Determination of Fumonisins B_1 and B_2 in Beer

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Abstract

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The aim of this study was to investigate the contamination of beer of Slovak origin with fumonisins. A suitable analytical procedure was suggested – the limit of detection at the level close to 1 µg/l was achieved for both fumonisins B_1 and B_2. The recovery was determined at 93% for fumonisin B_1 and at 78% for fumonisin B_2. Fluorescence detection was used after derivatisation with a mixture of *o*-phthaldialdehyde and 2-mercaptoethanol. Phosphate buffer usually applied resulted in a poor separation of derivatised fumonisins. Peak splitting was observed depending on the pH of the eluent. The pH value of 2.6 was found suitable for the peak splitting elimination. A convenient gradient elution metod was suggested avoiding the possible interference in fumonisin contents determination. For the preparation of samples, immunoaffinity cleaning procedure was applied. Beer samples from all domestic producers were analysed. The content of fumonisins determined was under the limit of detection in all cases. All the beers tested were produced from the barley grown in 2003.

Keywords: beer; fumonisin B_1; fumonisin B_2; immunoaffinity clean-up; HPLC

Fumonisins are mycotoxins produced by fungi of the genus *Fusarium*. The only species producing significant quantities of fumonisins are *Fusarium verticillioides* (*moniliforme)* and *Fusarium proliferatum* as published by Stroka *et al*. (2002). There are four structurally related mycotoxins produced by *Fusarium* species, fumonisins B_1, B_2, B_3 and B_4. Little is known about the natural occurrence of fumonisin B_4. Fumonisin B_4 was identified in 23 of 44 mouldy maize samples in the Republic of Korea at concentrations significantly lower than those of other three fumonisins (Seo & Lee 1999). In many studies, significant amounts were detected of other three fumonisins in decreasing concentrations in the order – fumonisin B_1, B_2, and B_3. These were found, in various commodities, not only in maize (Scott & Lawrence 1995; Smith & Thakur 1996; Siame *et al.* 1998; Da Silva *et al*. 2000). A comprehensive study of the occurrence of fumonisins in Europe was published recently (Report on Tasks for Scientific Cooperation 2003). Studies on fumonisins health impact were published by JECFA (FAO Food and Nutrition Paper 74 2001).

Analytical procedures applied for the fumonisins determination reached the detection limit of 50 µg per kg or below, and the recovery was better than 70% (FAO Food and Nutrition Paper 74, 2001). Fumonisins are derivatised with mixture of *o*-phthaldialdehyde (OPA) and 2-mercapthoethanol (MCE) before the separation by high performace liquid chromatography (Visconti *et al.* 2001a). In this case, immunoaffinity clean-up step was used (Visconti *et al.* 2001a). The application of

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immunoaffinity cleaning in the sample preparation process is given an increasing interest (Scott & Lawrence 1995; Trucksess *et al.* 1995; Visconti *et al.* 2001a; Papadopoulou-Bouraoui *et al*. 2002). Results were published applying only *o*-phthaldiadehyde for fumonisins derivatisation (Visconti *et al*. 2001b), the procedure was collaboratively studied. The matrix was corn and corn flakes spiked at the levels ranging from 0.05 mg/kg up to 1.05 mg/kg with fumonisin B_1, and from 0.05 mg/kg up to 0.46 mg/kg with fumonisin B_2, respectively. From the point of view of the detection level, the results are comparable to those received when the derivatisation mixture of OPA and MCE was used (Solfrizzo *et al.* 2001). An alternative based on the flow-injection liposome immunoanalysis procedure was also suggested (Ho & Durst 2003). In this case, the immunoaffinity clean-up step was substituted with a cleaning step based on ion interactions. Strong anion exchanger resin was applied. The detection level was 0.1 ng/kg with maize (Ho & Durst 2003). Another suitable tool for fumonisins analysis is enzyme-linked immunosorbent assay (ELISA) (Scott *et al.* 1997). ELISA is very useful in the case when high amounts of samples have to be tested. Another suitable tool for fumonisins determination is thin layer chromatography (TLC) providing results at levels of 0.1 mg/kg (Preis & Vargas 2000) or of 0.5 mg/kg (Shephard & Sewram 2004).

To improve the detection and the determination limits of fumonisins analysis, other derivatisation agents were tested (Scott & Lawrence 1992; Bennett & Richard 1994; Stroka *et al*. 2002). No real improvement was reached.

Another commodity suspicious of the contamination with fumonisins is beer. In this case, the separation of derivatised molecules of fumonisins is not as perfect as in the case of corn matrix (Scott & Lawrence 1995; Hlywka & Bullerman 1999). Either some residual interference influences the base line drift or the separation of the derivatised molecules of fumonisins is not at the base line level.

In the submitted paper, the application is demonstrated of the immunoaffinity cleaning step in the sample preparation for fumonisins B_1 and B_2 determination in beer samples. The influence of the mobile phase pH value on the separation of both fumonisins was studied. The main attention was paid to the elimination of any possible interference. The method was used to determine the contents of both fumonisins in the beer produced in Slovakia. Samples were taken from each domestic brewery and analysed. For the beer production, barley grown in 2003 was used. The results obtained are reported.

MATERIALS AND METHODS

Standards and chemicals. All chemicals were of analytical or HPLC purity grades. Fumonisin B_1 (FB_1) and Fumonisin B_2 (FB_2) from Sigma-Aldrich (Louisiana, USA) were used as the reference materials. Acetonitril Chromasolv and methanol Chromasolv were obtained from Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany). Potassium and sodium chlorides, potassium dihydrogen phosphate, disodium tetraborate, and *o*-phosphoric acid (min. 85%) were purchased from Lachema (Brno, Czech Republic). Other chemicals – disodium hydrogen phosphate and the reagents for derivatisation – ortho-phthadialdehyde and 2-mercaptoethanol – were obtained from Merck (Darmstadt, Germany). Hydrochloric acid (min. 35%) was delivered by ITES (Vranov n. T., Slovakia), glacial acetic acid (99.5%) from AFT (Bratislava, Slovakia). Immunoaffinity columns Fumoniprep for ther clean-up were received from R-Biopharm (Scotland).

Procedures. Stock standard solution FB_1 was prepared at the concentration of 1.257 mg/ml and stored at –18°C in the mixture of acetonitril: water, 1:1 (v/v). The working standard solution was prepared by dilution of the stock standard solution to the level of 31.4 µg/ml. The working standard was stored at 4°C. Stock standard solution of FB_2 was also diluted with the same solvent as FB_1 to the concentration of 0.252 mg/ml. The working standard was prepared at the level of 6.3 µg/ml. The storing conditions applied were the same as for FB_1.

Phosphorus buffer saline (PBS) was prepared from 8.0 g sodium chloride, 1.2 g disodium hydrogen phosphate, 0.2 g potassium dihydrogen phosphate, and 0.2 g potassium chloride. All these chemicals were dissolved in 990 ml of water (HPLC purity grade). Afterwards, the pH value was adjusted to 7.4 with hydrochloric acid and the total volume was made up to 1000 ml.

Samples. Beer samples were degassed in an ultrasonic bath and filtered. 15 ml of the beer was mixed with 15 ml of PBS. This diluted beer was ready for cleaning on immunoaffinity column.

Immunoaffinity cleaning. Fumoniprep immunoaffinity columns stored at 4°C were allowed to warm up to the laboratory temperature. It is recommended to keep immunoaffinity columns at laboratory temperature at least 6 h, otherwise the results are not reproducible. The best solution was to keep the immunoaffinity columns at laboratory temperature overnight before use. Immediately before the application, chemical conditioning was done. Conditioning was performed with 10 ml of PBS. Afterwards, the diluted sample was passed through the column under gravity (the support with the use of vacuum was not necessary). The interfering substances were washed with 10 ml of PBS. The column was then dried with air. Fumonisins were eluted with 3 ml of methanol. The eluate was evaporated on a vacuum rotary evaporator to the residual volume of ca. 0.5 ml. Drying was done with a milo stream of nitrogen. The residue was dissolved in 250 µl of the acetonitril:water, 1:1 (v/v) mixture and prepared for analysis.

Derivatisation. The derivatisation mixture was prepared from 40 mg *o*-phthaldialdehyde dissolved in 0.5 ml of methanol and diluted with 2.5 ml of 0.1 mol/l disodium tetraborate (3.8 g $Na_2B_4O_7 \times 10\ H_2O$ dissolved in 100 ml water). Afterwards, 50 µl of 2-mercaptoethanol was added and the mixture was thoroughly mixed. The mixture had to be stored in an amber glass vial at 4°C and could be used for 7 days. Then a new mixture had to be prepared.

Derivatisation was carried out with an autosampler. 20 µl of the prepared sample was diluted with 20 µl of the derivatisation mixture, mixed for 30 s, and after 3 min injected on the separation column.

Equipment and HPLC analysis conditions. Agilent Technologies 1100 Series (Halbron, Germany) with fluorescence detector, the wavelength settings excitation 335 nm and emission 460 nm, was used for the derivatised fumonisins molecules determination. The separation column was Zorbax SB-C18, 4.6 × 250 mm i.d. with particle size of the sorbent 5 µm, and the precolumn was Zorbax SB-C18, 12.5 × 4.6 mm i.d. with the same particle size Agilent Technologies (Halbron, Germany). Various mobile phases were tested. Isocratic separation was performed with the mobile phase prepared from a mixture of methanol:phosphate buffer (sodium dihydrogenphosphate 15.6 g dissolved in 1 l water, pH adjusted to 3.35 with 1 mol/l *o*-phosphoric acid) as applied in corn analysis (Visconti *et al.* 2001a). As satisfactory results were not obtained, gradient elution was also tested. In this case, phosphate buffer was substituted with glacial acetic acid solution and acetonitril was added. Various concentrations of acetic acid in water were evaluated. The best gradient separation programme was as follow: A – methanol; B – acetonitril; C – acidified water.

Time 0 min: A – 61%, B – 5%, C – 34%
Time 0–27 min: A – 61%, B – 5%, C – 34%
Time 27–30 min: A – 72%, B – 5%, C – 23%
Time 30–42 min: A – 72%, B – 5%, C – 23%

Equilibration time between each run was 10 min.

RESULTS AND DISCUSSION

Immunoaffinity columns were used for the sample preparation. The procedure described above was followed. It was in accordance with the producer's general recommendations. The flow of beer

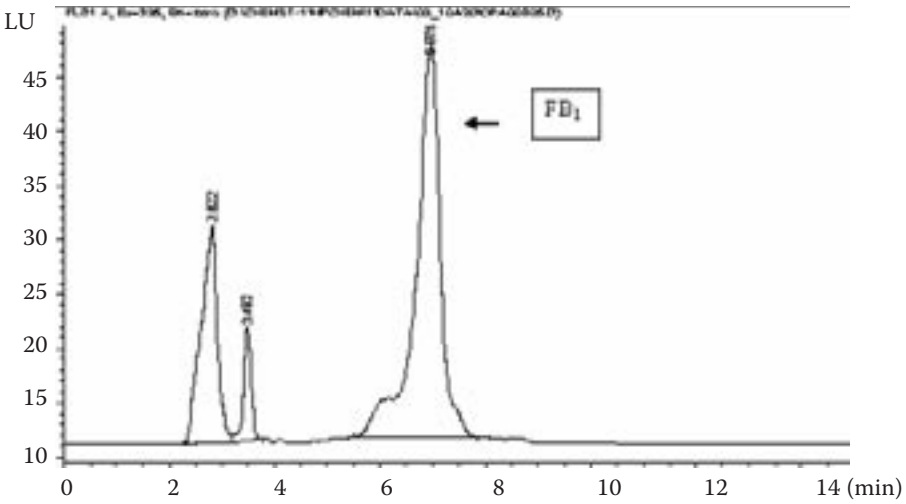


Figure 1. Chromatogram of isocratic separation with phosphate buffer at pH value 3.5. Fumonisin FB_1 standard

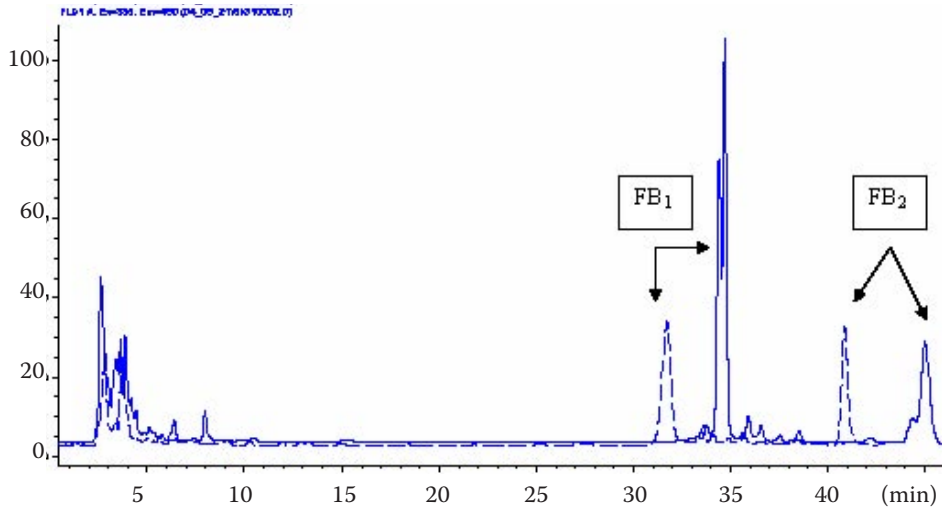


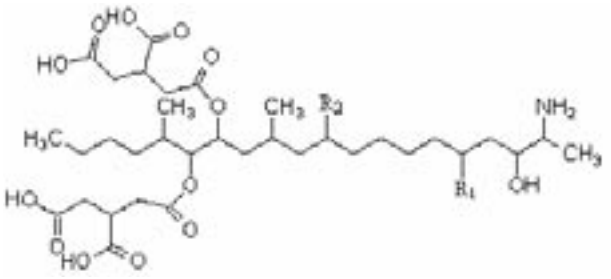
Figure 2. Chromatograms of split peaks at pH 2.9 (solid line) and non split peak at pH 2.6 (dashed line) in standard solution of FB_1 and FB_2

samples was sufficient so that the support with vacuum was not necessary.

For the separation of the derivatised molecules, the same composition of the mobile phase was applied in the first step as was suggested by Visconti *et al.* (2001b) in a collaborative study. There was a difference in the derivatisation procedure, OPA only was applied (Visconti *et al.* 2001b). In this study, a mixture of derivatisation agents was used. Isocratic separation with phosphate buffer at pH value 3.5 was not suitable. The presence was suspected of interference from the sample in the retention times of FB_1 and FB_2. A shoulder was observed on the FB_1 peak (Figure 1). That is why the composition of the mobile phase had to be changed. Acetic acid instead of phosphate buffer was used. Various concentrations of acetic acid were tested. Mixtures of acetic acid in water with pH values: 2.9, 2.7 and 2.6 were prepared. The mixture with pH value of 3.5 was not tested because of the bad results obtained in the case of phosphate buffer. The elution of both fumonisins was strongly pH dependent. At pH values above 2.6, an evident peak splitting occurred (Figure 2) with both fumonisins. The explanation of this phenomenon has roots in the molecular structure of the separated molecules – derivatised fumonisins. The molecules of fumonisins contain four carboxylic acid groups (Figure 3). One possibility for the explanation of the peak splitting at higher pH values can be the different dissociation of these carboxylic acid groups resulting in the two peaks observed. This different dissociation can be suppressed by decreasing the pH value. At pH 2.6 or less no peak splitting was observed (Figure 2).

The peak area of the first peak of the FB_1 twins was 910 $mAus^{-1}$, the second one was 1403 $mAus^{-1}$ (Figure 2). The area of FB_1 peak found at pH 2.6 was 915 $mAus^{-1}$. The comparison of these results indicates that at pH 2.6 only one form of the derivatised FB_1 survives while the second derivatised complex is broken – with no fluorescence observed. This idea is also supported by the observed behaviour of FB_2. In this case, FB_2 twins revealed the area of the first peak 162 $mAus^{-1}$ and of the second peak 800 $mAus^{-1}$. The peak area of FB_2 obtained at pH 2.6 was 720 $mAus^{-1}$. A direct comparison of the retention times was not possible because of their changes due to the different pH values of the mobile phase. Proper explanation of this phenomenon requires additional studies.

The separation procedure based on gradient elution was adopted. Acidified water containing 7% (vol.) of glacial acetic acid as constituent C of the mobile phase was used (the gradient is described in Experimental part). With this mobile phase no interference was scanned in the retention time of both fumonisins. A satisfactory base line was



FB_1 = R_1 – OH; R_2 – OH; FB_2 = R_1 – OH; R_2 – H

Figure 3. Chemical structure of FB_1 & FB_2

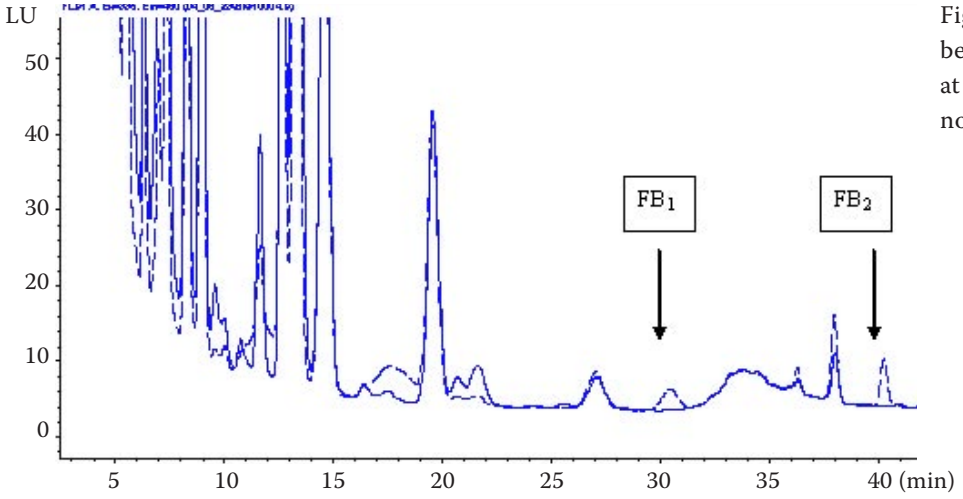


Figure 4. Chromatogram of beer spiked with FB_1 and FB_2 at level 4 µg/l (dashed line) and non spiked beer (solid line)

easily received under these conditions. A negative consequence of the good separation of the derivatised molecules of fumonisins is a relatively long time of the separation run (Figure 4).

Based on the acceptable separation conditions of the procedure suggested a partial single laboratory validation was performed. Various beer samples were tested. No sample was identified with natural fumonisins contamination. That is why spiked beer samples at suitable concentrations had to be used. Based on the literature data (Scott & Lawrence 1995), the selected spiked levels were around 4 µg/l and 10 µg/l (Table 1.) Minor differences exist between fumonisin B_1 and fumonisin B_2. The recovery factors are 0.94 for FB_1 and 0.79 for FB_2, respectively. This is also acceptable for the trace analysis. In the calculation of the combined extended uncertainty, the

Table 1. Selected validation characteristics in spiked beer (µg/l))

No.	I. Spiked level FB_1 and FB_2	Measured concentration		II. Spiked level FB_1 and FB_2	Measured concentration	
		FB_1	FB_2		FB_1	FB_2
1	4.08	3.90	3.00	10.34	9.65	9.31
2	4.08	3.85	3.0	10.34	9.40	9.29
3	4.08	4.11	3.46	10.34	9.45	9.32
4	4.08	4.07	3.58	10.34	9.58	9.16
5	4.08	3.53	3.46	10.34	9.62	9.12
6	4.08	3.48	3.21	10.34	11.44	10.65
7	4.08	3.79	3.14	10.34	10.67	10.87
8	4.08	4.07	3.26	10.34	10.59	10.18
9	4.08	3.79	2.98	10.34	9.20	8.96
10	4.08	4.07	3.02	10.34	9.25	9.37
Average value (µg/l)		3.82	3.21	_	9.88	9.62
Recovery (%)		93.8	78.9	_	95.6	93.0
Combined extended uncertainty (%)		11.4	9.5	_	7.2	5.6
Limit of detection (µg/l)		0.72	0.66			
Limit of determination (µg/l)		2.4	2.2			

increments derived from weighing, the diluting of standards, and sample dilution contributing into B increment of the combined extended uncertainty were taken into account. The value of the expansion factor *k* was 2. The resulting values of the extended combined uncertainty for FB_1 and FB_2 were 11.4% and 9.5%, respectively.

The content of fumonisins in beers produced by Slovak breweries was determined. More than 20 samples were analysed. In a close cooperation with the State Veterinary and Food Administration, samples were received produced from barley that was grown in 2003. No traces were observed of fumonisins in the beer samples analysed. The limit of detection of the method applied is 0.7 μg/l.

CONCLUSION

The procedure suggested is suitable for the determination of fumonisins B_1 and B_2 in beer samples. The detection level was 0.7 μg/l with both fumonisins. Acceptable recovery values 93% for fumonisin B_1 and 78% for fumonisin FB_2 were obtained. A suitable separation of the possible interfering material was achieved if the pH value of the eluent was 2.6 or less. Glacial acetic acid was a better constituent of the mobile phase than phosphate buffer to meet this goal. A good separation of both fumonisins without any interference was evident. The application of an appropriate concentration of acetic acid resulting in the pH value of the eluent given is important for the possible elimination of the peak splitting. The performance of the analytical method was demonstrated on the analysis of beers of the Slovak production. In none of them the concentration higher than 0.7 μg/l was detected.

References

Bennett G.A., Richard J.L. (1994): Liquid chromatographic method for analysis of the naphthalene dicarboxaldehyde derivative of fumonisins. Journal of the Official Association of Analytical Chemists International, **77**: 501–506.

Da Silva J.B., Pozzi C.R., Mallozzi M.A.B., Ortega E.M., Correa B. (2000): Mycoflora and occurrence of aflatoxin b_1 and fumonisin b_1 during storage of Brazilian sorghum. Journal of Agricultural and Food Chemistry, **48**: 4352–4356.

FAO Food and Nutrition Paper 74 (2001): Safety Evaluation of Certain Mycotoxins in Food. FAO, Rome.

Hlywka J.J., Bullerman L.B. (1999): Occurrence of fumonisin B_1 and B_2 in beer. Food Additives and Contaminants, **16**: 319–324.

Ho J.A., Durst R.A. (2003): Detection of fumonisin B_1: comparison of flow-injection liposome immunoanalysis with high-performance liquid chromatography. Analytical Biochemistry, **312**: 7–13.

Papadopoulou-Bouraoui A., Stroka J., Anklam E. (2002): Comparison of derivatisation agents for fumonisin determination in beer. In: Proceedings of the 24th Mycotoxin – Workshop, Berlin, 3–5 June, 2002.

Preis R.A., Vargas E.A. (2000): A method for determining fumonisin B_1 in corn using immunoaffinity column clean-up and thin layer chromatography/densitometry. Food Additives and Contaminants, **17**: 463–468.

Report on Tasks for Scientific Cooperation, Scoop Task 3.2.10, EC DG Health and Consumer Protection, April, 2003.

Scott P.M., Lawrence G.A. (1992): Liquid chromatographic determination of fumonisins with 4-fluoro-7-nitrobenzofurazan. Journal of the Official Association of Analytical Chemists International, **75**: 829–834.

Scott P.M., Lawrence G.A. (1995): Analysis of beer for fumonisins. Journal of Food Protection, **58**: 1379–1382.

Scott P.M., Yeung J.M., Lawrence G.A., Prelusky D.B. (1997): Evaluation of enzyme-linked immunosorbent assay for analysis of beer for fumonisins. Food Additives and Contaminants, **14**: 445–450.

Seo J.A., Lee Y.W. (1999): Natural occurrence of the series of fumonisins in mouldy corn. Applied and Environmental Microbiology, **65**: 1331–1334.

Shephard G.S., Sewram V. (2004): Determination of the mycotoxin fumonisin B_1 in maize by reversed-phase thin-layer chromatography: A collaborative study. Food Additives and Contaminants, **21**: 498–505.

Siame B.A., Mpuchane S.F., Gashe B.A., Allotey J., Teffera G. (1998): Occurrence of aflatoxin and fumonisin b_1, zearalenone in foods and feeds in Botswana. Journal of Food Protection, **61**: 1670–1673.

Smith J.S., Thakur R.A. (1996): Occurrence and fate of fumonisins in beef. Advances in Experimental Medicine and Biology, **392**: 39–55.

Solfrizzo M., De Girolamo A., Visconti A. (2001): Determination of fumonisins B_1 and B_2 in cornflakes by high performance liquid chromatography and immunoaffinity clean-up. Food Additives and Contaminants, **18**: 227–235.

Stroka J., Capelletti C., Papadopoulou-Bouraoui A., Pallaroni L., Ankla E. (2002): Investigation of alternative reagents to 2-mercaptoethanol for the pre-column derivatization of fumonisins with *o*-phthaldialdehyde, for HPLC analysis. Journal of Liquid Chromatography & Related Technologies, **25**: 1821–1833.

Trucksess M.W., Stack M.E., Allen S., Barrion N. (1995): Immunoaffinity column coupled with liquid chromatography for determination of fumonisin B_1 in canned and frozen sweet corn. Journal of the Official Association of Analytical Chemists International, **78**: 705–710.

Visconti A., Solfrizzo M., de Girolamo A., Boenke A. (2001a): Determination of fumonisins at levels of interest for future EU legislation: Development of an analytical method and interlaboratory validation for maize flour and cornflakes. Project Report, Contract No. SMT4-CT97-2193, EUR 19096 EN, edited by the European Commission, Directorate-General XII-Science, Research and Development: 95.

Visconti A., Solfrizzo M., de Girolamo A. (2001b): Determination of fumonisins B_1 and B_2 in corn and corn flakes by liquid chromatography with immunoaffinity column cleanup: Collaborative study. Journal of the Official Association of Analytical Chemists International, **84**: 1828–1838.

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