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Fumonisins in maize and gluten meal analysed in Argentinean wet milling industrial plants by ELISA compared with HPLC-FLD method

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ABSTRACT

Fumonisins are frequent contaminants of maize. Wet milling industrial plants in Argentina control not only maize, but the fractions of the process. The performance of two ELISA test kits (G and K) from different brands used in six plants to determine fumonisins in maize and gluten meal was evaluated by comparison with HPLC results. ELISA determinations in maize (n = 43) and gluten meal (n = 45) were carried out in the plants and HPLC analysis was done in our laboratory. No significant differences ($P > 0.05$) were found in most cases when fumonisins concentrations were within the ELISA quantification range. Thus, ELISA tests performed according to validated protocols are useful tools for screening purposes, but levels nearest the settled limits for rejection of lots should be confirmed by HPLC because of the high relative standard deviation of ELISA analyses. The contamination pattern of fumonisins in gluten meal was different from that of maize, with higher levels of FB2 than those of FB1.

1. Introduction

Fumonisins (FBs) are mycotoxins produced by species belonging to *Fusarium* genus, mostly by *Fusarium verticillioides*, followed by *F. proliferatum*, and *F. subglutinans* (Marasas, Miller, Riley, & Visconti, 2001; Rheeder, Marasas, & Vismer, 2002). FBs are natural contaminants of cereal grains and are frequently found in maize and products derived from maize (Jackson & Jablonski, 2004). *F. verticillioides* grows as an endophyte in maize, and infection in this crop can be asymptomatic or cause symptoms such as ear or kernel rot (Miller, 2001). Argentine maize is frequently contaminated by fumonisin (Garrido, Hernández Pezzani, & Pacin, 2012).

FBs chemical structures are similar to those of the sphingolipids, sphinganine and sphingosine, for that reason, FBs and particularly FB1 inhibit the enzyme ceramide synthase and interfere with the metabolism of sphingolipids resulting in cellular alterations that may lead to toxicity and carcinogenicity (Merrill, Sullards, Wang, Voss, & Riley, 2001; Riley et al., 2001). FBs cause leucoencephalomalacia and hepatosis in horses, pulmonary oedema in swine, nephrosis and hepatosis in sheep, and have carcinogenic and hepatotoxic effects in rats (Marasas et al., 2004). In humans, FBs are associated with defects of the neural tube (Gelineau-van Waes et al., 2005; Gelineau-van Waes, Voss, Stevens, Speer, & Riley, 2009; JECFA., 2011).

FB1 was classified as possibly carcinogenic to humans (Group 2B) by the International Agency for Research on Cancer (IARC., 2002). The Joint FAO/WHO Expert Committee on Food Additives established a provisional maximum tolerable daily intake of 2 μg/kg body weight/day for the total of FB1, FB2, and FB3, alone or in combination (JECFA., 2011).

The European Union established maximum levels of total FBs (sum of FB1 and FB2) in different products for human consumption. Thus, for unprocessed maize (except maize destined for wet milling), maximum admissible level is 4000 μg/kg. For fractions of milling not used for direct human consumption, 1400 μg/kg if particle size is bigger than 500 micron, and 2000 μg/kg if particle size is smaller than 500 micron. For maize and maize-based foods intended for direct human consumption, maize-based breakfast

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cereals and snacks, and maize-based baby foods, limits are 1000, 800, and 200 μg/kg, respectively (EC., 2006a, 2007).

The United States Food and Drug Administration (FDA) established a "FDA Mycotoxin Regulatory Guidance" regarding levels of FBs in corn and corn products intended for food and feed (NGFA., 2011). Total FBs in this case are considered as the sum of FB1, FB2 and FB3. Guidance levels are 2000 μg/kg for degermed dry milled corn products (e.g., flaking grits, corn grits, corn meal, corn flour with fat content of <2.25%, dry weight basis), 3000 μg/kg for cleaned corn intended for popcorn, 4000 μg/kg for whole or partially degermed dry milled corn products (e.g. flaking grits, corn grits, corn meal, corn flour with fat content of ≥2.25% dry weight basis), dry milled corn bran, and clean corn intended for masa production.

With reference to animal feed, the European Union recommended acceptable levels of total FBs in feed materials and complementary and complete feeding stuffs for different animals from 5 to 50 mg/kg depending on the animal (EC., 2006b). FDA guidance levels for total FBs in animal feed vary according to the species and the percentage of the contaminated material in the final feed product, from 5 to 100 ppm (NGFA., 2011). In Argentina there is no current legislation regarding FBs levels in food or feed.

Wet milling of maize involves the separation of the different parts of the maize kernel (mainly pericarp, germ, and endosperm) to obtain several products, such as starch, sweeteners, ethanol, maize oil, gluten feed, and gluten meal, products intended at either human or animal feed (Johnson & May, 2003; Saunders, Meredith, & Voss, 2001). Processing can either remove toxins from the maize or separate the components of the maize kernel into more-contaminated and less-contaminated fractions. Currently, wet milling industry uses ELISA (enzyme-linked immunosorbent assay) test kits for the screening of mycotoxins in maize and their products and by-products. Advantages of these kits are their easy-of-use, rapid results, minimal sample preparation, and affordability (Bowers, Hellmich, & Munkvold, 2014) in comparison with the analysis by the reference methods by high performance liquid chromatography (HPLC).

The aim of this study was to assess the performance of ELISA test kits used for the determination of total FBs in maize and gluten meal from the wet milling process of six industrial plants in Argentina, in samples with high and low levels of FBs, by comparison with HPLC analysis.

2. Materials and methods

2.1. Samples and sample preparation

Six industrial wet-milling plants provided samples of maize and gluten meal derived from their processes. Plants are located in different geographical points of the country: North-West, Centre, South-East, and South-West. Personnel of the plants were in charge of sampling and carried it out according to the industries' current sampling procedures for routine analysis of FBs.

The number of samples provided by each wet-milling plant was (maize; gluten meal): Plant A (3; 3), Plant B (12; 12), Plant C (5; 6), Plant D (11; 6), Plant E (6; 6), Plant F (6; 12). Total maize samples were 43, and total gluten meal samples were 45.

Maize samples were ground in each wet milling plant. Gluten meal samples were collected in the mills once they were processed. Ground maize and gluten meal samples were able to pass through a 20 mesh screen. As soon as ELISA analyses were performed in each plant, the rest of the samples were sent to the laboratory for HPLC analysis.

2.2. Analysis of fumonisins by competitive direct enzyme-linked immunosorbent assay (ELISA)

Determinations of FBs by ELISA in maize and gluten meal were performed in the industries by their operators, as done by routine. Test kits used by the industries were from two brands, which will be referred to as test kit G and test kit K. Three determinations per sample were done for the purposes of this study (industries normally perform one determination per sample). Research staff was present in at least one set of analyses to observe the procedure in the industry laboratories.

For both kits, extract for ELISA analysis is obtained by blending the sample with methanol:water (70:30) followed by filtration, pH adjustment, and dilution. In the ELISA test, dilution wells and antibody wells are used. Diluted extract is mixed with enzyme-labelled FB1, competitive binding with antibodies takes place during incubation, unbound conjugate is washed, afterwards substrate is added, and reaction is ended after incubation. Absorbance values (optical densities) are determined by reading the wells in a microwell spectrophotometer. For quantification, different concentrations of FB1 standard are run together with samples. All reagents needed for the ELISA are included in the test kits. Test results are calculated by using logit-log data transformation of absorbance values and linear regression. Minimum values of R^2 (0.985, test kit G) or R (0.980, test kit K) are needed for quantification. Most spectrophotometers are integrated with software and give the final result as concentration of total FBs.

Performance characteristics of the commercial test kits used are provider by the suppliers. Both suppliers determined as limit of detection (LOD) by the mean average of 10 fumonisin free samples plus 2 standard deviations; and limit of quantitation (LOQ) as the lowest concentration point on the calibration curve that this test can reliably detect fumonisin. Values of the *test kit G* LOD: 200 μg/kg, LOQ: 250 μg/kg, range of quantification: 250–5000 μg/kg; *test kit K*: LOD: 200 μg/kg, LOQ: 500 μg/kg, range of quantification: 500–6000 μg/kg. In case the resulting concentration of a sample is out of the quantification range of the test kit, both brands suggest a dilution of the extract, so that the concentration of the dilution is within the quantification range. Then, concentration of the sample can be calculated by considering the dilution factor.

2.3. Analysis of fumonisins by HPLC-FLD method

2.3.1. Chemicals, solutions, and reagents

FB1 (50 μg/mL), FB2 (30 μg/mL), and FB3 (15 μg/mL) standards in 50:50 acetonitrile:water were obtained from Trilogy Analytical Laboratory (Washington, MO, USA). Methanol and acetonitrile were HPLC grade, purchased from Sintorgan S.A. (Buenos Aires, Argentina). Acetic acid and hydrochloric acid (p.a. grade) were obtained from JT Baker Inc. (Phillisburg, NJ, USA), and orthophosphoric acid (p.a. grade) from Merck Inc. (Darmstadt, Germany). HPLC quality distilled water was purchased from Torbidoni y Cia. S.R.L. (Buenos Aires, Argentina). OPA (o-phthaldialdehyde) p.a. grade were obtained from Merck Inc. (PA, USA); 2-mercaptoetanol and $Na_2B_4O_7 \cdot 10H_2O$ (p.a. grade) was bought from Merck Inc. (Buenos Aires, Argentina). Salts used in the preparation of mobile phase and PBS were from Merck Inc. (Darmstadt, Germany) except NaN_3, which was obtained from JT Baker Inc. (Phillisburg, NJ, USA).

PBS (phosphate buffer solution) was prepared with 0.26 g NaH_2PO_4; 1.14 g Na_2HPO_4; 7.02 g NaCl; 0.20 g KCl and 0.50 g NaN_3 dissolved in 1 L H_2O, and pH was adjusted to 7.4. OPA derivatising reagent was prepared by dissolving 80 mg OPA in 1 mL methanol, mixing with 5 mL 0.1 M $Na_2B_4O_7 \cdot 10H_2O$, adding 50 μL 2-mercaptoethanol and vortexing. Approximately 4 L of mobile phase was prepared, mixing 1 L 0.1 M NaH_2PO_4, 3188 mL methanol, and

23 mL H_3PO_4. pH of mobile phase was adjusted to 3.33 with HCl 0.1 M.

2.3.2. Extraction and clean-up

Analyses of maize and gluten meal were done according to AOAC Official Methods 995.15 and 2001.04, respectively (AOAC International, 2005), with some modifications.

Maize and gluten meal samples (25 g) were extracted with 100 mL methanol:water (75:25, v/v). Mixture was blended for 3 min at maximum speed (Osterizer Cycle Blend 10 Pulse Matic, Sunbeam Products Inc., Boca Raton, FL, USA) and filtered afterwards (Whatman N° 4 filter).

Clean-up of maize samples: PuriTox® TC-F120 Fumonisin solid phase extraction (SPE) strong anion exchange columns for clean-up of maize samples were obtained from Trilogy Analytical Laboratory (Washington, MO, USA).pH of filtered extract was adjusted to 5.8–6.0 with HCl 0.1 M. SPE columns were conditioned by passing 5 mL methanol followed by 5 mL methanol:water (75:25, v/v). Extract (20 mL) was passed through the column at a flow rate of 1 drop per second. Column was washed with 5 mL methanol:water (75:25, v/v) followed by 5 mL methanol. FBs were eluted with 10 mL methanol:acetic acid (99:1, v/v). Extract was evaporated at 60 °C under vacuum.

Clean-up of gluten meal samples: Fumoniprep®immunoaffinity chromatography columns (IAC) for clean-up of gluten meal samples were obtained from R-BiopharmAG (Darmstadt, Germany). Filtered extract (5 mL) was mixed with 20 mL PBS solution. pH of diluted extract was adjusted to 7.0 with saturated NaOH solution, and 10 mL of this dilution were passed through the IAC at a flow rate of 1 drop per second. IAC was washed with 10 mL PBS, and FBs were eluted with 2 mL methanol. Extract was evaporated at 60 °C under vacuum.

2.3.3. HPLC analysis

The liquid chromatography equipment was an Agilent 1100 Series system equipped with a degasser (G1322A), a quaternary pump (G1311A), a temperature controller (G1316A), an autosampler (G1313A), and a fluorescence detector (G1321A). Phenomenex® Prodigy ODS3 column (5 μm, 250 × 4.6 mm) equipped with a Phenomenex® SecurityGuard guard column C18 (5 μm, 3 × 4 mm) was used. Integration software was ChemStation for LC (2001–2009 Agilent Technologies).

Dried extracts of maize and gluten meal were resuspended in 4.0 and 0.4 mL acetonitrile:water (50:50, v/v), respectively. Derivatization was done automatically in the HPLC system by mixing the resuspended extract (20 μL) with OPA reagent (34 μL) prior to injection. Injection volume was 54 μl. Isocratic elution was done at a flow rate of 1 mL/min. Excitation and emission wavelengths were 335 and 440 nm, respectively. Temperature of column and guard column was maintained at 30 °C.

If FBs concentration levels of samples were above the quantification range of the calibration curves, the analysis of the sample was repeated after diluting the filtered extract of the sample at 1:5 with methanol:water (75:25, v/v), previous to the addition of PBS (gluten meal samples) and pH adjustment (maize samples). Clean-up was afterwards carried out as previously described.

Certified reference material was included in the analysis sets. Reference material (TR-F 1000, Lot number F–C-438) was maize naturally contaminated with FB1 at 2700 μg/kg, FB2 at 700 μg/kg, and FB3 at 200 μg/kg obtained from Trilogy Analytical Laboratory (Washington, MO, USA). Total FBs levels (calculated as the sum of FB1, FB2, and FB3) was 3600 μg/kg±700 μg/kg according to the provider's certificate.

2.4. Statistical analysis

Normality of distributions was tested by the Shapiro–Wilk test. Differences were evaluated by the t test for paired samples ($\alpha = 0.05$) for HPLC and ELISA results of the same samples, and by the t test for independent samples ($\alpha = 0.05$) for FBs ratios in maize and gluten meal. Correlations were tested by the Pearson coefficient. InfoStat software (2015 version, InfoStat Group, Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Argentina) was used for the analysis.

3. Results and discussion

3.1. HPLC-FLD method performance

Linearity ranges of calibration curve utilised for maize samples for FB1, FB2, and FB3 were (μg/kg): 5–8000; 5–3200; 4–3200, respectively. Coefficient of determination (R^2) for FB1, FB2, and FB3 was 0.9999, 0.9967, and 0.9992, respectively. Limits of detection (LOD) for FB1, FB2, and FB3 were 1 μg/kg for each fumonisin. Limits of quantification (LOQ) were 5, 5, and 4 μg/kg, respectively. Total FBs were calculated as the sum of FB1, FB2, and FB3. Repeatability was assessed in the same day (n = 7) using a control sample contaminated at the levels of 2000, 600, and 300 μg/kg for FB1, FB2 and FB3, respectively. Relative standard deviation (RSD) was 5.3% for total FBs. Recovery rate was higher than 80% for the three FBs.

Linearity ranges of calibration curve utilised for gluten meal samples for FB1, FB2, and FB3 were (μg/kg): 3–6000; 1–3000; 1–2000, respectively. Coefficient of determination (R^2) for FB1, FB2, and FB3 was 0.9985, 0.9987, and 0.9992, respectively. LODs for FB1, FB2, and FB3 were respectively 1, 0.4, and 0.3 μg/kg. LOQs for FB1, FB2, and FB3 were 3, 1, and 1 μg/kg, respectively. Repeatability was assessed in the same day (n = 7) using a control sample contaminated at the same levels for maize. RSD was 9.3% for total FBs. Recovery rate was nearby 100% for the three FBs.

3.2. Levels of fumonisins as determined by ELISA and HPLC

Tables 1 and 2 list FBs levels in maize and gluten meal, respectively, as determined by ELISA and HPLC. Plants A, B, and C used test kit G, whereas plants D, E, and F used test kit K.

A maize sample analysed in plant D had a total FBs mean concentration (317 μg/kg) below the LOQ of the test kit used (500 μg/kg). The rest of the samples had FBs levels above the LOQ of the test kits.

It is necessary to point up that, industrial plants involved in this study normally perform only one ELISA determination per sample of maize or gluten meal, and replicates were specially done for the purposes of this work. ELISA determinations were done in triplicate, except when indicated. Relative standard deviations (RSDs) varied from very low values as 1% up to 56% in maize and 37% in gluten meal. Variability of results by ELISA test kits can be increased if procedures of analysis indicated by the kits' providers are modified. Certain deviations from those instructions were observed in the ELISA analyses at the wet milling plants, such as modification of the volumes of extraction solvents and its relation to the weight of samples, use of methanol of low quality for the extraction, lack of pH adjustment of the extracts, use of a calibration curve for more than one set of analysis, and no further dilution of extracts if the results were out of the quantification range of the test kits; these modifications could impact on the results.

HPLC determinations were one per sample, and results were not corrected by recovery. Each run of fumonisins (sequence at the HPLC) included a certificate sample. If the certificate sample showed more than one standard deviation it was analyzed again.

Table 1
Fumonisins levels in maize samples (n = 43) in the different wet milling plants.

Fumonisins in maize (μg/kg)							
ELISA				HPLC			
Plant	Test kit	Mean	SD	FB1	FB2	FB3	FBs Total
A	G	437	112	352	261	26	640
A	G	4660	312	2294	722	126	3142
A	G	6000	680	3686	1380	327	5393
B	G	3290	501	6544	2470	738	9752
B	G	5779	198	3391	1420	541	5351
B	G	3539	215	2376	1277	171	3824
B	G	4527	247	3152	1193	299	4644
B	G	7372	405	5699	2733	694	9126
B	G	5316	307	4014	2144	376	6535
B	G	6097	248	3763	1595	341	5699
B	G	3333	416	6797	2626	729	10,151
B	G	2933	473	5956	2176	646	8778
B	G	3767	208	3993	1528	427	5947
B	G	3067	833	5008	1718	405	7132
B	G	3333	252	6487	2332	581	9401
C*	G	8450	–	5316	1763	355	7434
C*	G	9370	–	6866	2449	570	9885
C*	G	4010	–	1180	399	111	1690
C*	G	7560	–	4541	1268	510	6320
C*	G	5450	–	1935	667	236	2839
D	K	967	58	460	167	49	677
D	K	6333	1976	4610	2033	224	6867
D	K	7067	503	4212	1645	316	6173
D**	K	3367	971	1078	717	136	1932
D**	K	317	160	132	111	ND	244
D	K	1566	208	644	232	74	950
D	K	1533	854	665	277	54	996
D	K	1700	681	1180	486	99	1765
D	K	1567	462	636	253	47	935
D	K	1967	346	906	303	77	1285
D*	K	2300	–	759	352	32	1144
E	K	5067	252	4031	1391	376	5799
E	K	7967	115	10,352	3845	668	14864
E	K	4200	265	2538	679	343	3560
E	K	4743	191	3594	1104	403	5101
E	K	3900	200	2909	955	292	4156
E	K	4333	306	4951	1893	476	7320
F	K	7167	231	8406	2948	769	12124
F	K	2233	58	1284	330	99	1713
F	K	5067	58	3838	1399	324	5562
F	K	6333	115	6434	2074	536	9045
F	K	2533	58	1368	741	125	2234
F	K	4033	58	2452	872	210	3534

Replicates: * = 1 for ELISA analysis; ** = 6 for ELISA analysis.
ND = non detected.

Table 2
Fumonisins levels in gluten meal samples (n = 45) in the different wet milling plants.

Fumonisins in gluten meal (μg/kg)							
ELISA				HPLC			
Plant	Test kit	Mean	SD	FB1	FB2	FB3	FBs Total
A	G	3350	87	961	939	163	2063
A	G	6833	709	2072	2554	446	5071
A	G	6663	916	4046	5845	845	10,736
B	G	2117	789	1551	1551	390	3492
B	G	4145	168	2187	2653	476	5315
B	G	2562	268	2215	1667	393	4275
B	G	3443	608	1515	1907	286	3707
B	G	4825	458	3552	3222	653	7426
B	G	3976	408	4378	5494	1037	10,908
B	G	5046	778	2321	1569	371	4260
B	G	2367	551	4788	3689	673	9150
B	G	2467	231	5409	4593	958	10,960
B	G	3000	400	3259	3234	606	7099
B	G	3567	513	4346	3471	777	8594
B	G	2967	503	4842	4570	897	10,308
C*	G	3640	–	7593	8348	1480	17,420
C	G	3113	25	6409	7820	1064	15,292
C	G	2530	20	5367	7280	979	13,626
C	G	2960	60	5310	6636	877	12,823
C	G	2430	30	6263	6395	1087	13,744
C	G	1627	32	3974	4198	722	8894
D	K	9703	606	3928	10,808	998	15,734
D	K	9990	772	4193	12,344	965	17,501
D	K	10377	834	3771	13,602	981	18,354
D	K	9103	415	2573	8475	727	11,774
D	K	9137	486	2687	9445	856	12,987
D	K	7860	1597	1936	5830	562	8327
E**	K	13,733	1353	10,938	12,097	1212	24,246
E	K	14,933	1026	6465	8810	1590	16,865
E	K	11,600	529	7322	10,825	1998	20,144
E	K	16,267	2212	12,295	10,205	1530	24,030
E	K	17,600	1039	10,951	10,713	1564	23,228
E	K	9867	379	13,434	12,346	1826	27,606
E	K	2733	416	961	939	163	2063
E	K	4100	520	2072	2554	446	5071
E	K	4067	231	1551	1551	390	3492
E	K	6233	723	2187	2653	476	5315
E	K	5500	100	2215	1667	393	4275
E	K	4567	321	2321	1569	371	4260
F	K	9000	100	8112	11,041	1200	20,353
F	K	13,667	115	7511	7952	964	16,426
F	K	11,733	306	5636	9324	1021	15,980
F	K	13,667	231	6431	9392	1072	16,895
F	K	8600	400	3442	7097	692	11,231
F	K	6267	115	4673	8684	1035	14,391

Replicates: * = 1 for ELISA analysis; ** = 9 for ELISA analysis.

Total FBs were calculated as the sum of the levels of FB1, FB2, and FB3. Chromatograms of FBs in maize and gluten meal samples overlaid with a standard are shown in Fig. 1.

From other hand, all samples of maize and gluten meal, selected for this study, were naturally contaminated with FBs in a wide range of concentrations, but gluten meal samples ratios of FB1/FB2, FB1/FB3 and FB2/FB3 were significantly different ($P < 0.0001$) from those observed in maize samples (Fig. 2).

The ratio FB1/FB2 for gluten meal was in most of the cases lower than 1.00, which means that FB2 levels were higher than FB1 levels. This may be due to a higher solubility of FB1 in water, which is present in the wet milling steps of maceration and subsequent separations of fractions or other reason not yet studied.

3.3. Comparison of ELISA with HPLC results

Based on the concentrations of total FB determined by HPLC, only 22 maize samples and 11 gluten meal samples had FBs concentrations within the ELISA quantification ranges of the test kits used. When comparing the results obtained by both methods for this concentrations range, ELISA values were higher than HPLC values in a 68% for maize (from 1.1 to 2.4 fold) and 73% for gluten meal (from 1.1 to 1.6 fold) samples. Other studies also found that ELISA results for FBs were in most cases higher than HPLC results in maize samples (Bowers et al., 2014; Ono, Kawamura, Ono, Ueno, & Hirooka, 2000; van Rensburg, Flett, Mc Laren & Mc Donald, 2011). Immunochemical methods are based on the specificity of the antibodies used, and antibodies are characteristic of each kit brand. Cross-reactivity of compounds structurally related to the target will lead to false positives or overestimation of results (Bird et al., 2002; Jackson & Jablonski, 2004; M. Zachariasova, Cuhra, & Hajslova, 2014). Cross-reactivity can occur with structurally related mycotoxins as well as with structurally related matrix components co-extracted (Alldrick, 2014; Goryacheva & De Saeger, 2012; Zachariasova et al., 2008, 2014). Regarding FBs, Dall'Asta, Galaverna, Aureli, Dossena, and Marchelli (2008) demonstrated the cross-reactivity of anti-fumonisin antibodies to some FBs-

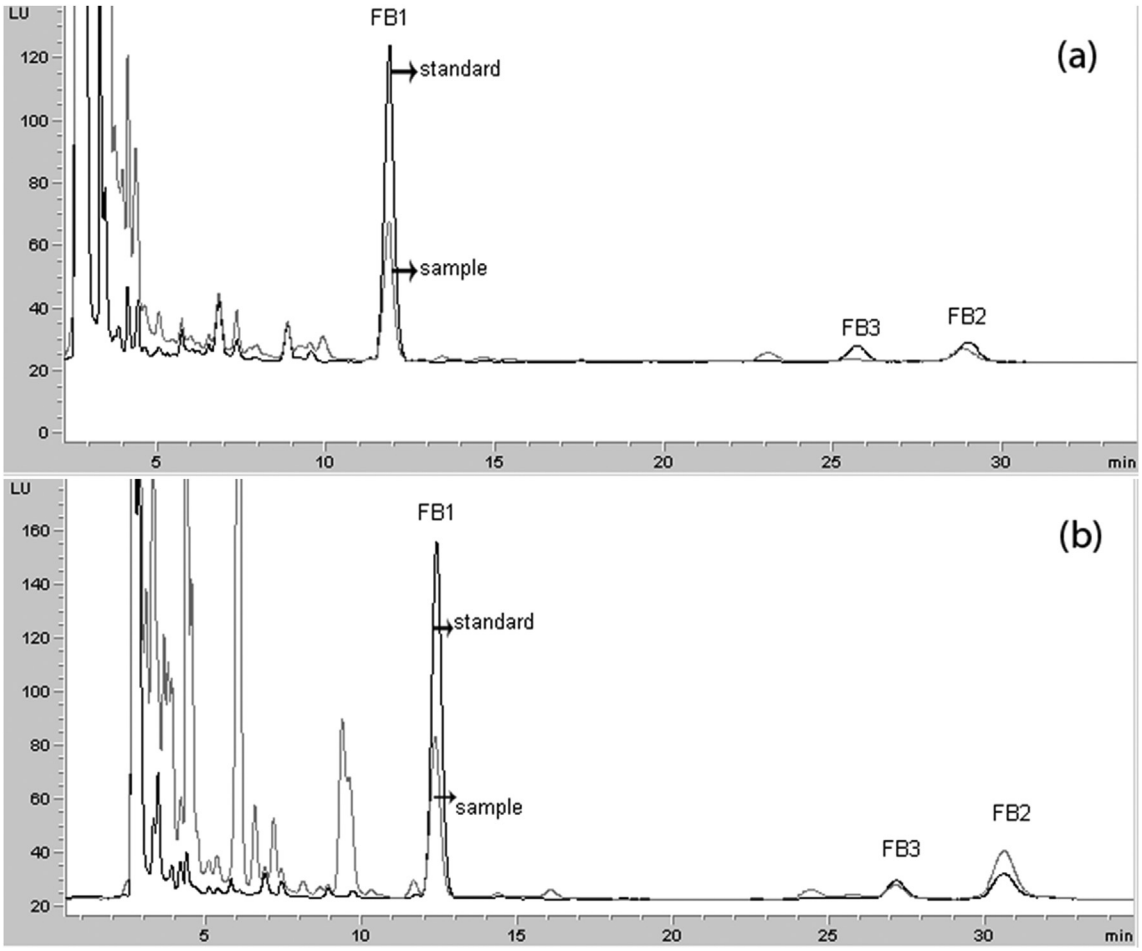

Fig. 1. Chromatograms of fumonisins in maize (a: FB1 = 759 μg/kg, FB3 = 32 μg/kg, and FB2 = 352 μg/kg) and gluten meal (b: FB1 = 876 μg/kg, FB3 = 207 μg/kg, and FB2 = 1099 μg/kg) samples overlaid with a concentration of standard equivalent in a sample to FB1 = 2000 μg/kg, FB3 = 300 μg/kg, and FB2 = 600 μg/kg.

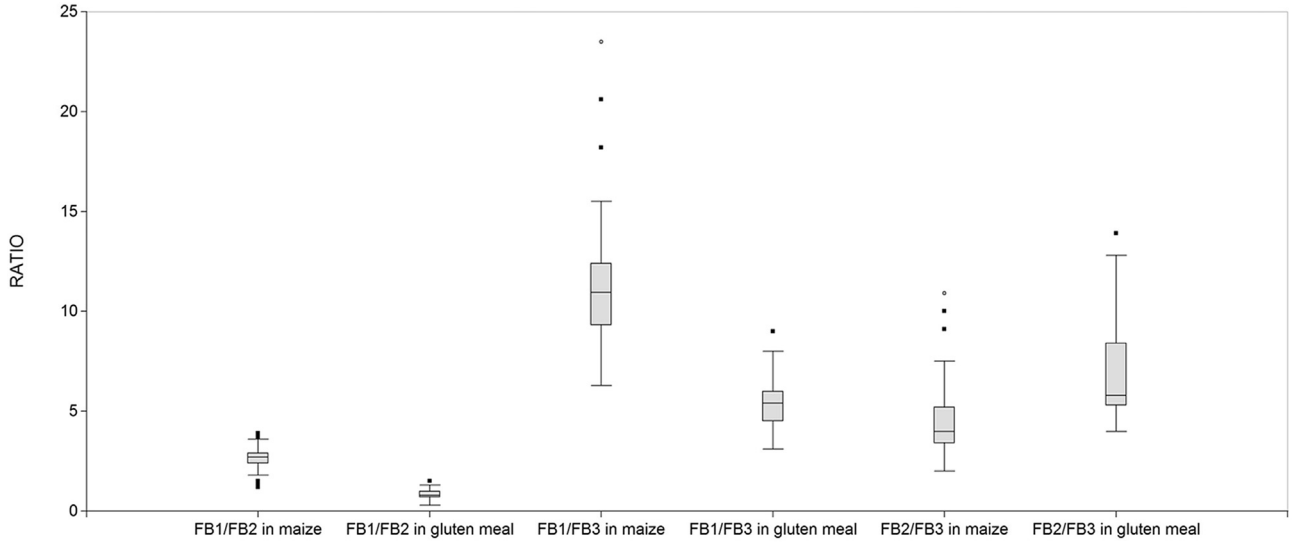

Fig. 2. Box-plots of the ratios FB1/FB2, FB1/FB3, FB2/FB3 in maize and gluten meal.

conjugates derived from food processing, and suggested the detection of hidden FBs by ELISA as a cause of the overestimation of results when comparing them to those obtained by chromatographic methods. Therefore, higher concentration levels by ELISA analyses in this work were not unexpected, as test kits quantify total FBs, which comprise other fumonisin analogues in

addition to FB1, FB2, and FB3 detected by HPLC.

Considering the results that were out of the ELISA quantification ranges, the opposite was found: ELISA values were lower than HPLC values (30% for maize and 97% for gluten meal samples). As observed during ELISA analyses in all the industrial plants involved, when results were above the quantification ranges of the test kits, no further dilutions of the extracted samples were done in order to obtain a quantifiable level, and the first result was considered as definitive. As noted by Alldrick (2014), problems with both accuracy and precision can occur, particularly when the levels of contamination are extended beyond the original criteria used in the test kit's validation processes.

Differences and correlations of ELISA *versus* HPLC results were statistically tested, and FBs concentration levels were split according to the kits' quantification ranges. Mean values of ELISA replicates were used in the comparisons with HPLC values (Table 3).

These results indicate that for both test kits, G and K, with exception of the maize samples analysed with kit K, values obtained by ELISA and HPLC were not significantly different (t test) when levels were within ELISA quantification ranges (even though most ELISA values were higher than HPLC values), whereas when levels were above those ranges, significant differences were found.

Pearson correlation was used to identify the degree of linear dependence between both sets of data. Correlations were positive (*i.e.* as the value of HPLC analysis increases, so does the value of the ELISA analysis) within ELISA quantification ranges for both kits and matrices (Table 3). The strength of association was high (Pearson coefficient > 0.5) excepting for test G in gluten meal, where the correlation was small (Pearson coefficient < 0.3). When values were above the quantification range, for test kit G, weak negative correlations (i.e. as the value of HPLC analysis increases, the value of the ELISA analysis decreased) were observed, while for test kit K, strong positive correlations were obtained. However, only in two cases (kit K), the linear correlation was statistically significant as p-value was a lot lesser than 0.05 ($P << 0.05$). Ono et al. (2000) also found different correlation coefficients for different concentration levels of FBs (FB1 + FB2) in maize determined by ELISA and HPLC. Ranges were 80–10,000 μg/kg and above 10,000 μg/kg, and correlation coefficients were 0.91 and 0.66, respectively. Bowers et al. (2014) analysed maize samples for FBs (FB1 + FB2 + FB3). ELISA quantification range was 250–5000 μg/kg, and samples exceeding 5000 μg/kg were subject to additional extract dilution to bring the extract concentration within the range of the test kit. FBs levels ranged from zero to 48,000 μg/kg by ELISA and from LOD to 34,870 μg/kg by HPLC, and a strong positive correlation between ELISA and HPLC results was observed in all the range of concentrations ($r = 0.95$, $P < 0.0001$). Correlations in that study could be properly evaluated in the complete range of concentrations found, because the adequate dilutions were done for the ELISA analyses. In other studies involving ELISA and HPLC analysis and regarding correlations as well, positive correlations were found: Kim, Shon, Chung, and Kim (2002) determined FB1 in maize-based food products. Levels ranged from non-detected to 1210 μg/kg by ELISA, and from non-detected to 1010 μg/kg by HPLC. A positive correlation was observed between results by linear regression analysis ($r^2 = 0.992$). Ghali, Ghorbel, and Hedilli (2009) analysed FBs (FB1 + FB2) in food and feed samples (maize, rice, sorghum and wheat). When considering the results of positive samples for both methods (range of concentrations found: 25–2800 μg/kg), they found no significant differences between them, as well as a positive correlation ($r^2 = 0.978$, $P < 0.005$). FBs concentration levels in these two studies were below the upper limits of the quantification ranges of the kits used in this work. Correlation coefficients (within the ELISA quantification ranges) in this work were similar to those found in the cited studies.

Wet milling industries process maize to obtain several products, one of them being gluten meal, which is used as an ingredient in the formulation of animal feed. Even though FBs maximum levels in gluten meal are not regulated in Argentina, commercial limits are usually set by the buyers of this product. As the contamination of wet milling products is due to the contamination present in maize, wet milling plants also set limits to their maize providers. In both cases (wet milling and feed industries), levels above the settled limits lead to rejection of lots. Considering the results in this study, most values obtained by ELISA above the quantification ranges of the test kits were lower than the obtained by HPLC.

4. Conclusions

Industries involved in this work showed a good performance for both matrices for their purpose regarding the use of ELISA test kits, since there were no statistically significant differences among ELISA and HPLC results when triplicates were done within ELISA quantification ranges. Although rapid tests gave slightly overestimated values in most cases, attributed to cross-reactivity with structurally related matrix components co-extracted. Only for one kit it was possible to find a positive correlation between HPLC and ELISA determinations out of the quantification range, but the ELISA response was less sensitive than the obtained within the quantification range of the kit. When the levels of contamination were extended beyond the range used in the test-kit's approval/validation processes, significant differences raised between results of HPLC analysis and test kits; in general, values of fumonisins contamination were under-estimates by ELISA based assays in this range.

These results indicated that ELISA test kits can be used as a screening method for fumonisin contamination in maize and gluten meal if test procedures are used adequately. As normally in

Table 3
Differences and correlations between ELISA mean values and HPLC values, by matrix, kit brand, and fumonisins concentration.

Matrix	n	Test kit	ELISA	t test	Correlation analysis	
			Quantification range	P-value	Pearson coefficient	P-value
Maize	6	G	250–5000 μg/kg	0.1436	0.67	0.1484
Maize	14	G	>5000 μg/kg	0.0162	−0.14	0.6296
Gluten meal	5	G	250–5000 μg/kg	0.6844	0.18	0.7744
Gluten meal	16	G	>5000 μg/kg	<0.0001	−0.36	0.1747
Maize	16	K	500–6000 μg/kg	0.0365	0.96	<0.0001
Maize	6	K	>6000 μg/kg	0.0562	0.61	0.1954
Gluten meal	6	K	500–6000 μg/kg	0.2056	0.80	0.0569
Gluten meal	18	K	>6000 μg/kg	<0.0001	0.55	0.0171

t test: Null hypothesis indicates that means from different distributions are equal, and it is rejected if P-value is less or equal to the significance value ($\alpha = 0.05$).
Pearson correlation: Null hypothesis indicates that the correlation coefficient was zero. If P-value is less or equal to the significance value ($\alpha = 0.05$), the correlation coefficient is statistically significant.

the Argentinean wet milling industries only one ELISA determination is done, when levels determined by ELISA would lead to rejection of lots, such levels should be confirmed by HPLC quantification because of the high RSD of ELISA analyses (up to 56% in maize and 37% in gluten meal samples).

For all samples of maize and gluten meal selected for this study, naturally contaminated with FBs in a wide range of concentrations, the contamination pattern of fumonisins in gluten meal was different from that of maize, with higher levels of FB2 than those of FB1.

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