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Inter-laboratory validation of liquid chromatography–tandem mass spectrometry multi-mycotoxin determination in animal feed – method transfer from the reference laboratory to regional laboratories

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Abstract

Introduction: The results are presented of the inter-laboratory validation of a liquid chromatography–tandem mass spectrometry method for the determination of eight mycotoxins (aflatoxin B1, deoxynivalenol, fumonisin B1, fumonisin B2, ochratoxin A, toxin T-2, toxin HT-2 and zearalenone) in animal feeds. **Material and Methods:** This study was an essential part of the method's transfer from the National Reference Laboratory to six regional laboratories in Poland working in the official survey of mycotoxins in feed. The laboratories received a batch of standard solutions, blank samples and quality control materials on which to perform analysis with one procedure and different liquid chromatography–tandem mass spectrometry conditions. **Results:** The validation results show good precision (reproducibility coefficient of variation 3.7–20.5%) and accuracy of the method (recovery 89–120% and trueness 94–103%) and sufficient skills of the laboratory personnel. **Conclusion:** The study is an example of the successful transfer of the method among laboratories.

Keywords: mycotoxins, mass spectrometry, feed, validation, inter-laboratory comparison.

Introduction

The xenobiotic contamination of food of animal origin often has a source in the animal's feed. Mycotoxins seem to be some of the most challenging among the wide range of hazardous and toxic compounds. These compounds produced by moulds in specific environmental conditions affect animal and human health and cause significant economic losses. Carcinogenicity, hepatotoxicity, genotoxicity, oestrogenicity, nephrotoxicity, and other harmful effects as well as the causation of reproductive disorders are related to mycotoxin intake (14).

The adverse effects of mycotoxins can be intensified by their co-occurrence in food and feed (10). In animal food production and husbandry, feed contamination with mycotoxins can play an important role. Main food animals like swine, bovines and poultry are susceptible to the presence of mycotoxins in feed (9, 16, 27). The toxins affect animal health, cause suffering and decrease production efficiency. Another consequence of such animal feed contamination is the transfer of some mycotoxins to food, particularly aflatoxin M1 to milk and ochratoxin A to tissues (12, 21). Multi-year studies conducted in various countries showed a significant percentage of feed samples to be contaminated with mycotoxins. Specific toxins occur to degrees varying by latitude. While in Europe deoxynivalenol and zearalenone are the most common, in Asia aflatoxin B1 dominates, and in the Americas a high percentage of fumonisins is found in feed (22).

For the above reasons, maximum levels (for aflatoxin B1) or recommended levels of mycotoxins in animal feed were established. For example,

the European Union regulations require the determination of eight mycotoxins (aflatoxin B1, deoxynivalenol, fumonisin B1 and B2, ochratoxin A, toxin T-2 & HT-2, and zearalenone) in different feed samples (4, 5, 6, 8). It is worth noting that other regions or organisations use different limits, recommended concentrations, or guidance values for mycotoxins in feed, which shows that balance between consumer health (humans and animals) and feed (and grain) producers interests is hard to find. Despite no consensus existing about limit values, most countries agree that official monitoring needs to be conducted of the mycotoxin in food and feed and have implemented appropriate programmes using different analytical techniques.

The feed heterogeneity and the diversity of mycotoxins' chemical properties make the analysis challenging. For many years, liquid chromatography with fluorescence or UV detection was the gold standard in mycotoxin control. Most of the published and normalised methods were based on immunoaffinity clean-up (24, 25, 26), and performed acceptably in specific, single-analyte analysis. Nowadays, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) makes multi-analyte analysis possible with high sensitivity and good accuracy and precision. Acceptable validation results can be achieved with generic sample preparation, often based only on sample extraction and dilution (1). Matrix effects, often very high, have to be compensated for by the use of labelled internal standards. In most cases, validation of the multi-mycotoxin method can be performed as a single-laboratory experiment based on the analysis of a series of spiked samples (19).

The official monitoring performed by laboratories has to be based on reliable methods. If the analytical norms are unavailable, in-house developed methods have to be used. The role of National Reference Laboratories (NRL) is the development of analytical methods and their transfer to regional laboratories. In most cases, an NRL organises the training for regional laboratories, and after the implementation period, a proficiency test is undertaken. Another way of method transfer is inter-laboratory validation. Some methods for mycotoxin were verified in this way, but most of them were single-analyte methods, *e.g.* for aflatoxin M1 (11) or single-mycotoxin-group methods *e.g.* for aflatoxins (28) or fumonisins (24). In this particular case, it was decided for the Polish NRL at the National Veterinary Research Institute (NVRI) to verify regional laboratories' performance of a multi-analyte method to determine mycotoxins in the feed.

Material and Methods

Reagents, standards and standards solutions used by the Polish NRL. Acetonitrile (ACN, analytical grade), methanol (MeOH, LC-MS grade), and acetic acid were supplied by JT Baker (part of Avantor Performance Materials, Deventer, the Netherlands). Formic acid and ammonium acetate (LC/MS grade) were purchased from Sigma-Aldrich (Schnelldorf, Germany). The water was purified with a Milli-Q apparatus (Millipore Sigma, Burlington, MA, USA). The standards of aflatoxin B1 (AF B1), deoxynivalenol (DON), toxins T-2 (T-2) and HT-2 (HT-2), ochratoxin A (OTA), fumonisin B1 (FB1) and B2 (FB2), zearalenone (ZEN) and isotopically labelled internal standards (aflatoxin B1 $^{13}C_{17}$ (AF B1-IS), deoxynivalenol $^{13}C_{15}$ (DON-IS), toxin T-2 $^{13}C_{24}$ (T-2-IS), toxin HT-2 $^{13}C_{22}$ (HT-2-IS), ochratoxin A $^{13}C_{20}$ (OTA-IS), fumonisin B1 $^{13}C_{34}$ (FB1-IS), and zearalenone $^{13}C_{18}$ (ZEN-IS) were obtained from Sigma-Aldrich and stored according to their manufacturer's recommendations. Primary standard stock solutions of DON, ZEA, T-2, HT-2, and all internal standards were prepared in acetonitrile, those of AF B1 and OTA in methanol, and FB1 and FB2 solutions in 50% ACN.

Laboratories seeking method validation also purchased and prepared a mixture of internal standards of the compounds to be tested for on their own, as shown in Table 1. Each laboratory was also instructed to prepare the FB1 and FB2 reference solutions separately at 5 µg/mL on site. The working solutions, designated MIX6 validation level 1(1VL) for standards (Table 2), MIX2 for FB1FB2 VL and MIX7 IS for internal standards were stored at 2–8°C and were stable for at least three months.

Table 1. Isotopically labelled mycotoxin internal standard mixture designated MIX7 IS

Internal standard	Concentration	Solvent	Final concentration
AF B1 $^{13}C_{17}$	0.5 µg/mL		0.02 µg/mL
DON $^{13}C_{15}$	25 µg/mL		1 µg/mL
FB1 $^{13}C_{34}$	25 µg/mL		1 µg/mL
OTA $^{13}C_{20}$	10 µg/mL	acetonitrile	0.4 µg/mL
ZEN $^{13}C_{18}$	25 µg/mL		1 µg/mL
T-2 $^{13}C_{24}$	25 µg/mL		1 µg/mL
HT-2 $^{13}C_{22}$	25 µg/mL		1 µg/mL

AF B1 – aflatoxin B1; DON – deoxynivalenol; FB1 – fumonisin B1; OTA – ochratoxin A; ZEN – zearalenone; T-2 – toxin T-2; HT-2 – toxin HT-2

Experimental procedure. The original method was developed in the European Union Reference Laboratory for Mycotoxins in Food and Feed (Joint Research Centre, Geel, Belgium) and validated in inter-laboratory comparison in 2016 as a part of the preparation of the EN 17194:2019 standard (7). This method was modified by the authors in the NVRI and transferred to regional labs. The six regional laboratories of the Veterinary Inspectorate in Poland (Białystok, Bydgoszcz, Katowice, Kraków, Szczecin and Wrocław) took part in the inter-laboratory validation. Including the NVRI laboratory, all laboratories were randomly numbered from 1 to 7.

Table 2. Mycotoxin standard mixture designated MIX6 1VL

Analyte	Concentration (µg/mL)	Volume of solution (mL)	Concentration in solution (µg/mL)	Solvent	Final volume (for one lab)
AF B1	1	1	0.1	acetonitrile	10 mL
DON	100	1.8	18		
OTA	10	1	1		
ZEN	10	2	2		
T-2	10	1	1		
HT-2	10	1	1		

AF B1 – aflatoxin B1; DON – deoxynivalenol; OTA – ochratoxin A; ZEN – zearalenone; T-2 – toxin T-2; HT-2 – toxin HT-2

Table 3. The list of minor changes to the sample preparation

	Sample weight	Volume of the extraction	Extraction mixture	Volume of the extract taken for evaporation	Reconstitution of the dry residue	Centrifugation
Laboratory 1	1g	4 mL	acetonitrile:water:formic acid (79:20:1, v/v/v)	0.1 mL	0.05 mL of methanol and 0.05 mL of water	+
Laboratory 2	1g	4 mL	acetonitrile:water:formic acid (48:50:2, v/v/v)	0.1 mL	0.05 mL of methanol and 0.05 mL of 0.01 M ammonium acetate + 0.1% acetic acid	+
Laboratory 3	1g	4 mL	acetonitrile:water:formic acid (79:20:1, v/v/v)	0.1 mL	0.1 mL of mobile phase B	–
Laboratory 4	1g	4 mL	acetonitrile:water:formic acid (79:20:1, v/v/v)	0.1 mL	0.1 mL of mobile phase B	+
Laboratory 5	1g	4 mL	acetonitrile:water:formic acid (79:20:1, v/v/v)	0.1 mL	0.05 mL of methanol and 0.05 mL of 0.01 M ammonium acetate + 0.1% acetic acid	+
Laboratory 6	1g	4 mL	acetonitrile:water:formic acid (79:20:1, v/v/v)	0.2 mL	0.2 mL of mobile phase B	+
Laboratory 7	1g	4 mL	acetonitrile:water:formic acid (79:20:1, v/v/v)	0.1 mL	0.05 mL of methanol and 0.05 mL of 0.01 M ammonium acetate + 0.1% acetic acid	+

After a two-day training session for personnel at the NVRI, the laboratories received the standards for spiking and three blank samples. After three months of method development in the laboratories, the preliminary study results were sent to the NVRI and used to qualify these laboratories for further validation.

Sample preparation. A 1 g mass of previously ground feed was placed into a 50 mL polypropylene tube and extracted with a mixture (4 mL) of acetonitrile:water:formic acid (79:20:1, v/v/v) using a vertical shaker (200 cycles/min) for 30 min. The samples were centrifuged at 3,500 rpm for 15 min. Next, 0.01 mL of the internal standard mixture was added to 0.1 mL of supernatant and samples were evaporated to dryness in a stream of nitrogen at 40°C. The dry residue was reconstituted in 0.05 mL of mobile phase A and 0.05 mL of mobile phase B. The sample was centrifuged in the 1.5 mL Eppendorf tubes at 14,000 rpm for 30 min and transferred to autosampler vials. The laboratories were allowed to modify the procedure, making minor changes after consultation (Table 3). One of the laboratories changed the proportion of constituents in the extraction mixture (from acetonitrile:water:formic acid 79:20:1, v/v/v to acetonitrile:water:formic acid 48:50:2, v/v/v). Another changed the volume of the evaporated extract, and a further laboratory changed the mixture of the sample reconstitution.

Instrumental parameters. The NRL used the following conditions: detection and quantification were performed with a Nexera X2 system with an LCMS-8050 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan), which was operated in positive and negative electrospray modes, with Q1 and Q3 resolution of 1 unit, nebulising gas flow of 2 L/min, heating gas flow of 10 L/min, drying gas flow of 10 L/min, interface temperature of 300°C, desolvation line temperature of 250°C and heat block temperature –

400°C. Two multiple reaction monitoring (MRM) transitions for each analyte were monitored. Lab Solution software was used for the analysis. Chromatographic separation was performed at 40°C on a Kinetex Biphenyl column of 100 × 2.1 mm for 2.6 µm particle size, coupled with a Biphenyl security guard cartridge (all from Phenomenex, Torrance, CA, US) using 0.3 mL/min of constant flow. The separation was performed using a gradient elution of mobile phase A consisting of 0.01 M ammonium acetate and 0.1% of acetic acid in water/MeOH (95:5, v/v) and mobile phase B consisting of 0.01 M ammonium acetate and 0.1% of acetic acid in water/MeOH (5:95, v/v). Separation was performed in the following gradient conditions: (1) 0–9 min linear gradient to 95% solvent B; (2) 9–13.1 min isocratic step at 95% solvent B; (3) reconditioning for 2.9 min with the initial composition of the mobile phase. The injection volume was 5 µL, and the duration of a single run was 16 min. The detailed conditions of the MS/MS analysis applied in the NVRI laboratory are presented in Table 4.

Most of the changes were to LC-MS/MS conditions such as the analytical column, gradient profile, mobile phase, or brand of the liquid chromatograph and mass spectrometer (Table 5). All the laboratories used core-shell columns (with C18 or Biphenyl). The mobile phase for most laboratories consisted of methanol (except one, where acetonitrile was used) with ammonium acetate or formic acid solutions.

Validation. Each laboratory received a standard mixture of mycotoxins designated MIX6 1VL, which was needed to produce enriched samples at validation levels of 0.5 VL, 1 VL and 1.5 VL (Table 6) and to plot a six-point calibration curve (Table 7). This solution did not contain fumonisins B1 and B2 because of their low stability. Each laboratory was instructed to prepare a reference solution of FB1 and FB2 at 5 µg/mL on site.

Each laboratory received four pre-ground blank samples of feed (15 g of each) designated P1, P2, P3 and P4 which had been previously tested by triple analysis of each sample for the presence of mycotoxins with an LC-MS/MS method. Because it is currently challenging to find a mycotoxin-free feed sample owing to the low limits of detection of LC-MS/MS methods, samples containing small amounts of DON and ZEN were used for validation (Table 8).

Table 4. The tandem mass spectrometry parameters of mycotoxin analysis applied by the Polish National Veterinary Research Institute (NVRI)

Analyte	Parent ion	Daughter ions	Dwell time (msec)	Collision energy
		Positive ionization		
Aflatoxin B1	$(M+H)^+$ 313	**285***, 241, 269	52, 52, 52	−23, −38, −32
Aflatoxin B1 (IS)	$(M+H)^+$ 330	301	79	−24
Fumonisin B1	$(M+H)^+$ 722	**352**, 334	65, 65	−37, −41
Fumonisin B2	$(M+H)^+$ 706	**336**, 318	65, 65	−37, −40
Fumonisin B1 (IS)	$(M+H)^+$ 756	357	42	−42
Ochratoxin A	$(M+H)^+$ 404	**239**, 358	65, 65	−24, −15
Ochratoxin A (IS)	$(M+H)^+$ 424	250	133	−24
T-2 toxin	$(M+Na)^+$ 489	**327**, 387	42, 42	−24, −22
T-2 toxin	$(M+NH_4)^+$ 484	**215**, 305	65, 65	−20, −16
T-2 toxin (IS)	$(M+NH_4)^+$ 508	198	65	−23
HT-2 toxin	$(M+Na)^+$ 447	**345**, 285	65, 65	−20, −21
	$(M+NH_4)^+$ 442	**263**, 215	65, 65	−14, −14
HT-2 toxin (IS)	$(M+NH_4)^+$ 464	278	65	−14
		Negative Ionisation		
Zearalenone	$(M-H)^-$ 317	**175**, 131	71, 71	25, 30
Zearalenone (IS)	$(M-H)^-$ 335	140	71	31
Deoxynivalenol	$(M-CH_3COO)^-$ 355	295, **265**, 59	247, 247, 247	11, 16, 20
Deoxynivalenol (IS)	$(M-CH_3COO)^-$ 370	310	247	11

* – bold ions were used for quantitation analysis

Table 5. Liquid chromatography–tandem mass spectrometry conditions used in inter-laboratory validation

	Liquid chromatograph	Column	Mobile phase	Flow rate temperature	Mass spectrometer
Laboratory 1	Eksigent ekspert ultra LC 100-XL	Phenomenex Kinetex Biphenyl, 50 × 2.1 mm, 2.6 µm	A – methanol B – 2 mM ammonium formate + 0.1% formic acid	0.35 mL/min 40°C	Sciex 5500
Laboratory 2	Agilent 1260	Agilent Poroshell 120 EC-C18, 4.6 × 50 mm, 2.7µm	A – 0.05 mM ammonium formate + 0.1% formic acid B – methanol + 0.05 mM ammonium formate + 0.1% formic acid	0.5 mL/min 40°C	Sciex 5500
Laboratory 3	Agilent 1200	Agilent Poroshell 120 EC-C18, 3.0 × 50 mm, 2.7 µm	A – methanol (5%) + 0.02 M ammonium acetate + 0.1% acetic acid, 0.25% ammonium fluoride (95%) B – methanol (95%) + 0.02 M ammonium acetate, 0.1% acetic acid, 0.25% ammonium fluoride (5%)	0.8 mL/min 30°C	Agilent 6410
Laboratory 4	Shimadzu Prominence	Phenomenex Kinetex Biphenyl 2.6 µm, 100 × 2.1 mm	A – 0.01 M ammonium acetate + 0.1% of acetic acid/ methanol (95:5, v/v) B – 0.01 M ammonium acetate + 0.1% of acetic acid/methanol (5:95, v/v)	0.3 mL/min 40°C	Shimadzu LCMS-8040
Laboratory 5	Agilent 1260	Agilent Poroshell 120 EC-C18, 4.6 × 50 mm, 2.7µm	A – 1 mM ammonium formate + 0.1% formic acid B – methanol + 0.1% formic acid + 1 mM ammonium formate	0.8 mL/min 40°C	Sciex 4500
Laboratory 6	Agilent 1260	Agilent Poroshell 120 EC-C18, 3.0 × 50 mm, 2.7 µm	A – methanol/0.02 M ammonium acetate + 0.1% acetic acid, (5:95, v/v) B – methanol/0.02M ammonium acetate + 0.1% acetic acid, (95:5, v/v)	0.8 mL/min 30°C	Agilent LC/MS 6460
Laboratory 7	Shimadzu Nexera X2	Phenomenex Kinetex Biphenyl 2.6 µm, 100 × 2.1 mm	A – 0.01 M ammonium acetate + 0.1% acetic acid/methanol (95:5, v/v) B – 0.01 M ammonium acetate + 0.1% of acetic acid/methanol (5:95, v/v)	0.3 mL/min 40°C	Shimadzu LCMS-8050

Table 6. Scheme of the inter-laboratory validation

Samples	Laboratory						
	1	2	3	4	5	6	7
Calibration curve (sample P1, n = 6)	(blank, 0.25 × VL, 0.5 × VL, 1.0 × VL, 1.5 × VL and 2.0 × VL)						
Repeatability (sample P1, n = 6)	0.5 × VL	1.0 × VL	1.5 × VL	0.5 × VL	1.0 × VL	1.5 × VL	
Reproducibility + recovery (sample P2, n = 2; P3, n = 2; P4, n = 2)	1.5 × VL	1.0 × VL	0.5 × VL	1.5 × VL	1.0 × VL	0.5 × VL	
Trueness (quality control material QCM, n = 3)	all labs						

VL – validation level

Table 7. Levels of analytes in calibration curve (µg/kg)

Analyte	0.25 × VL	0.5 × VL	1.0 × VL	1.5 × VL	2.0 × VL
AF B1	1.25	2.5	5	7.5	10
DON	225	450	900	1350	1800
FB1	62.5	125	250	375	500
FB2	62.5	125	250	375	500
HT-2	12.5	25	50	75	100
OTA	12.5	25	50	75	100
T-2	12.5	25	50	75	100
ZEN	25	50	100	150	200

VL – validation level; AF B1 – aflatoxin B1; DON – deoxynivalenol; FB1 – fumonisin B1; FB2 – fumonisin B2; HT-2 – toxin HT-2, OTA – ochratoxin A; T-2 – toxin T-2; ZEN – zearalenone

Table 8. Mycotoxin contamination of blank samples

Feed sample code	Type of feed	DON (µg/kg)	ZEN (µg/kg)
P1	Fish feed	~50	~20
P2	Broiler feed	~25	-
P3	Fish feed	-	-
P4	Swine feed	-	~20

DON – deoxynivalenol; ZEN – zearalenone

It was considered that relatively low concentrations of mycotoxins in selected samples would not significantly impact the validation carried out. At the same time as they received the feed samples, laboratories also received a quality control sample (QC), which was a material produced and evaluated by Fapas (Mycotoxins in animal feed, Test 04303; Fapas, Food and Environment Research Agency, York, UK) containing the following concentrations of mycotoxins: 16.9 µg/kg of aflatoxin B1, 1028 µg/kg of deoxynivalenol, 46.7 µg/kg of ochratoxin A and 661 µg/kg of zearalenone. These concentrations were values attributed to the sample from the results of Fapas proficiency tests.

The aim of the inter-laboratory validation study was an evaluation of the transferred method by calculation of linearity (calibration curves), precision (repeatability and reproducibility) and accuracy (trueness. *i.e.* spiked samples analysis in the case of QC analysis and recovery) in each of the six participating laboratories. The VLs used in the inter-laboratory study are presented in Table 6. The six-point matrix calibration curve was performed by spiking P1 sample with a mycotoxin standard mixture, and comprised blank samples and 0.25 × VL, 0.5 × VL, 1.0 × VL, 1.5 × VL and 2.0 × VL.

The repeatability test was performed by analysis of P1 spiked samples (n = 6) at one level and its results were expressed as percentages of relative standard deviation (RSD_r). The reproducibility test was performed by analysis of P2, P3 and P4 spiked sample (n = 2) at one level and analysis of P2, P3 and P4 as blank (n = 1) and its results were also expressed as percentages of relative standard deviation (RSD_R). The recovery of the method was calculated as the ratio between concentration determined in the reproducibility study and the spiking level. This parameter was expressed as a percentage (Rec). It was calculated for all analytes. Trueness was calculated based on the difference between the obtained concentration in the QCM material analysis and the QCM assigned values. It was expressed as bias in a per cent (Tr). These parameters were calculated for aflatoxin B1, deoxynivalenol, ochratoxin A and zearalenone. Uncertainty calculation was based on a top-down approach that used laboratory performance data (validation with the MUkit (Measurement Uncertainty Kit) (17), which is a measurement uncertainty software application in which calculations are based on the Nordtest TR537 handbook (15).

Quantitative analysis and criteria for the identification of mycotoxins. The analyte concentration in the sample was calculated using a matrix calibration curve showing the relationship of the peak area ratio of a more intensive fragmentation reaction of the analyte (quantitative ion) to the peak area of the internal standard in the enriched sample. The calculated concentrations (area or peak height) was required to be within the reference curve's following range. Based on document SANTE/12089/2016 (23) which served as the guide for Ok *et al.* (18), the criteria for identifying mycotoxins were applied in feed. Qualitative analysis was performed by comparing peak retention times on the chromatogram in test samples with retention times of the peaks in standard solutions. The retention time of the internal standard was expected to be within the tolerance range ± 0.05 min relative to the appropriate standard. Any change in retention times of mycotoxins for the test sample was expected to be within a tolerance of ± 0.1 min concerning the retention times of the standard mycotoxins solution. Identification was additionally confirmed by selecting at least two characteristic fragmentary ions: comparison was made of the ion ratio of two characteristic MRM transitions of analyte in the real sample to that of analyte in the standards solution. Requirements for identification are described in document SANTE/12089/2016 (23). Ion ratio differences should not exceed 30% (relative).

Statistical analysis. The results were statistically evaluated with the Grubbs test and one-way analysis of variance test. The Horwitz ratio (HorRat) was used to evaluate the acceptability of analysis methods concerning inter-laboratory precision (13).

Results

The calibration curves from the matrix proved to be linear over the whole concentration range for all analytes in all laboratories. Values greater than 0.99 were reached by the regression factor of the calibration curve from the matrix reached, and the low contamination of the blank sample did not influence the method's performance. Low values of the coefficient of variation RSD (0.29–10.7%) confirmed the results of the repeatability test. Low RSDr values (3.76–20.5%) were also obtained in the reproducibility tests. The HorRat parameter was lower than 1, which confirmed the high precision of the applied test procedure, and in

many cases, its value was lower than 0.5, which was proof of the excellent training and extensive experience of the personnel at the participating laboratories (15). The accuracy of the method, expressed as recovery, reached high values, in the range of 89–120% (Tables 9 and 10).

Analysis of quality control material showed the sufficient accuracy of the method and the good performance of the laboratories (Fig. 1). The results obtained as the range of QCM uncertainty declared by laboratories complied with the material datasheet. The overall precision (coefficient of variation (CV), %) and recovery (%) were CV = 8.5%, Rec = 95% for AF B1; CV = 6.5%, Rec = 94% for DON; CV = 12.6%, Rec = 103% for ZEN; and CV = 10.4%, Rec = 101% for OTA.

Table 9. Validation results for aflatoxin B1 (AFB1), deoxynivalenol (DON), zearalenone (ZEN) and ochratoxin A (OTA)

	AF B1			DON			ZEN			OTA		
VL (µg/kg)	2.5	5	7.5	450	900	1350	50	100	150	25	50	75
X (µg/kg)	2.40	5.08	7.24	401	883	1217	52.2	107	148	23.2	48.8	71.4
Rec (%)	95.9	102	96.5	89.0	98.1	90.2	104	107	98.7	92.9	97.6	95.2
RSD_R (%)	3.76	5.13	5.41	9.17	5.76	7.48	16.4	10.6	6.67	5.92	8.90	6.51
PRSD	39.4	35.5	33.4	18.0	16.3	15.3	25.1	22.6	21.3	27.9	25.1	23.6
HORRAT	0.1	0.1	0.2	0.5	0.4	0.5	0.7	0.5	0.3	0.2	0.4	0.3
U (%; k = 2)		15			17			35			25	

VL – validation level; X – concentration; Rec – recovery; RSD_R – reproducibility; PRSD – predicted relative reproducibility of standard deviation; HORRAT – Horwitz ratio; U – extended uncertainty

Table 10. Validation results for toxin HT-2 (HT-2), toxin T-2 (T-2), fumonisin B1 (FB1) and fumonisin B2 (FB2)

	HT-2			T-2			FB1			FB2		
VL (µg/kg)	25	50	75	25	50	75	125	250	375	125	250	375
X (µg/kg)	23.9	51.4	71.2	23.7	52.7	78.8	127	300	363	126	263	359
Rec (%)	95.6	103	94.9	94.6	105	105	101	120	96.8	101	105	95.8
RSD_R (%)	14.0	9.54	9.92	11.6	6.82	14.4	5.45	8.22	4.44	8.32	20.5	8.30
PRSD	27.9	25.1	23.6	27.9	25.1	23.6	21.9	19.7	18.5	21.9	19.7	18.5
HORRAT	0.2	0.4	0.3	0.2	0.4	0.3	0.2	0.4	0.2	0.4	1.0	0.4
U (%, k = 2)		28			23			48			59	

VL – validation leve;l X – concentration; Rec – recovery; RSD_R – reproducibility; PRSD – predicted relative reproducibility of standard deviation; HORRAT – Horwitz ratio; U – extended uncertainty

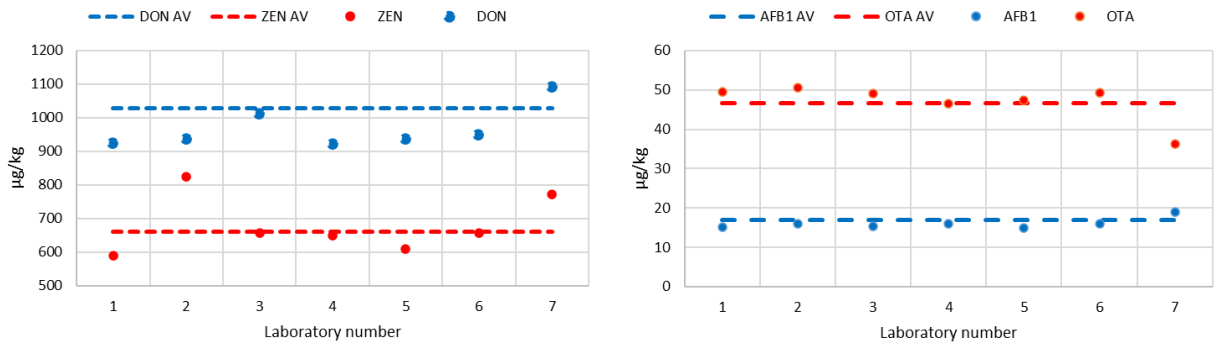

Fig. 1. Individual results of analysis of quality control materials
DON AV – average deoxynivalenol; ZEN AV – average zearalenone; ZEN – zearalenone; DON – deoxynivalenol; AF B1 AV – average aflatoxin B1; OTA AV – average ochratoxin A; AF B1 – aflatoxin B1; OTA – ochratoxin A

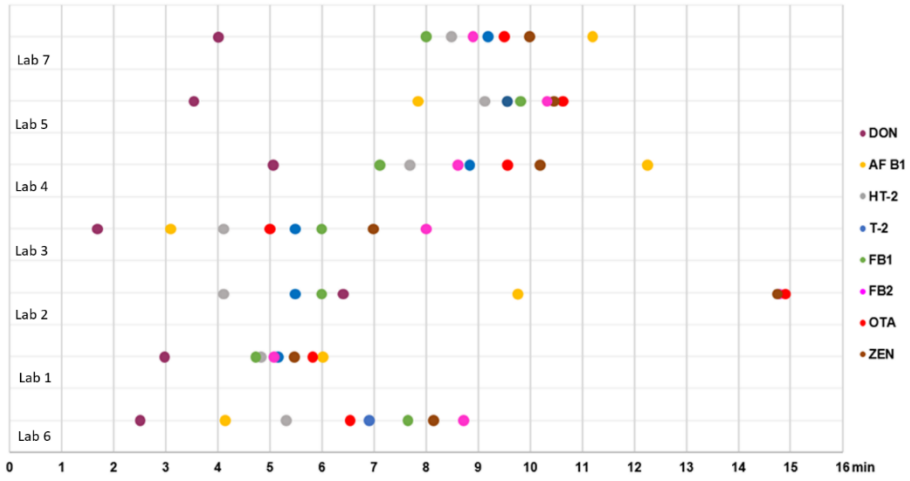

Fig. 2. Retention times of mycotoxins in LC-MS/MS analysis performed by participants and the NVRI
DON – deoxynivalenol; AF B1 – aflatoxin B1; HT-2 – toxin HT-2; T-2 – toxin T-2; FB1 – fumonisin B1; – FB2 – fumonisin B2; OTA – ochratoxin A; ZEN – zearalenone

The changes in sample preparation and LC-MS/MS analysis in laboratories did not affect the performance of the method and showed its good robustness. However, the choice of the chromatographic conditions has a significant influence on the retention order of analytes. As an example, aflatoxins B1 was eluted as a last analyte with (bi)phenyl columns and as second (after the deoxynivalenol) when the C18 column was used (Fig. 2).

Discussion

In this study, the scheme of inter-laboratory validation was organised for six regional laboratories of the Veterinary Inspectorate in Poland. We chose a batch of low contaminated feed samples and decided to use a spiking experiment in the calibration, recovery and precision tests. The laboratories also received Fapas quality control material to verify the trueness of the method.

It is worth noting that the scheme of inter-laboratory validation is not standardised. In the described study, we developed an original scheme of validation based on a calculation of validation parameters (precision and accuracy) from the results of all laboratories. Each laboratory had to analyse a series of spiked sample at three different levels and calculate the concentration from the matrix calibration curve. Additionally, laboratories received a sample of reference material with an unknown amount of mycotoxins. The scheme of the inter-laboratory validation study was designed to minimise the number of determined samples per laboratory and obtain enough validation data to characterise the procedure. For this reason, each laboratory analysed 20 samples overall in a batch: 5 samples for the calibration curve (P1), 6 samples for the repeatability study (P1), 6 samples for reproducibility and recovery (P2, P3, P4) and 3 samples as quality control material (QCM). Such a simple design makes one-day sample preparation of the batch possible in the laboratory and shortens the instrumental analysis.

The VL were chosen based on the concentration of aflatoxin B1 and the guidance values recommended by the European Union (3, 6). The wide range of feed levels for different animal species suggested that the authors decide to choose the lowest value for a mycotoxin. The exceptions were fumonisin B1 and B2, for which the authors decided to choose VL = 250 µg/kg. The reason for the choice was the high guidance value for these mycotoxins (5–60 mg/kg) and, in consequence, the large amounts of expensive standards required for a validation experiment. Moreover, the animal feed survey results in Poland (13) show a relatively low mean concentration of fumonisins contamination (in the range of 10–600 µg/kg).

One of the essential tasks of an NRL working in the food and feed safety area is a transfer of the methods (described as standard operating procedures) to regional laboratories. The transfer involves training in the procedure scheme and verifying the regional laboratory's performance, the latter of which proficiency testing can provide. A less frequently used option is organising inter-laboratory validation. Such a study is much more complicated and time-consuming, but it has added value: not only does the NRL receive information about laboratory performance but also about the characteristics of the method itself. Such validation parameters as reproducibility or ruggedness/robustness are better estimated thanks to inter-laboratory comparisons.

Published reviews show the different approaches to the scheme and range of inter-laboratory validation. It was undertaken most often for 8–14 participants (2, 8, 11) but was also for 3 (28). Samples for validation

studies were often naturally contaminated (15) and also often spiked with standards (18). In some studies, the organisers also sent certified reference materials to verify the trueness of the validated method (24, 28).

The results of the inter-laboratory validation obtained were within the limits indicated in the criteria for determining mycotoxins in feed (3, 6). These criteria differ for different mycotoxins, and their levels are currently under discussion (20). New, stricter criteria for repeatability and within laboratory reproducibility were proposed (RSD < 25%) for European Union Reference Laboratories (draft document). The results of the inter-laboratory validation reported here were also compliant with these criteria advocated for recently. Besides satisfactory repeatability and reproducibility, also for the estimated uncertainty of the applied methods acceptable values were obtained. The scheme of the experiment, involving analysis of spiked samples and quality control samples, ensures good characteristics both of the method and of laboratory performance. The obtained results of the inter-laboratory validation show proper implementation of the method in the regional laboratories and confirm the ruggedness of the method. The process presented in this report is an excellent example of the successful transfer of the method from a reference laboratory to regional laboratories.

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