4 mL saturated with NaCl), heated to 37 °C, mixed and centrifugated. The organic phase was removed, evaporated, re-dissolved in acetonitrile (0.6 mL) and extracted twice with petroleum ether (0.4 mL) to remove lipids. The acetonitrile phase was blown to dryness and the residue dissolved in methanol for analysis.

Table 1
Experimental design to evaluate the best conditions for mycotoxin extraction by the LLE–LTP method.

Independent variables	Independent variable levels		
	(−)	(0)	(+)
Sonication (X_1)	5 min	10 min	15 min
Ionic strength (X_2)	0.0 g/mL NaCl	0.1 g/mL NaCl	0.2 g/mL NaCl
Solvent composition (X_3)[a]	A	B	C

[a] Total of 4 mL; A = 3.75 mL acetonitrile (0.1% formic acid) + 0.25 mL methanol; B = 4.0 mL acetonitrile (0.1% formic acid); C = 3.25 mL acetonitrile (0.1% formic acid) + 0.75 mL ethyl acetate.

In the LLE–LTP procedure, 2 mL of breast milk were transferred to a falcon tube, 4 mL of a solvent mixture added, and the tube taken to sonication and centrifugation (3500 rpm – 5 min). Samples were stored in a freezer (−18 °C) for 12 h, and the liquid supernatant (organic phase) was passed through a syringe barrel containing anhydrous sodium sulfate and filtered through a syringe filter (0.45 μm). The extract was completely dried under nitrogen flow at 40 °C, and the residue dissolved in 100 μL of methanol for analysis.

Tests for each procedure were conducted in triplicate with blank breast milk samples fortified with AFM1, AFB1, AFG1 and OTA at 0.15 ng/mL, and with AFB2 and AFG2 at 0.03 ng/mL. Mycotoxin concentrations were chosen considering their fluorescence characteristics, which affect their sensitivity to the detector. AFB2 and AFG2 were the most fluorescent molecules, in comparison to the other mycotoxins [33].

2.3. Optimization of the LLE–LTP method

A 2^3 factorial design with a central point was used to determine the best extraction conditions for the LLE–LTP method. The effects of sonication time (X_1), ionic strength (X_2), and solvent composition (X_3) were evaluated based on the recovery rates of the mycotoxins from the breast milk. The independent variables were tested on 3 levels, represented by (−), (0) and (+), as shown in Table 1. The proportions of solvents and salt concentration (NaCl) used were chosen in order to alter the polarity/ionic strength of the extracting mixture without disturbing the freezing process [34,35]. A total of 11 tests were conducted, corresponding to all possible combinations between levels (−) and (+) without replicates, and the central point (0) was performed in triplicate to allow calculation of the experimental error. The experimental design was conducted with breast milk blank samples fortified with AFM1, AFB1, AFG1 and OTA (0.10 ng/mL), and AFB2 and AFG2 (0.03 ng/mL).

Experimental data from the factorial design for each mycotoxin were fitted to a polynomial model and analyzed using the response surface methodology (RSM). If the linear model goodness-of-fit was shown not to be satisfactory following analysis of variance (ANOVA) and Fisher's F test, a quadratic term was introduced to the equation and the model re-evaluated. The statistical significances of the models were evaluated using ANOVA. Statistica® was used to estimate the goodness-of-fit of the regression model and the parameter significances.

2.4. Chromatographic conditions

The HPLC-FLD analyses were carried out in a Shimadzu LC-20AT system (Kyoto, Japan), consisting of a quaternary pump, a degasser (DGU-20A5), an auto sampler (SIL-20A), a column oven (CTO-20A), a system controller (CBM-20A), and a fluorescence detector (RF-10AXL). The chromatographic separation was

Table 2
Parameters on the *m/z*, declustering potential (DP), collision energy (CE), collision cell exit potential (CXP) and ion ratio (IR) of ions selected for mycotoxins analysis.

Mycotoxin	Structure	Parent ion (+H *m/z*)	Product ions (*m/z*)[a]	DP (V)	CE (V)	CXP (V)	IR (RSD, %), $n = 15$
AFM1		329.11	273.1;	81	33	14	2.2 (10.9)
			259.0		33	14	
AFG2		331.13	313.0;	86	35	24	1.6 (9.7)
			245.0		43	18	
AFG1		329.08	243.1;	76	41	18	3.1 (6.5)
			128.0		79	20	
AFB2		315.11	287.1;	91	37	18	1.1 (11.4)
			259.0		41	20	
AFB1		313.11	285.1;	101	33	22	1.3 (9.2)
			241.0		53	18	
OTA		404.23	239.0;	51	33	18	1.6 (6.6)
			358.1		21	10	

[a] Quantifier ion; qualifier ion; IR = ion ratio (quantifier/qualifier); RSD = relative standard deviation.

performed with a Gemini C18 analytical column (150 × 4.6 mm, 5 μm) preceded by a C18 security guard cartridge (4.0 × 3.0 mm, 5 μm), both from Phenomenex (Torrance, CA, USA). A photochemical post-column reactor (PHRED; Aura) was used to enhance the AFB1 and AFG1 responses [36]. The mobile phase consisted of a gradient of methanol, acetonitrile and water (1% acetic acid), as follows: acetonitrile:methanol:water (13:13:74) for 25 min; change to acetonitrile:methanol:water (40:10:50) in 3 min; held for 20 min; change to acetonitrile:methanol:water (13:80:7) in 1 min; held for 2 min; return to the initial conditions in 1 min and held for until 8 min (total run time of 60 min). Flow rate was set at 0.8 mL/min, with oven temperature at 40 °C, and a injection volume of 10 μL. Excitation and emission wavelengths of the fluorescence detector were set at 360/430 nm for aflatoxins, and at 333/470 nm for ochratoxin A.

Samples shown to be positive in the HPLC/FLD were confirmed in a LC–MS/MS system comprised of a Shimadzu LC-20AD chromatograph (Kyoto, Japan) with a binary pump, system controller (CBM-20A), degasser (DGU-20A3), auto sampler (SIL-20AC) and column oven (CTO-20A), coupled to a 4000QTRAP triple quadrupole mass spectrometer equipped with a TurboIonSpray (TISP) source (ABSciex, Foster City, EUA). The analyses were performed on the same analytical column used in the HPLC-FLD at 40 °C. The mobile phase consisted of a gradient of methanol/water (20:80; 0.1% formic acid; A) and acetonitrile (0.1% formic acid; B). Gradient program – A:B (75:25) for 0.2 min; change to A:B (50:50) in 10 min; change to A:B (30:70) in 2 min; held for 3 min; change to A:B (70:30) in 1 min; return to the initial conditions and re-equilibrate the column for 9 min. Flow rate was set at 0.8 mL/min and injection volume was 10 μL. Data were acquired in Multiple Reaction Monitoring (MRM) mode and the electrospray ionization (ESI) was performed in positive mode. The full scan and product ion scan analyses were performed by direct infusion of mycotoxins solutions (200 ng/mL) in the mass spectrometer, at 10 μL/min, and the optimized parameters are shown in Table 2. Ion source parameters were optimized by Flow Injection Analysis (FIA), with a 20 ng/mL

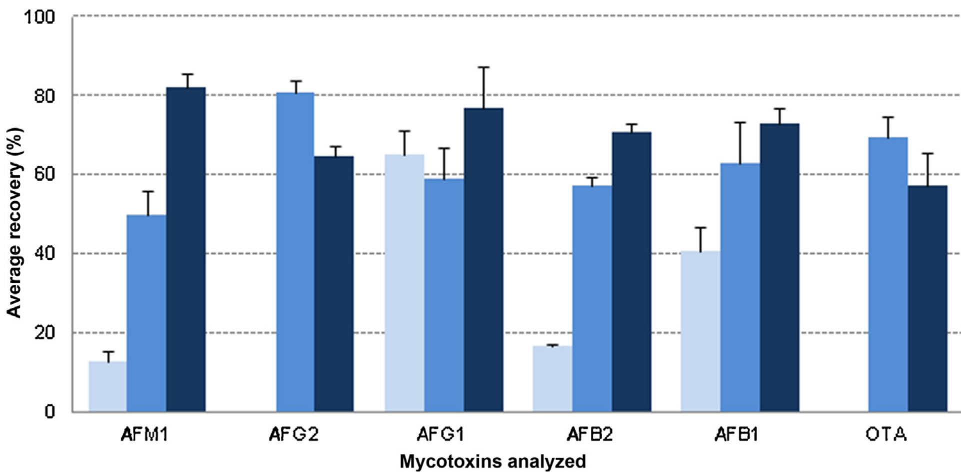


Fig. 1. Recoveries of AFM1, AFG2, AFG1, AFB2, AFB1 and OTA in fortified breast milk samples. AFG2 and OTA were not detected in the SPE extraction.

standard solution, at 0.8 mL/min, using the same mobile phase of the method. The MS/MS ion source was operated at 700.0 °C, entrance potential at 10 V and ion source gas at 40 psi (GS1) and 50 psi (GS2). Ion spray voltage was set to 3500 V, curtain gas at 15 psi, and collision gas as high.

2.5. Method validation

The best extraction procedure was optimized and submitted to a full validation process. *Selectivity* was evaluated by analyzing the HPLC-FLD chromatographic profile of the blank and fortified breast milk samples, checking for interferences eluting at the same retention time as the mycotoxins of interest [37]. The presence of *matrix effects* was investigated by comparing the responses (areas) in the HPLC-FLD system of a matrix matched curve and a calibration curve made in methanol [38], both constructed within the range of 0.2–15 ng/mL for AFM1, AFG1, AFB1 and OTA, and 0.06–5 ng/mL for AFG2 and AFB2, with six calibration points each. *Linearity* was verified with three in- methanol standard curves with seven calibration points each, within the range of 0.1–15 ng/mL for AFM1, AFB1, AFG2 and OTA, 0.3–45 ng/mL for AFG1, and 0.025–3.75 ng/mL for AFB2. The least square method was used to estimate the linear regression parameters, the Grubbs test to verify the presence of outliers, the Cochran test for the homogeneity of variances, and the ANOVA to calculate the coefficient of determination (R^2) and significance of the regression [37].

Trueness was determined by the % of recovery obtained from experiments conducted with blank breast milk samples spiked at 3 different fortification levels (low, intermediate and high), with eight replicates each, performed on the same day by the same analyst [37]. *Repeatability* was expressed as the relative standard deviations (RSD%) obtained from the analysis performed in the recovery study. *Intermediate precision* was evaluated through the analysis of 5 replicates of spiked samples performed by different analysts on the same day. For each mycotoxin, the LOQ was defined as the lower level at which the method was satisfactorily validated (recovery between 70 and 110% and RSD <20%). *Robustness* was evaluated by investigating the impact of small changes in the LLE–LTP method (Section 2.3) [39].

The stability of the sample extract was assessed by analyzing blank breast milk samples spiked at 0.05 ng/mL for AFM1, AFB1, AFG1 and OTA, and at 0.0125 ng/mL for AFB2 and AFG2. Triplicate samples were extracted, evaporated until complete dryness, and kept at −20 °C until analysis on days 1, 2, 3, 7, 21, and 30 of storage. ANOVA was used to determine whether there were statistically significant differences among the estimated concentration levels during storage.

2.6. Breast milk samples

The number of breast milk samples that should be collected in this study (362 samples, 95%) was based on the total number of donors at sixteen human milk banks and two collection posts in the Federal District in 2010 (n = 6005). However, as only eight Banks joined the project, 224 samples (93.5% significance level) were collected (May 2011 to February 2012). Samples were kept at −18 °C until analyzed. Information on the mother's age and address, and the infant's date of birth was collected at the human milk bank. Information on the consumption of peanuts and Brazil nuts in the week prior to milk donation was obtained through a questionnaire applied at the mother's residence. The project was approved by the Ethics Committee on Human Research of the Faculty of Health Science of the University of Brasilia (n° 027/11), and all mothers signed a consent form before donating samples.

Samples were analyzed according to the optimized analytical method. Three fortified breast milk samples (at the intermediate level) were included in each batch of extraction as an internal quality control to verify the reliability of the extraction procedure (30 samples/batch). The identity of the mycotoxin detected in the HPLC/FLD was confirmed by LC–MS/MS and re-analyzed in the HPLC/FLD (duplicate sample) for quantification.

3. Results and discussion

3.1. Sample extraction

The average % recoveries of the mycotoxins obtained with different extraction procedures are shown in Fig. 1. AFG2 and OTA were not detected in the fortified samples analyzed after purification with C18 SPE, and this procedure was not further considered in this study. The LLE and LLE–LTP methods showed the best recoveries for all mycotoxins, with higher recoveries using LLE–LTP for most of these, mainly for AFM1 (82.3%). Mean OTA recovery was lower using LLE–LTP (57.2%). When the acetonitrile used in extraction was acidified (0.1% formic acid), OTA recovery increased to over 80%, with no effect on the other mycotoxins (data not shown). As the LLE–LTP procedure had fewer steps and the overall best recoveries, it was the extraction method chosen for analysis of aflatoxins and OTA in breast milk.

3.2. Optimization of the LLE–LTP method

The effects of 3 independent variables on mycotoxin recoveries were simultaneously evaluated during the optimization of the LLE–LTP method. Table S1 (Supplementary data) shows the average recoveries, the effects of each factor on recovery, as well as their interactions. Extraction time in the ultrasonic bath (X_1) was only statistically significant ($p < 0.05$) for AFG2 and OTA, with a lower recovery for a longer extraction time. Ionic strength (X_2) was statistically significant for all mycotoxins analyzed, with a decrease in recoveries with increasing ionic strength. Changes in polarity (X_3; from a mixture of acetonitrile with methanol to a mixture with ethyl acetate) significantly increased AFM1 and AFG2 recoveries. Most interactions were not significant, and those considered significant were kept in the regression model. As not all degrees of freedom were used in the regression, it was possible to calculate the lack-of-fit for each model.Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2012.11.066.

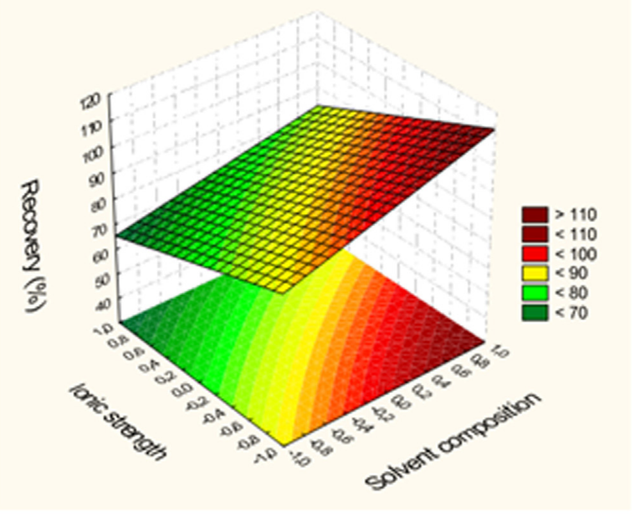
Significant regression values and non-significant lack-of-fit values showed that the quadratic model calculated for AFM1, AFG2, AFB2 and OTA was valid for the present study. Lack-of-fit was significant for AFG1 and AFB1, meaning that the recoveries of these mycotoxins were not adequately explained by the equation determined by the model. As the main mycotoxin of interest in breast milk was AFM1, the optimized LLE–LTP method was chosen in this study for presenting the best performance for AFM1 analysis. The response surface plots (Fig. 2) show the effects of the independent variables on the recovery of AFM1. The best conditions of extraction of AFM1 were: 10 min in the ultrasonic bath, no added salt, and mixture of solvent comprised of 3.25 mL of acetonitrile (0.1% formic acid) + 0.75 mL of ethyl acetate. These conditions did not significantly impact the recoveries of the other mycotoxins analyzed (Table S1).

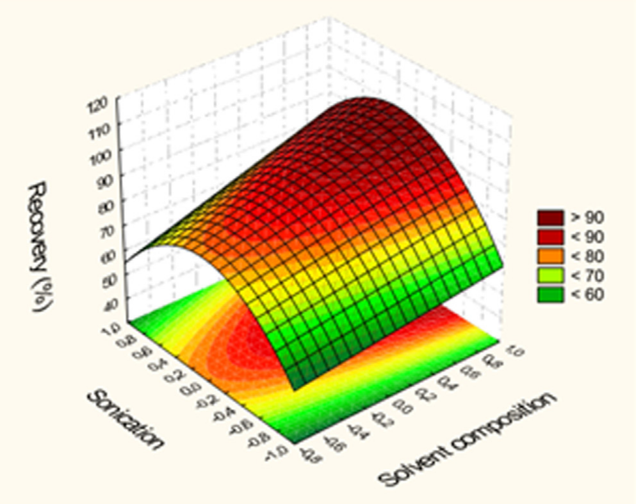
3.3. LLE–LTP method validation

The chromatograms of a blank and a fortified breast milk sample did not show any interfering peaks eluting in the same retention time for any of the mycotoxins analyzed (Fig. 3), confirming that the chromatographic conditions ensured satisfactory selectivity of the method. For both solvent and matrix matched standard curves, the coefficients of determination (R^2) were higher than 0.999, and linear regression of all curves were significant ($p < 0.05$). The Fischer *F*-test showed that the residual variances of the solvent and matrix matched curves were homogeneous ($\alpha = 0.05$) and the *t*-test (combined variances) was used to evaluate matrix effects. No significant differences in responses (areas) were obtained for the solvent and the matrix matched curves ($\alpha = 0.05$), indicating no matrix effects. Hence, the solvent standard curves were used for the quantification of mycotoxins in this study. No outliers ($p > 0.05$) were found at any calibration level in the three solvent standard curves analyzed. The behavior of the residues resulting from the adjustment of the analytical curves obtained by the least squares method was homoscedastic ($C_{calculated} < C_{critical;7;3}$) for all mycotoxins.

The LOQs found with the optimized method were 0.01 ng/mL for AFM1, AFG2 and OTA, 0.03 ng/mL for AFG1, 0.02 ng/mL for AFB1 and 0.005 ng/mL for AFB2. These LOQs are in same range of those found by other authors who used LLE and HPLC-FLD in breast milk analyses [12,16,22], but higher than those reported using immunoaffinity or C18 SPE for clean up (0.0005–0.007 ng/mL) [13–15].

Table 3 shows the recoveries for three different levels of fortification for each mycotoxin. Outliers for each fortification level were identified with the construction of box plots and were removed from the overall results. Mean recoveries at the LOQ levels varied from 73.0% (AFG2) to 99.5% (AFG1), while for intermediate and high levels of fortification, average recoveries ranged between 70.1 and 95.8%. Precision was evaluated both as repeatability and intermediate precision (Table 3). RSD obtained from analyses performed in the recovery tests under repeatability conditions (same analyst, same day) ranged from 3.8% (AFB2) to 17.3 (OTA) at the LOQ





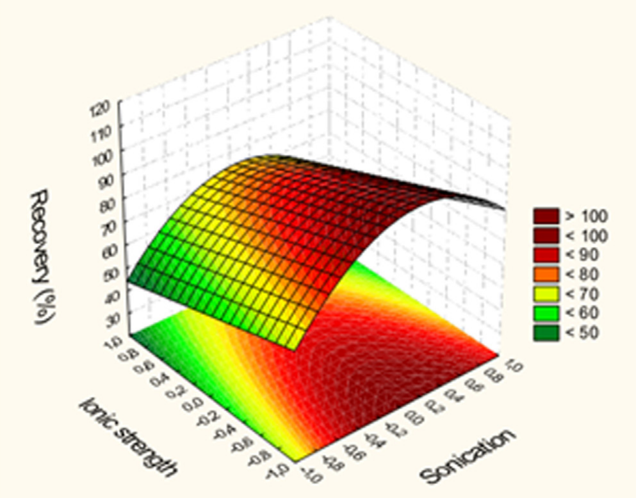


Fig. 2. Response surface plots on the recoveries of AFM1, varying the ionic strength, solvent composition and sonication time.

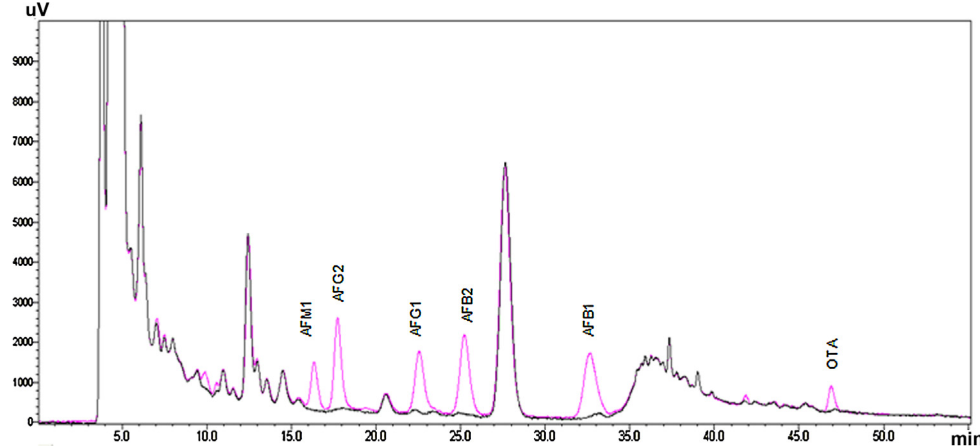


Fig. 3. HPLC/FLD chromatograms of a blank and fortified breast milk sample (2 ng/mL for AFM1, AFG2, AFB1 and OTA; 6 ng/mL for AFG1; 0.5 ng/mL for AFB2).

levels, and from 1.8 to 6.3% at the other two fortification levels, which are within the acceptable range [39,40]. Intermediate precision obtained by different analysts through the analysis of fortified breast milk samples are also shown in Table 3. The Grubbs test did not show any outliers ($p > 0.05$) in the recoveries obtained by each analyst and no statistical differences were found between the mean recoveries obtained by the analysts. RSDs were considerably higher for analyst 2 and, therefore, all the human milk samples were analyzed only by analyst 1.

Robustness of the LLE–LTP method was evaluated during the method optimization. Among other factors, changes in the extraction period (5/10/15 min) showed small variations in recoveries for most mycotoxins analyzed. However, ionic strength had a significant impact on mycotoxin recovery, indicating the need to control this parameter in the procedure.

The stability of AFs and OTA in sample extracts over the period of storage (1–30 days, at −20°C) is shown in Fig. S1 (Supplementary data). There was a considerable reduction in mycotoxin concentration after 14 days of storage. AFG1 and AFM1 had the highest decreases (47.0 and 40.0%, respectively), while OTA had the lowest reduction (19.0%). Most of the mean recoveries obtained from the extract at 21 and 30 days of storage were statistically different ($p < 0.05$) from those obtained during the first days of the experiment. In light of these results, a maximum storage period of 14 days was established for the extract before analysis of the mycotoxins in the breast milk by HPLC/FLD.Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2012.11.066.

In summary, the optimized extraction method met the performance criteria required for the validation of methods of analysis and, therefore, may be used to investigate the presence of aflatoxins and OTA in breast milk.

3.4. Mycotoxins in breast milk samples

A total of 224 breast milk samples were collected from human milk banks in the Federal District between May/2011 and February/2012. Samples were obtained from 213 different donors, 38.3% of them during the first month of breastfeeding. The mothers' ages were, on average, 28.4 years (±6.55) and the babies' mean weight at birth ($n = 176$) was 3.19 kg (±0.55). Only 47% of the donors answered the questionnaire on the consumption of peanuts, peanut products, Brazil nuts, and their products in the week before the milk samples were donated. Of the 99 respondents, 23% reported having consumed peanuts or Brazil nuts in the week prior to donation, and only 7 of them (30%) had consumed both products in the

Table 3
Recoveries, repeatability and intermediate precision for AFM1, AFG2, AFG1, AFB2, AFB1 and OTA in breast milk.

Recoveries and repeatability					Intermediate precision					
Analyst 1					Analyst 1			Analyst 2		
Mycotoxins	Level (ng/mL)	n^a	Mean ± SD (%)	RSD (%)	n	Mean ± SD (%)	RSD (%)	n	Mean ± SD (%)	RSD (%)
AFM1	0.01	8	74.5 ± 4.4	5.9	5	75.5 ± 3.9	5.1	5	69.2 ± 17.6	25.5
	0.05	8	70.1 ± 4.0	5.7						
	0.5	6	72.6 ± 1.3	1.8						
AFG2	0.01	8	73.0 ± 11.0	15.1	5	79.5 ± 4.0	5.0	5	78.2 ± 13.1	16.8
	0.05	7	77.3 ± 2.5	3.2						
	0.5	7	85.2 ± 2.5	3.0						
AFG1	0.03	8	99.5 ± 14.0	14.1	5	85.6 ± 6.5	7.6	5	83.7 ± 14.6	17.5
	0.15	8	95.8 ± 3.3	3.5						
	1.5	8	89.7 ± 5.6	6.3						
AFB2	0.005	6	73.1 ± 2.8	3.8	5	74.4 ± 5.8	7.8	5	80.6 ± 16.6	20.6
	0.0125	8	71.2 ± 4.0	5.7						
	0.125	7	81.7 ± 3.0	3.7						
AFB1	0.02	8	77.4 ± 7.1	9.1	5	74.0 ± 1.2	1.7	5	71.2 ± 14.8	20.8
	0.05	8	88.0 ± 4.9	5.6						
	0.5	8	79.6 ± 4.4	5.5						
OTA	0.01	8	77.6 ± 13.4	17.3	5	74.4 ± 1.2	1.6	5	62.1 ± 15.6	25.1
	0.05	7	76.9 ± 1.9	2.5						
	0.5	7	88.5 ± 2.7	3.1						

[a] Outliers identified through box plots were removed.

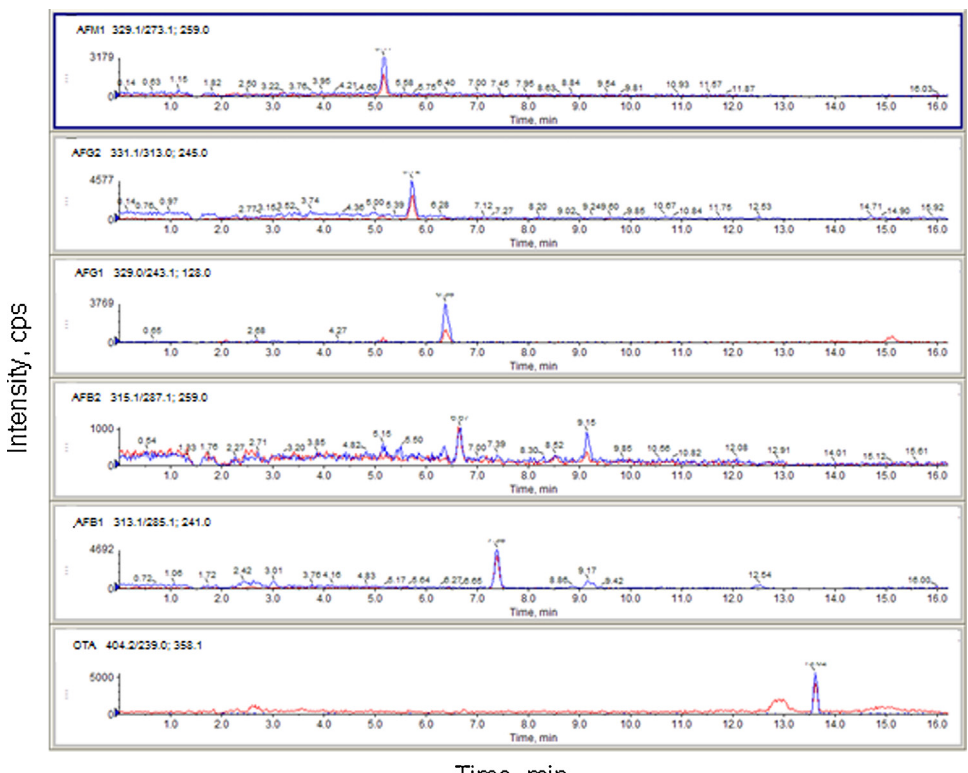


Fig. 4. LC–MS/MS ion chromatograms of a blank breast milk sample spiked at 2 ng/mL for AFM1, AFG2, AFB1 and OTA, 6 ng/mL for AFG1 and 0.5 ng/mL for AFB2.

same period. Breast milk samples were analyzed using the validated LLE–LTP – HPLC/FLD method. In each batch of extraction, three fortified samples (intermediate level) were included. Mean concurrent recoveries of fortified samples were also within the acceptable range (60–120%, $n = 24$) [39,40], ensuring the quality of the extraction procedure.

Of all 224 samples analyzed, only two were positive for the mycotoxins investigated in this study, both for AFB2 at the LOQ level (0.005 ng/mL). Considering a recovery correction for losses during sample extraction (mean recovery of 73.1% at the LOQ level), samples found to be contaminated with AFB2 had a corrected concentration of 0.007 ng/mL. This result corroborated the dietary information that indicated that the breast milk donors were low consumers of the food that may be potentially contaminated with aflatoxins. However, due to extremely low number of contaminated samples, it was not possible to make a statistical correlation between the presence of mycotoxins in breast milk and nut consumption. The presence of AFB2 in the breast milk positive samples was confirmed by LC–MS/MS. Fig. 4 shows the chromatograms of a fortified breast milk sample obtained with this technique.

Aflatoxin contamination in food commercialized in the Federal District has decreased considerably in recent years [41]. Aflatoxin intake calculated for the total population ranged from 0.06 to 0.08 ng/kg bw/day and cancer risk values obtained were extremely low (0.0006–0.0009 cancers/year/10^5 individuals), showing that the population of the Federal District is not at great risk. OTA has been reported in coffee, rice, wheat flour, and chocolate sold in Brazil, although the levels of contamination found were mostly low (0.01–109 μg/kg) [42–45].

Contamination levels of AFM1 and OTA in breast milk from human milk banks donors have been previously assessed in Brazil (São Paulo), and similar to the present study, the incidence was low [23]. Of the 50 samples analyzed, only three were found to be contaminated, one with AFM1 (0.024 ng/mL), and two with OTA (0.011–0.024 ng/mL). Higher incidences and levels have been found around the world. In Egypt, 56% of the 443 breast milk samples analyzed were positive for AFM1 (0.0042–0.889 ng/mL) [13]. In Italy, Galvano et al. [15] found 5 positive samples for AFM1 (0.007–0.14 ng/mL), and 61 positive for OTA (0.005–0.405 ng/mL) from 82 breast milk samples collected in the first month of breastfeeding. Gürbay et al. [16,20] found all 75 samples of breast milk in Turkey positive for AFM1 (0.06–0.29 ng/mL), AFB1 (0.09–4.123 ng/mL) and OTA (0.6–13.1 ng/mL). In Chile, all 11 samples collected in the first 6 days postpartum were positive for OTA (0.044–0.184 ng/mL) [22].

4. Conclusions

The method developed for the simultaneous analysis of AFM1, AFG2, AFG1, AFB2, AFB1 and OTA in breast milk samples using HPLC-FLD was satisfactorily validated. The LLE–LTP extraction was shown to be simple and cost-effective, since no columns are needed for clean-up. The breast milk samples analyzed did not have significant mycotoxin levels, and therefore infants who consumed milk from the human milk banks were not at risk from exposure to these mycotoxins. Children are considered more vulnerable than adults to the effects of toxicants due to their higher metabolic rate, lower body weight, immature metabolic pathways, and incomplete development of organs and tissues [46]. Considering this high sensitivity and the importance of breastfeeding for the development of newborns, the presence of mycotoxins and other contaminants in breast milk should be continuously evaluated. Hence, the development of analytical methods to identify and quantify the mycotoxins is essential to assess the exposure to these chemicals through breastfeeding.

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