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Sphinganine and sphingosine levels and ratio in urine and blood samples from a Catalanian population (Spain).

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Abstract:	<p>Fumonisin occurs mainly in maize, and produces alterations in sphingolipid metabolism, unbalancing sphinganine (Sa) / sphingosine (So) ratio. This alteration has been proposed as a biomarker of fumonisin exposure.</p> <p>The objective of the present work was to study the urinary and plasmatic levels of Sa, So as well as, the ratio Sa/So from a sample of Catalanian population exposed at low levels of fumonisin. Firstly, plasmatic and urinary Sa and So levels and the ratio Sa/So were compared between two population groups, and after, urinary Sa and So levels from corn-food consumers and a control group were monitored during two weeks under controlled intake of corn-foods. Sa and So levels were determined in urine and blood samples using validated methods using HPLC with fluorescence detection.</p> <p>Significant differences were not found in urine samples when Sa/So ratios were compared from corn-food consumers and non</p>

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Sphinganine and sphingosine levels and ratio in urine and blood samples from a Catalanian population (Spain).

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Keywords: fumonisin; biomarker; sphinganine; sphingosine; urine; blood.

Abbreviations: Enzyme Ceramide (CER), Food and Agriculture Organization (FAO), Food and Drug Administration (FDA), food frequency questionnaire (FFQ), fumonisin B₁(FB₁), fumonisin B₂ (FB₂), total fumonisins (FB_T), high performance liquid chromatography (HPLC), International Agency for Research on Cancer (IARC), Joint FAO/WHO Expert Committee on Food Additives (JECFA), limit of detection (LOD), limit of quantification (LOQ), non observed effect level (NOEL), *o*-phthaldialdehyde (OPA), provisional maximum tolerable daily intake (PMTDI), three days dietary record (R3), sphinganine (Sa), sphingosine (So), World Health Organization (WHO).

Abstract

Fumonisin occurs mainly in maize, and produces alterations in sphingolipid metabolism, unbalancing the sphinganine (Sa) / sphingosine (So) ratio. This alteration has been proposed as a biomarker of fumonisin exposure. The objective of this study was to establish the urinary and plasmatic levels of Sa, So as well as, the ratio Sa/So from a sample of Catalan population exposed to fumonisins at low levels. Firstly, plasma and urinary Sa and So levels and the ratio Sa/So were compared between two population groups, and after, urinary Sa and So levels from corn-food consumers and a control group were monitored for two weeks under controlled intake of corn-foods. Sa and So levels were determined in urine and blood samples using validated methods using HPLC with fluorescence detection. Significant differences were not found in urine samples when Sa/So ratios were compared from corn-food consumers and non-consumers, while significant differences were found in urine and plasma samples but evidence of the mechanism of action of fumonisins was not apparent. Through a time-course study, we have narrowed down the day in which the maximum alteration of Sa/So ratio should be expected in humans. In this paper we have reported some useful information to improve the design of studies to validate the ratio Sa/So as a possible biomarker of fumonisin exposure.

Introduction

Fumonisin B₁ (FB₁) and B₂ (FB₂) are mycotoxins produced by *Fusarium verticillioides* and *F. proliferatum* that commonly contaminate maize (Nelson et al. 1992). Fumonisin occurs mainly in maize and maize-based foods, thus populations with high maize consumption can be exposed to significant amounts of these mycotoxins via the ingestion of fumonisin contaminated maize (Marasas 1996; Shephard et al. 1996; Visconti et al. 1996).

Acute and chronic toxicity of fumonisin has been demonstrated in several animal species, including carcinogenicity and cardiovascular toxic effects (Gelderblom et al. 1988, 1991; Howard et al. 2001; Shephard et al. 2007). FB₁ is a potent cancer promoter in rats after initiation with diethylnitrosamine and aflatoxin B₁ (Gelderblom et al. 1996). Human exposure to fumonisin contaminated commodities has been correlated with high rates of esophageal and liver cancer in South Africa and China (Sydenham et al. 1990; Yoshizawa et al. 1994) and more recently, with neuronal tube defects on the Texas-Mexico border with other possible risk factors (Hendricks et al. 1999).

Based on toxicological evidence, the International Agency for Research on Cancer (IARC) classified FB₁ as a possible human carcinogen (group 2B) (IARC 2002). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) evaluated fumonisins and allocated a provisional maximum tolerable daily intake (PMTDI) of 2 µg/kg body

weight/day of fumonisins. This value was determined on the basis of the overall non observed effect level (NOEL) of 0.2 mg/kg body weight/day for renal toxicity in rats, and the safety factor was 100 (JECFA 2001).

Natural occurrence of fumonisins in maize or maize-based foods has been widely studied worldwide (Castelo et al., 1998) and several surveys have been conducted in maize-food for human consumption marketed in Spain (Sanchis et al. 1994, 1995; Torres et al. 1998; Castellá et al. 1999; Velluti et al. 2001; UdL-ACSA 2009). In the latest study conducted in Catalonia to assess the incidence of mycotoxins in food for human consumption, 928 samples were purchased from the Catalonian market (in 2008 and 2009) and pooled in 370 composite samples to be analyzed. The commodities analyzed were beer, sweet-corn, corn snacks, corn flakes, free-gluten pasta and bread and ethnic food. The authors reported that the highest occurrence of fumonisins was found in beer (90 % of positive samples), however those levels were low, while the incidence of these mycotoxins in other cereal-based foods was moderated. The mean values of positive samples of corn snacks and corn flakes were 119.1 ± 83.1 and 78.9 ± 27.9 $\mu\text{g/kg}$, respectively (UdL-ACSA 2009).

Fumonisin have a remarkable structural similarity to sphingolipids (Merrill and Sweeley 1996; Riley et al. 2001). This group of mycotoxins, especially FB₁, potently inhibits the enzyme ceramide (CER) synthase which catalyzes the acylation of sphinganine (Sa) and reacylation of sphingosine (So). The inhibition of CER synthase increases the intracellular Sa and other sphingoid bases, highly cytotoxic compounds. This imbalance

has been proposed as the main responsible for the toxicity, and possibly carcinogenicity, of FBs, based on mechanistic studies with cells cultures, and borne out by animal studies (Wang et al. 1991; Norred et al. 1992; Merrill et al. 1993, 2001; Yoo et al. 1996; Riley et al. 2001; Voss et al. 2006; Zitomer et al. 2009). Based on this biological perturbation, elevation of Sa to So in tissues, urine and blood have been proposed as potential biomarkers of fumonisin exposure in various animal species (Wang et al. 1992; Riley et al. 1993; Morgan et al. 1997; Wang et al. 1999; van der Westhuizen et al. 2001; Kim et al. 2006; Tran et al. 2006; Cai et al. 2007). Several studies have been conducted to assess the effectiveness of this biomarker in humans, but results did not allow an accurate validation (Hendricks et al. 1999; van der Westhuizen et al. 1999, 2008, 2010; Abnet et al. 2001; Qiu and Liu 2001; Solfrizzo et al. 2004; Nikiema et al. 2008; Silva et al. 2009; Xu et al. 2010). The individual Sa and So basal levels, as well as, the basal Sa/So ratio vary depending on unknown parameters, being related with nutrition factors (Abnet et al. 2001; Shephard et al. 2007). The sensitivity of the correlation between fumonisin intake and Sa/So has been demonstrated to be poor at low and very low doses in animals (< 1 mg/kg bw/day). Considering that the PMTDI is $2 \mu\text{g/kg bw/day}$, low sensitivity should be expected when we apply this biomarker in human population (Kim et al. 2006; Voss et al. 2006; Cai et al. 2007).

The objective of the work reported here was to study the urinary and plasma levels of Sa, So as well as the ratio Sa/So from a sample of Catalanian population, exposed at low levels of fumonisins, as a means to assess this ratio as a possible biomarker of fumonisin intake in the region. This work is structured in two experimental sections: in the first,

plasma and urinary Sa and So levels and the ratio Sa/So were compared between two population groups, in the second, urinary Sa and So levels from maize-food consumers and a control group were monitored during two weeks under controlled intake of maize-foods.

Materials and methods

Study design and sampling

This research project did not involve any risks for the volunteer donors; neither harmful modification of usual dietary habits nor administration was included in methodology for the subjects. Each participant was informed about the study rules and a signed authorization was requested individually.

Part 1. Urinary and plasmatic Sa/So ratio point estimates.

The first attempt to know the urinary and plasmatic Sa and So levels, and the ratio Sa/So from Catalanian population was designed to compare Sa, So levels and ratio Sa/So between high consumers of maize food and low/non consumers.

Part 1.1. Study of plasmatic sphingoid bases levels

Blood samples were collected from 136 healthy adult volunteers during 2008, from Catalanian population following approval from University of Lleida Ethical Council and informed consent. Blood was extracted and stored (less than 2 hours) in Vacutainers® with anticoagulant (EDTA) followed by centrifugation at 1000 g for 10 min, and finally, the plasma was stored at -20°C until analysis (same month). Maize-food intake was requested with a Food Frequency Questionnaire (FFQ) in order to determine the

approximate individual fumonisin intake. Fumonisin intake was estimated through the combination of the consumption data with contamination data provided by UdL-ACSA (2009). Two population groups were made depending on their estimated fumonisin intake: high exposed and low or none exposed.

Part 1.2. Urinary sphingoid base levels

First morning urine from 89 volunteers was collected in sterile containers, during 2009, according to Declaration of Helsinki. Urine samples were transported under refrigeration and they were stored at -20°C until analysis. In order to estimate the individual fumonisin intake, maize-food intake was requested through a FFQ and a 3 days record (R3) during the days prior to the sample collection day. Fumonisin intake was estimated through the combination of the consumption data with contamination data provided by UdL-ACSA (2009). The population was grouped in high consumers and low consumers depending on their maize dietary estimates. Finally, 7 urine samples were provided by esophageal cancer sufferers from University Hospital Arnau de Vilanova (Lleida). These samples were analyzed to determine Sa and So levels, and compared with the healthy group.

Part 2. Study of the urinary Sa and So time-course.

To know the changes of urinary sphingolipid levels over time, two groups of volunteers were monitored during 16 days. One group was composed of maize-food consumers (exposed group n=24), and the other, by non consumers (control group n=12). The exposed group was restricted of maize-food consumption during 16 days, with the exception of the seventh day after restriction, when the maize-food intake was completely

free. The food items consumed were: home-made Mexican “tortillas”, corn snacks, maize-based cake, sweet corn and beer purchased from a Catalonian market. A representative sample of each maize-food consumed during that day, was kept and they were analyzed by duplicated to determine the FB levels. The control group was restricted to maize-food during the entire study period (See Fig 1).

[Insert Figure 1 about here, if possible]

(Title: Fig. 1. Diagram of urine sampling design and restriction periods performed in the study to assess urinary Sa and So time-course.

Footnote: US: urine sample, R24: 24 hours record, FFQ: Food Frequency Questionnaire).

At the beginning of the experiment, dietary habits of the individuals were requested via a FFQ. Maize-food intake during the free consumption day (day 0) by the exposed group was requested with a 24 hour dietary using household sizes previously standardized. First morning urine was sampled according to Declaration of Helsinki, from control group at days -7, 0, 4 and 8, while in the exposed group, two sampling days (1 and 6) were added in order to increase the accuracy. Urine samples were transported under refrigeration, and they were stored at -20°C until analysis (during the same month). Sa and So levels were determined for each urine samples.

Fumonisin analysis in food

Fumoniprep[®] immunoaffinity cleanup columns (IAC) (R-Biopharm, Rhône LTD Glasgow, UK) were used to extract FB₁ and FB₂ from beer samples. A volume of 5 mL

of beer previously degassed in ultrasonic bath during 40 minutes was mixed with 15 mL of phosphate buffer solution (PBS; 0.8 % NaCl, 0.12% Na₂HPO₄, 0.02% KH₂PO₄, 0.02% KCl) and drained through the IAC. The column was washed with 20 mL of PBS solution and fumonisins were eluted with 1.5 mL of methanol grade HPLC and 1.5 mL of milli-Q water. Regarding solid maize-based samples, 10 g of ground sample was mixed with 1 g NaCl, and 50 mL of extract solution (50% water, 25% methanol, 25% acetonitrile) for 20 minutes and filtered. 10 mL of filtered solution was diluted with 40 mL of PBS and drained through the IAC and follows as described previously.

Fluorescent derivatives of FB₁ and FB₂ were obtained using pre-column derivatization with an *o*-phthaldialdehyde (OPA) solution prepared diluting 40 mg of *o*-phthaldialdehyde with 1 mL of methanol HPLC grade and mixed with 5 mL of Na₂B₄O₇·10H₂O (0.1M) and 50 µL de 2-mercaptoethanol. Derivatization was conducted mixing 200 µL of eluate with 200 µL of OPA solution for 30 seconds in vortex. Chromatography equipment: Separations Module Alliance 2695 Waters®, analytical column Waters Spherisorb® 5µm ODS2, 4.6 x 150 mm, Multi λ Fluorescence Detector Waters 2475®, kept at 35 °C and a flow-rate maintained at 1 mL/min. Mobile phase was based on a methanol and 0.1M sodium dihydrogen phosphate (77:23, v/v) solution. Excitation and emission wavelength was 335 nm and 440 nm, respectively.

Sphinganine and Sphingosine analysis in plasma

Plasma (500 µL) was deproteinized with methanol (2 mL) and the protein precipitate was centrifuged down at 1200 g for 10 min at 10 °C. An aliquot of the sobrenatant (1.5 mL) was mixed with 1.5 mL potassium chloride solution (0.8 %) and 50 µL potassium

hydroxide (1 M). The mixture was extracted with 4 mL of ethyl acetate by gentle rotation in a blender for 20 min and the phases were separated by centrifugation at 1100 g for 15 min, as described by Castegnaro et al. (1998). The organic phase was evaporated to complete dryness at 55°C under nitrogen. Dried samples were redissolved by vortex shaking in 275 µL methanol-water solution (88:12) and derivatized for 35 min by addition of 25 µL of OPA mixture. The derivatization mixture consisted of 50 mg OPA dissolved in 1 mL of ethanol and mixed with 50 µL of mercaptoethanol and 48.95 mL of boric acid solution (3 %) adjusted to pH 10.5 with potassium hydroxide (1 M) to obtain a final volume of 50 mL. The derivatives were analyzed by HPLC with fluorescence detection (excitation wavelength of 340 nm, emission wavelength of 455 nm), using a Waters Spherisorb® 3 µm ODS2 4.5x250 mm column, kept at 35 °C and a flow-rate maintained at 1 mL/min of methanol-water (88:12, v/v).

Sphinganine and sphingosine analysis in urine

Urinary samples were stored at -20°C in the dark before the analysis. Extraction of Sa and So was performed using a method adapted from Castegnaro et al. (1996). To sum up, 20 mL of urine thawed sample were centrifuged at 2000 g for 15 minutes at 10 °C, in order to isolate exfoliated cells. Cell pellets were re-suspended in 2 ml distilled water with 50 µl of potassium hydroxide (1 M). Following, 2 ml of ethyl acetate were added and mixed vigorously using the vortex for 1 minute. Then, the mixture was centrifuged at 2000 g for 15 minutes, and the upper solvent layer was kept, while the aqueous phase was extracted again. Finally, the mixed solvent layers containing sphingolipids were dried under a

nitrogen stream. Dried samples were analyzed as described for plasma samples, under the same chromatographic conditions.

Validation of the analytical methods

The analytical methods used for fumonisins, Sa and So were assessed for selectivity, linearity, and precision. Selectivity was checked by injecting 50 µl of mycotoxin standard solutions three times before injecting extracted samples and comparing the peak retention times and the fluorescence spectra of the substances that produced these peaks. Standard curves were generated by linear regression of peak areas against concentrations.

Accuracy and recovery were established by determination of FB₁ and FB₂ levels, spiked in samples of corn snacks, beer, and sweet corn; in the case of Sa and So, they were spiked in urine and blood samples. Recovery was determined by comparing the absolute responses of fumonisins, Sa and So, with the absolute responses of calibration standards.

The limit of detection (LOD) was considered as the mycotoxin and the sphingolipid concentration that provides a signal equal to $b+3S_b$, where b is the intercept of the calibration curve and S_b is the standard error of the estimate assuming to be the blank, and the limit of quantification (LOQ) was considered equal to $3 \times \text{LOD}$.

Recovery data, repeatability, limit of detection (LOD) and limit of quantification (LOQ) of FB₁ and FB₂ in sweet corn, corn snacks and beer are shown in Table 1. These values are in accordance to performance criteria established by Commission Regulation (EC) N° 401/2006 (European Commission 2006a).

[Insert Table 1 about here, if possible]

Method performance characteristics for Sa and So in blood are shown in Table 2. This method showed recovery rates of So ranging from 92.2 ± 19.7 to 104.0 ± 12.8 %, while the recovery rates for Sa were between 93.1 ± 13.4 and 98.3 ± 11.2 %.

[Insert Table 2 about here, if possible]

The method to determine the sphingoid bases in urinary samples was optimized in order to obtain a low detection limit, due to the low concentration of sphingolipids expected to be found in this matrix. Recovery rates, RSDr, LOQ and LOD are shown in Table 3.

[Insert Table 3 about here, if possible]

Statistical Analysis

Sa/So ratios were calculated individually by division of Sa and So levels from each volunteer, and expressed as medians, means and standard deviations of ratios for each group.

Mann-Whitney *U* test was used for two-group comparison and Kruskal-Wallis test was used to compare more samples. Principal Component Analysis was conducted to obtain matrix correlation from Sa/So ratio data and associated factors. Software SAS Enterprise guide v2.0.0.417[®] and SAS v9.0.[®] were used in statistical analysis.

Results

Study of sphingoid base levels and ratios in plasma

In this first study, 136 blood donors were grouped in high maize-based food consumers (68) and non consumers (68). The mean exposure to fumonisin estimated for the first group was 0.23 ± 0.11 $\mu\text{g/kg bw/day}$. Medians were 0.53 and 0.46 for maize-based food consumers and non consumers, respectively. Although significant differences were observed when the Sa/So ratios were compared, non statistically significant differences were found between sphinganine levels ($P > 0.05$), the sphingosine decrease being the most probable responsible of ratio variation in the exposed group (See Table 4).

Study of the sphingoid base levels and ratios in urine

In this cross-sectional study, 78 volunteers were selected to assess the urinary Sa and So levels. Each volunteer was asked about dietary habits, through a FFQ and a R3. Considering the estimated fumonisin intake, the population was divided in high and low consumers in order to compare Sa and So levels and their ratios. The mean fumonisin intake estimated through the R3, was 0.013 and 0.046 $\mu\text{g/kg bw/day}$ for males and females, respectively, while these respective estimates were 0.089 and 0.057 $\mu\text{g/kg bw/day}$ when the estimation was made using the FFQ. The most important bias sources were that males overestimated significantly the beer consumption in comparison with the R3 and the females underestimated the usual corn snacks consumption with this method. Levels of Sa, So and Sa/So ratio and fumonisin intake estimated through the R3 are shown in the Table 4.

[Insert Table 4 about here, if possible]

Significant differences were found between median ratios Sa/So from high and low consumers ($P<0.05$), but no differences were found in Sa and So levels.

The urinary samples from esophageal cancer sufferers showed mean levels of Sa and So to be 0.376 ± 0.471 and 0.208 ± 0.484 ng/mL, respectively, while the mean Sa/So ratio was 0.363 ± 0.458 , no significantly different from that of healthy population.

Urinary Sa/So ratio time-course

Fumonisin dietary intake of individuals was assessed combining food consumption data with fumonisin levels on the maize-food consumed. Consumption data was recorded during the day 0 (free consumption day), using previously standardized portions. Mean levels of fumonisin contamination in corn snacks, Mexican “tortillas”, corn-based cake, and sweet corn samples were 133.9, 99.3, 110.1 $\mu\text{g/kg}$ and non detectable level, respectively. These values were far from EU limits of 400 $\mu\text{g/kg}$ (European Commission 2006b). Volunteers were classified in three groups, depending on total fumonisin intake estimated during the “free maize-food consumption day”: high consumers, H, (>0.6 $\mu\text{g/kg bw/day}$, mean 0.84 ± 0.26 $\mu\text{g/kg bw/day}$); low consumers, L, (<0.6 $\mu\text{g/kg bw/day}$, mean 0.43 ± 0.12 $\mu\text{g/kg bw/day}$) and non consumers, C, (control group, $n=12$). The high consumers did not exceed the tolerable daily intake of 2 $\mu\text{g/kg bw/day}$. The volunteer population was 18 males and 18 females. 55 % of volunteers presented a body mass index between 18.5 and 24.9 kg/m^2 (normal) and 45 % were overweight. Tobacco was consumed by 32 % of the individuals.

Mean levels of Sa and So, and mean ratios in urine samples from volunteer donors collected during the restriction period, are shown in Table 5. The two volunteers excluded

from the study showed So and Sa basal levels markedly higher than the rest of the group (40 fold greater than the mean group level).

[Insert Table 5 about here, if possible]

Median Sa levels at the beginning of the study were 0.27, 0.28 and 0.14 ng/mL, for non consumers, low consumers and high consumers, respectively, and So median levels were 1.22, 0.68 and 0.33 ng/mL, respectively. The median Sa/So ratios were quite similar between exposition groups, without statistically significant differences for these values (0.25 to 0.70). During the first week of maize-food restriction, we did not observe significant differences in the Sa/So ratios for any group, however, after the free maize-food consumption day, statistically significant differences were observed in exposed groups while no differences were observed in the control group with time. The maximum increase of the Sa/So ratio was observed the fourth day after the free consumption day, with mean values of 1.96 ± 2.24 and 2.52 ± 2.00 ng/mL for low and high consumers, respectively, while the mean ratio for the control group was 0.67 ± 0.49 ng/mL (see figure 2). After the fourth day, the stabilization of the ratios was observed for these groups, recovering initial values, without statistical differences among all groups (See Table 5). This fact was confirmed by means of the correlation matrix of the ratios against the estimated daily intake during the free consumption day. Principal component analysis of Sa, Sa, Sa/So and fumonisin intake showed that the higher correlation should be expected between estimated fumonisin intake and Sa/So ratio from day 4 ($r=0.3322$; $P<0.01$) with

low correlation with the other days. The mean Sa/So ratios through the time are represented in the figure 2.

[Insert Figure 2 about here, if possible]

Title: Fig. 2. Time-course of mean Sa/So ratio for high exposed (H, \blacklozenge), low exposed (L, \blacksquare) and non exposed (C, \blacktriangle).

Sa levels decreased during the first restriction week in each group, but increased after the free consumption day in exposed groups (high and low consumers), while the level slightly decreased in the control group. So levels decreased during the first week, after day 0 the median values decreased slightly but no significant differences were found (See Table 5).

The absolute modification of the sphingoid bases, as well as of the ratio, after the free consumption day was quantified as the absolute difference (maximum – minimum) among day 0 and day 8 (Table 6). Sa and So levels were slightly modified during this period, without differences among exposure groups, neither significant differences were observed in the increase of the Sa/So ratio.

[Insert Table 6 about here, if possible]

4. Discussion

Based on the mechanism of action, it has been observed that fumonisins inhibit CER synthase, a disruption that leads to an increase of Sa levels and Sa/So ratio (Riley et al.

2001). It is due to the rapid elimination and low bioavailability of fumonisins, that it is necessary to find an indirect indicator of human exposure to these toxins. Sa/So and Sa 1-phosphate / So 1-phosphate ratios in tissues, urine and blood, have been proposed as potential biomarkers in various animals (Wang et al. 1992, 1999; Morgan et al. 1997; van der Westhuizen et al. 2001; Tran et al. 2006), these ratios being validated in F344 rats by Cai et al. (2007), obtaining more sensitive results in urine than in serum for acute and sub-chronic exposure to FB₁. However, no successful results have been found when this biomarker has been assessed in human population, due to the low sensitivity when it is applied over individuals (van der Westhuizen et al. 1999, 2008, 2010; Abnet et al. 2001; Qiu and Liu 2001; Solfrizzo et al. 2004; Nikiema et al. 2008; Silva et al. 2009; Xu et al. 2010).

In Catalonia, **maize**-based food is not highly consumed; therefore the exposure of the population to fumonisins is expected to be low, as reported in the Technical Report from UdL-ACSA (2009). In the present study, we have estimated that fumonisin intake of the volunteers from this region was in all cases below the PMTDI of 2 µg/kg bw/day, including the high consumers, who showed maximum estimates of 1.04 and 1.42 µg/kg bw/day. Other previous studies were conducted in regions where **maize** is highly consumed, and estimated fumonisin intake has been estimated to be quite high; for example, in some regions of South Africa the mean fumonisin intake was estimated to be between 5.8 and 3.8 µg/kg bw/day (van der Westhuizen et al. 1999, 2008, 2010), and the 93 % of 43 volunteers from Huian (China) had their daily FB intakes above the PMTDI of 2 µg/kg bw/day (Xu et al. 2010).

Concerning our cross-sectional studies, the mean plasmatic Sa and So levels were higher than urinary levels, as reported previously (van der Westhuizen et al. 2008), while mean ratios were slightly higher in urinary samples.

In both cross-sectional studies we have found significant differences between ratios from exposed and non exposed groups, however no differences were found in sphinganine levels. In the study performed with plasma, the main responsible of ratio increase was elucidated through a decrease of So levels, with significant differences, therefore no evidences of mechanism of action of fumonisins were found.

Esophageal cancer rates have been correlated with fumonisin exposure in China and South Africa, to regions highly exposed to fumonisins (Chu and Li 1994; Zhang et al., 1999; Wang et al., 2000; Rheeder et al., 1992). In northern Italy region, maize consumption was correlated with higher rates of esophageal cancer than other regions (Rossi et al., 1982; Franceschi et al., 1990), and presence of fumonisin-producing *Fusarium* species in maize and polenta was lately reported (Logrieco et al., 1995; Pascale et al., 1995). In this study seven urine samples from esophageal cancer sufferers were analyzed and compared with healthy groups, and non differences were found in any case. In our latest study, we have monitored the expected alteration of Sa and So levels in urine from maize-food consumers after a free maize-food consumption day within a maize-food restriction period. Significant differences were observed for the ratio Sa/So after the free consumption day (day 0) for both exposed groups, while no differences were observed in the control group. The maximum values of the ratios were observed at day 4 after the free consumption day. Previous studies conducted with animal species dosed with fumonisins showed variable results concerning the day of maximum Sa/So ratio. For

example, the maximum peak of Sa/So in weaned piglets dosed with 5 mg/kg bw/day showed the peak at 12 h (Dilkin et al. 2010), in rats dosed with 10 mg/kg bw/day the maximum was observed at day 3 and day 5 (Garren et al. 2001; Cai et al. 2007), while in vervet monkeys dosed with 1 mg/kg bw/day the maximum was found to be the day 3 (Van der Westhuizen et al. 2001). The time period between fumonisin intake and maximum peak of the ratio Sa/So is an important data to validate a human biomarker that will permit a better design of sampling and dietary exposure assessment.

To date, the cross-sectional studies have shown poor correlation between fumonisin dietary intakes and Sa/So ratio in humans, however, successful results have been found in several animal studies. Thus, there are several drawbacks which prevent this biomarker to be applied to humans for epidemiologic purposes:

- 1) The individual Sa and So basal levels, as well as, the basal Sa/So ratio vary depending on unknown parameters, being related with nutrition factors (Abnet et al. 2001; Shephard et al. 2007). Therefore, the absolute ratio could not be a good predictor of fumonisin intake.
- 2) The sensitivity of the correlation between fumonisin intake and Sa/So has been demonstrated to be poor at low and very low doses in animals (< 1 mg/kg bw/day). Considering that the PMTDI is 2 µg/kg bw/day, low sensitivity should be expected when we apply this biomarker in human population. The Sa-P and So-P have been proposed to monitor the exposure of fumonisins, being more sensitive than the original sphingoid bases, therefore, could be suitable to use in human epidemiological studies for low-level exposed population (Kim et al. 2006; Voss et al. 2006; Cai et al. 2007).

- 3) To reach a realistic correlation between sphingoid base levels and fumonisin intake in human populations, it is required to use reliable analytical and consumption data (Willet 1998). Improved analytical methods to determine Sa and So are reliable in urine and blood and likewise the methods to determine the FB levels in food. However, the dietary intake assessment methods used in previous studies do not report on their accuracy or reliability.
- 4) Finally, the most commonly used method to assess dietary intake has been the food frequency questionnaire (FFQ); it is the most comfortable method for researchers and volunteers. If we consider that the maximum Sa/So ratio has been closely correlated with a specific consumption day in animals (dose day) (Garren et al. 2001; van der Westhuizen et al. 2001; Dilkin et al. 2010), and reversible after that point, the dietary intake methods should assess rigorously those foods consumed 4-5 days before the urine sampling. Therefore, the dietary record could be a more accurate method to assess the fumonisin intake than de FFQ.

Conclusions

To our knowledge, this is the first study conducted in Spain to assess the sphingoid base levels and ratios in plasma and urine from a maize-food consumer population. We have proved that the volunteers were not exposed to high levels of fumonisins, in all cases below PMTDI of 2 µg/kg bw/day (maximum value of 1.4 µg/kg bw/day). The analytical method to determine Sa and So in urine and plasma was reliable, showing good recovery and reproducibility. The results showed higher Sa and So levels in plasma than in urine, and significant differences were shown when males were compared to females.

Concerning Sa/So ratios from maize-food consumers and non-consumers, significant differences were found in urine and plasma samples but evidences of mechanism of action of fumonisins were not apparent. Through time-course study, we have narrowed down the day in which the maximum alteration of Sa/So ratio should be expected in humans.

In this paper we have reported some useful information to improve the design of studies to validate the ratio Sa/So as a possible biomarker of fumonisin exposure. However, more studies are required to better understand the use of this biomarker with human population, mainly, to improve the accuracy at low levels of exposure.

Acknowledgements

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Table 1. Method performance characteristics for fumonisin B₁ and B₂

Food Matrix	n	FB ₁				FB ₂			
		LOD/LOQ	Spiked level	Recovery*	RSDr	LOD/LOQ	Spiked level	Recovery	RSDr
		µg/kg	µg/kg	%	%	µg/kg	µg/kg	%	%
Sweet Corn	3	5.2/15.6	266	101.5±11.8	11.60	5.2/15.6	133	104.0±7.3	7.04
Corn Snacks	3	5.2/15.6	266	76.5±11.5	15.01	5.2/15.6	133	109.7±12.3	11.30
Beer	5	3.9/11.7	200	93.0±14.0	15.00	3.9/11.7	100	108.0±10.0	10.00

*: Mean ± Standard Deviation

Table 2. Method performance characteristics for sphingosine and sphinganine in plasma

	LOQ/LOD	Spiked level	n	Recovery*	RSDr
	ng/mL	ng/mL		%	%
Sphingosine	0.15/0.048	5	4	98.9±6.4	6.4
	0.15/0.048	20	4	92.2±19.7	21.3
	0.15/0.048	40	4	104.0±12.8	12.3
Sphinganine	0.14/0.047	2.5	4	96.8±9.7	9.3
	0.14/0.047	12	4	98.3±11.2	11.4
	0.14/0.047	24	4	93.1±13.4	14.3

*: Mean ± Standard Deviation

Table 3. Method performance characteristics for sphingosine and sphinganine in urine

	LOD ng/mL	Spiked level ng/mL	n	Recovery* %	RSDr %
Sphingosine	0.04	5	5	122.9±5.5	4.5
	0.04	40	5	126.9±18.2	14.4
Sphinganine	0.02	5	5	107.6±5.6	5.2
	0.02	40	5	104.3±9.3	8.9

*: Mean ± Standard Deviation

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Table 4. Sphinganine (Sa) and sphingosine (So) levels in urine and plasma, and the Sa/So ratio in population from Catalonia (Spain), from the cross-sectional studies.

Group	n	Mean Fumonisin intake µg/kg bw/day	Sa* ng/mL	So* ng/mL	Ratio* Sa/So
<i>Levels in urine</i>					
Low and non consumers	43	0.02±0.02	0.38 (0.95±2.15) ^A	0.83 (2.57±5.02) ^A	0.40 (0.55±0.47) ^A
Consumers	35	0.14±0.83	0.26 (1.29±2.15) ^A	0.57 (2.59±0.85) ^A	0.56 (0.62±0.47) ^B
<i>Levels in plasma</i>					
Non consumers	68	0.00	4.12 (6.5±9.2) ^A	8.51 (14.3±16.5) ^A	0.46 (0.45±0.12) ^A
Consumers	68	0.23±0.11	3.14 (4.1±3.6) ^A	5.89 (7.8±6.8) ^B	0.53 (0.54±0.16) ^B

*Median (Mean ± Standard Deviation).
(^A) Capital letter: different letters mean significant differences between groups, when we compare non consumers with consumers ($P < 0.05$; Mann-Whitney U test)

Table 5. Time-course of median sphinganine and sphingosine levels, and Sa/So ratios (in ng/mL).

	Day -7	Day 0	Day 1	Day 4	Day 6	Day 8
<i>Ratio Sa/So</i>						
Control	0.25 ^{aA}	0.62 ^{aA}		0.52 ^{aA}		0.45 ^{aA}
Low exposed	0.70 ^{aAB}	0.26 ^{bA}	0.27 ^{aAB}	1.10 ^{abB}	0.43 ^{aA}	0.47 ^{aA}
High Exposed	0.51 ^{aA}	0.39 ^{abA}	0.80 ^{aA}	2.43 ^{bB}	0.98 ^{bAB}	0.45 ^{aAB}
<i>Sphingosine</i>						
Control	1.23 ^{aA}	0.53 ^{aA}		0.30 ^{aA}		0.21 ^{aA}
Low exposed	0.68 ^{aA}	0.45 ^{aA}	0.36 ^{aA}	0.19 ^{aA}	0.28 ^{aA}	0.32 ^{aA}
High Exposed	0.33 ^{aA}	0.20 ^{aA}	0.15 ^{aA}	0.08 ^{aA}	0.12 ^{aA}	0.31 ^{aA}
<i>Sphinganine</i>						
Control	0.27 ^{aA}	0.14 ^{aA}		0.12 ^{aA}		0.16 ^{aA}
Low Exposed	0.28 ^{aA}	0.13 ^{aA}	0.12 ^{aA}	0.24 ^{aA}	0.19 ^{aA}	0.15 ^{aA}
High Exposed	0.14 ^{aA}	0.10 ^{aA}	0.19 ^{aA}	0.19 ^{aA}	0.12 ^{aA}	0.16 ^{aA}

Control group (non consumers), Low exposed (< 0.6 µg/kg bw/day), High exposed (> 0.6 µg/kg bw/day).

^(A) Capital letter: in each row, different letters mean significant differences among days ($P < 0.05$, Kruskal-Wallis test)

^(a) Lower case letter: in each column, for each category, different letters mean significant differences between groups ($P < 0.05$, Kruskal-Wallis test)

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Table 6. Absolute variation of sphinganine and sphingosine urinary levels from volunteers under restricted conditions, variation was accounted between day 0 and day 8 (in ng/mL).

Group	Sa			So			Sa/So		
	max	min	median (mean±sd)	max	min	median (mean±sd)	max	min	median (mean±sd)
Control	1.30	0.02	0.17 (0.27±0.35) ^a	2.31	0.01	0.28 (0.56±0.67) ^a	1.76	0.09	0.96 (0.90±0.55) ^a
Low Exposed	0.62	0.11	0.23 (0.28±0.18) ^a	1.77	0.10	0.81 (0.83±0.51) ^a	7.56	0.12	0.98 (2.09±2.65) ^a
High Exposed	0.80	0.10	0.15 (0.26±0.22) ^a	1.69	0.08	0.27 (0.45±0.48) ^a	6.56	0.44	1.06 (2.73±2.32) ^a

Control group (non consumers), Low exposed (< 0.6 µg/kg bw/day), High exposed (> 0.6 µg/kg bw/day).
^(a) Lower case letter: different letters mean significant differences when we compare categories ($P < 0.05$, Kruskal-Wallis test)

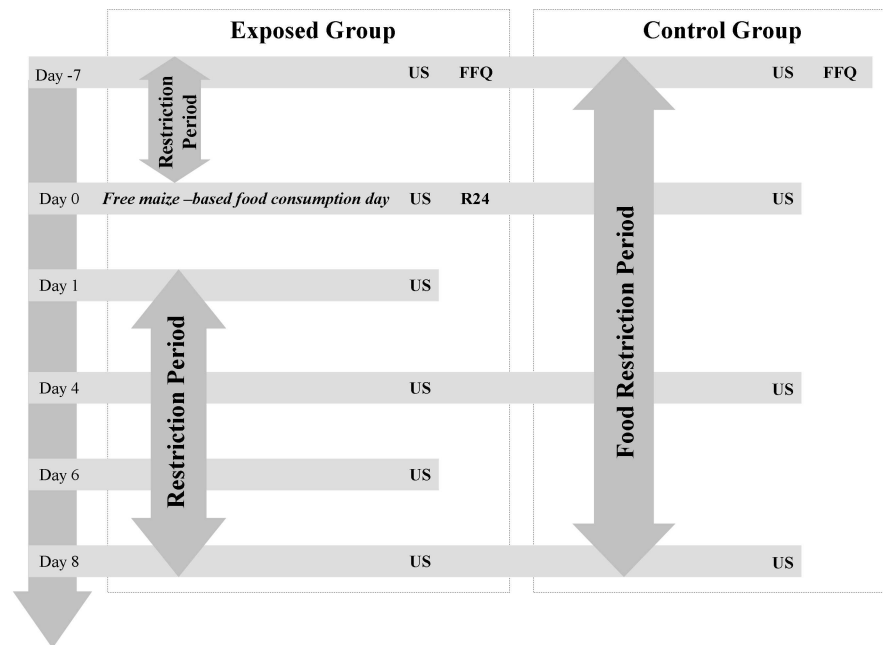


Figure 1.

Title: Diagram of urine sampling design and restriction periods performed in the study to assess urinary Sa and So time-course.

Footnote: US: urine sample, R24: 24 hours record, FFQ: Food Frequency Questionnaire

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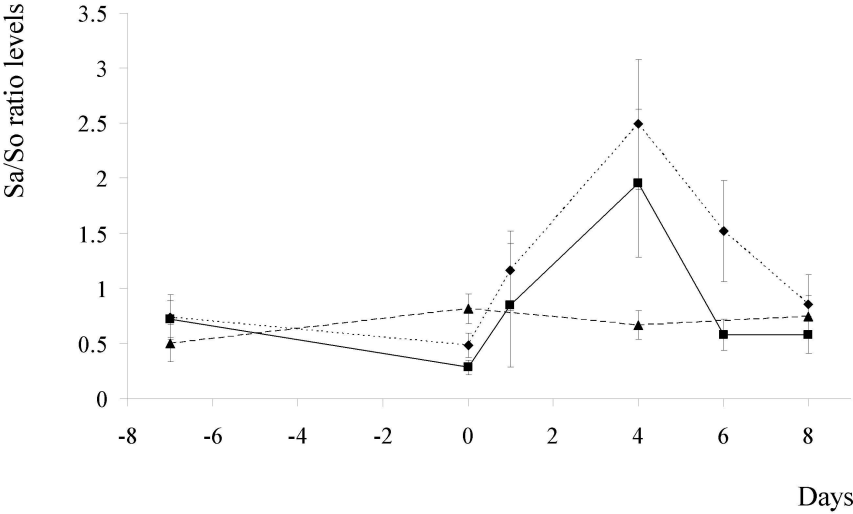


Figure 2
Title: Time-course of mean Sa/So ratio for high exposed (H,), low exposed (L,) and non exposed (C,).

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Figure Captions

Figure 1

Title: Diagram of urine sampling design and restriction periods performed in the study to assess urinary Sa and So time-course.

Footnote: US: urine sample, R24: 24 hours record, FFQ: Food Frequency Questionnaire

Figure 2

Title: Time-course of mean Sa/So ratio for high exposed (H, $\cdots\blacklozenge\cdots$), low exposed (L, $\text{---}\blacksquare\text{---}$) and non exposed (C, $\text{---}\blacktriangle\text{---}$).