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Assessment of aflatoxin exposure using serum and urinary biomarkers in São Paulo, Brazil: A pilot study

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ABSTRACT

The aim of this study was to evaluate the human exposure of individuals from Pirassununga, Brazil, to dietary aflatoxins B_1 (AFB_1) and M_1 (AFM_1) by determination of serum AFB_1-lysine and urinary aflatoxin biomarkers (AFM_1 and AFB_1-N^7-guanine). The participants were recruited among employees from a Campus of the University of São Paulo, which provided food samples from their homes, as well as serum and urine samples four times every three months, from June 2011 until March 2012. The probable daily intake (PDI) of aflatoxin was estimated by using the results from analysis of food products collected by the time of samples collection, and data from a 24-hour dietary recall questionnaire. Analyses of AFB_1 and AFM_1 in food samples were conducted by high-performance liquid chromatography with fluorescence detection. Biomarkers in serum and urine were determined by tandem mass spectrometry. AFB_1 and AFM_1 were detected in 38 samples of cereals (28%, $N = 136$) and 31 milk products (36%, $N = 86$), respectively. AFB_1-lysine and AFB_1-N^7-guanine and were not detected in serum or urine samples, respectively. However, AFM_1 was found in 74 urine samples (65%), at mean levels in the 4 sampling times ranging from 0.37 ± 0.23 to 1.70 ± 2.88 pg/mg creatinine. The mean PDI varied among different sampling times, ranging from 0.09 ± 0.09 to 1.35 ± 5.98 ng/kg body weight/day. A modest though significant correlation ($r = 0.45$; $p = 0.03$; $N = 23$) was found for the first time in Brazil between the AFM_1 concentration in urine and the PDI for total aflatoxins ($AFB_1 + AFM_1$) in sampling 1 (June 2011). Urinary AFM_1 was confirmed as very sensitive for monitoring the human exposure to dietary aflatoxin. Further studies using serum and urinary biomarkers are needed to estimate the aflatoxin exposure of populations in higher risk areas in Brazil.

1. Introduction

Aflatoxin B_1 (AFB_1) is an hepatocarcinogen produced mainly by strains of *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius* during growth on several food and feed materials (Murphy et al., 2006). The occurrence of AFB_1 in food products is a public health concern because of its involvement in the aetiology of human liver cancer and classification as Group 1 (human carcinogen) by the International Agency for Research on Cancer (2002). AFB_1 is activated in the liver by cytochrome P-450 associated enzymes to the reactive form AFB-8,9-epoxide, which binds to DNA and generate the AFB_1-N^7-guanine adduct (Essigmann et al., 1977), or react with albumin to produce AFB_1-albumin adduct by covalent binding mainly with the amino acid lysine (Sabbioni et al., 1987). These compounds are frequently used as biomarkers for evaluation of dietary exposure to AFB_1 in epidemiological studies, as they are excreted in urine (AFB_1-N^7-guanine) or present in blood (AFB_1-lysine). While the excretion of AFB_1-N^7-guanine adduct in urine occurs after a short period of aflatoxin ingestion, the concentration of AFB_1-lysine in serum indicates that an individual has been exposed to AFB_1 for period of 2 to 3-month (Wild et al., 1992). Therefore AFB_1-N^7-guanine and AFB_1-lysine have been validated and employed as short and long-term biomarkers of exposure to AFB_1 -through the diet, respectively (Gan et al., 1988; Groopman

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et al., 1992a, 1992b). The urinary excretion of AFB_1-N^7-guanine serves as a biomarker of the biologically effective dose of aflatoxin and high levels are associated with increased risk of liver cancer (Qian et al., 1994).

Liver enzymes can form hydroxylated metabolites as well, including aflatoxin M_1 (AFM_1) (Hsieh and Atkinson, 1991), which can also be excreted in urine and used as biomarker of short-term exposure to AFB_1. Furthermore, AFM_1 is also found in milk of lactating cows and other mammalian species, including humans that have consumed food products contaminated with AFB_1. AFM_1 is also classified as a Group 1 carcinogen (International Agency for Research on Cancer, 2002). The consumption of dairy foods contaminated with AFM_1 may contribute for the overall aflatoxin intake, and therefore increase the excretion of AFM_1 in human urine (Redzwan et al., 2012). Several studies reported correlations between the ingestion of AFB_1 and excretion of AFM_1 in urine samples from populations highly exposed to aflatoxin-contaminated foods, such as Guangxi province, People's Republic of China, Egypt and in Gambia, West Africa (Groopman et al., 1992a, 1992b; Polychronaki et al., 2008; Zhu et al., 1987).

The occurrence of aflatoxins in Brazilian foodstuffs has been frequently reported, mainly in peanut and corn products at variable levels and incidences from up to 50% of the samples analyzed (Magrine et al., 2011; Ono et al., 2010; Rodriguez-Amaya and Sabino, 2002). High incidences of AFM_1 in fluid milk have also been described, but at levels usually below the limit (0.50 μg/L) established by Brazilian regulations (Agência Nacional De Vigilância Sanitária, 2011). Assessment of aflatoxin intake by Brazilian population has been usually estimated in terms of probable daily intake (PDI) by analyzing the occurrence of AFB_1 in food products and the consumption data (Magrine et al., 2011; Jager et al., 2013). However, the human exposure to dietary aflatoxins by the biomarker approach have been assessed in only three studies conducted in the State of São Paulo, two describing the excretion of AFM_1 in human urine at levels ranging from 1.8 to 39.9 pg/mL (Romero et al., 2010) and from 0.19 to 12.7 pg/mg creatinine (Jager et al., 2014), and one reporting the AFB_1-albumin adduct in blood samples in concentrations ranging from up to 57.3 pg/mg albumin (Scussel et al., 2006). There is no information available on the occurrence of urinary AFB_1-N^7-guanine associated to aflatoxin intake by the population in Brazil. Although the previous studies on aflatoxin biomarkers indicated a low short-term aflatoxin exposure of the Brazilian populations studied, the correlations between the biomarker levels found with the estimated intake of aflatoxins were not investigated. The objective of the present study was to conduct a first evaluation of human exposure of individuals from Pirassununga, Brazil, to dietary aflatoxins by determination of PDI values for total aflatoxins (AFB_1 and AFM_1), serum AFB_1-lysine and urinary aflatoxin biomarkers (AFM_1 and AFB1-N^7-guanine).

2. Material and methods

2.1. Sampling procedures

The study was approved by the Ethics Committee of the School of Medicine at Ribeirão Preto of the University of São Paulo (USP). Employees (mean age 33 years, range 18–60 years) from the USP *Campus* at Pirassununga, State of São Paulo, Brazil, were kindly invited to provide food samples from their homes, as well as serum and urine samples four times every three months, from June 2011 until March 2012. Each participant signed a written and approved consent form prior to inclusion in the study. The number of participants varied in each sampling time: 33, 31, 27 and 22 individuals in June 2011, September 2011, December 2011 and March 2012, respectively. All participants were apparently healthy throughout the study, as assessed by clinical examination in sampling times by a qualified nurse, and had no previous liver or kidney diseases. In each sampling time, all participants were instructed to collect the following foods susceptible to aflatoxin contamination and regulated in Brazil (Agência Nacional De Vigilância Sanitária, 2011), if available on their residences on the day of samples collection: peanut products (unprocessed, salted peanut and *paçoca*), corn flour, popcorn, white hominy, beans, fluid milk, milk powder and milk products (yoghurt and cheese). Food samples were packed in polyethylene bags or vessels and delivered by the participants in the Public Health Unit of the USP *Campus*, and finally sent to the laboratory. Solid samples were homogenized, finely ground and kept frozen along with liquid samples at −20 °C until analysis. The number of food products provided by participants in samplings conducted in June 2011, September 2011, December 2011 and March 2012 was 74, 59, 49 and 40, respectively, totaling 222 food samples analyzed in the study.

On each day of food samples collection, all participants were instructed to collect the early morning first urine (minimum 50 mL) in a polyethylene vessel previously supplied, and deliver the sample in the Public Health Unit of the USP *Campus*. Urine samples were maintained in a cool box (nearly 5 °C) until transport to the laboratory, where they were separated in 50 mL aliquots and kept frozen at −20 °C until analysis. Upon delivery of urine samples by the participants, blood samples were also collected by a qualified nurse. The serum was immediately separated by centrifugation and stored at −80 °C until analysis. The number of urine or serum samples collected in June 2011, September 2011, December 2011 and March 2012 was 33, 31, 27 and 22, respectively (total number of urine or serum samples: 113).

2.2. 24-Hour dietary recall

A 24-hour dietary recall (DR) was elaborated to estimate the consumption of the food products provided by the participants. Portion sizes were estimated for each type of food according to usual measures, such as tablespoons for corn flour or cups for beans, milk and dairy products. A blank space was also available if the participant wanted to express a different consumption quantity for a specific food item. The mean consumption of each food product by each participant was estimated from the portion sizes as declared in the 24-hour DR, and used to estimate the individual probable daily intake (PDI). The body weight (b.w.) of each volunteer was also recorded to calculate the PDI value by the following expression: PDI = (mean concentration of AFB_1 and AFM_1 x mean consumption of food type per day/(each individual b.w.).

2.3. Determination of aflatoxins B_1 and M_1 in food products

Analyses of AFB_1 in bean, peanuts and corn products, and AFM_1 in liquid or milk powder, cheese and yoghurt were performed using immunoaffinity columns (Aflatest®, Vicam, Watertown, MA, USA) and high performance liquid chromatography with fluorescence detection (HPLC-FD) as described by Jager et al. (2013). Derivatization of AFB_1 was obtained by adding 200 μL of *n*-hexane and 200 μL of trifluoroacetic acid. The mixture was kept at 40 °C for 10 min, evaporated to near-dryness and diluted in 1 mL of methanol:water (50:50, v/v). Final extracts were filtered through a 0.45 μm PTFE membrane prior to injection into HPLC column. AFB_1 and AFM_1 standards were purchased from Sigma (Sigma, St Louis, MO, USA), prepared in toluene:acetonitrile (9:1, v/v) and stored in an amber glass vial at −20 °C. Aflatoxin working solutions were prepared at concentrations of 0.5, 1.0, 5.0, 10.0 and 20.0 μg/L for AFB_1, and 0.25, 0.50, 1.5, 5.0, 7.5 and 10.0 μg/L for AFM_1.

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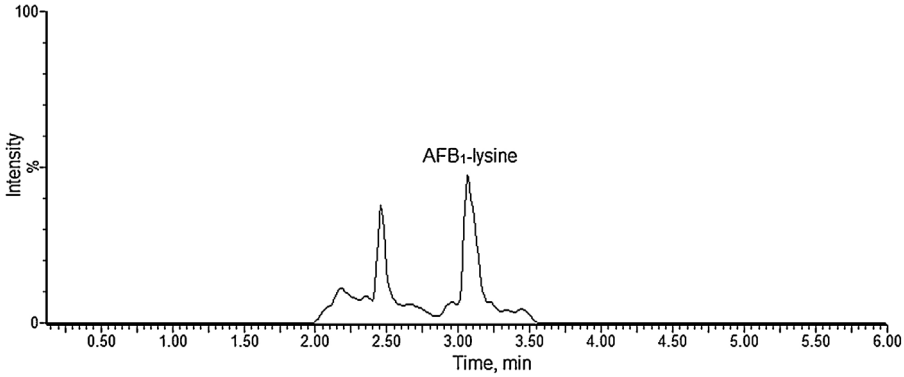

Fig. 1. Chromatogram (quantification transition) of AFB_1-lysine standard prepared in blank serum spiked with 5 ng/mL.

2.4. Determination of aflatoxin B_1-lysine in serum

The digestion and extraction procedures for AFB_1-lysine analyses in serum samples were performed as described by McCoy et al. (2005) with minor modifications. Serum was thawed at room temperature and vortexed until completely homogeneized. An aliquot of 250 μL was digested with 250 μL of Pronase (EMD Millipore, San Diego, CA) solution (13 mg/mL in deionized water) in water bath at 37 °C for 5 h. After incubation, 500 μL of deionized water were added and the mixture loaded onto the Waters Oasis® MAX cartridge 30 mg/1 mL (Milford, MA, United States), previously conditioned with 1 mL of methanol and 1 mL of water. After eluting the sample, cartridge was washed with 1 mL of water, 1 mL of methanol:water (70:30, v/v) and 1 mL of 1% ammonium hydroxide in methanol. AFB_1-lysine was eluted with 0.8 mL of freshly prepared 2% formic acid in methanol. The eluate was evaporated to dryness using a rotational vacuum concentrator (MartinChrist, Osterode am Harz, Germany), and diluted in 200 μL of methanol:water (25:75, v/v).

AFB_1-lysine standard was synthesized, purified and characterized as originally described by Scholl and Groopman (2004), with modifications proposed by Sass et al. (2015). Calibration curves were prepared by the extraction of a blank serum samples previously analyzed for the albumin content, and fortified with the AFB_1-lysine standard at levels of 20 to 200 pg/mg albumin. Final extracts were injected into a Waters Acquity I-Class ultra-performance liquid chromatographic (UPLC) system (Waters, Milford, MA, USA) equipped with a BEH C_{18} column (2.1 × 50 mm, 1.7 μm) and coupled to a Xevo TQ-S mass spectrometer (Waters, Milford, MA, USA). The column was kept at 40 °C during analyses, and samples were maintained at 15 °C. Ten microliters of extracted samples and standards were injected. Gradient elution was accomplished with mobile phase composed by water (eluent A) and acetonitrile (eluent B), both containing 0.1% of formic acid. After an initial period of 0.5 min at 95% A, the percentage of B was linearly raised to 25% over 4.5 min (5.0 min). Then, eluent B was increased to 90% over 0.5 min, followed by a hold time of 0.25 min (5.5 min). Following this, the percentage of B was reduced to 5% over 0.5 min (6.0 min) and the column re-equilibrated to initial conditions for 0.5 min. Total chromatographic run time was 6.5 min, and the mobile phase flow rate was maintained at 0.5 mL/min. Under these conditions, the retention time for AFB_1-lysine was 3.08 min, as shown in Fig. 1. The mass spectrometer was operated in Multi Reaction Monitoring (MRM) mode using electrospray ionization in positive ion mode, with a capillary voltage of 0.75 kV, source temperature of 150 °C, and desolvation temperature of 500 °C. Desolvation gas flow and cone gas flow were maintained at 800 L/h and 150 L/h, respectively. Cone voltage, collision energy and MRM transitions (major precursor ion > fragment ion) were manually optimized. Quantification and confirmatory MRM transition for AFB_1-lysine were m/z 457 > 394 and 457 > 376, respectively. Data collection and processing was performed using software MassLynx version 4.1.

2.5. Determination of aflatoxin B_1-N^7-guanine in urine

An in-house analytical method was developed and validated for determination of AFB_1-N^7-guanine in urine. Samples were thawed at room temperature and centrifuged at 3500 rpm for 5 min. Extraction of AFB_1-N^7-guanine from urine was performed by using solid phase extraction (SPE) columns (Phenomenex, StrataX 200 mg/6 mL, Torrance, CA). A 10-mL aliquot of urine was diluted with 10 mL of phosphate buffered saline (PBS), and the mixture passed through the SPE column at flow rate of 1 drop per second. After washing with 6 mL of methanol:water (60:40, v/v), AFB_1-N^7-guanine was eluted with 6 mL of methanol with 1% formic acid. The eluate was evaporated to dryness using a rotational vacuum concentrator (MartinChrist, Osterode am Harz, Germany), and the residue dissolved in 500 μL of acetonitrile:water (1:9, v/v) containing 0.5% of formic acid.

AFB_1-N^7-guanine standard was purchased from Toronto Research Canada (Toronto, ON, Canada). Standard stock solutions were prepared in methanol:1% acetic acid in water (50:50, v/v) and stored at −20 °C. Calibration curves were prepared by the extraction of a blank urine sample fortified with the AFB_1-N^7-guanine standard at 0.2 to 4.0 ng/mL. Final extracts were injected in the same UPLC system coupled to a Xevo TQ-S mass spectrometer (Waters, Milford, MA, USA) as previously described, keeping the same temperature conditions and injection volumes. The gradient elution was accomplished with water (eluent A) and acetonitrile (eluent B), both containing 0.1% of formic acid. After an initial period of 0.5 min at 90% A, the percentage of B was linearly raised to 30% over 1.5 min (2.0 min). Then, eluent B was increased to 90% over 0.1 min, followed by a hold time of 0.2 min (2.3 min). Following this, the percentage of B was reduced to 10% over 0.1 min (2.4 min) and the column re-equilibrated to initial conditions for 0.6 min. Total chromatographic run time was 3.0 min and the mobile phase flow rate was maintained at 0.5 mL/min. The retention time for AFB_1-N^7-guanine was 1.42 min, as shown in Fig. 2. Electrospray ionization in positive ion mode was used in MRM mode, with a capillary voltage of 0.75 kV, a source temperature of 150 °C and a desolvation temperature of 500 °C. Desolvation gas flow and cone gas flow were maintained at 800 L/h and 150 L/h, respectively. Cone voltage, collision energy and MRM transitions (major precursor ion > fragment ion) were automatically optimized using Intellistart program of Acquity UPLC console. Quantification and confirmatory MRM transition for AFB_1-N^7-guanine were m/z 480 > 152 and 457 > 135, respectively.

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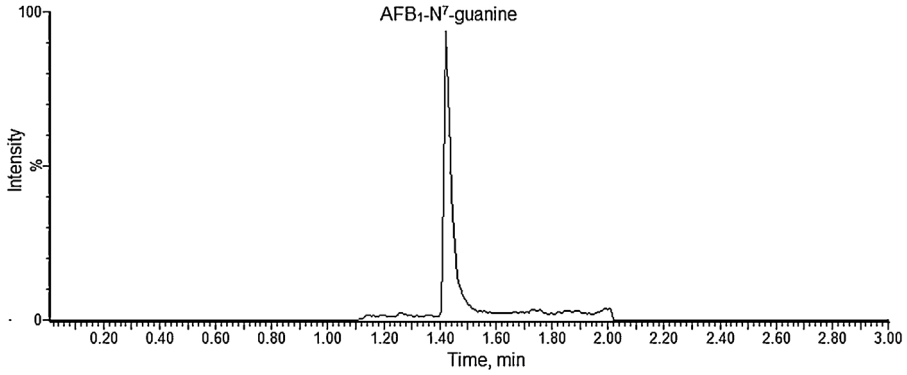

Fig. 2. Chromatogram (quantification transition) of AFB_1-N^7-guanine standard prepared in blank urine spiked with 100 pg/mL.

Data collection and processing were performed as previously described.

2.6. Determination of aflatoxin M_1 in urine

Urine samples were thawed at room temperature and centrifuged at 3500 rpm for 10 min. Extraction and purification of AFM_1 in urine samples were performed by using immunoaffinity columns (Aflatest®, Vicam, Watertown, MA, USA) according to Jager et al. (2014). AFM_1 standard (Sigma, St Louis, MO, USA), prepared as previously described, was diluted to prepare calibration curves at levels of 0.25 to 5.0 pg/mL.

Final extracts from urine samples were analyzed in the same UPLC system coupled to a Xevo TQ-S mass spectrometer (Waters, Milford, MA, USA) as previously described, using equal temperatures for column (40 °C) and samples (15 °C) during analysis, and injection volumes for standards and samples (10 μL). Gradient elution consisted of water (eluent A) and acetonitrile (eluent B), both containing 0.05% of aqueous ammonia. After an initial period of 0.2 min at 90% A, the percentage of B was linearly raised to 60% over 1.1 min (1.3 min). Then, eluent B was increased to 90% over 0.05 min, followed by a hold time of 0.25 min (1.6 min). Following this, the percentage of B was reduced to 10% over 0.1 min (1.7 min) and the column re-equilibrated to initial conditions for 0.8 min. The mobile phase flow rate was maintained at 0.6 mL/min during a total chromatographic run time of 2.5 min, and the retention time of AFM_1 was 1.03 min (Fig. 3). The mass spectrometer was operated in MRM mode using electrospray ionization in positive ion mode, with a capillary voltage of 0.75 kV, a source temperature of 150 °C and a desolvation temperature of 600 °C. Desolvation gas flow and cone gas flow were maintained at 500 L/h and 150 L/h, respectively. Cone voltage, collision energy and MRM transitions (major precursor ion > fragment ion) were automatically optimized using the Intellistart program of Acquity UPLC console. The most intense transition was set for quantification and the second most intense was used as confirmatory transition. Quantification and confirmatory MRM transitions for AFM_1 were m/z 329 > 273 and 329 > 229, respectively.

2.7. Validation of analytical methods

The performance of methods used for determination of AFB_1 and AFM_1 in food products were previously evaluated by Jager et al. (2013). The analytical methods for aflatoxin metabolites in serum and urine were validated in the present study for determination of recovery and precision, as follows: a blank serum sample was fortified with the AFB_1-lysine standard at 100 pg/mg albumin; AFM_1 and AFB_1-N^7-guanine standards were added to blank urine samples until reaching the concentrations of 0.25, 2.5 and 5.0 pg/mL (AFM_1), and 10, 100 and 200 pg/mL (AFB_1-N^7-guanine). Given the limited availability of the AFB_1-lysine standard, only one spiking level was used in the validation procedure. Fortified samples of serum (with AFB_1-lysine) and urine (with AFM_1 and AFB_1-N^7-guanine) were analyzed strictly following the same procedures as previously described. Triplicate injections of each concentration level were performed. The limits of detection (LOD) and quantification (LOQ) were calculated for each analytical method based on a signal:noise ratio of 3:1 and 10:1, respectively. Linearity was evaluated by verifying the coefficient of determination (r^2) and visual inspection of residual.

2.8. Statistical analyses

The results found in serum and urine samples in each sampling time were used to determine Pearson's Correlation Coefficient

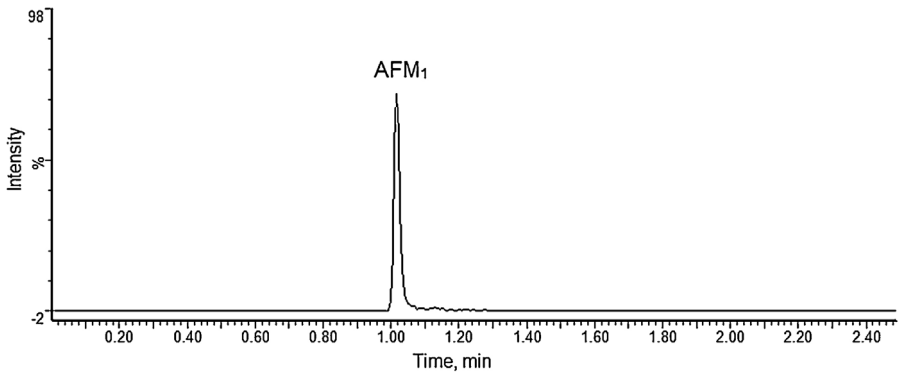

Fig. 3. Chromatogram (quantification transition) of AFM_1 in a positive urine sample containing 2.55 pg/mg creatinine.

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Table 1
Performance of analytical methods for determination of aflatoxin metabolites in serum and urine.

Aflatoxin metabolite	Matrix	LOD	LOQ	Spiking level[a]	Mean recovery (%)	CV[b] (%)
Aflatoxin B_1-lysine (pg/mg albumin)	Serum	6	20	100	85	10
Aflatoxin B_1-N^7-guanine (pg/mL)	Urine	3	10	10	70	15
				100	72	15
				200	78	15
Aflatoxin M_1 (pg/mL)	Urine	0.075	0.25	0.25	87	27
				2.5	70	6
				5.0	79	4

LOD: Limit of detection. LOQ: Limit of quantification.
[a] $N = 6$ for each spiking level of aflatoxicol or aflatoxin M_1; $N = 3$ for each spiking level of aflatoxin B_1-N^7-guanine or aflatoxin B_1-lysine.
[b] Coefficient of variation.

(r) between the PDI values for total aflatoxins (AFB_1 and AFM_1) and the concentration of aflatoxin metabolites. A Student t test, with normal approximation, was used to compare the correlation values at a 5% level of significance (Snedecor and Cochran, 1967).

3. Results and discussion

3.1. Methods performance

Table 1 shows the results for LOD, LOQ, recovery and coefficient of variation obtained for the aflatoxin metabolites analyzed in serum and urine samples. AFB_1-lysine in serum had LOD and LOQ values of 6.0 and 20.0 pg/mg albumin, respectively. For urine samples, the LOD values for AFM_1 and AFB_1-N^7-guanine were 0.075 and 3.0 pg/mL, respectively, while LOQ values were 0.25 and 10.0 pg/mL, respectively. Recoveries values were higher than 70% for all analytes, and coefficient of variation ranged from 2 to 27%, hence indicating that the analytical methods were successfully validated, providing satisfactory results for the determination of AFB_1-lysine in serum, and AFM_1 AFB_1-N^7-guanine in urine. The coefficients of determination for all analytes were higher than 0.99, and residuals plot did not show tendency or deviation from linearity.

Methods performance for AFB_1 and AFM_1 in food samples has been presented elsewhere (Jager et al., 2013), describing LOD and LOQ values for AFB_1 of 0.015 and 0.05 μg/kg, respectively, and 0.0025 and 0.0080 μg/L for AFM_1 in fluid milk, respectively.

3.2. Analyses of aflatoxins B_1 and M_1 in food products

Table 2 presents the levels of AFB_1 found in 222 samples of cereal and milk products analyzed in the 4 samplings conducted in this study. No sample had AFB_1 or AFM_1 above the tolerance limits established by Brazilian regulations for peanut, corn or dairy products (Agência Nacional De Vigilância Sanitária, 2011). Peanuts products showed the highest values for AFB_1 contamination when compared with the other samples analyzed, which is in agreement with previous reports on the incidence of aflatoxins in those products (Jager et al., 2014; Magrine et al., 2011). Corn flour had the lowest AFB_1 mean concentration among corn products, but with the highest incidence of positive samples (>60%). These data indicate that peanut and corn products were the main source of aflatoxin intake by the participants, although the concentrations of AFB_1 in peanuts and corn products varied markedly between different sampling times, from June 2011 (sampling 1) to March 2012 (sampling 4). Seasonal variations may have contributed for the differences in the AFB_1 levels, since higher values were found in corn and peanut products in December 2011 and March 2012, respectively, coinciding with the rainfall period in Brazil. AFB_1 concentration in beans was low in all samplings (Table 2). However, beans are largely consumed in Brazil, and therefore it may contribute significantly for the aflatoxin intake.

AFM_1 was quantified in 40% out of 65 samples of fluid milk, and in 30% of 10 cheese samples analyzed. Although the number of milk powder samples provided by the participants was low ($N = 4$), 50% of samples were positive for AFM_1. These findings confirm that liquid or powder milk and cheeses are the main sources for

Table 2
Aflatoxin B_1 (AFB_1) and M_1 (AFM_1) levels in food samples provided by the participants at 4 sampling times.

Type of food	Sampling 1 (June 2011)			Sampling 2 (September 2011)			Sampling 3 (December 2011)			Sampling 4 (March 2012)		
	N	*n*	Level[a]	*N*	*n*	Level[a]	*N*	*n*	Level[a]	*N*	*n*	Level[a]
Samples analyzed for AFB_1												
Peanut products	6	2	1.70 ± 2.30	6	3	7.00 ± 6.10	4	1	0.06	7	2	9.00 ± 12.00
Corn flour	11	5	0.20 ± 0.10	7	5	0.18 ± 0.25	5	3	0.30 ± 0.20	5	3	0.70 ± 0.53
Corn (popcorn)	10	0	<LOQ	10	2	1.60 ± 1.90	7	1	7.70	4	2	1.80 ± 0.60
White hominy	2	2	1.40 ± 1.60	1	1	0.06	0	0	<LOQ	0	0	<LOQ
Bean	14	2	0.05 ± 0.03	14	2	0.05 ± 0.01	13	2	0.05 ± 0.01	10	0	<LOQ
Samples analyzed for AFM_1												
Liquid milk	19	12	0.037 ± 0.018	17	6	0.018 ± 0.003	16	5	0.012 ± 0.004	13	3	0.015 ± 0.002
Milk powder	2	1	0.50	2	1	0.81	0	0	–	0	0	–
Cheese	5	2	0.2 ± 0.1	1	0	<LOQ	3	1	0.091	1	0	<LOQ
Yoghurt	5	0	<LOQ	1	0	<LOQ	1	0	<LOQ	0	0	–
Total	74	26	0.31 ± 0.79	59	20	1.3 ± 3.2	49	13	0.68 ± 2.11	40	10	2.1 ± 4.8

N: Number of samples analyzed for each food product. *n*: Number of samples with concentrations above the limit of quantification (LOQ) (0.05 μg/kg for AFB_1; 8.0 ng/L or kg for AFM_1).
[a] Results are expressed as mean ± standard deviation of samples with concentrations above LOQ. Units: μg/kg for AFB_1; ng/L or kg for AFM_1.

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Table 3
Aflatoxin M_1 levels in urine samples provided by the participants, and mean probable daily intake (PDI_M) for the sum of aflatoxins B_1 and M_1 at 4 sampling times.

	Sampling 1 (June 2011)	Sampling 2 (September 2011)	Sampling 3 (December 2011)	Sampling 4 (March 2012)
Number of samples analyzed	33	31	27	22
Number of positive samples (%)[a]	23 (70)	20 (64)	20 (74)	11 (50)
Mean ± SD (pg/mg creatinine)	1.46 ± 2.04	1.70 ± 2.88	0.92 ± 0.78	0.37 ± 0.23
Median (pg/mg creatinine)	0.91	0.79	0.49	0.28
Minimum (pg/mg creatinine)	0.25	0.26	0.25	0.25
Maximum (pg/mg creatinine)	2.79	12.68	2.45	0.86
PDI_M (ng/kg body weight/day)	0.51 ± 1.12	1.35 ± 5.98	0.09 ± 0.09	0.86 ± 2.59

[a] Samples with aflatoxin M_1 concentration above the limit of quantification (0.25 pg/mg creatinine).

AFM_1 exposure in Brazil, hence contributing for the overall aflatoxin intake by the participants in the present study, similar to data reported in Malaysia by Redzwan et al. (2012).

3.3. Analyses of aflatoxin B_1 metabolites in serum and urine

None of the 113 serum samples collected in this pilot survey showed detectable levels of AFB1-lysine. The LOD of the analytical method (6.0 pg/mg albumin) in the present work was higher than those reported in other studies, such as Johnson et al. (2010) (1.0 pg/mg albumin) or Leong et al. (2012) (0.4 pg/mg albumin). The higher LOD values obtained in this study may be caused by matrix effect due to sample components that could remain in the final extract after clean-up. Results for AFB_1-lysine were different from those reported by Scussel et al. (2006), who found AFB_1-albumin adduct in 62% of 50 blood samples collected in the city of Sao Paulo in 1999 at mean concentration of 14.9 pg/mg albumin. The absence of positive serum samples for AFB_1-lysine in the present study indicates a low long-term exposure to AFB_1 through the diet by the participants in the 4 sampling times.

AFB_1-N^7-guanine was not detected in any urine sample, which corroborates the low exposure indicated by the absence of detectable levels of serum AFB_1-lysine. Previous studies indicated that AFB_1-N^7-guanine levels in urine of individuals from highly exposure populations is variable, with values reported in China ranging from 300 to 1810 pg/mL (Qian et al., 1994), 64.9 to 1789.0 pg/mg creatinine (Wang et al., 2001) or up to 2150 pg/mL (Mykkänen et al., 2005). Our study indicates a low biologically effective dose of aflatoxin in the participants by the time of sample collections.

AFM_1 was determined in 74 (65%) urine samples, as presented in Table 3. The mean concentrations of AFM_1 in urine samples collected in the 4 sampling times varied from 0.37 ± 0.23 to 1.70 ± 2.88 pg/mg creatinine, which are similar to the urinary AFM_1 levels (1.2 ± 2.0 pg/mg creatinine) reported in São Paulo by Jager et al. (2014). The comparison between the results of the present study and those reported by other authors is difficult, because mostly of them expressed the urinary AFM_1 levels without correcting the value for the excreted creatinine. However, our results were lower than those reported previously for urinary AFM_1 in Brazil by Romero et al. (2010) and Giolo et al. (2012), who found mean values of 5.96 ± 6.39 pg/mL and 2.74 ± 1.98 pg/mL, respectively. In the present study, the maximum AFM_1 level in urine (12.68 pg/mg creatinine, or 6.85 pg/mL urine) was found in sampling 2 (September 2011), although the maximum concentrations in the other sampling times ranged from 0.86 to 2.79 pg/mg creatinine (1.60 to 5.24 pg/mL urine, respectively). Thus the AFM_1 levels described in this study indicate low exposure to aflatoxins in Brazil, when compared to data reported in areas of high aflatoxin-contamination of China. Early assessments conducted in several Chinese regions described AFM_1 concentrations in human urine as high as 5200 pg/mL (Qian et al., 1994; Zhu et al., 1987). In a study conducted latter in China by Tang et al. (2008), AFM_1 levels ranged from 0.42 to 141.9 pg/mg creatinine. Mykkänen et al. (2005) found urinary AFM_1 levels in China ranging from 10 to 33 pg/mL, which are much lower than the levels reported in former studies but still higher than those observed in the present study. In Malaysia, Redzwan et al. (2012) reported an association between the consumption of milk and dairy products and high urinary excretion of AFM_1, with a mean concentration of 23.4 ± 17.7 pg/mL, which is also higher than the mean levels found in this study.

Considering the mean levels of total aflatoxins (AFB_1 + AFM_1) in food products obtained in the present study in each sampling time, as well as the consumption values obtained from the 24-hour dietary recall, the mean PDI of aflatoxin was calculated and the results were presented in Table 3. Mean PDI varied among different sampling times, ranging from 0.09 ± 0.09 to 1.35 ± 5.98 ng/kg b.w./day, which are similar to data reported previously in the States of Paraná (Magrine et al., 2011) and São Paulo (Jager et al., 2013). There is no consensus for tolerable daily intake of AFB_1 or AFM_1. However, Kuiper-Goodman (1998) indicated a provisional maximum tolerable daily intake for aflatoxin (PMTDI) of 1.0 ng/kg b.w./day for adults and children without hepatitis B virus and 0.4 ng/kg b.w./day for adults carrying hepatitis B virus. The mean PDI values obtained for the participants in this study were higher than PMTDI for both carriers and non-carriers hepatitis B virus, except in sampling 3 (December 2011), thus indicating health risks associated to human exposure to aflatoxins in Brazil.

Based on the untransformed data from urine and food analyses, a modest though significant correlation ($r = 0.45$; $p = 0.03$; $N = 23$) was found in the first sampling (June 2011) between the AFM_1 concentration in urine (normalized with creatinine) and the PDI for total aflatoxins (AFB_1 + AFM_1), as shown in Fig. 4. The correlations between urinary levels of AFM_1 and PDI obtained in samplings 2, 3 and 4 were not significant ($p > 0.05$), possibly because of seasonal variations in the consumption of food products. Sampling 1 was conducted in June, when peanut and corn products are largely consumed in typical Brazilian countryside parties celebrated during this month. Although the mean PDI for aflatoxins was not higher in June 2011 (0.51 ± 1.12 ng/kg b.w./day), the frequent consumption

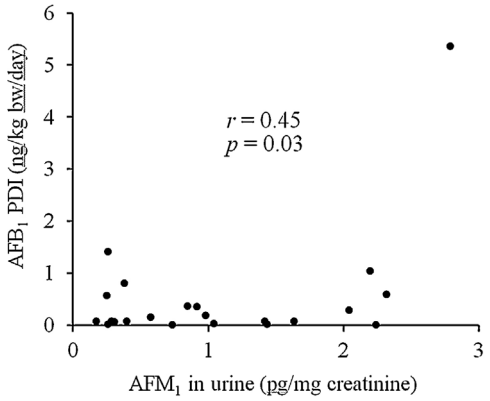

Fig. 4. The relationship between aflatoxin M_1 (AFM_1) in urine and aflatoxin B_1 (AFB_1) probable daily intake (PDI) at sampling 1 (June 2011, $N = 23$).

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of peanut and corn products in this month could have contributed for the correlation between the urinary AFM_1 levels and the PDI found in sampling 1. Taking into account the mean PDI values and the mean AFM_1 levels excreted in urine by participants in June 2011, as well as the mean volume of urine collected (300 mL) and the mean body weight (70 kg), it was estimated that 1.3% of the total intake of aflatoxins could be found in the urine samples. The carry-over rates of ingested AFB_1 or AFM_1 in foods to AFM_1 in urine may vary according to the level of toxin ingested and length of exposure, among other factors (Gan et al., 1988). However, the percentage found in our study was close to the mean carry-over rates estimated by Zhu et al. (1987) in high aflatoxin-contaminated areas in China (1.7% for males and 1.5% for females).

4. Conclusion

Results of this trial indicate both low long-term and short-term aflatoxin exposure by the participants over a period of one year, as confirmed by the absence of detectable levels of serum AFB_1-lysine and urinary AFB_1-N^7-guanine, respectively. AFB_1 and AFM_1 levels in food samples consumed by the participants were below the tolerance limits adopted in Brazil, leading to low PDI values for total aflatoxins ($AFB_1 + AFM_1$). However, AFM_1 was found in 65% of urine samples analyzed, and a positive correlation was determined for the first time in Brazil between AFM_1 levels in urine and PDI estimated in June 2011. Urinary AFM_1 was confirmed to be very sensitive for monitoring the actual human exposure to dietary aflatoxin. Further studies using serum and urinary biomarkers are needed to estimate the aflatoxin exposure of populations in higher risk areas in Brazil.

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