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## Comparison of methods and optimization of the analysis of fumonisins B1 and B2 in masa flour, an alkaline cooked corn product

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**Comparison of methods and optimization of the analysis of fumonisins B1 and B2 in masa flour, an alkaline cooked corn product**

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3 **Comparison of methods and optimization of the analysis of fumonisins B<sub>1</sub> and B<sub>2</sub>**  
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6 **in masa flour, an alkaline cooked corn product**  
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37 **Running title:** Fumonisin analysis in corn masa flour  
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**Abstract**

A comparison study of different extraction and clean-up procedures for the liquid chromatographic analysis of fumonisins B<sub>1</sub> (FB<sub>1</sub>) and B<sub>2</sub> (FB<sub>2</sub>) in corn masa flour has been performed. They included extraction (heat or room temperature) with acidic conditions or EDTA containing solvents, and clean-up by immunoaffinity or C18 solid phase extraction columns. Thereafter an analytical method has been optimized using extraction with an acidic mixture of methanol-acetonitrile-citrate/phosphate buffer, clean-up through immunoaffinity column and fumonisins determination by liquid chromatography with automated pre-column derivatization with o-phthalaldehyde reagent. Recovery experiments performed on yellow, white and blue masa flours at spiking levels of 400, 800 and 1200 µg kg<sup>-1</sup> FB<sub>1</sub>, and 100, 200 and 300 µg kg<sup>-1</sup> FB<sub>2</sub> gave overall mean recoveries of 99% (± 6%) for FB<sub>1</sub> and 88% (± 6%) for FB<sub>2</sub>. Good recoveries (higher than 90% for both FB<sub>1</sub> and FB<sub>2</sub>) were also obtained with corn tortilla chips. The limit of quantification of the method (signal to noise ratio of 10) was 25 µg kg<sup>-1</sup> for FB<sub>1</sub> and 17 µg kg<sup>-1</sup> for FB<sub>2</sub>. The method was tested on different commercial corn masa flours as well as on white and yellow corn tortilla chips, showing fumonisins contamination levels (FB<sub>1</sub> + FB<sub>2</sub>) up to 1800 µg kg<sup>-1</sup> (FB<sub>1</sub> + FB<sub>2</sub>) in masa flour and 960 µg kg<sup>-1</sup> in tortilla chips. Over 30% of masa flours originating from Mexico exceeded the EU maximum permitted level.

**Keywords:** fumonisin B<sub>1</sub>, fumonisin B<sub>2</sub>, corn, nixtamalization, masa flour, tortilla chips, high performance liquid chromatography (HPLC).

## Introduction

Fumonisin B<sub>1</sub> (FB<sub>1</sub>) and B<sub>2</sub> (FB<sub>2</sub>) are mycotoxins produced mainly by *Fusarium verticillioides* and *Fusarium proliferatum*, which frequently contaminate maize and maize products worldwide. Fumonisin have been implicated in several animal diseases and suspected to be involved in some human diseases (EFSA 2005). The International Agency for Research on Cancer (IARC) classified FB<sub>1</sub> as a Group 2B carcinogen (possibly carcinogenic to humans) (IARC 2003), and the Joint FAO/WHO Expert Committee on Food Additives and the Scientific Committee for Food of the European Commission (SCF) established a group tolerable daily intake (TDI) of 2 µg/kg body weight for FB<sub>1</sub>, FB<sub>2</sub>, and fumonisin B<sub>3</sub> (FB<sub>3</sub>), alone or in combination (JECFA 2001; SCF 2003).

Nixtamalization is a major way of processing maize in Mexico and Central America based on boiling maize in a lime solution to produce masa for tortillas. After boiling, the grain is separated from the steep liquor, washed to remove residual lime and extraneous pericarp materials, and then stone-ground to produce maize masa dough. The masa is then dried and milled into (instant) masa flour, which can be used for future reconstitution into fresh masa dough at food service establishments. Alternatively, the masa can be moulded, cut or extruded, and baked or fried to make tortillas, taco shells, tortilla chips or corn chips (Bello-Perez et al. 2003; Rosentrater 2006). During nixtamalization many physical, structural and chemical changes are known to occur inside the maize kernels, leading to softened and swollen kernels that can be readily ground via attrition milling during downstream processing.

Studies on combined effect of nixtamalization and cooking on fumonisin levels showed that fumonisin levels in the final product are lower than in the uncooked maize. Maize cooking and steeping under mild alkaline conditions, followed by water rinsing, are critical steps where fumonisin reduction occurs, while masa sheeting, baking and frying to make fried tortilla chips have little additional effect on fumonisin levels (De La Campa et al. 2004; Dombrink-Kurtzman et al. 2000; Sydenham et al. 1995; Voss et al. 2001). Although fumonisin are relatively heat stable and persist through most conditions used in food manufacturing, the high pH in the alkaline processing may give rise to hydrolysis of one or both tricarballic acid groups (TCA) side chains, yielding hydrolyzed (HFBs) or partially hydrolyzed fumonisin

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3 (PHFBs) (Maragos et al. 1997). Both HFBs and PHFBs can occur in nixtamalized maize products, including  
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5 masa and tortilla chips, but at lower levels with respect to the native forms (FBs) as they are mainly retained  
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7 in the cooking/steeping liquid and solid waste (Hartl and Humpf 1999; Hopmans and Murphy 1993; Maragos  
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9 et al. 1997; Park et al. 2004; Scott and Lawrence 1996; Stack 1998; Voss et al. 2001).

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14 The EU has set limits for total fumonisins ( $FB_1+FB_2$ ) at  $1000 \mu\text{g kg}^{-1}$  for maize and maize-based foods for  
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16 direct human consumption and at  $800 \mu\text{g kg}^{-1}$  for maize-based breakfast cereals and snacks (European  
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18 Commission 2007).

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23 Several surveys on the occurrence of fumonisins in alkaline processed foods, including masa, tortillas and  
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25 tortilla chips, have been carried out in North America showing fumonisins levels generally lower than  $1000$   
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27  $\mu\text{g kg}^{-1}$  (Dombrink-Kurtzman and Dvorak 1999; Dombrink-Kurtzman et al. 2000; Hartl and Humpf 1999;  
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29 Hopmans and Murphy 1993; Maragos et al. 1997; Park et al. 2004; Scott and Lawrence 1996; Stack 1998). A  
30  
31 variety of methods have been used for the analysis of fumonisins in these products. They usually involve  
32  
33 extraction of fumonisins with aqueous methanol and/or acetonitrile, sometime with the inclusion of  
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35 ethylenediaminetetraacetic acid (EDTA) or another acid, and clean-up by reversed phase (C18), strong anion  
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37 exchange (SAX) or immunoaffinity (IMA) columns (Cortez-Roca et al. 2002; Dombrink-Kurtzman and  
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39 Dvorak 1999; Dombrink-Kurtzman et al. 2000; Maragos et al. 1997; Park et al. 2004; Scott & Lawrence  
40  
41 1996; Stack 1998; Sydenham et al. 1995; Voss et al. 2001). Detection and quantification of fumonisins are  
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43 usually performed by high-performance liquid chromatography (HPLC) with fluorometric detection (FD)  
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45 and pre-column derivatization with ortho-phthalaldehyde (OPA).  
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51 Only three of the aforementioned methods have been specifically proposed for the analysis of fumonisins in  
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53 masa (Dombrink-Kurtzman and Dvorak 1999; Stack et al. 1998; Voss et al. 2001) and only one of these  
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55 (Dombrink-Kurtzman and Dvorak 1999) provided satisfactory recoveries for both  $FB_1$  and  $FB_2$ . Recently an  
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57 advanced LC-MS/MS method has been proposed for accurate fumonisins quantification in masa, maize and  
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59 derived products (Pietri et al. 2009).  
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3 In the present paper we report the results of a comparison study of different procedures for the extraction and  
4 clean-up from maize masa flour of the two fumonisins (FB<sub>1</sub> and FB<sub>2</sub>) which are regulated in the EU for  
5 maize based products destined to human consumption. Thereafter, an analytical method using LC for  
6 fumonisins quantification has been optimized and validated in-house for blue, white and yellow maize masa  
7 flours. The application of the method to different samples of commercial maize masa flour and tortilla chips  
8 is also reported.  
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## 18 **Materials and methods**

### 19 *Test materials*

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22 Twenty-six samples of different commercial instant masa flours, obtained from yellow maize (4), white  
23 maize (17) or blue maize (5), were collected from retail stores in Italy, Mexico and USA. Sixteen of them  
24 contained also natural fibers and/or vitamins and/or minerals. Thirteen samples of commercial maize tortilla  
25 chips (from white and yellow maize) from retail stores in USA, Canada and Europe were also analyzed.  
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### 33 *Chemicals and materials*

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35 Analytical grade acetonitrile (ACN), methanol (MeOH), *o*-phthalaldehyde (OPA), 2-mercaptoethanol, di-  
36 sodium hydrogen phosphate (Na<sub>2</sub>HPO<sub>4</sub>), sodium phosphate monobasic monohydrate (NaH<sub>2</sub>PO<sub>4</sub> · H<sub>2</sub>O), citric  
37 acid (C<sub>6</sub>H<sub>8</sub>O<sub>7</sub> · H<sub>2</sub>O), phosphate buffered saline (PBS) tablet, sodium tetraborate (Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>), EDTA,  
38 hydrochloric acid and glacial acetic acid were purchased either from Mallinckrodt Baker (Milan, Italy) or  
39 Sigma (St. Louis, MO, USA). Ultrapure water was produced by a Millipore Milli-Q system (Millipore,  
40 Bedford, MA, USA). Certified calibrant solutions of FB<sub>1</sub> (50.9 and 51.0 µg ml<sup>-1</sup>) and FB<sub>2</sub> (50.5 µg ml<sup>-1</sup>) in  
41 ACN-water (50+50, v/v) were purchased from Biopure (Romer Labs Diagnostic GmbH, Tulln, Austria).  
42 FumoniTest™ Wide Bore IMA columns were purchased from Vicam L.P. (Milford, MA, USA). C18  
43 columns containing 500 mg of sorbent were from Waters Corporation (Waters, Milford, MA). OPA reagent  
44 solution was prepared by dissolving 40 mg OPA in 1 ml MeOH and diluting with 5 ml 0.1 M sodium  
45 tetraborate solution. Then, 50 µl 2-mercaptoethanol were added and the solution was mixed for 1 min and  
46 stored in the dark up to one week at room temperature (Visconti *et al.*, 2001).  
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### *Preparation of standards*

A mixed fumonisins stock solution for spiking purposes with a final concentration of  $40.7 \mu\text{g ml}^{-1}$  FB<sub>1</sub> and  $10.1 \mu\text{g ml}^{-1}$  FB<sub>2</sub> was prepared in ACN-water (50+50, v/v) by appropriate dilution of the certified calibrant solutions. A mixed fumonisins standard solution for HPLC was prepared at the concentration of  $4.07 \mu\text{g ml}^{-1}$  FB<sub>1</sub> and  $1.01 \mu\text{g ml}^{-1}$  FB<sub>2</sub> by evaporating under a nitrogen stream at 50°C adequate amounts of the stock solution and dissolving with ACN-water (30+70, v/v). Mixed fumonisins working standard solutions for calibration purposes were prepared in the range of  $0.05$ - $1.27 \mu\text{g ml}^{-1}$  (FB<sub>1</sub>+FB<sub>2</sub>) by diluting adequate amounts of the stock solution with ACN-water (30+70, v/v).

### *Determination of fumonisins in maize masa flour: comparison of methods*

Fumonisins were extracted from maize masa flour using 5 different analytical methods (I-V, Table 1). Method I was based on the AOAC International official method 995.15 (Sydenham et al. 1996) consisting of extraction with MeOH-water and clean-up by SAX columns. Method II was based on the AOAC International official method 2001.04 (Visconti et al. 2001) consisting of two consecutive extractions with a mixture of ACN-MeOH-water and clean-up by IMA column. Method III was based on the procedure described by Dombrink-Kurtzman and Dvorak (1999) with minor modifications; in particular, a 20 g aliquot of masa flour was extracted with 50 ml methanol plus 50 ml of 0.073 M EDTA (aq), then the extract was cleaned-up as described by Dombrink-Kurtzman and Dvorak (1999). Method IV was the procedure described by (De Girolamo et al. 2010) consisting of extraction at 55°C with an acidic mixture of ACN-MeOH- citrate/phosphate buffer followed by clean-up through IMA column. Method V was an optimization of method IV as following. A 20 g aliquot of sample was extracted with 100 ml of ACN-MeOH- citrate/phosphate buffer (25+25+50, v/v/v) by shaking for 1 h at room temperature. After filtration through filter paper (Whatman n. 4), a 10 ml aliquot of filtrate was diluted 5-fold with water, filtered through glass microfibre filter (Whatman GF/A), and 10 ml were purified through IMA column. The IMA column was washed with 10 ml PBS followed by 2 ml water at a flow rate of 1-2 drops/ second until air came through the column and the eluate was discarded. Fumonisins were eluted from IMA columns by passing 2x1 ml MeOH, followed by 2x1 ml pure water. The eluted extracts were evaporated under a nitrogen stream at 50°C,



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3 dissolved in 0.5 ml ACN-water (30+70, v/v) by mixing on a vortex mixer for 1 min and retained at ca. 4°C  
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5 until HPLC analysis. Dilution with ACN-water (30+70, v/v) was performed in case fumonisin concentration  
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7 in the derivatized extract was higher than the maximum concentration of the calibration range.  
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### 10 11 ***Recovery experiments***

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13 Recovery experiments were performed in triplicate on white, yellow and blue maize masa flour and on white  
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15 and yellow maize tortilla chips by spiking fumonisins at levels inclusive of the EU maximum permitted  
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17 levels. In particular spiking levels for masa flour were 400, 800 and 1200  $\mu\text{g kg}^{-1}$  FB<sub>1</sub>, and 100, 200 and 300  
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19  $\mu\text{g kg}^{-1}$  FB<sub>2</sub>, while for tortilla chips they were 320, 640 and 960  $\mu\text{g kg}^{-1}$  FB<sub>1</sub> and 80, 160 and 240  $\mu\text{g kg}^{-1}$   
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21 FB<sub>2</sub>. Test portions (20 g) were spiked with appropriate amounts of spiking solution (200-600  $\mu\text{l}$ ) and left 1 h  
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23 at room temperature to allow solvent evaporation, then analysed according to method V. Recovery values  
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25 were calculated by subtraction of the fumonisin concentration measured in unfortified material (i.e. 40  $\mu\text{g kg}^{-1}$   
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27 <sup>1</sup> FB<sub>1</sub> in blue masa flour; 175  $\mu\text{g kg}^{-1}$  FB<sub>1</sub> and 40  $\mu\text{g kg}^{-1}$  FB<sub>2</sub> in white masa flour; 390  $\mu\text{g kg}^{-1}$  FB<sub>1</sub> and 100  
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29  $\mu\text{g kg}^{-1}$  FB<sub>2</sub> in yellow masa flour; 45  $\mu\text{g kg}^{-1}$  FB<sub>1</sub> in white tortilla chips) from the relevant fumonisin  
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31 concentration measured in fortified material and expressed as percent of the amount added.  
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### 33 ***Chromatographic conditions***

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35 The HPLC apparatus was an Agilent 1100 series (Agilent, Waldbronn, Germany) equipped with a binary  
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37 pump, autosampler, column thermostat set at 30°C and a spectrofluorometric detector with excitation and  
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39 emission wavelengths offset at 335 nm and 440 nm, respectively. The analytical column was a Symmetry  
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41 Shield (150 mm×4.6 mm, 5  $\mu\text{m}$ ) (Waters, Milford, MA) with a 3 mm i.d., 0.45  $\mu\text{m}$  pore size guard filter  
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43 (Rheodyne, Cotati, CA). Quantification of fumonisins was performed by measuring peak areas at FB<sub>1</sub> and  
44  
45 FB<sub>2</sub> retention times and comparing them with the relevant calibration curves. The autosampler was  
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47 programmed to mix 50  $\mu\text{l}$  of sample extracts or standard with 50  $\mu\text{l}$  of OPA reagent, mix for 50 sec, incubate  
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49 for 2 min and then inject all the derivatized mixture. The mobile phase was a mixture of acetonitrile-acetic  
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51 acid (99+1, v/v, solvent A) and water-acetic acid (99+1, v/v, solvent B) eluted at a flow rate of 0.8 ml min<sup>-1</sup>.  
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53 The program of the solvent delivery model was as follows: 43% (solvent B) for 5 min; then up to 54% at 21  
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55 min, to 58% at 25 min and kept constant up to 30 min. With this mobile phase retention times of FB<sub>1</sub> and  
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57 FB<sub>2</sub> were about 19 min and 28 min, respectively.  
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### ***Statistical analysis***

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3 Data were subjected to ANOVA (one-way analysis of variance). Significant differences ( $p < 0.05$ ) between  
4 mean values were identified by the General Linear Model (GLM) procedure with the Duncan's post-hoc  
5 tests. Data were processed using the statistical software package Statistics for Windows (StatSoft, Tulsa, OK,  
6 USA).  
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## 11 **Results and discussion**

### 12 ***Comparison of methods***

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14 Table 1 summarizes the extraction and clean-up procedures considered for the comparison study of the  
15 HPLC determination of FB<sub>1</sub> and FB<sub>2</sub> in maize masa flour. They include the official or validated methods for  
16 fumonisins analysis in maize and maize products and methods that have been used for the analysis of masa  
17 and other nixtamalized food products. In particular, we tested the AOAC official methods 995.15 (Sydenham  
18 et al. 1996) for the determination of fumonisins in maize (Method I) and the AOAC 2001.04 (Visconti et al.  
19 2001) for the determination of fumonisins in both maize and cornflakes (Method II); both methods have also  
20 been adopted by European Committee for Standardization (CEN) as standards EN 13585 and EN 14352,  
21 respectively.  
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37 Method III (Table 1) was a modification of the procedure described by Dombrink-Kurtzman and Dvorak  
38 (1999), including EDTA in the extraction solvent. Several reports indicated that the inclusion of the chelating  
39 agent EDTA in the extraction solvent enhanced the extraction of fumonisins from nixtamalized maize  
40 products (Sydenham et al. 1995; Dombrink-Kurtzman and Dvorak 1999; Dombrink-Kurtzman et al. 2000).  
41 In the present study we used an amount of EDTA that exceeded by far the calcium content generally  
42 occurring in commercial masa flours.  
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53 Method IV (Table 1) was a recently developed method for the determination of FB<sub>1</sub> and FB<sub>2</sub> in processed  
54 maize-based baby foods at levels close to the EU maximum permitted limit (De Girolamo et al. 2010;  
55 Solfrizzo et al. 2010). This method has been collaboratively validated and proposed to CEN to be adopted as  
56 Technical Specification (FprCEN/TS 16187). The method proved to be effective for the quantitative  
57 extraction of fumonisins from different maize baby food products (De Girolamo et al. 2010). Baby food is a  
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3 complex food matrix that may contain a variety of ingredients such as sugar, milk and/or minerals, salt,  
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5 vitamins, tapioca, or other cereals, such as rice, wheat, rye, barley, oat. Likewise, the composition of maize  
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7 masa flour can vary considerably according to the maize variety due to the different proteins and  
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9 carbohydrates levels, pigmented components (i.e. white, yellow or blue maize), and fortifying compounds  
10  
11 (such as vitamins, protein, minerals, or fibers). Therefore the method described by De Girolamo et al. (2010)  
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13 was expected to be suitable for the analysis of fumonisins in a complex food matrix such as instant maize  
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15 masa flour.  
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20 Finally Method V (Table 1) was an improvement of method IV providing satisfactory results without the  
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22 need of heated extraction.  
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26  
27 Table 2 provides a comparison of the mean fumonisins ( $FB_1+FB_2$ ) content in different instant maize masa  
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29 flours analyzed by the above mentioned methods I-V. The amount of fumonisins detected in the 11 tested  
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31 samples varied according to the method used ranging from not detected to about  $2000 \mu\text{g kg}^{-1}$ . By comparing  
32  
33 the two AOAC Official Methods (methods I and II) it resulted that method II gave fumonisin results  
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35 significantly higher (about 2.5 fold,  $p<0.05$ ) than those obtained with method I. Furthermore, 3 out of 10  
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37 samples contained undetectable fumonisins when analysed with method I indicating the absolute  
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39 ineffectiveness of this method (validated for fumonisins in maize) for the analysis of instant maize masa.  
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41 Poor recoveries had been previously reported when the same procedure was used for the analysis of tortillas  
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43 (Stack et al. 1998). It is likely that lime in the masa flour negatively affects either the extraction efficiency of  
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45 MeOH-water mixture or SAX column clean up. The extraction solvent of method II (ACN-MeOH-water) in  
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47 combination with IMA (Park et al. 2004; Scott et al. 1999), SAX (Scott et. al. 1999) or C18 (Hartl and  
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49 Humpf 1999; Scott and Lawrence 1996) column clean up was previously used for the analysis of tortilla  
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51 chips or dried tortillas. When method III was used, a big increase ( $p<0.05$ ) of fumonisin concentrations was  
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53 observed in several masa samples (Table 2), confirming the enhancement effect of EDTA inclusion towards  
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55 fumonisin extraction (Dombrink-Kurtzman and Dvorak 1999; Dombrink-Kurtzman et al. 2000; Sydenham et  
56  
57 al. 1995). However, clean-up by C18 column in method III resulted in unsatisfactory chromatographic  
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59 profiles in some masa samples due to interfering peaks at the retention time of  $FB_1$ , as shown in Figure 1 for  
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3 both blue and white masa flours. These problems of interfering peaks were not observed with method IV,  
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5 using IMA clean up, that provided comparable fumonisin results and better chromatographic profiles with  
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7 respect to method III (Table 2, Figure 1).  
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11 Based on these findings, it was concluded that method IV, performing extraction with ACN-MeOH-  
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13 citrate/phosphate buffer at 55°C, offered a good compromise between fumonisin extraction efficiency and  
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15 chromatographic resolution, but was still less practical due to the need of heating during the extraction. This  
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17 drawback was overcome by the optimization obtained with method V after evaluating the effect of  
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19 temperature on fumonisin extraction. Method V, involving extraction at room temperature, gave similar  
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21 results to method IV (Table 2) indicating that the heating step, that was indispensable for the analysis of  
22  
23 maize baby foods, was not necessary for maize masa flour. These findings led us to select method V as  
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25 method of choice for in-house validation and its use for a survey of fumonisin occurrence in masa flour and  
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27 maize tortilla chips from different countries.  
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34 Recently a LC-MS/MS method using phosphate buffer extraction has been proposed for the analysis of masa  
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36 flours with good recoveries for both FB<sub>1</sub> and FB<sub>2</sub> (Pietri et al. 2009). Results obtained by HPLC-FD analysis  
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38 of FB<sub>1</sub> and FB<sub>2</sub> on 8 masa flour samples from the present study using the extraction procedure described by  
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40 Pietri et al. (2009) were not significantly different ( $p > 0.05$ ) from those obtained with method V.  
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#### 44 ***In-house validation of method V***

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46 To evaluate the effect of spiking time on fumonisin recovery from maize masa flour, six sub-samples of a  
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48 masa flour sample were individually spiked with fumonisins (240 µg kg<sup>-1</sup> FB<sub>1</sub>, 60 µg kg<sup>-1</sup> FB<sub>2</sub>). Three of  
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50 them were left 18 h at room temperature to evaporate spiking solution, whereas the other three were left in  
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52 the same conditions for only 1 h. Then, all samples were analysed with method V. No significant differences  
53  
54 were observed in recovery rates (100% FB<sub>1</sub>, 91% FB<sub>2</sub> vs 105% FB<sub>1</sub>, 91% FB<sub>2</sub>, respectively) using different  
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56 spiking times, indicating that no interaction between fumonisins and masa flour occurred during this time.  
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60 On the contrary, a decrease in fumonisins levels in spiked maize starch, white rice flour and maize baby  
foods over a drying time up to 24 h was previously reported probably due to a possible interaction of

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fumonisin with starch or other matrix components (De Girolamo et al. 2010; Kim et al. 2002; Scott et al. 1999).

The performance of the proposed method were evaluated by recovery experiments performed with blue, white and yellow masa flours spiked with fumonisins at levels equivalent to 0.5, 1.0 and 1.5 times the EU maximum permitted level for total fumonisins in maize intended for direct human consumption (i.e. 1000  $\mu\text{g kg}^{-1}$ ); results are reported in Table 3. Mean recoveries ranged from 90% to 109% for  $\text{FB}_1$  and from 79% to 98% for  $\text{FB}_2$ . The relative standard deviation (RSD) of spiked samples ranged from 1% to 7% for both  $\text{FB}_1$  and  $\text{FB}_2$ . The limit of quantification of the method (signal to noise ratio of 10) was 25  $\mu\text{g kg}^{-1}$  for  $\text{FB}_1$  and 17  $\mu\text{g kg}^{-1}$  for  $\text{FB}_2$ . Recoveries and repeatability data of the present method fulfil the criteria established by the EC 401/2006 for the acceptability of the analytical method (European Commission 2006).

Furthermore, the ANOVA test showed a variance homogeneity of recoveries for both  $\text{FB}_1$  and  $\text{FB}_2$  at the different spiking level. The calculated overall recovery was 99% ( $\pm 6\%$ ) for  $\text{FB}_1$  and 88% ( $\pm 6\%$ ) for  $\text{FB}_2$  (Table 3).

The applicability of method V to the analysis of fumonisins in another nixtamalized product, tortilla chips, was investigated by performing recovery experiments on white and yellow maize tortilla chips. Overall recoveries were 108% ( $\pm 10\%$ ) for  $\text{FB}_1$  and 94% ( $\pm 11\%$ ) for  $\text{FB}_2$  with RSD values less than 12%, and met the EU criteria established by the EC 401/2006 (European Commission 2006). Also for tortilla chips the limit of quantification of the method was 25  $\mu\text{g kg}^{-1}$  for  $\text{FB}_1$  and 17  $\mu\text{g kg}^{-1}$  for  $\text{FB}_2$ .

#### ***Occurrence of fumonisins in masa flour and maize tortilla chips***

The occurrence of  $\text{FB}_1$  and  $\text{FB}_2$  in 25 masa flour samples and in 13 samples of maize tortilla chips are reported in Table 4. Fumonisin levels ( $\text{FB}_1 + \text{FB}_2$ ) in masa flours ranged from not detected (only 1 sample of blue masa) to 1800  $\mu\text{g kg}^{-1}$ , with 36% of samples exceeding the EU maximum permitted level. All samples exceeding the EU limit were originated from Mexico. Mean fumonisin level of masa flour samples from Mexico was 885  $\mu\text{g kg}^{-1}$  as compared to 389  $\mu\text{g kg}^{-1}$  for samples from the USA. Differences in fumonisin levels may be related to either fumonisin levels in the unprocessed maize and processing

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3 conditions (i.e. calcium concentration, cooking temperature and time, steeping time, pericarp removal) used  
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5 in different plants. Based on an average consumption of 35 g/day of maize in the USA and 337 g/day in  
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7 Mexico (FAO 2007), the intake of a 65-kg consumer of maize could be estimated at 4.6  $\mu\text{g kg}^{-1}$  body  
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9 weight/day in Mexico and 0.21  $\mu\text{g kg}^{-1}$  body weight/day in the USA. Based on this limited set of data, the  
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11 exposure to fumonisins in Mexico could exceed the PMTDI (2  $\mu\text{g kg}^{-1}$  body weight/day) by a factor of 2.3,  
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13 thus indicating the high risk of exposure to fumonisins associated to the consumption of masa flour in this  
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15 country. Furthermore, the high number of samples exceeding the EU limit may represent a relevant problem  
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17 also from the economical and trading point of view.  
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23 A difference between maize phenotypes used for masa production was observed, with fumonisin levels in  
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25 blue masa flours (70  $\mu\text{g kg}^{-1}$ ) being lower ( $p < 0.05$ ) than in yellow (702  $\mu\text{g kg}^{-1}$ ) and white (986  $\mu\text{g kg}^{-1}$ )  
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27 masa flours. Differences in protein, lipid and starch contents, as well as in polyphenolic and antioxidant  
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29 contents among blue and white maize genotypes (Del Pozo-Insfran et al. 2006; Hernandez-Urbe et al. 2007)  
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31 could have influenced the resistance of maize to fungal infection and/or mycotoxin production.  
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34 Fumonisin levels in maize tortilla chips samples were generally low with only one sample exceeding 500  $\mu\text{g}$   
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36  $\text{kg}^{-1}$ , supporting previous reported data (Dombrink-Kurtzman and Dvorak 1999; Hartl and Humpf 1998;  
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38 Maragos et al. 1997; Hopmans and Murphy 1993; Park et al. 2004; Stack 1998), and indicating a very low  
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40 risk of exposure to fumonisins through the consumption of maize tortilla chips.  
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## 44 Conclusions

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46 A comparison study of different extraction and clean up procedures for the analysis of FB<sub>1</sub> and FB<sub>2</sub> in  
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48 different commercial masa flours has been performed. Thereafter, a precise and accurate method for the  
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50 determination of FB<sub>1</sub> and FB<sub>2</sub> in different commercial instant masa flours has been optimized and in-house  
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52 validated on blue, white and yellow masa flours as well as in white and yellow tortilla chips. Recovery and  
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54 repeatability data fulfil the EU criteria for the acceptability of an analytical method for fumonisins  
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56 determination. The method can be used by private and public laboratories for an adequate quality control in  
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58 commercial trading and to ensure a good level of consumer safety particularly in countries with high risk of  
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60 exposure to fumonisins.

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The high fumonisin levels found in masa flours from Mexico indicated a high risk of exposure to fumonisins associated to the consumption of masa flour in this country.

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**Figure caption**

Figure 1. HPLC chromatograms of fumonisin B<sub>1</sub> (FB<sub>1</sub>) and B<sub>2</sub> (FB<sub>2</sub>) standard solution (1 ng FB<sub>1</sub> injected, 0.25 ng FB<sub>2</sub> injected) (trace A); blue masa flour (MF) sample naturally contaminated with fumonisins and analyzed with method V (MF 14, trace B); white (MF 1, trace C) and blue (MF 5, trace D) masa flour samples naturally contaminated with fumonisins and analyzed with method III.

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Table 1. Summary of the analytical methods (I-V) parameters used to analyze different masa flours.

Method	Extraction solvent	Extraction time (min)	Column clean-up <sup>a</sup>	LOQ <sup>b</sup>	Reference
I	MeOH-H <sub>2</sub> O	5	SAX	FB <sub>1</sub> 45 µg kg <sup>-1</sup> FB <sub>2</sub> 15 µg kg <sup>-1</sup>	AOAC 995.15 (Sydenham <i>et al.</i> 1996)
II	ACN-MeOH-water <i>two consecutive extractions</i>	2 x 20 (at room temperature)	IMA	FB <sub>1</sub> 10 µg kg <sup>-1</sup> FB <sub>2</sub> 5 µg kg <sup>-1</sup>	AOAC 2001.01 (Visconti <i>et al.</i> 2001)
III	MeOH-aq. EDTA (0.073M)	60 (at room temperature)	C18	FB <sub>1</sub> 45 µg kg <sup>-1</sup> FB <sub>2</sub> 40 µg kg <sup>-1</sup>	Dombrink-Kurtzman and Dvorak (1999) ( <i>modified</i> )
IV	MeOH-ACN-citrate buffer solution	60 (at 55°C) 30 (at room temperature)	IMA	FB <sub>1</sub> 10 µg kg <sup>-1</sup> FB <sub>2</sub> 5 µg kg <sup>-1</sup>	FprCEN/TS 16187 (De Girolamo <i>et al.</i> 2010)
V	MeOH-ACN-citrate buffer solution	60 (at room temperature)	IMA	FB <sub>1</sub> 15 µg kg <sup>-1</sup> FB <sub>2</sub> 10 µg kg <sup>-1</sup>	Optimized method ( <i>present study</i> )

<sup>a</sup>SAX, strong anion exchange; IMA, immunoaffinity. <sup>b</sup>LOQ, limit of quantification (signal to noise ratio 6) obtained in the present study.

Table 2. Comparison of fumonisins (FB<sub>1</sub> + FB<sub>2</sub>) levels in commercial instant masa flour (MF) analyzed by different analytical methods (I-V).

Samples	Method I (AOAC 995.15)	Method II (AOAC 2001.04)	Method III (Dombrink-Kurtzman and Dvorak 1999)	Method IV (CEN/TS 16187)	Method V (Present)
MF 1	520 ± 30 a	1024 ± 28 b	1194 ± 199 b	1177 ± 65 b	1210 ± 53 b
MF 2	891 ± 76 a	1589 ± 79 b	1625 ± 124 b	1661 ± 111 b	1530 ± 24 b
MF 3	961 ± 29 a	1444 ± 172 b	1762 ± 42 c	1920 ± 136 c	1741 ± 10 c
MF 5	n.d. * a	(92±105) ** ab	(56 ± 18) ** b	35 ± 3 a	70 ± 6 b
MF 14	n.d. a	32 ± 4 b	n.d. a	20 ± 10 ab	40 ± 20 b
MF 17	444 ± 47 a	849 ± 38 b	1294 ± 147 c	1203 ± 88 c	1163 ± 111 c
MF 18	n.d. a	111 ± 43 b	626 ± 15 c	462 ± 12 d	468 ± 22 d
MF 21	84 ± 21 a	328 ± 12 b	400 ± 35 c	397 ± 47 c	449 ± 6 c
MF 22	415 ± 35 a	938 ± 100 c	950 ± 14 c	690 ± 49 b	756 ± 64 b
MF 23	122 ± 24 a	247 ± 20 b	284 ± 1 b	283 ± 15 b	252 ± 27 b
MF 26	42 ± 4 a	172 ± 15 b	1002 ± 75 c	905 ± 36 c	--***

Note: Different letters in the row are significantly different ( $p < 0.05$ ). Data refers to the mean fumonisin levels of three replicates ± 1 standard deviation.

\* n.d., not detected, less than the detection limit. \*\* measured together with an interfering peak. \*\*\* not performed.

Table 3. Mean recoveries of fumonisin B<sub>1</sub> (FB<sub>1</sub>) and B<sub>2</sub> (FB<sub>2</sub>) obtained with method V from samples of different instant masa flours spiked with fumonisins<sup>a</sup>.

Spiking FB <sub>1</sub> level ( $\mu\text{g kg}^{-1}$ )	FB <sub>1</sub> recovery (%) <sup>b</sup>		
	Blue masa flour	White masa flour	Yellow masa flour
400	105 $\pm$ 2	96 $\pm$ 1	109 $\pm$ 7
800	91 $\pm$ 3	96 $\pm$ 3	90 $\pm$ 6
1200	98 $\pm$ 6	102 $\pm$ 4	100 $\pm$ 5
Overall mean $\pm$ SD	99 $\pm$ 6		
Spiking FB <sub>2</sub> level ( $\mu\text{g kg}^{-1}$ )	FB <sub>2</sub> recovery (%) <sup>b</sup>		
	Blue masa flour	White masa flour	Yellow masa flour
100	92 $\pm$ 4	88 $\pm$ 2	98 $\pm$ 6
200	83 $\pm$ 3	81 $\pm$ 2	79 $\pm$ 4
300	88 $\pm$ 6	94 $\pm$ 5	88 $\pm$ 1
Overall mean $\pm$ SD	88 $\pm$ 6		

<sup>a</sup>Spiking levels were equivalent to 0.5, 1.0 and 1.5 times the EU maximum permitted level for corn intended for direct human consumption (i.e. 1000  $\mu\text{g kg}^{-1}$ ). <sup>b</sup>Data refers to the mean of three replicates  $\pm$  1 standard deviation (SD).

Table 4. Fumonisin B<sub>1</sub> (FB<sub>1</sub>) and B<sub>2</sub> (FB<sub>2</sub>) content in samples of commercial instant masa flour (MF) and corn tortilla chips (TC) from different countries<sup>a</sup>.

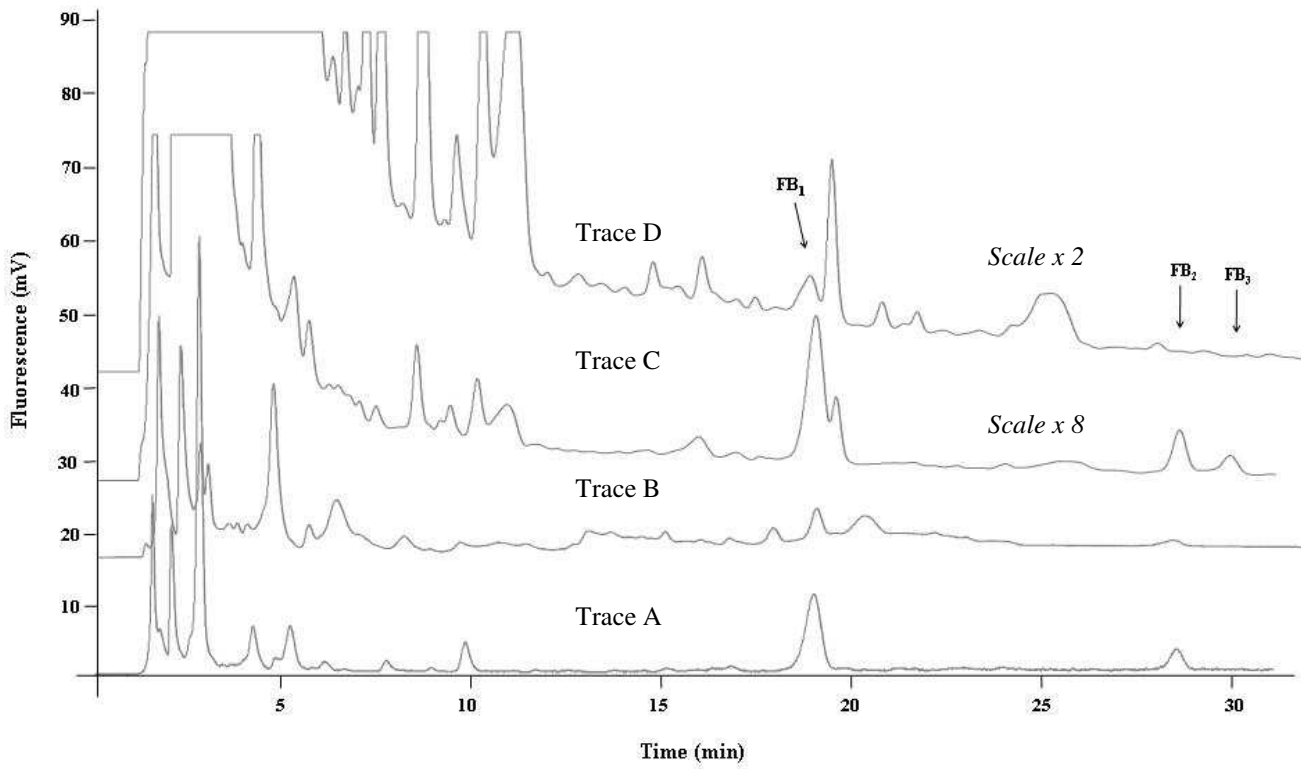
Samples	Country	Corn phenotype	Other ingredients	FB <sub>1</sub> (µg kg <sup>-1</sup> )	FB <sub>2</sub> (µg kg <sup>-1</sup> )	FB <sub>1</sub> +FB <sub>2</sub> (µg kg <sup>-1</sup> )
MF 1	Mexico	White	Fibers	973	323	1295
MF 2	Mexico	White	Vitamins and minerals	1252	349	1600
MF 3	Mexico	White	Vitamins and minerals	1366	434	1800
MF 4	Mexico	Blue	Vitamins and minerals	n.d. <sup>b</sup>	n.d.	-
MF 5	Mexico	Blue	Vitamins and minerals	55	20	75
MF 6	Mexico	Blue	--	76	27	103
MF 7	Mexico	White	Fibers	742	277	1019
MF 8	Mexico	White	Vitamins and minerals	1295	453	1748
MF 9	Mexico	White	Vitamins and minerals	1219	406	1624
MF 10	Mexico	White	Vitamins and minerals	1075	397	1472
MF 11	Mexico	White	Vitamins and minerals	1088	416	1504
MF 12	Mexico	Yellow	Vitamins and minerals	697	267	964
MF 13	Mexico	Blue	--	95	29	124
MF 14	Mexico	Blue	Vitamins and minerals	39	14	48
MF 15	Mexico	Yellow	Vitamins and minerals	373	124	498
MF 16	Mexico	Yellow	Vitamins and minerals	369	121	490
MF 17	Mexico	Yellow	Vitamins and minerals	785	297	1081
MF 18	Mexico	Yellow	Vitamins and minerals	375	102	477
MF 19	USA	White	--	243	86	330
MF 20	USA	White	--	151	41	191
MF 21	USA	White	--	388	108	496
MF 22	USA	White	--	610	152	762
MF 23	USA	White	--	184	50	234
MF 24	USA	White	--	171	52	223
MF 25	USA	White	--	368	119	487
TC 1	USA	White	--	32	n.d.	32
TC 2	USA	White	--	69	46	115
TC 3	USA	Yellow	Lime juice powder	48	26	74
TC 4	USA	Yellow	--	73	33	106
TC 5	Canada	White	--	124	54	178
TC 6	Canada	Yellow	--	305	147	451
TC 7	Europe	White	Cheese	n.d.	n.d.	-
TC 8	Europe	White	--	n.d.	n.d.	-
TC 9	Europe	White	Chilli flavour, onion, garlic and tomato powder	n.d.	n.d.	-
TC 10	Europe	White	--	n.d.	n.d.	-
TC 11	Europe	White	--	769	191	960
TC 12	Europe	Yellow	Cheese	n.d.	n.d.	-
TC 13	Europe	Yellow	--	n.d.	n.d.	--

<sup>a</sup>Values are calculated considering the overall recovery factor. <sup>b</sup>Not detected, less than the detection limit.

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Figure 1.



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