Single laboratory-validated HPLC methods for determination of ochratoxin A, fumonisin B1 and B2, zearalenone and deoxynivalenol in cereals and cereal-based foods

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Summary

The aim of this study was to optimize analytical methods for determination of ochratoxin A (OTA), fumonisins B1 and B2 (FB1, FB2), zearalenone (ZON), and deoxynivalenol (DON) in cereals and cereal-based food products. The chromatographic separation was performed by reversed phase high performance liquid chromatography (HPLC) with fluorescence detection (FLD) and ultraviolet-diode array detection (UV-DAD). Optimized sample preparation methods utilizing extraction with solvent and clean-up on immunoaffinity column (IAC) were used. The procedures were validated in accordance with single laboratory validation principles. The limit of detection (*LOD*) for OTA, FB1, FB2 and ZON was 0.081, 50, 40 and 12 $\mu g \cdot kg^{-1}$, respectively, whereas the limit of quantification (*LOQ*) for DON was 45 $\mu g \cdot kg^{-1}$ for processed cereals (group A) and 85 $\mu g \cdot kg^{-1}$ for unprocessed cereals (group B). Average recoveries varied, in the range of 92–94% for OTA, 93–87% for FB1, 83–96% for FB2, 89–103% for ZON, and 97–87% for DON at defined spiking levels. The accuracy was additionally estimated for OTA, ZON and fumonisins using a certified reference material (CRM). All methods showed good sensitivity, accuracy and precision comparable to those published recently. The methods were successfully applied to the determination of mycotoxins in cereals at examination of occurrence and content of these toxins.

Keywords

ochratoxin A; fumonisins; zearalenone; deoxynivalenol; cereal; HPLC

The most important *Fusarium* mycotoxins that frequently occur in cereals are fumonisins (FB), zearalenone (ZON) and trichothecenes – deoxynivalenol (DON), nivalenol (NIV) and T-2 toxin. The most expanded mycotoxins of genera *Aspergillus* and *Penicillium* are ochratoxin A (OTA) and aflatoxins (AFL) [1]. Some of them can unfavourably affect human and animal health, especially when present at high contents in food and feed [2]. In order to protect human health, it is essential to keep these toxins at levels, which are toxicologically acceptable. The maximum residual levels for mycotoxins in food were set in the latest European directive 1126/2007 in details [3].

According to a review on research of mycotoxins in last years, many cereal food commodities were investigated using high performance liquid chromatography (HPLC) [4–17] or gas chromatography (GC) using various detection systems [18–20]. Both HPLC and GC are non-screening methods usually used as confirmative ones. An alternative method of enzyme-linked immuno-assay (ELISA) is widely employed for screening purposes [11, 21]. The method is relatively accurate, highly sensitive and appropriate in cases when high numbers of samples have to be tested. Another suitable analytical tool for identification of mycotoxins is thin layer chromatography (TLC), but it suffers from low selectivity and sensitivity reflected in high detection limits [22]. Currently, a continuous attention is paid to the analytical methodology for detection and quantification of mycotoxins. It is generally accepted that any analytical procedure may be applied if it meets the required performance characteristics, aside from valid European standards (CEN) or standards of International Organization for Standardization (ISO).

In this paper, individual HPLC methods with fluorescence and ultraviolet-diode array detection

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were adopted for OTA, FB1, FB2, ZON and DON determination in cereal products. These methods involve preliminary solvent extraction and clean-up on specific immunoaffinity columns. The procedures were validated with respect to general requirements of single laboratory validation. In addition, the methods were applied to determine the mycotoxins contamination in cereal foods available on the market in Slovakia.

MATERIALS AND METHODS

Chemicals

Reference substances of fumonisin B1 (FB1 98% purity), fumonisin B2 (FB2), OTA (99% purity) and ZON (98% purity) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). DON (97% purity) was from Fluka (St. Louis, Missouri, USA).

All used chemicals were of analytical or HPLC purity grade. Sodium chloride, potassium dihydrogen phosphate, potassium chloride, hydrochloric acid min. 35%, disodium tetraborate decahydrate and sodium hydroxide were from Lachema (Brno, Czech Republic); glacial acetic acid and toluene min. 99% were from AFT (Bratislava, Slovakia); acetonitrile (ACN) and methanol (MeOH) Chromasolv were from Sigma-Aldrich Laborchemikalien (Seelze, Germany); disodium hydrogen phosphate, ortho-phthadialdehyde and 2-mercaptoethanol were from Merck (Darmstadt, Germany).

The following certified reference materials (CRM) for determination of matrix effect and accuracy were used: Ground corn Zearalenone, (104.5 ± 10) $\mu g \cdot kg^{-1}$; Ground corn Fumonisin, 2.5 $mg \cdot kg^{-1}$, including FB1 (1.6 ± 0.3) $mg \cdot kg^{-1}$, FB2 (0.7 ± 0.2) $mg \cdot kg^{-1}$, and FB3 (0.2 ± 0.1) $mg \cdot kg^{-1}$; Wheat Ochratoxin A, (5 ± 1.5) $\mu g \cdot kg^{-1}$. CRM were from R-Biopharm Rhône (Glasgow, United Kingdom). Deionized water was prepared in Analyst HP (Purite, Thame, United Kingdom).

Preparation of standard solutions

The stock standard solution of DON and ZON with a concentration of 1.0 $mg \cdot ml^{-1}$ was prepared in acetonitrile and methanol, respectively. The working standard solutions of DON were prepared in the mobile phase used in DON analysis in a concentration range of 0.1–4.0 $\mu g \cdot ml^{-1}$. The working standard solutions of ZON were prepared in methanol at concentrations from 0.01 $\mu g \cdot ml^{-1}$ to 1.0 $\mu g \cdot ml^{-1}$. The stock standard solution of FB1 and FB2 was prepared at the primary concentration of 1.26 $mg \cdot ml^{-1}$ for FB1 and 0.25 $mg \cdot ml^{-1}$ for FB2, both in the mixture of acetonitrile : water (50 : 50, v/v). This solution was diluted with the same mixture to the concentrations ranging from 0.019 $\mu g \cdot ml^{-1}$ to 3.14 $\mu g \cdot ml^{-1}$. The stock standard solution of OTA with a concentration of 1.25 $mg \cdot ml^{-1}$ was prepared in a mixture of toluene : acetic acid (99 : 1, v/v). The solution was diluted with the mobile phase used in OTA analysis to OTA concentration ranging from 0.2 $ng \cdot ml^{-1}$ to 42 $ng \cdot ml^{-1}$. All stock standard solutions were stored at –18 °C, the working standard solutions at 4 °C.

Additional solutions

Phosphate-buffered saline (PBS) consisted of 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) and the pH value was adjusted to 7.4 with hydrochloric acid. The solution was filled up to the volume of 1 000 ml.

The derivatization mixture (DM) necessary in the analysis of fumonisins was prepared as follows: 40 mg of ortho-phthaldialdehyde was dissolved in 0.5 ml of methanol and diluted with 2.5 ml of 0.1 $mol \cdot l^{-1}$ disodium tetraborate (3.8 g disodium tetraborate decahydrate in 100 ml of deionized water). Afterwards, 50 μl of 2-mercaptoethanol was added and the mixture was thoroughly mixed. The prepared mixture was stored in an amber glass vial at 4 °C for a maximum of 7 days (after this period, a new mixture was prepared).

Equipment

The device Ultra-Turrax T 25 blender (Janke & Kunkel, IKA-Labortechnik, Staufen i. Br., Germany) and OS-20 orbital shaker (Boechel & Co., Scientific Equipment, Hamburg, Germany) were used for sample extraction. The immunoaffinity columns (IAC) for the clean-up of extracts were obtained from R-Biopharm Rhône – Fumoniprep for FB1/FB2, Easi-Extract Zearalenone for ZON, Ochraprep for OTA and Donprep for DON.

The liquid chromatograph Agilent Technologies 1100 Series (Waldbronn, Germany) was used for chromatographic separation, equipped with a quaternary pump, auto-sampler, diode array detector (DAD) and fluorescence detector (FLD). One type of analytical column was used for all analyses – Zorbax SB C-18, 4.6 × 250 mm i.d., 5 μm conjugated with the pre-column Zorbax SB C-18, 4.6 × 12.5 mm i.d., 5 μm (Agilent Technologies). Technical conditions for chromatographic analysis were adjusted in order to achieve a maximal simplicity of operation and rapid manipula-

tion, e.g. coincident instrument configuration, one analytical column type, almost no replacement of mobile phase containers in a quaternary delivery system (three fixed containers of methanol, acetonitrile, and water; the fourth of acidic water changeable).

The comprehensive description of procedures for sample extraction, clean-up and chromatographic conditions for mycotoxins analyses is in the final Tab. 1 and 2.

Indentification and calculations

Identification of mycotoxins was accomplished by simple comparison of retention times, eventually through absorption spectra in the spotted wavelength range. The original absorption spectra were created using pure standard solutions of mycotoxins, which were then compared with those from cereal samples.

Each sample was analysed in duplicate. The acquired data were processed by chromatographic software Agilent Chem Station (Agilent Technologies). For quantification of concentration c (in $\mu g \cdot kg^{-1}$) in a tested portion, the following equation was used:

$$c = c_{inj} \cdot F \quad (1)$$

where c_{inj} is concentration of mycotoxin assigned from calibration curve in $\mu g \cdot ml^{-1}$ (injection concentration), F is conversion factor including portion of sample taken into analysis in grams, a dilution or concentration sample volume in ml, as well as a coefficient representing the concentration in $\mu g \cdot kg^{-1}$ (F = 250 for OTA and ZON, 625 for FB1

Tab. 1. Final sample preparation procedures for the determination of mycotoxins.

Step of sample preparation	FB1, FB2	OTA	ZON	DON
1. Sample weight	25 g of finely ground sample			
2. Extraction mixture	125 ml ACN : MeOH : water (25 : 25 : 50, v/v/v)	100 ml ACN : water (60 : 40, v/v)	125 ml ACN : water (75 : 25, v/v)	100 ml ACN : water (10 : 90, v/v)
3. Addition of salt to extraction mixture	2.5 g NaCl	None	None	None
4. Blending	1. 341.7 Hz, 2 min (Ultra-Turrax blender) 2. 3.7 Hz, 10 min (orbital shaker)	3.7 Hz, 5 min (orbital shaker)	3.7 Hz, 10 min (orbital shaker)	1. 341.7 Hz, 5 min (Ultra-Turrax blender) 2. 3.7 Hz, 1 h (orbital shaker)
5. Filtration or centrifugation	Paper filter	Paper filter	Paper filter	Centrifugation at 489 *g*, 30 min
6. Volume of the filtrate to be diluted	10 ml	4 ml	20 ml	None
7. Volume of dilution solution	40 ml PBS	44 ml PBS	80 ml PBS	None
8. IAC pre-washing	10 ml PBS	None	None	None
9. Volume of the filtrate applied on IAC	10 ml of the diluted filtrate	48 ml (entire volume)	25 ml of the diluted filtrate	10 ml (group A[a]) 2 ml (group B[b])
10. IAC post-washing	10 ml of PBS	20 ml of PBS	20 ml of water	10 ml of water
11. IAC drying	10 min under vacuum			
12. Elution of mycotoxin from IAC	3 ml MeOH	2 ml MeOH	1.5 ml ACN + 1.5 ml water	2 ml MeOH
13. Evaporation of eluate to dryness	Under vacuum at water bath temperature 50 °C			
14. Reconstitution of dried residue	0.25 ml ACN : water (50 : 50, v/v)	0.25 ml ACN : acidic water (20 ml of glacial acetic acid in one litre of water) (50 : 50, v/v)	0.25 ml ACN : water (60 : 40, v/v)	0.25 ml ACN : water (10 : 90, v/v)

Note: an analytical parameter in grey cells means modification done in our laboratory.
a – processed cereals intended for direct human consumption (cereal bran, flour, pasta, bread including small bakery wares) and cereal-based foods for infants and young children, b – unprocessed wheat, barley, maize and maize-based products.

Tab. 2. Final HPLC conditions for the determination of mycotoxins.

HPLC analysis	FB1, FB2	OTA	ZON	DON
Analytical column type	Zorbax SB-C18, 250 × 4.6 mm, 5μm particle size			
Pre-column type	Zorbax SB-C18, 12.5 × 4.6 mm, 5μm particle size			
Mobile phase	A – MeOH, B – ACN, C – acidic water (70 ml of glacial acetic acid in one litre of water)	ACN : acidic water (20 ml of glacial acetic acid in one litre of water; 50 : 50, v/v)	ACN : acidic water (12 ml of glacial acetic acid in one litre of water; 60 : 40, v/v)	ACN : water (10 : 90, v/v)
Mobile phase elution mode	Gradient	Isocratic	Isocratic	Isocratic
Gradient programme	0 min: 61% A, 5% B, 34% C; 0. – 27. min: 61% A, 5% B, 34% C; 27. – 30. min: 72% A, 5% B, 23% C; 30. – 42. min: 72% A, 5% B, 23% C			
Flow rate	1 ml·min^{-1}	1 ml·min^{-1}	0.5 ml·min^{-1}	1 ml·min^{-1}
Detection	Fluorescence Exc/Em 335/460 nm	Fluorescence Exc/Em 333/460 nm	Fluorescence Exc/Em 274/440 nm	UV-DAD 220 nm
Sample volume for injection	0.04 ml (0.02 ml of sample + 0.02 ml of DM); pre-column derivatization of FB using auto-sampler	0.1 ml	0.02 ml	0.05 ml
Column temperature	25 °C controlled			

Note: an analytical parameter in grey cells means modification done in our laboratory.
Exc – excitation wavelength, Em – emission wavelength, DAD – diode array detection, DM – derivatization mixture, FB – fumonisins (FB1 and FB2).

and FB2, 100 for DON in processed products, 500 for DON in unprocessed products).

Data processing

For validation of the methods, detection and quantification limits, linearity, precision, accuracy and uncertainty were determined.

The limit of detection (*LOD*) and quantification (*LOQ*) were calculated as $X_0 + 3SD$ and $X_0 + 10SD$, respectively, where X_0 was the average response of blank samples analysed in ten replicates and *SD* was the standard deviation of an average response.

The within-laboratory reproducibility (precision) was expressed as repeatability relative standard deviation (RSD_r) of the mean of ten replicates. The precision was assessed using cereal extracts from ground corn for FB1 and FB2, corn flour for ZON, ground wheat for OTA, and ground maize + wheat for DON. The extracts were spiked with mycotoxins standard solution at two levels each (FB1 at 50 μg·kg^{-1} and 800 μg·kg^{-1}, FB2 at 50 μg·kg^{-1} and 500 μg·kg^{-1}, ZON at 15 μg·kg^{-1} and 400 μg·kg^{-1}, OTA at 0.124 μg·kg^{-1} and 4.800 μg·kg^{-1}, and DON at 60 μg·kg^{-1} and 1 000 μg·kg^{-1}).

The measurement uncertainty was evaluated as extended combined uncertainty U_C (multiplied by factor 2) at 95% confidence interval. The estimation of combined uncertainty U_C includes the components arising from systematic effects. It was calculated as association of Type A (U_A) and Type B (U_B) uncertainty as follows:

$$U_C = \sqrt{(U_A{}^2 + U_B{}^2)} \quad (2)$$

where U_A was evaluated from the statistical distribution of the values from a series of measurements ($n = 10$) and was characterized by experimental standard deviation, U_B involved systematic factors which most influence the result and was also expressed by standard deviation (sample weighing, standard and sample dilution, and calibration curve linearity). The measurement uncertainty was considered only for the analytical process, not for sampling.

For the recovery test, an extract of wheat flour was used, spiked with mycotoxins standard at two concentrations of 80 μg·kg^{-1} and 140 μg·kg^{-1} for FB1, 150 μg·kg^{-1} and 345 μg·kg^{-1} for FB2, 75 μg·kg^{-1} and 390 μg·kg^{-1} for ZON, 0.800 μg·kg^{-1} and 4.850 μg·kg^{-1} for OTA, and 60 μg·kg^{-1} and 2010 μg·kg^{-1} for DON in triplicate. The recovery rate (*R*) was calculated according to the following formula, where *c* was the concentration of mycotoxins in μg·kg^{-1}:

$$R\,[\%] = \frac{c_{\text{spiked sample}} - c_{\text{nonspiked sample}}}{c_{\text{added}}} \times 100 \quad (3)$$

Under our conditions, the data for recoveries were not corrected by the recovery factor.

The linearity of the methods was verified by analysing six standard solutions and six CRM extracts (including the blank sample) for each mycotoxin. The concentrations used were in the working range of 16–2000 $\mu g \cdot kg^{-1}$ for FB1 and FB2, 2.5–420 $\mu g \cdot kg^{-1}$ for ZON, 0.010–5.2 $\mu g \cdot kg^{-1}$ for OTA, and 10–2000 $\mu g \cdot kg^{-1}$ for DON. Each concentration was analysed in duplicate. The linearity achieved for pure standard solutions and CRM extracts was tested for difference significance of calibration lines. In this test, the null hypothesis was evaluated by the Chow test. The main criterion $F_{c(m,n-2m)}$ was determined on the basis of squared residuals from linear regression, at $\alpha = 0.05$, following the equation:

$$F_{c(m,n-2m)} = \frac{(SSE - SSE_1 - SSE_2)(n-2m)}{(SSE_1 + SSE_2)m} \quad (4)$$

where, SSE is the error sums of squares when all data were used within the combined model, SSE_1 is the error sums of squares for the first group (pure standard solution) and SSE_2 is the error sums of squares for the second group (CRM); n is the sum of the data of both groups; m is number of parameters tested, including intercept. Linear regression diagnostics was carried out by ADSTAT version 1.25 (TriloByte Statistical Software, Pardubice, Czech Republic).

Samples

In total, 24 cereal products were collected from retails and farms in the vicinity of Bratislava, Slovakia, that involved common cereals and derived cereal products (unprocessed grains, cereal flour, grits, bran, pasta, bread, snacks, etc.) as well as baby wheat-based food. Just prior to analysis, the whole volume of each sample was grounded, homogenized and sub-samples of 25 g were taken for the extraction procedure. Remains of samples were stored in plastic bags at –18 °C. From the bulk of samples, 38% was analysed for fumonisins, 75% for OTA, 71% for ZON and 67% for DON.

RESULTS AND DISCUSSION

Optimization of extraction and clean-up procedures

In order to optimize extraction of mycotoxins from the cereal matrix, various procedures were performed, modifying the conditions previously used and published. The main information sources for sample extraction, including the extract clean-up, were the procedures described in instructions for use of selective IAC (from R-Biopharm Rhône) as well as other literature sources (mentioned below), which were slightly modified in our laboratory. Minimally five independent procedures were tested for each mycotoxin, which comprised variable sample weight, volume and composition of the extraction mixture (EM), as well as extraction duration. The efficacy of particular procedures was assessed on the basis of FLD and DAD responses of mycotoxins which were also compared with the responses of pure substances of mycotoxins. The extraction procedures mentioned, which were verified and modified in our laboratory, are listed in Tab. 3.

The factor that markedly affected the recovery rates of mycotoxins was the proportion of EM (generated from acidified water, methanol and acetonitrile) and its volume used for extraction of the specified sample amount. As can be seen from Tab. 3, application of 50 ml EM volumes resulted in low yields of mycotoxins and, in addition, samples were not easy to filtrate. Following the recoveries achieved, the volumes of 100 ml and 125 ml seemed appropriate for extraction of 25 g sample portions. According to publications [25, 34], addition of sodium chloride to the sample can improve the extraction rate of mycotoxins. By our experience, higher recoveries were reached after application of sodium chloride at fumonisins extraction (increase by 25%), especially when ACN : MeOH : water 25 : 25 : 50 (v/v/v) was used as EM. Methanol and water as constituents of EM, applied together with this salt, led to a low FB1 yield, and to an undetectable concentration of FB2 (Tab. 3).

At DON estimation, more modifications were applied, which covered nearly all variable factors (Tab. 3). As a result, two collateral preparation techniques were adjusted for both processed (group A) and unprocessed cereal foods (group B). In case of DON only, the extraction mixture and mobile phase composition were identical.

As regards the duration of the extraction process, high speed blending of the sample with EM was followed by shaking using an orbital shaker in order to maximize the release of mycotoxins from the cereal matrix. Prolonged shaking was used especially at fumonisins and DON extractions (Tab. 3).

Acetonitrile, methanol and water were applied as elution agents in the clean-up procedure on IAC, either individually or in a mixture. The optimal volume of the elution agent was estimated on the basis of the highest analytical response expressed as recovery rate. As shown in Tab. 4 the results were comparable. The procedure

Tab. 3. Mycotoxins recovery rates achieved by various extraction procedures verified and/or modified in our laboratory.

Mycotoxin	Sample tested	Variable factors						Recovery rate ± *SD* [%]	Source
		SW [g]	EM volume [ml]	EM composition	NaCl addition	Blending	Volume and solvent for MTX elution from IAC		
FB1, FB2	Corn flakes FB1 100 $\mu g \cdot kg^{-1}$; FB2 80 $\mu g \cdot kg^{-1}$	25	125	ACN : MeOH : water 25 : 25 : 50 (v/v/v)	Yes	HS mix 2 min	1.5 ml MeOH + 1.5 ml water	94 ± 9.6 (FB1) 64 ± 9.9 (FB2)	R-Bio [23]
		20	50	ACN : MeOH : water 25 : 25 : 50 (v/v/v)	No	Shake 20 min	3 ml MeOH	31 ± 2.9 (FB1) 0 (FB2)	[24], Lab
		25	100	ACN : MeOH : water 25 : 25 : 50 (v/v/v)	No	HS mix 5 min, shake 10 min		79 ± 9.9 (FB1) 21 ± 1.9 (FB2)	Lab
		25	50	MeOH : water 80 : 20 (v/v)	Yes	HS mix 2 min, shake 10 min		13 ± 0.1 (FB1) 0 (FB2)	Lab
		25	100	MeOH : water 50 : 50 (v/v)	Yes	HS mix 5 min, shake 10 min		19 ± 0.8 (FB1) 0 (FB2)	Lab
		25	125	ACN : MeOH : water 25 : 25 : 50 (v/v/v)	Yes	HS mix 2 min, shake 10 min		105 ± 11.4 (FB1) 81 ± 7.9 (FB2)	[25], Lab
ZON	Corn flakes 35 $\mu g \cdot kg^{-1}$	25	125	ACN : water 75 : 25 (v/v)	No	HS mix 10 min	1.5 ml ACN + 1.5 ml water	90 ± 5.2	R-Bio [26]
		5	25	ACN : water 75 : 25 (v/v)	Yes	Shake 10 min	2.1 ml MeOH + 0.9 ml water	62 ± 3.2	[11], Lab
		20	50	ACN : water 90 : 10 (v/v)	Yes	HS mix 2 min, shake 10 min	1.5 ml MeOH	not detected	[27], Lab
		25	125	ACN : water 90 : 10 (v/v)	Yes	HS mix 3 min, shake 5 min	1.5 ml MeOH + 1.5 ml water	46 ± 1.2	[25], Lab
		25	125	ACN : water 75 : 25 (v/v)	No	Shake 5 min	1.5 ml ACN + 1.5 ml water	88 ± 5.5	R-Bio, Lab
OTA	Ground wheat 4.2 $\mu g \cdot kg^{-1}$	50	200	ACN : water 60 : 40 (v/v)	No	HS mix 2 min	1.5 ml MeOH + 1.5 ml water	89 ± 8.4	R-Bio [28]
		20	100	ACN : water 84 : 16 (v/v)	No	Shake 10 min	1 ml MeOH	31 ± 2.2	[29], Lab
		25	100	ACN : MeOH 60 : 40 (v/v)	No	Shake 5 min	2 ml MeOH	86 ± 10.1	[30], Lab
		50	100	MeOH : water 80 : 20 (v/v)	Yes	Shake 5 min	1.5 ml MeOH + 1.5 ml water	71 ± 4.8	[31], Lab
		25	100	MeOH : 1% $NaHCO_3$ 70 : 30 (v/v)	No	Shake 10 min	2 ml MeOH	69 ± 8.0	[32], Lab
DON group A	Wheat flour 104 $\mu g \cdot kg^{-1}$	25	200	Water	No	HS mix 3 min	1.5 ml MeOH	88 ± 4.5	R-Bio [33]
		25	100	ACN : water 10 : 90 (v/v)	No	HS mix 5 min, shake 1 hour	2 ml ACN	63 ± 4.0	Lab
		25	100	ACN : water 10 : 90 (v/v)	No	HS mix 5 min, shake 1 hour	2 ml MeOH	98 ± 4.0	Lab
DON group B	Ground wheat + maize 448 $\mu g \cdot kg^{-1}$	25	100	Water	No	HS mix 5 min, shake 1 hour	2 ml MeOH	92 ± 1.0	Lab
		25	100	water + 5 g polyethylene glycol	No	HS mix 5 min, shake 1 hour	2 ml MeOH	85 ± 6.5	[5], Lab

SW – sample weight, EM – extraction mixture, MTX – mycotoxin, IAC – immunoaffinity column, HS – high speed, R-Bio – R-Biopharm Rhône procedure, Lab – own modification, *SD* – standard deviation of two measurements ($n = 2$). Group A – processed cereals, group B – unprocessed cereals.

Tab. 4. Recovery rates data obtained for the clean-up procedure using different elution agents.

Mycotoxin	Extraction mixture	Elution agent	Recovery rate±*SD* [%]
FB1, FB2	125 ml ACN : MeOH : water 25 : 25 : 50 (v/v/v)	1.5 ml MeOH + 1.5 ml water[a] 2 ml MeOH 3 ml MeOH[b]	94.9±1.3 (FB1); 63.5±5.2 (FB2) 93.0±2.7 (FB1); 62.1±8.3 (FB2) 99.2±4.3 (FB1); 81.6±5.3 (FB2)
ZON	125 ml ACN : water 75 : 25 (v/v)	1.5 ml ACN + 1.5 ml water[a,b] 1.5 ml MeOH + 1.5 ml water	99.2±8.1 89.9±9.6
OTA	100 ml ACN : water 60 : 40 (v/v)	1.5 ml MeOH + 1.5 ml water[a] 2 ml MeOH[b] 3 ml MeOH	95.0±6.9 101.9±4.9 94.1±8.4
DON	100 ml ACN : water 10 : 90 (v/v)	1.5 ml MeOH[a] 2 ml MeOH[b] 2 ml ACN	88.3±4.2 97.4±5.4 72.0±6.7

SD – standard deviation of two measurements (n = 2). a – elution agent suggested by R-Biopharm Rhône, b – elution agent chosen by our laboratory.

with the highest efficiency was selected for next experiments. Under our conditions, the eluates from IAC were evaporated to dryness in a vacuum evaporator and the residues were solubilized in 0.25 ml of the mobile phase or in another appropriate solvent. This was a distinctive modification of the R-Biopharm procedure, after which the eluates could be directly injected onto the analytical column. The definitive modified conditions for sample preparation are summarized in Tab. 1.

Conditions for analytical separation

In the initial stage, a selected mobile phase composition and flow rate of the mobile phase were verified in experiments with pure mycotoxin substances. Analysing mycotoxins extracted from the cereal matrix, only minor modifications of chromatographic conditions were needed. The definitive chromatographic conditions are summarized in Tab. 2. An isocratic mode of elution was used, followed by fluorescence or diode-array detection. At the determination of fumonisins, gradient elution was employed after pre-column derivatization of fumonisins with ortho-phthaldialdehyde and 2-mercaptoethanol, using a programmed auto-sampler, on the basis of our previous experience [35]. As shown in Figs. 1–4, analytical separation combined with optimized extraction procedures (listed in Tab. 1) did not reveal any substantial co-extractives originating from the cereal matrix. Moreover, any shift of the baseline was minimal in the area of elution time of a specific mycotoxin when the matrix sample was analysed.

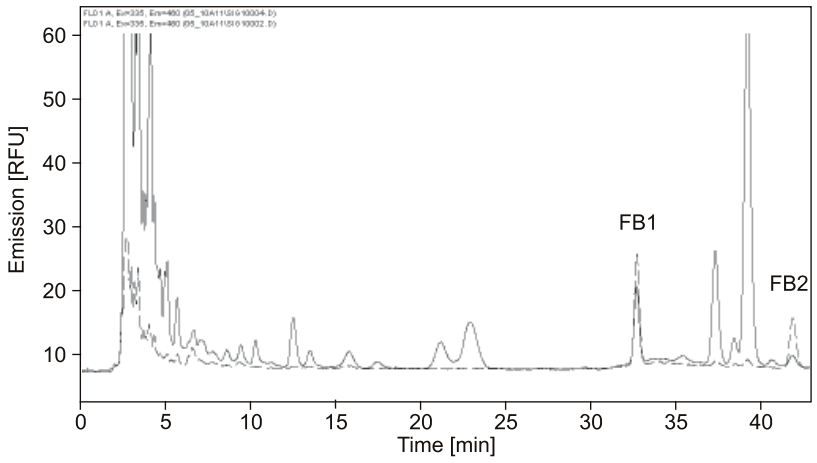

Fig. 1. HPLC-FLD record of a naturally contaminated sample of maize flakes with fumonisins overlaid with standard solution.

Solid line – maize flakes, concentration of FB1 and FB2 of 151 $\mu g \cdot kg^{-1}$ and 52 $\mu g \cdot kg^{-1}$, respectively, dashed line – standard solution, concentration of FB1 and FB2 of 0.314 $\mu g \cdot ml^{-1}$.

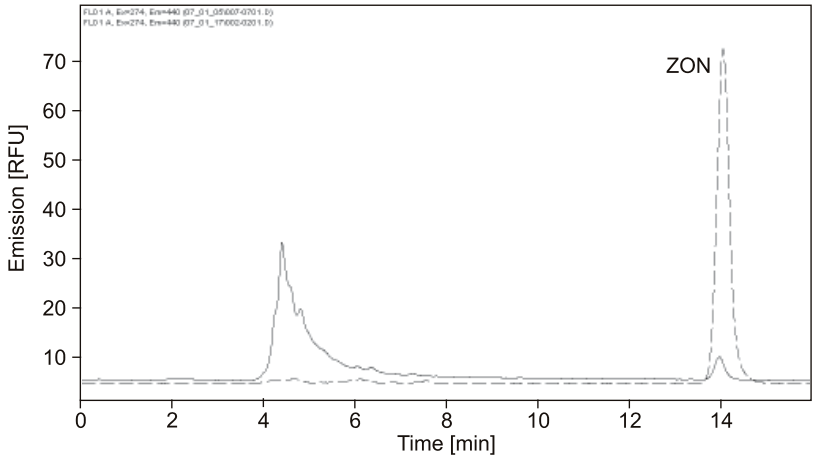

Fig. 2. HPLC-FLD record of a naturally contaminated sample of maize flakes with ZON overlaid with standard solution.
Solid line – maize flakes, ZON concentration of 53.5 $\mu g \cdot kg^{-1}$, dashed line – standard solution, ZON concentration of 0.1 $\mu g \cdot ml^{-1}$.

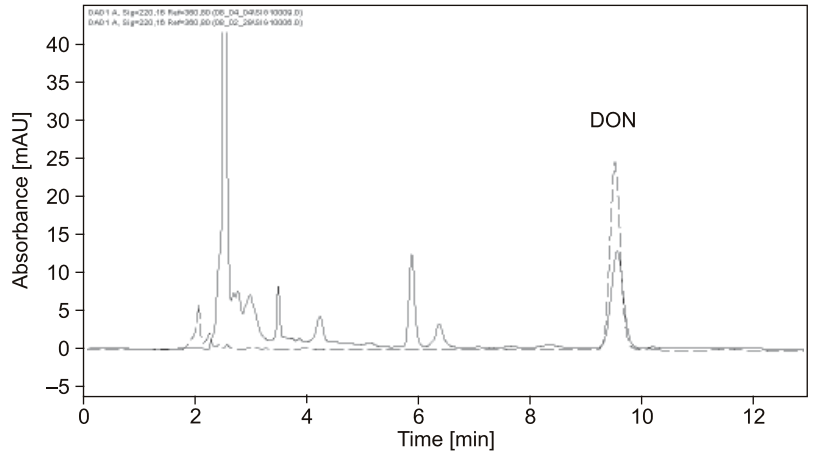

Fig. 3. HPLC-DAD record of a naturally contaminated sample of maize grains with DON overlaid with standard solution.
Solid line – maize grains, DON concentration of 396 $\mu g \cdot kg^{-1}$, dashed line – standard solution, DON concentration of 5.0 $\mu g \cdot ml^{-1}$.

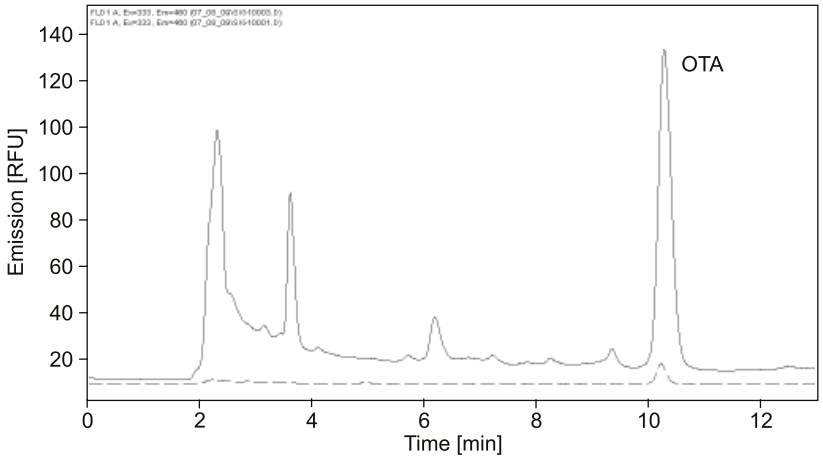

Fig. 4. HPLC-FLD record of a naturally contaminated sample of wheat grains with OTA overlaid with standard solution.
Solid line – wheat grains, OTA concentration of 8.2 $\mu g \cdot kg^{-1}$, dashed line – standard solution, OTA concentration of 0.32 $\mu g \cdot ml^{-1}$.

Tab. 5. Performance characteristics of HPLC methods for the determination of mycotoxins.

Parameter	FB1		FB2		OTA		ZON		DON	
Limit of detection [$\mu g \cdot kg^{-1}$]	41		31		0.014		5.5		30 (A); 55 (B)	
Limit of quantification [$\mu g \cdot kg^{-1}$]	50		48		0.081		12		45 (A); 85 (B)	
Linearity range [$\mu g \cdot kg^{-1}$]	50–1 963		48–1 963		0.081–4.870		12–416		45–1 000 (A); 85–2 000 (B)	
Precision RSD_r [%]	12.3	5.5	8.6	3.9	16.1	15.8	8.8	4.7	13.3 (A,B)	10.9 (A,B)
at level [$\mu g \cdot kg^{-1}$]	50	1 000	50	500	0.124	4.800	15	400	60 (A,B)	1 000 (A,B)
and expanded uncertainty $2U_C$ [%]	45	12	10	23	16	21	15	7	30 (A,B)	33 (A,B)
Recovery rate R [%]	93	87	83	96	92	94	89	103	97	87
at spike level [$\mu g \cdot kg^{-1}$]	80	140	150	345	0.800	4.850	75	390	60	2010
Trueness (using CRM) ± RSD [%]	107 ± 8.4		78 ± 4.7		96.4 ± 15.8		99.3 ± 5.2		not determined	

RSD_r – repeatability relative standard deviation, RSD – relative standard deviation of three measurements ($n = 3$).
A – group A (processed cereals), B – group B (unprocessed cereals),

Performance of methods

The procedures described in this paper were validated for a range of cereal foods and maize. Generally, an accepted concept simultaneously developed by IUPAC [34], Eurachem [36] and the European Union [37] was used for single laboratory validation. The validation data of the methods are listed in Tab. 5.

The *LOD* values recently published for HPLC methods have been in the range of 0.5–100 $\mu g \cdot kg^{-1}$ for DON [4–6], 3–50 $\mu g \cdot kg^{-1}$ for ZON [4, 11], 5–50 $\mu g \cdot kg^{-1}$ for FB1 [4, 13, 16, 17, 25], 5–25 $\mu g \cdot kg^{-1}$ for FB2 [13, 16, 17], and 0.01–0.08 $\mu g \cdot kg^{-1}$ for OTA [7, 8, 25]. The *LOD* values estimated by us fell within the described intervals, except of FB2 (Tab. 5).

In calibration measurements, standard solutions of pure mycotoxin substances were prepared in an appropriate concentration range covered by six calibration points, including the blank sample. Starting at step 2 of Tab. 1, an accurate volume of the standard solution was added to the extraction mixture and the sample was further treated following next steps. Compliant concentrations were also prepared using the CRM extracts (ground maize and wheat) for matrix effect testing.

The linear calibration lines obtained by standard solution and CRM were assessed upon significance of difference by the Chow test. This test is based on examination of null hypothesis and F-distribution of the sum of squared residuals from a linear regression. The main criterion F_c, which is calculated from Eq. 4, should be lower than the critical value F_{crit}, in order to accept the null hypothesis. According to the results presented in Tab. 6, the regression compatibility was achieved only for FB1. The rest of regression relations showed differences either in slope or in intercept.

The precision of methods was determined as within-laboratory reproducibility. Ten extracts were prepared from ground corn for FB1 and

Tab. 6. The results of statistical assessment of conformity of two linear regressions by Chow test.

Data of regression analysis ($y = Ax + B$)		Fumonisin B1	Fumonisin B2	Ochratoxin A	Zearalenone
Slope (A)	Regression 1 (standard solution)	0.001029	0.001461	0.000030	0.000859
	Regression 2 (CRM)	0.001043	0.001589	0.000019	0.000767
	Regression 1+2	0.001075 $m = 2, n = 13$	0.001465 $m = 2, n = 14$	0.000024 $m = 2, n = 9$	0.000852 $m = 2, n = 13$
Intercept (B)	Regression 1 (standard solution)	0.028800	0.023569	–0.004862	–0.008846
	Regression 2 (CRM)	0.035989	0.006292	–0.000698	–0.007314
	Regression 1+2	0.030184 $m = 2, n = 13$	0.026720 $m = 2, n = 14$	–0.002575 $m = 2, n = 9$	–0.012583 $m = 2, n = 13$
Critical value $F_{crit(m,n-2m)}$ [a]		4.26	4.10	5.79	4.26
Testing criterion $F_{c(m,n-2m)}$ [b]		0.414	4.933	14.361	27.582

n – sum of the data of both groups (standard solution + CRM), m – number of parameters tested (including intercept).
a – critical value was drawn from F-distribution with m and $n-2m$ degrees of freedom (table value), b – calculated value of Chow criterion.

Tab. 7. Content of mycotoxins in cereals and cereal-based products available on the market in Slovakia.

Mycotoxin	Cereals	Number of samples analysed / number of positive samples	Min.–max. concentration in positive samples [$\mu g \cdot kg^{-1}$]	Range of EU limits for various cereal food commodities [a] [$\mu g \cdot kg^{-1}$]
OTA	processed	16/8	0.014–0.094	0.5–5.0
	unprocessed	4/1	8.2* mean	
FB1, FB2	processed	7/2	150–195 (FB1); 52–54 (FB2)	200–1 000
	unprocessed	2/0	–	
ZON	processed	13/4	4.7–56*	20–400
	unprocessed	4/1	16.4 mean	
DON	processed	12/11	60–325	200–1 750
	unprocessed	4/4	90–391	

Processed cereals: cereal flour, grits, bran, pasta, bread, snacks, biscuits, instant mush, maize-based products, etc.; unprocessed cereals: wheat, barley, maize. * – over-limit concentration. a – according to the reference [7].

FB2, corn flour for ZON, ground wheat for OTA, and ground maize + wheat for DON. The extracts were spiked with standard solutions at two different concentration levels for each mycotoxin, as indicated in Tab. 5. As can be seen, the relative standard deviations (RSD_r) calculated from ten analyses did not exceed the allowable values stated by the European Council Directive [37].

The accuracy (trueness) of the assay procedures was estimated by two techniques:

1. Analysing the matrix certified reference material (CRM) and expressing the trueness in terms of biases;
2. Spiking wheat flour extract with mycotoxin standard solutions at two concentration levels and calculating the recovery rate for each mycotoxin. In this case, the concentration levels were different from those used in precision estimation.

In both cases, the material was analysed in ten replicates from which the mean value and relative standard deviation (*RSD*) were calculated.

The bias (laboratory bias + method bias) was calculated in percentage as the difference between the experimental result and the reference value. The biases estimated in triplicates of CRM were +7.0% for FB1, –22% for FB2, –0.7% for ZON and –3.6% for OTA (Tab. 5 lists the complying values of trueness). A controversial result was obtained for FB2, which was out of the acceptable limit of ±10% for trueness. Applying the second technique, the recovery rates met the official acceptable range of 50–120% for OTA and 80–110% for FB1, FB2, ZON and DON. The competent values of *RSD* (not listed in Tab. 5) were also within the required interval of ±10% [36, 37].

Survey results

The suggested procedures were applied to the analysis of mycotoxins in cereals and derived cereal products from the market in Slovakia. The primary purpose was to demonstrate the applicability of the extraction and analysis procedures as well as to determine actual levels of the toxins in foods. The results are summarized in Tab. 7. Within the samples analysed, 74% were contaminated with at least one mycotoxin.

FB1 and FB2 were present in all maize samples, though below the EU allowable limit. No fumonisins were detected in foods for children. OTA was found in a fraction of processed products at concentrations between *LOD* and *LOQ*. One over-limit OTA concentration of 8.2 $\mu g \cdot kg^{-1}$ was detected in unprocessed wheat (permitted limit of 5 $\mu g \cdot kg^{-1}$). As reported by Czerwiecki et al. [10], multiple higher amounts of OTA were found in wheat grain samples from conventional farms, varying from 0.6 $\mu g \cdot kg^{-1}$ to 1 024 $\mu g \cdot kg^{-1}$. The frequency of ZON occurrence was 29% in the batch of samples analysed, with 100% incidence in maize samples. DON was the most prevalent mycotoxin in the samples tested. The frequency of sample contamination was 94%, however, DON concentration did not reach the maximal limit. Baby foods were also contaminated with DON, still safely outlying from the legal acceptable limit 200 $\mu g \cdot kg^{-1}$. In general, our results were comparable with the published ones [18–20].

In a majority of samples, natural co-occurrence of mycotoxins was indicated. FB1 and FB2 as well as DON occurred with ZON at determinable concentrations, especially in maize-based products. These results correlate with the observation that

ZON occurs in cereal grains with other *Fusarium* toxins including trichothecenes and fumonisins [38]. Similar situation was observed with infant foods, that contained either ZON or DON along with OTA at concentrations higher than *LOQ*.

CONCLUSIONS

The analytical methods presented in this paper match the general requirements for method performance and are shown to be sensitive, accurate and precise for determination of fumonisin B1 and B2, ochratoxin A, zearalenone and deoxynivalenol in cereals as well as cereal-based products. For application of these methods, classic liquid chromatographic equipment is needed, with the exception of automation of the derivatization procedure by an auto-sampler in fumonisins analysis, which is necessary for reproducible operation. The results of a small survey revealed that cereals and cereal-based foods available in Slovakia are contaminated with mycotoxins at different levels and showed some natural co-occurrence of these toxins.

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