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Collaborative investigation of matrix effects in mycotoxin determination by high performance liquid chromatography coupled to mass spectrometry

A. Malachova[1], M. Sulyok[1], R. Schuhmacher[1], F. Berthiller[1], J. Hajslova[2], Z. Veprikova[2], M. Zachariasova[2], V.M.T. Lattanzio[3], S. De Saeger[4], J. Diana Di Mavungu[4], S.V. Malysheva[4], S. Biselli[5], O. Winkelmann[5], A. Breidbach[6], S. Hird[7] and R. Krska[1]

[1]University of Natural Resources and Life Sciences, Vienna, Department for Agrobiotechnology (IFA-Tulln), Konrad Lorenz Str. 20, 3430 Tulln, Austria; [2]Institute of Chemical Technology, Prague, Faculty of Food and Biochemical Technology, Department of Food Analysis and Nutrition, Technicka 3, 166 28 Prague 6, Czech Republic; [3]Institute of Sciences of Food Production, National Research Council of Italy (CNR), via G. Amendola 122/O, 70126 Bari, Italy; [4]Ghent University, Department of Bioanalysis, Laboratory of Food Analysis, Harelbekestraat 72, 9000 Ghent, Belgium; [5]Eurofins/WEJ-Contaminants GmbH, Neuländer Kamp 1, 21079 Hamburg, Germany; [6]European Commission, Joint Research Centre, Institute for Reference Materials and Measurements, Retieseweg 111, 2440 Geel, Belgium; [7]The Food and Environment Research Agency, Sand Hutton, YO41 1LZ York, United Kingdom; michael.sulyok@boku.ac.at

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Abstract

Liquid chromatography coupled to mass spectrometry (LC-MS) is a commonly used technique for mycotoxin determination in food and feed. However, accuracy and reliability of the obtained results can suffer from ion suppression/enhancement caused by co-eluting matrix components. Inter-laboratory study concerning relative and absolute matrix effects (MEs) in various food and feed matrices was carried out. The applicability of commonly used strategies in ME reduction were tested in the quantitative determination of nivalenol, deoxynivalenol, fumonisin B_1 and B_2, and zearalenone in complex feed matrices. 'In house' validated LC-MS methods were applied. The relative MEs were assessed on the different food/feed sample sets. Cereal based- and hay/silage feed were used as complex model matrices for absolute ME assessment. The relative MEs within the different sample sets were in range of 3-72%. The absolute MEs for all analytes were in the range of 60-100% and 7-187% for cereal based and hay silage feed, respectively. The use of any purification technique helped to improve absolute MEs for some analytes. Changes in LC conditions did not eliminate ME occurrence. Shifting from electrospray ionization in positive mode (ESI^+) to electrospray ionization in negative mode (ESI^-) improved MEs for early eluting analytes in hay/silage feed. The choice of ion source highly depended on the analyte-matrix combination.

Keyword: complex matrix, *Fusarium* toxins, LC-MS, matrix effects, sample preparation

1. Introduction

Due to its high selectivity and sensitivity, liquid chromatography coupled to mass spectrometry (LC-MS) is considered to be a powerful technique for the quantitative determination of organic compounds in complex matrices. Mycotoxins are poisonous secondary metabolites of moulds, which can often be found in food and feed. Concerning their determination, the use of LC-MS based methods has increased nowadays. This statement is illustrated by the number of published LC-MS applications for mycotoxin determination which increased twenty times over the past ten years (Thomson Reuters, 2011). High performance (HPLC) or ultra-high performance (UHPLC) liquid chromatography coupled to tandem mass spectrometry (MS/MS) has gained the highest popularity because of the effectiveness for the quantitative determination even at trace levels (Cappiello *et al.*, 2008). Moreover, thanks to the

possibility of retrospective data mining, the combination of HPLC or UHPLC with high resolution MS is becoming increasingly popular (Lattanzio *et al.*, 2011; Lehner *et al.*, 2011; Zachariasova *et al.*, 2010a,b).

Furthermore, LC-MS based methods allow the highly selective and sensitive quantitative determination of analytes in complex matrices with little or no sample preparation. However, the accuracy and reliability of the obtained results can suffer from unexpected signal suppression or enhancement (SSE) caused by co-eluting matrix components. The exact mechanism of so-called matrix effects (MEs) is still unknown. It is speculated that MEs originate from the competition of analytes and co-eluting interfering compounds for charges during ionization (reviewed by Gosetti *et al.*, 2010). Depending on the conditions of ionization and ion evaporation, this competition may lead to a decrease (ion suppression) or an increase (ion enhancement) of the efficiency in the formation of the analyte ions. It is supposed that the extent of the ionization reaction depends on the ionization energy as well as on the proton affinity of all the molecules present in the interface. Therefore, the efficiency of the formation of the desired ions also depends on the competition of the ionized analytes with the interfering compounds (Gosetti *et al.*, 2010). The extent of MEs is both analyte- and matrix-dependent. To prevent erroneous quantitation, two basic approaches leading to reduction or elimination of MEs have been developed: (1) reduction of the amount of matrix components in the sample; and (2) selection of an optimal calibration strategy (Gosetti *et al.,* 2010; Hajslova and Zrostlikova, 2003). Adequacy of these approaches depends on the analyte-matrix combination to be analysed and the purpose of the intended method.

Since the first approach often includes selective extraction and specific clean-up, it is mostly applied when a limited number of analytes are comprised in the method. Another option to reduce the amount of matrix components is a 'dilute-and-shoot' approach: raw extracts are diluted with solvents (often in ratios from 1:1 to 1:10 depending on the type of matrix) prior to the final LC-MS determination. The dilution of the extract significantly reduces MEs and thus enables an efficient determination of a wide range of compounds (Sulyok *et al.*, 2006). However, this approach requires highly sensitive LC-MS instrumentation to achieve sufficiently low detection limits.

Recent trends in food analysis have led to the development of multi-residual analytical methods covering chemically unrelated analytes. The main challenges are the choice of an optimal extraction solvent and appropriate clean-up in order to minimize the losses of the investigated analytes during the sample preparation. In most cases the clean-up step is completely omitted, hence the amount of matrix components in an injected sample is high. The most common approach used for the ME compensation in multi-residual analysis is matrix-matched calibration, i.e. the preparation of standards in a blank extract of the respective matrix. Several rules must be followed to make this approach reliable: (1) the blank matrix for the preparation of the matrix-matched standards should be free of target analytes; and (2) it must match the samples to be investigated as closely as possible (Gosetti *et al.*, 2010).

Another approach used in mycotoxin quantitation is the standard addition method. This technique is based on an addition (spiking) of known amounts of target analytes to the sample prior to analysis at several increasing levels supplying a calibration curve. Standard addition is a valuable technique in particular when many samples of different matrices are needed to be analysed. The main disadvantage is the high consumption of standards per sample and the time-consuming sample preparation (Gosetti *et al.*, 2010).

Stable isotope dilution assays (SIDA) represent another highly effective solution in the compensation of MEs as well as in control of analyte losses during the sample preparation. The principle of this approach is based on the existence of stable isotope labelled analogues of the target analytes. Until recently only a small number of isotopically labelled analytes were commercially available and SIDA methods were considered too costly in mycotoxin determination (Haeubl *et al.*, 2006; Rychlik and Asam, 2008). With an increasing number of isotopically labelled mycotoxins on the market together with more sensitive equipment these limitations seem to be overcome now (Varga *et al.*, 2012).

The high variability of MEs was firstly described by Matuszewski *et al.* (2003) among different lots of plasma. On the basis of these observations, it was recommended to investigate 'two types' of MEs during method development to avoid erroneous quantification. The term 'absolute MEs' represents the difference in response between the solvent and matrix-matched standards and 'relative MEs' which show the difference in response among various lots of matrix-matched standards (Matuszewski *et al.*, 2003).

The present research paper describes for the first time an inter-laboratory study dealing with the assessment of MEs in LC-MS analysis of food and feed. Apart from 'absolute' MEs which are commonly investigated within method development, also an extent of 'relative' MEs among the seemingly identical food and feed matrices was studied by each participating laboratory. The second part of this study was focused on the compensation of the MEs during the analysis of two model feed matrices, namely cereal-based feed and hay/silage based feed. Different commonly applied approaches to cope with MEs were tested individually in each laboratory.

2. Materials and methods

Assessment of relative matrix effects

The initial experiments of this study were focused on the assessment of the relative MEs, i.e. the extent of ME variability in the analysis of seemingly identical food matrices. Each participating institution chose a model group of mycotoxins and matrices, employed routinely used 'in-house' validated analytical methods and evaluated the relative MEs. Various matrices, including sample sets of raw cereals (different cultivars and lots of maize, different cultivars of wheat, different cultivars of rye and triticale), feedstuffs (silage and cereal based feedstuffs of different compositions of individual ingredients) and foodstuffs (different types of wheat flours, crackers, rusk and biscuits) were analysed. The individual experiments performed by each laboratory are summarised in Supplementary Table S1. The LC-MS conditions of the 'in-house' validated methods are given in Supplementary Table S2. The experimental procedure has been adopted from Matuszewski *et al.* (2003). Briefly, all samples within a chosen matrix were extracted according to the sample preparation protocol of the respective laboratory (Supplementary Table S1) and the final extracts were spiked at 50, 100 and 150% of the regulatory limit of the model analyte(s) in the respective matrix. If no regulatory limit was set by the EU, the lowest used concentration was at least ten times the limit of quantification. Additionally, a three-point calibration curve in neat solvent was prepared at levels corresponding to the spiked samples. Furthermore, both solvent and extract blanks were prepared to complete the experimental set. All three spiked extracts were analysed consecutively in the order of increasing concentrations and the related blank extract preceded and followed respective sample set. Neat solvent standards were included at the beginning of each sequence and after each third sample set.

For each extract, a linear 1/x weighted calibration curve was constructed. MEs expressed as a SSE were calculated by comparison of the slope of extracts to the slope deriving from external calibration using pure solvent standards according to Equation 1:

$$\text{SSE (\%)} = 100 \times \frac{\text{slope}_{\text{spiked extract}}}{\text{slope}_{\text{liquid standards}}} \quad (1)$$

The extent of MEs was expressed as the relative standard deviation (RSD) from the obtained SSEs across the sample set for any given matrix (Matuszewski *et al.* 2003).

Assessment of absolute matrix effects

The second part of the study was focused on the assessment of the absolute MEs using different LC-MS conditions. Firstly, the 'initial' level of MEs was evaluated in each participating laboratory. For this purpose, all tested materials, standard solutions as well as spiked extracts were prepared at the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium) and distributed among all participants. Each participant analysed them under their routinely applied chromatographic and mass spectrometric conditions and assessed the 'reference' MEs for all investigated analytes. As in the initial experiments, the MEs were expressed as SSEs and calculated according to Equation 1. Obtained SSE levels were taken as reference values for the evaluation of further experiments focused on the compensation of MEs when any reduction strategy was chosen and carried out by each participating laboratory.

Model matrices and analytes

Two feed commodities were chosen as model matrices for our experiments. Test material I, a cereal based feed composed of 16% rye, 20% wheat, 11% maize, 11% rape, 26% soy and 16% rice was provided by IRMM. Test material II, a hay/silage based feed, consisted of 8.6% hay, 74% maize silage, 17% forage and 0.4% minerals, provided by the Institute of Chemical Technology Prague (ICT, Prague, Czech Republic).

As model analytes, mycotoxins of different polarities were chosen ranging from polar nivalenol (NIV), deoxynivalenol (DON), fumonisins B_1 (FB_1) and B_2 (FB_2) to the relatively apolar zearalenone (ZEA).

Reagents and materials

All analytical standards were purchased from Romer Labs (Tulln, Austria). NIV, DON and ZEA were supplied as solid standards, whereas FB_1 and FB_2 were delivered as acetonitrile/water solutions. For the spiking experiments working standard solutions were prepared as follows: (1) standard solution 1 (STD1) which was a mixture containing 3.2 µg/ml DON, 3.2 µg/ml NIV and 0.26 µg/ml ZEA in acetonitrile; and (2) standard solution 2 (STD2) which was a mixture containing 2.6 µg/ml FB_1 and 2.6 µg/ml FB_2 in acetonitrile:water (50:50, v/v).

LC grade methanol and acetonitrile, glacial acetic acid, formic acid (98-100%) ammonium acetate, ammonium formate (both MS grade), ethyl acetate, n-hexane, NaCl (extra pure <99.5%), anhydrous $MgSO_4$, anhydrous Na_2SO_4 were purchased from local branches of VWR International GmbH or Sigma Aldrich. A Purelab ultra system (ELGA 118 LabWater, Celle, Germany) was used for further purification of reverse osmosis water. Phosphate-buffered saline tablets for the preparation of a pH 7.4 buffer were purchased from Sigma-Aldrich (Munich, Germany).

Octadecyl (C18) solid phase extraction (SPE) columns (500 mg/6 ml) were supplied by Grace Discovery Sciences (Lokeren, Belgium). Multisep®226 AflaZON+

multifunctional SPE columns and Myco6in1® immunoaffinity columns were purchased from Coring System Diagnostics (Gernsheim, Germany) and VICAM (Watertown, MA, USA), respectively. Whatman glass microfilters with 125 mm diameter were provided by VWR International (Zaventem, Belgium).

Establishment of 'initial' levels of matrix effects

MEs were investigated at three levels corresponding to 50% (level 1), 100% (level 2) and 150% (level 3) of legislative limits in foodstuffs laid down in EC No 1881/2006 (EC, 2006). Maximum legislative levels for DON and ZEA in unprocessed cereals are 1,250 µg/kg and 100 µg/kg, respectively. As no limits have been established for NIV so far, the same tentative limits as for DON were assumed. The maximum limit of 1,000 µg/kg for the sum of FB_1 and FB_2 in maize products for human consumption was taken from EC No 1126/2007 which amended the levels for *Fusarium* toxins in maize and maize products (EC, 2007).

The spiked extracts of test material I and test material II were prepared at IRMM using IRMM (Spiked extract I) and ICT (Spiked extract II) sample preparation protocols.

Each participant received the same test set, containing test material I, test material II, STD1, STD2, three vials of Spiked extract I (level 1, 2 and 3) and three vials of Spiked extract II (level 1, 2 and 3). Both spiked extracts were ready for injection. External calibration standards were prepared by each of 6 participants at levels corresponding to those in spiked extracts. The final measurements and the expression of MEs were performed as described above.

Spiked extract I

Four times 1 g of test material I were each suspended in 4 ml water:glacial acetic acid (90:10, v/v). After addition of 8 ml ethyl acetate, the mixtures were sonicated for 30 min. Then 4 g anhydrous sodium sulphate was added. After a ten-minute rest, the sample was centrifuged at 3,200 rpm for 2 min to separate the organic from the aqueous phase. The organic phases were all combined, mixed and 1 ml was aliquoted into 21 vials. To 7 of these vials, 25 µl STD1 and 25 µl STD2 were added (level 1). Another set of 7 vials was fortified with 50 µl STD1 and 50 µl STD2 (level 2), and to the last 7 vials, 75 µl STD1 and 75 µl STD2 (level 3) were added. All vials were then evaporated to dryness under a gentle stream of nitrogen at laboratory temperature (23 °C). The dry residues were reconstituted with 500 µl methanol and vortexed for 10 s. Then 500 µl water were added and the solution was again vortexed for 10 s.

Spiked extract II

Three times 2 g of test material II were suspended in 10 ml water:formic acid (99:1, v/v). Then 10 ml acetonitrile were added and the suspension was shaken hard for 3 min. After addition of 1 g sodium chloride and 4 g magnesium sulphate the suspension was again vigorously shaken for 1 min. Finally, the acetonitrile and aqueous phases were separated through centrifugation for 10 min at 3,200 rpm. The upper layers were combined, mixed and aliquoted into 21 aliquots of 850 µl. To 7 of these vials 25 µl STD1, 25 µl STD2, and 100 µl acetonitrile were added (level 1). 7 other vials were fortified with 50 µl STD1, 50 µl STD2 and 50 µl acetonitrile (level 2), and to the last 7 vials, 75 µl STD1 and 75 µl STD2 (level 3) were added.

'Initial' LC-MS conditions

The LC-MS conditions used for the establishment of the 'reference' MEs in the respective laboratories are summarized in Supplementary Table S3.

Strategies for matrix effect reduction/elimination

In the second phase of our study, each laboratory applied approaches in order to reduce MEs. The used approaches included: (1) changes in sample preparation protocol (dilution of extract, different extraction solvent, involvement of clean-up); and (2) changes in LC-MS conditions (different gradient, different type of ionization, polarity switching).

Dilution experiments

Effects of extract dilution on the level of MEs were tested in two different ways. LABs 3 and 6 investigated dilution effects on the reference extracts provided by the IRMM. All received extracts as well as external calibration standards were fivefold diluted with water. LAB 5 applied an 'in-house' validated protocol for the preparation of new extracts which were twofold and fourfold diluted by a dilution solvent consisting of acetonitrile:water:acetic acid (20:79:1, v/v/v).

Application of 'in-house' sample preparation protocols

Only LABs 1, 2 and 4 and 5 applied their own 'in-house' sample preparation protocols. The method descriptions are given in Supplementary Table S4.

The use of different gradients

The gradient used by LAB 6 in the initial measurement (see Supplementary Table S3) was changed in the further experiment. The new gradient started with 5% B, followed by a linear increase within 5 min to 80% B and a jump to 95% B at 5.01 min. Isocratic conditions at 95% B were

held for another 1.5 min. Equilibration of the column was performed at 5% B for 1.5 min.

Switching from HPLC to UHPLC

The HPLC column used by LAB 2 (see Supplementary Table S3) was replaced by an UHPLC column (Acquity UPLC BEH C18; 1.7 µm, 2.1 × 100 mm; Waters, Milford, MA, USA). Apart from the column, eluents and gradient had to be adapted as follows: both eluents consisted of 0.3% formic acid in water (eluent A) and in methanol (eluent B), and the gradient started with 95% A for 3 min, then linear change to 70% A for another 2 min, 5-9 min linear change to 40% A, 9-11 min linear change to 95% A, the last 9 min column equilibration at 95% A. A flow rate of 0.5 ml/min was used. A mixture of water/methanol (60/40, v/v) containing 0.3% formic acid was used as injection solvent for UHPLC-MS/MS.

Polarity switching

Apart from ESI^+, the IRMM extracts of test material II as well as 'in-house' prepared extracts were measured using ESI^- by LAB 2. All other LC-MS conditions remained identical.

Different ionization types

The effects of different ionization type on the reduction of MEs were investigated by LAB 6. After gradient changing (see above), IRMM extracts of both model matrices were analysed using atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI).

3. Results and discussion

Accurate quantitative LC-MS analysis of mycotoxins in food and feed is often hampered by MEs due to the complexity of the samples. In practice, the most common approach is to assess only absolute MEs on one respective matrix sample during method validation. Often, one set of matrix-matched standards is used for quantification, regardless of matrix variability among analysed samples. On this account, we decided to take into consideration both aspects of MEs, i.e. the extent of MEs (absolute MEs) and the variability of MEs among the seemingly identical samples (relative MEs). An overview of the performed experiments is given in Figure 1.

Assessment of relative matrix effects

To obtain comprehensive knowledge about the variability of MEs among the seemingly identical food and feed matrices, each participating laboratory chose different matrix/analyte combination and employed own 'in-house' validated method.

Since a broad set of results was obtained within this study, we decided to bring only a brief overview of some experiments to demonstrate that the variability of MEs can be a low or moderate source of error in quantitative analysis of one sample set, but a significant source of error for another batch of samples. The obtained levels of relative MEs of the experiments described in Supplementary Table S2 are summarized in Table 1.

Variability of the MEs in the determination of aflatoxins (B_1, B_2, G_1 and G_2) in maize was assessed by LAB 5. Altogether 60 maize samples of two genotypes belonging to four batches differing in the ways of growing conditions were analysed. Interestingly, despite low concentration of aflatoxins (corresponding to 2.5, 5.0 and 7.5 µg/kg of matrix), the RSDs across the entire sample set were below 10%. These findings are in agreement with our previously published study (Sulyok *et al.*, 2007). The variability of MEs for aflatoxins among four different maize samples (different batches of the same variety) was below 15%.

Furthermore, relative MEs in ergot alkaloids determination in wheat (set A), rye (set B) and triticale (set C) sample sets were assessed (LAB 2). The highest variability of MEs (19-28%) in all studied sample sets was observed for the early eluting ergometrine. This ergot alkaloid was also highly suppressed by co-eluting matrix (SSEs in all samples were in range of 18-38%) which obviously caused the higher variability in MEs among the samples within a given set. No significant differences in relative MEs were observed either among varieties of given cereal species or in different batches of the same variety.

Low ME variability in different cultivars of wheat was observed also in the determination of *Fusarium* mycotoxins using the quick easy cheap effective rugged safe (QuEChERS) type clean-up (LAB 4, set A). Although strong signal enhancement was observed for the polar mycotoxins NIV, DON and deoxynivalenol-3-glucoside (DON-3G) (average SSE 350%, 163% and 1,562%, respectively), it was consistent for all investigated wheat cultivars. Therefore, the relative MEs calculated across 7 cultivars were below 19%. The enormous SSE levels obtained for DON-3G might be attributed to another phenomenon, which is caused by similar processes as in hot splitless injectors used in gas chromatography. DON-3G injected in pure solvent is adsorbed and/or thermally degraded on catalytically active sites of the interface unless a protective matrix is present (Zachariasova *et al.*, 2010a). Similarly, when silage samples consisting of different components representing a complex matrix were analysed using the same method (LAB 4, set B), MEs occurred (SSEs 68-963%) depending on the respective analyte, but the relative MEs were below 10%, except for NIV and DON-3G.

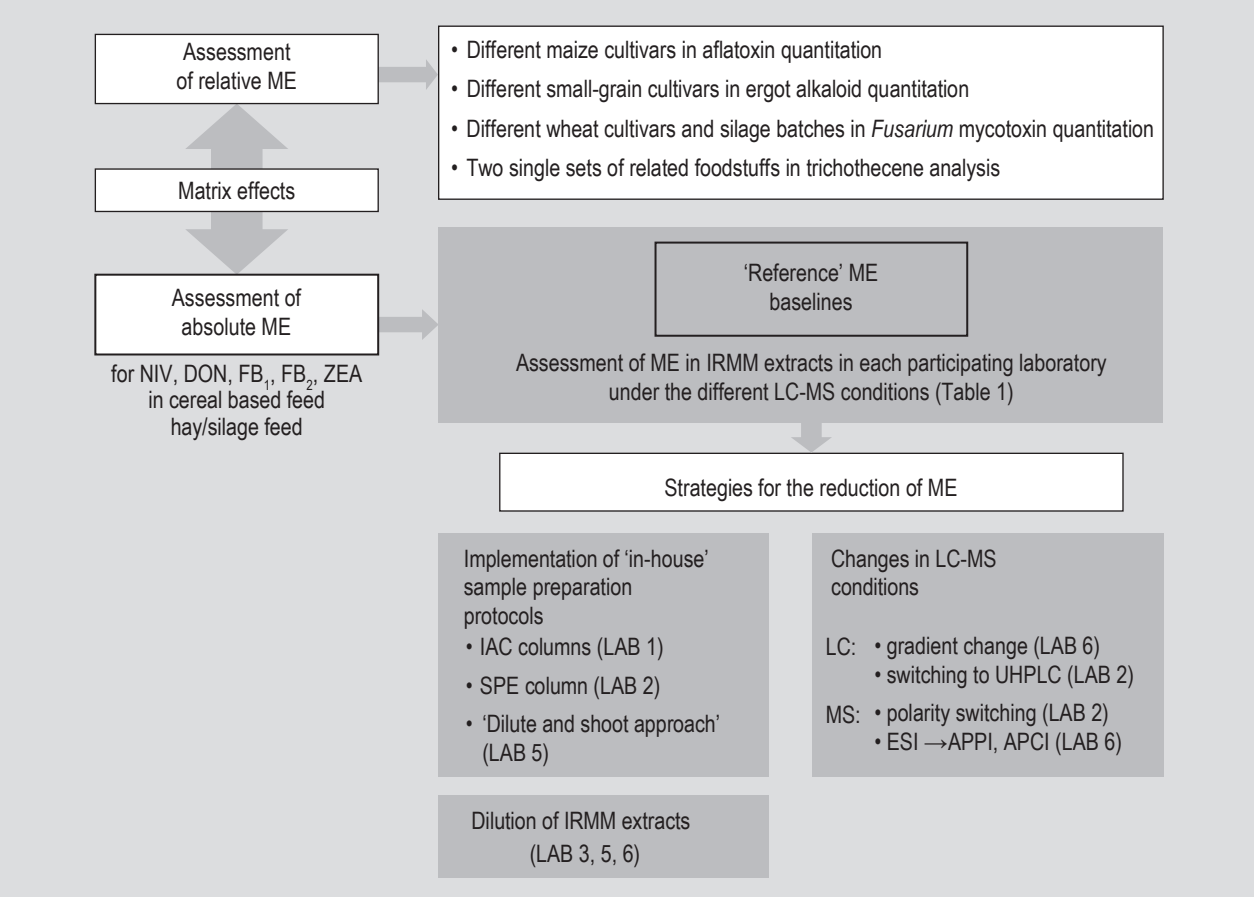

Figure 1. Overview of inter-laboratory experiments. APCI = atmospheric pressure chemical ionization; APPI = atmospheric pressure photoionization; DON = deoxynivalenol; ESI = electrospray ionization; FB_1 = fumonisin B_1; FB_2 = fumonisin B_2; IAC = immunoaffinity chromatography; IRMM = Institute for Reference Materials and Measurements; LC-MS = liquid chromatography coupled to mass spectrometry; ME = matrix effect; NIV = nivalenol; SPE = solid phase extraction; UHPLC = ultra-high performance liquid chromatography; ZEA = zearalenone.

Table 1. Summary of relative matrix effects (relative standard deviation; %) obtained within the given sample sets.

Laboratory code	Analyte						
LAB 1	NIV	DON	HT-2	T-2			
	(A/B)	(A/B)	(A/B)	(A/B)			
	56/10	72/12	45/8	41/14			
LAB 2	ergometrine	ergosine	ergotamine	ergocornine	ergocryptine	ergocristine	
	(A/B/C)	(A/B/C)	(A/B/C)	(A/B/C)	(A/B/C)	(A/B/C)	
	20/19/28	8/10/16	12/16/11	6/12/9	5/9/8	15/13/15	
LAB 4	NIV	DON	FUS-X	HT-2	T-2	ZEA	DON-3G
	(A/B)	(A/B)	(A/B)	(A/B)	(A/B)	(A/B)	(A/B)
	13/18	8/10	8/4	10/5	5/3	11/9	19/25
LAB 5	AFB_1	AFB_2	AFG_1	AFG_2			
	9	7	10	10			

AFB_1 = aflatoxin B_1; AFB_2 = aflatoxin B_2; AFG_1 = aflatoxin G_1; AFG_2 = aflatoxin G_2; DON-3G = deoxynivalenol-3-glucoside; DON = deoxynivalenol; FUS-X = fusarenon-X; HT-2 = HT-2 toxin; NIV = nivalenol; T-2 = T-2 toxin; ZEA = zearalenone.
A/B or A/B/C experiments stated in Supplementary Table S1.

LAB 1 focused on the assessment of relative MEs for NIV, DON, HT-2 and T-2 toxins within the two sample sets consisting of related foodstuffs. SSEs across all analytes in the first sample set (A) involving wheat flour, crackers and rusks were extremely variable, the relative MEs ranged between 41-72%, while biscuits (sample set B) differing in the type of ingredients showed the relative MEs for all analytes below 14%.

To sum up, the variability in MEs (relative MEs) among different batches of the seemingly identical matrix were in general lower than previously expected. Therefore, as mentioned above, the second part of the study focused on the assessment of efficacy of strategies commonly used for the reduction/elimination of the absolute MEs.

Assessment of absolute matrix effects

The level of the absolute MEs in *Fusarium* mycotoxin analysis of two model materials, test material I (cereal based feed) and test material II (hay/silage feed) (extracted according to the IRMM protocols), observed under different LC-MS conditions of the respective laboratories described in Supplementary Table S3 is shown in Figure 2. MEs are expressed as signal suppression (levels of SSE below 100%) or signal enhancement (levels of SSE above 100%).

The extent of MEs varied significantly depending on the tested material and the LC-MS conditions. ME occurrence was much higher in hay/silage feed (Figure 2B) than in cereal based feed (Figure 2A). On the other hand, different extraction solvents were used for each of the tested matrices. Therefore, a definite statement cannot be drawn from the obtained results. For example, LAB 3, 4 and 5 did not report the results for fumonisins spiked into acetonitrile extract of hay/silage feed. As it occurred only in case of the hay/silage feed extract, it is obvious that pure acetonitrile is an unsuitable solvent for fumonisins stored in glass vials. The absence of water in this extract could lead to an undesirable sorption of fumonisins on the glass of the vials and/or on the matrix particles in extract (e.g. mentioned by Lacina *et al.*, 2012; Varga *et al.*, 2012). Rather than fumonisin sorption, huge peak distortion caused by the strong injection solvent has been responsible for this phenomenon here. This assumption is supported by the fact that those three laboratories (LAB 1, LAB 2 and LAB 6), which reported SSEs for fumonisins did not inject the IRMM extracts directly. LAB 1 and 2, evaporated and reconstituted the extracts in 'in-house' injection solvents containing water and LAB 6 diluted the acetonitrile extracts with water in ratio of 1:1 prior to injection.

Concerning cereal based feed, signal suppression was observed in almost all cases. SSEs were in the range of 42-125%. Data for NIV in cereal based feed were not reported by LAB 6 due to huge peak distortion and high occurrence of co-eluting compounds. Contrary to cereal based feed, the ranges of SSEs for respective mycotoxin in hay/silage feed varied significantly. Besides strong signal suppression, i.e. LAB 1 and LAB 5 reported high signal suppression for NIV (7% and 26%), DON (25% and 20%) and ZEA (11% and 13%), respectively, high signal enhancement was observed under the LC-MS conditions applied in the LAB 6 (NIV = 187%, ZEA = 181%). As mentioned above, FB_1 and FB_2 were reported only by three participants. The extent of MEs observed under the different LC-MS conditions ranged from 7-187%, 25-107%, 11-181%, 45-100%, 51-88% for NIV, DON, ZEA, FB_1 and FB_2, respectively. Lab 3 did not report results for hay/silage feed due to incompatibility of injecting pure acetonitrile with their 'in-house' validated LC method.

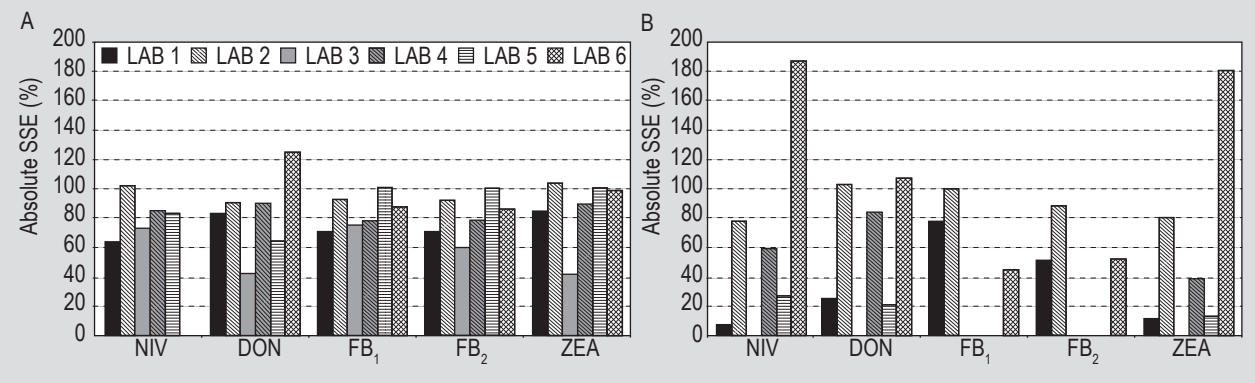

Figure 2. The level of absolute matrix effects in *Fusarium* mycotoxin analysis of (A) cereal based feed (test material I) and (B) hay/silage feed (test material II) extracted according to the Institute for Reference Materials and Measurements protocols and evaluated in the participating laboratories (LAB 1-6). The results for hay/silage feed were not reported by LAB 3. DON = deoxynivalenol; FB_1 = fumonisins B_1; FB_2 = fumonisins B_2; NIV = nivalenol; ZEA = zearalenone.

Strategies for the reduction of matrix effects

Comparison between IRMM and 'in-house' sample preparation protocols

Four out of six participants used own 'in-house' validated methods developed for mycotoxin determination. Spiked extracts of both model matrices were prepared at the same levels as the IRMM ones following the respective 'in-house' sample preparation protocol described in Supplementary Table S4. Figure 3 summarizes the SSE observed in 'in-house' prepared extracts of test material I and II. The collected results indicate the importance of clean-up in control of MEs. The highest MEs were reported by LAB 5 which did not involve any clean-up step within sample preparation. SSE values obtained in this laboratory also showed the different occurrence of MEs during the chromatographic run depending on the analysed matrix. Contrary, the use of any clean-up such as immunoaffinity columns (LAB 1), multifunctional SPE columns (LAB 2) or liquid-liquid partitioning in QuEChERS (LAB 4) diminished differences in MEs between both model matrices to a great extent.

Immunoaffinity clean-up

LAB 1 applied an 'in-house' validated method involving immunoaffinity chromatography (IAC) clean-up. As the used Myco6in1® columns contain antibodies only against DON, FB_1 and FB_2 and ZEA, they cannot be used for purification of extracts containing NIV in 'real-life' analysis. However, the extracts in our experiments were spiked by toxins after purification hence the following discussion involves also NIV. Surprisingly, IACs did not completely eliminate MEs. On the other hand, IAC allowed a high pre-concentration of samples. Hence, high amounts of matrix could be injected to achieve almost the same ME occurrence as in non-concentrated extracts. Therefore, this approach can be recommended for trace analysis or when only older, less-sensitive LC-MS equipment is available.

Concerning SSEs for cereal based feed, slight improvement for DON and FB_1 were achieved after IAC clean-up. The SSEs for DON in extracts (containing 4 mg of matrix) were improved from 83% to 104% compared to IRMM extracts. Further increase of injected matrix (40 mg) did not have any negative effect on the occurrence of MEs (SSE for DON remained at 83%). However, the matrix content in IAC extracts did not seem to have any impact on the SSE observed for FB_1. The use of IAC decreased the signal suppression by 18% in both cases (4 and 40 mg of matrix injected into LC-MS). Interestingly, signal suppression for ZEA drastically increased in IAC extracts. The SSE level of 85% was decreased to 25% and 34% for 4 mg and 40 mg of matrix in the final extracts. As the target analytes were spiked into the purified extract, this phenomenon cannot be attributed to the losses of ZEA during IAC clean-up. A slight increase of signal suppression was observed in IAC extracts for FB_2. SSE levels were decreased by 11% and 19% when 4 mg and 40 mg of matrix were injected. Concerning NIV, signal suppression observed in IRMM extract of cereal based feed did not show any improvement when 40 mg of matrix were injected, but it improved from SSE 64% to 89% in diluted IAC extract (4 mg of matrix).

The use of IACs improved the MEs for DON in analysis of hay/silage feed. 25% SSE for DON was increased to 78% and 88% in IACs extracts containing 4 mg and 40 mg of matrix,

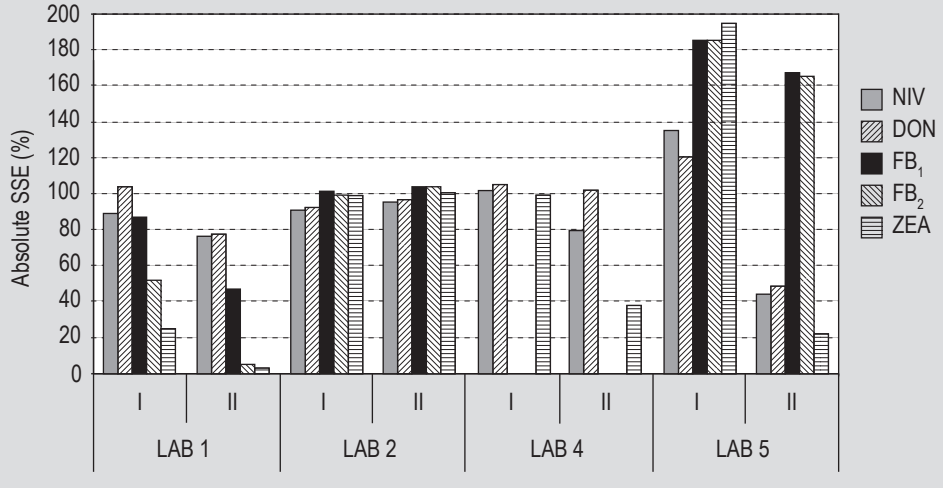

Figure 3. Signal suppression or enhancement (SSE, %) observed in extracts of cereal based feed (test material I) and hay/silage feed (test material II) prepared according to the 'in-house' validated protocols (described in Supplementary Tables S3 and S4) of four laboratories (LAB1, 2, 4 and 5). DON = deoxynivalenol; FB_1 = fumonisins B_1; FB_2 = fumonisins B_2; NIV = nivalenol; ZEA = zearalenone.

respectively. However, the suppression of FB_1 was even higher (SSEs decreased from 78% to 47%) when IACs extracts of 4 mg matrix were analysed. A similar phenomenon was observed in case of FB_2. SSE for FB_2 in IRMM hay/silage extract of 51% were decreased to 5% and 20% in '4 mg' and '40 mg' IAC extracts. IAC clean-up did not have any effects on ZEA determination in the silage samples. Almost complete suppression of NIV improved to SSE of 76% and 50% in '4 mg' and '40 mg' IAC extracts, respectively.

Solid phase extraction (SPE) column clean-up

Almost no MEs in the IRMM extract of cereal based feed (test material I) were observed under the LC-MS conditions used in LAB 2 (Figure 2A). The application of C18 SPE and hexane defatting followed by Multisep®226 AflaZON$^+$ clean-up resulted in a very low level of MEs (Figure 3).

Also in case of hay/silage feed (test material II), only slight signal suppression was observed in the IRMM extract for NIV (77%), FB_2 (88%) and ZEA (80%). These were improved to 85%, 104% and 101%, respectively, using SPE purification. As mycotoxins in both IRMM extracts and 'in-house' prepared extracts suffered from MEs only to a little extent under the LAB 2's LC-MS conditions, it is obvious that sample preparation does not play an important role in the control of MEs in this case.

Liquid-liquid partitioning (QuEChERS)

The approach used by LAB 4 is identical with the sample preparation protocol used for the extraction of hay/silage feed at IRMM. A detailed description of LAB 4's samples preparation protocol is given in Supplementary Table S4. Figure 3 shows that SSE for FB_1 and FB_2 were reported neither for cereal based feed nor for hay/silage feed, which confirms our previous findings stated in the section 'Assessment of absolute matrix effects'. Acetonitrile is obviously an inconsistent injection solvent for the LC conditions used by LAB 4. Comparing the IRMM cereal based feed extract with LAB 4's extract, increases of SSE for NIV and DON were observed from 85% and 90% to 102 and 105%, respectively. No effect was visible for ZEA.

Interestingly, despite the identical sample preparation protocol of extract of hay/silage feed from IRMM and LAB 4, 20% higher SSEs were observed for NIV (SSE 80%) and DON (SSE 102%) in freshly prepared LAB 4's spiked extracts by LAB 4 'dilute-and-shoot' approach.

In the analysis of the cereal based feed IRMM extract using LAB 5's LC-MS setup, ME occurrence was observed only for the polar compounds NIV and DON. The application of an 'in-house' validated method led to signal enhancement for all investigated analytes (Supplementary Table S4). The SSE values ranged from 135% (NIV) to 196% (ZEA). Decreases in these enhancement effects were achieved by a further twofold dilution of the spiked extracts (final dilution of the raw extract was fourfold). Further twofold dilution did not have any more effect on the levels of SSE. The average SSEs calculated for diluted extracts were 95%, 82%, 123%, 118% and 127% for NIV, DON, FB_1, FB_2 and ZEA, respectively.

The analysis of 'dilute-and-shoot' extracts confirmed that MEs are more pronounced in the hay/silage matrix. Direct injection of the IRMM extracts led to an extensive peak distortion caused by the high amount of organic solvent. Using the 'in-house' validated protocol the peak shape was improved, enabling evaluation of the acquired data. Still, MS signals of NIV, DON and ZEA were highly suppressed, while signal enhancement was observed for both fumonisins. Signal suppression for NIV was decreased almost two times (from 44% to 77% of SSE) by further twofold dilution of spiked extracts (fourfold diluted raw extract). Following twofold dilution (eightfold diluted raw extract) did not have any effect on the improvement of NIV suppression. A 50% suppression of DON observed in 'in-house' validated protocol extracts was improved neither after fourfold dilution nor after eightfold dilution of raw extracts. High enhancement of FB_1 and FB_2 were decreased to 85% and 93% in fourfold diluted raw extracts, respectively. Further dilution did not lead to any changes in SSE levels of FB_1 and FB_2. Concerning ZEA, the lowest level of MEs was achieved in eightfold diluted raw extracts in which almost no MEs were observed (SSE of 102%).

Dilution of 'in-house' prepared extracts significantly helped to reduce MEs for all analytes except for DON. As the ratio water/organic solvent was not changed during the dilution of extracts, reduction of the MEs was caused only by dilution of co-extracted compounds.

Dilution approaches were also used by LAB 3 and LAB 6. IRMM extracts were diluted with water in order to increase the polarity of an injected sample. Concerning the cereal based feed, a significant trend in ME reduction was observed in fivefold diluted extracts compared to undiluted ones. The most pronounced improvement was achieved for DON (SSE undiluted extract = 42%, SSE diluted extract = 93%) and for ZEA (SSE undiluted extract = 42%, SSE diluted extract = 108%). The differences in SSE of diluted extracts and undiluted extracts for NIV, FB_1 and FB_2 were less than 20%, which can be attributed to the uncertainty of the method. Similarly, data provided by LAB 6 showed an improvement of NIV and DON peak shape, which led to a significant reduction of MEs for NIV. SSE of fivefold diluted extracts for NIV was 84%, whereas the signal to noise ratio of the NIV peak was too low to allow its evaluation in undiluted extracts. On the other hand, no ME reduction was observed for FB_1, FB_2 and ZEA.

A fivefold dilution of the IRMM hay/silage extract improved the peak shape of all analytes sufficiently to allow the evaluation of the peaks, which was impossible in raw extracts (LAB 3). The SSE values obtained in diluted silage/hay extracts were 116, 82, 123, 111 and 63% for NIV, DON, FB_1, FB_2 and ZEA, respectively. Concerning LC-MS conditions used in the LAB 6, decrease of organic solvent to 20% of acetonitrile in the sample solution did not help to decrease signal enhancement for NIV (SSE undiluted and diluted extracts = 187%). On the other hand, dilution of matrix led to the decline of signal suppression for FB_1 (SSE undiluted extract = 45%, SSE diluted extract = 84%), FB_2 (SSE undiluted extract = 52%, SSE diluted extract = 82%) and signal enhancement for ZEA (SSE undiluted extract = 132%, SSE diluted extract = 181%). No effect was observed for DON.

Changes in LC-MS conditions by different gradients

Although both gradients used by LAB 6 were designed to achieve a good separation of matrix compounds from target analytes, MEs were observed to a different extent in both cases. The obtained levels of SSE are given in Figure 4. Concerning cereal based feed the SSE differed slightly with the exception of NIV and FB_1. The peak for NIV was not found after the elution using gradient I while almost no ME influence on NIV was observed employing gradient II. FB_1 was highly suppressed (SSE = 31%) by changing gradient conditions. In case of hay/silage feed analysis, the use of steeper and faster gradient II led to more than eightfold higher enhancement of ZEA compared to the measurement using gradient I (SSE = 181% with gradient I and SSE = 927% with gradient II).

The differences in the occurrence and the extent of MEs caused by changes in gradients strongly depended on the respective analyte. Therefore, the gradient setup should be taken into account in the control of MEs already during method development.

Switching from HPLC to UHPLC

The impact of chromatography on the MEs on IRMM as well as on the 'in-house' prepared extracts was tested by LAB 2. Regarding the IRMM extracts, significant improvements from 77% and 88% of SSE for NIV and FB_2, respectively, to 100% were observed. Switching to UHPLC did not have any impact on DON and FB_1. The analysis of 'in-house' prepared extract showed no differences to the HPLC method.

Polarity switching

LAB 2 investigated the impact of the ESI-ionization mode on the occurrence of the MEs in IRMM extract of hay/silage feed. The use of negative ionization led to the reduction of signal suppression of NIV. SSE of 77% obtained in ESI^+ mode was increased to 105% by the switching to ESI^- mode. No significant differences in SSE values were observed for other analytes. As negative mode is usually considered as more specific and consequently less subjected to the MEs, our observation in case of NIV was in agreement with some other studies concerning the method development for trichothecenes B determination (Berthiller *et al.*, 2005; Santini *et al.*, 2009; Zachariasova *et al.*, 2010a).

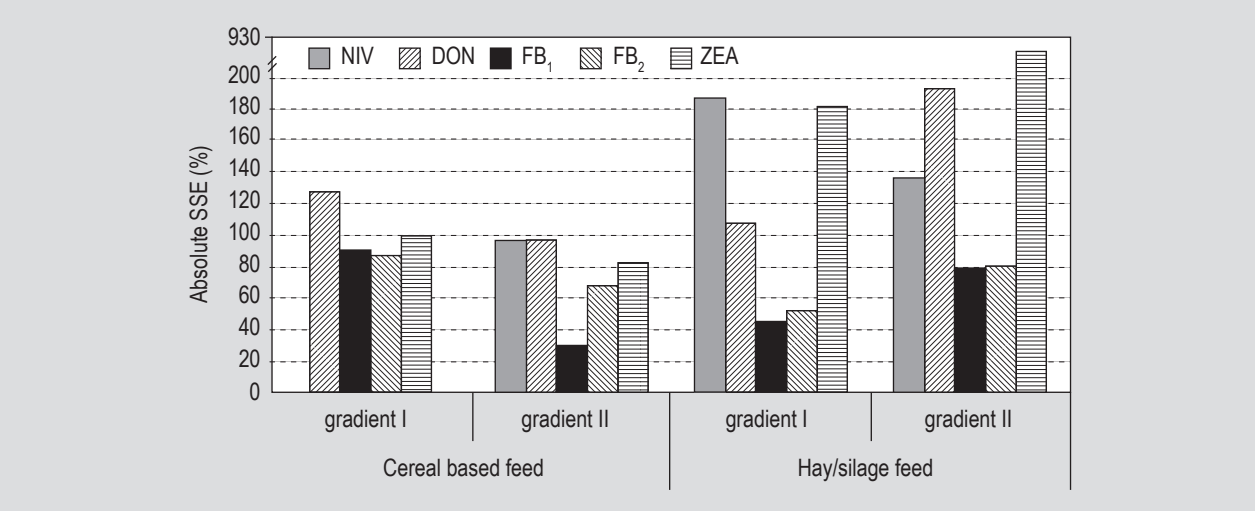

Figure 4. Signal suppression or enhancement (SSE, %) observed in Institute for Reference Materials and Measurements extracts of cereal based feed and hay/silage feed using two different gradients (gradient I and gradient II). DON = deoxynivalenol; FB_1 = fumonisins B_1; FB_2 = fumonisins B_2; NIV = nivalenol; ZEA = zearalenone.

Efffect of different ionization techniques

The effect of ionization on the MEs in mycotoxin analysis was tested by LAB 6. In literature, different extent of MEs are reported for ESI and APCI (Dams *et al.*, 2003; Lagana *et al.*, 2003; Souverain *et al.*, 2004; Zachariasova *et al.*, 2010a). With some exceptions (Lagana *et al.*, 2003; Sangster *et al.*, 2004), ESI is more prone to signal suppression than APCI (Dams *et al.*,2003; Liang *et al.*, 2003; Zachariasova *et al.*, 2010a). However, the use of ESI is mandatory in some cases because not all analytes form ions under APCI conditions in suitable abundance. APPI is more selective and sensitive compared to APCI (Robb *et al.*, 2000). Since photoionization is not based on charge affinity, ion suppression phenomena are generally lower than in APCI or ESI. The major disadvantage of this ion source is the limited range of its application (Robb *et al.*, 2000). Figure 5 shows the SSE values calculated for respective type of ionization used in the analysis of both model matrices.

Concerning the cereal based feed, the most suitable interface for the analysis of NIV and DON in cereal based feed was APPI (SSE for both toxins were 100%). No significant differences in SSE were observed for ZEA regarding all ionization types. Fumonisins were suppressed to some extent in all ion sources (SSE ranged from 51% to 87%), but the lowest ion suppression (SSE>80%) was obtained with ESI.

The use of ESI in hay/silage feed analysis resulted in strong enhancement of NIV and ZEA to 187% and 181%, respectively, and high suppression of fumonisins (SSE<50%). Neither employment of APCI nor APPI helped to reduce the MEs observed in hay/silage feed. No fumonisin peaks were found when APCI was employed. FB_1 ionization using APPI resulted in 200% SSE and FB_2 was suppressed to 56%. Moreover, the SSE of ZEA observed in ESI was fivefold increased using APPI (to 250% SSE).

While ESI offers the highest range of analyte ionization, other sources like APCI or APPI should be considered already during method development for individual toxins, if available.

4. Conclusions

The conclusions resulting from our inter-laboratory study concerned with the assessment of the MEs in the LC-MS determination of mycotoxins in the complex matrices can be summarised as follows:

- The use of matrix-matched standards can be recommended for the accurate quantitative determination of mycotoxins in the samples belonging to the different lots of the same commodity, various cultivars of cereals and different composition of the same type of one product (biscuits containing different ingredients or feed consisting of different content of the same ingredient). Even if the absolute MEs are high for the respective matrix, they are consistent for most of the samples of given matrix (the relative MEs are low, below 20%).
- Employment of various LC-MS instruments in the analysis of the same spiked extract of cereal-based feed showed a low variability in the ME extent among individual measurements. However, hay/silage feed extract analysed in the same way as cereal-based extract revealed large differences among the respective SSE values reported by participating laboratories.
- Modification of the sample preparation procedure helped to reduce MEs to some extent. The use of SPE

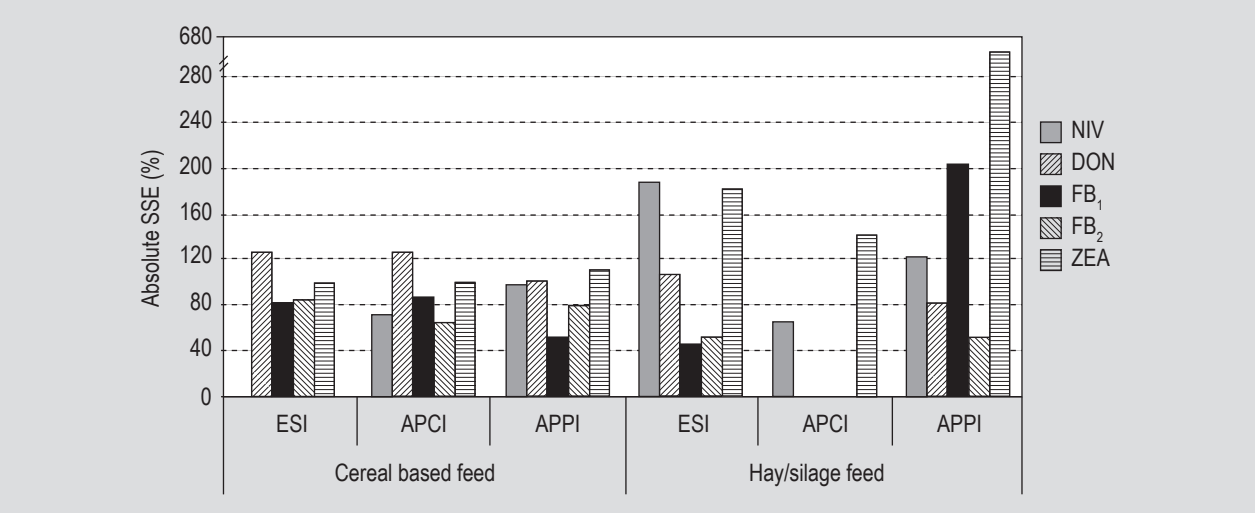

Figure 5. Signal suppression or enhancement values (SSE, %) obtained in the analysis of cereal based feed and hay/silage feed using electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) interfaces. DON = deoxynivalenol; FB_1 = fumonisins B_1; FB_2 = fumonisins B_2; NIV = nivalenol; ZEA = zearalenone.

helped to improve MEs, and also IAC had an effect on the extent of MEs. In cereal based feed IAC decreased ion suppression for NIV, DON and FB_1, whereas FB_2 and ZEA were more suppressed. Concerning the hay/silage feed IAC decreased ion suppression for NIV and DON but increased ion suppression for FB_1, FB_2 and ZEA. These two approaches can be recommended in particular in low level analysis of complex matrix, where 'dilute-and-shoot' approaches are limited due to sensitivity loss.

- Another possibility is the modification of LC-MS conditions. Chromatographic conditions (gradient changes, switching from HPLC to UHPLC) led to an improvement of separation efficiency and thus decreased the extent of the MEs for several analytes. Also the modification of mass spectrometric conditions can have significant impact on the behaviour of the MEs. Choosing the optimal ionization polarity can also reduce signal suppression. The efficacy of different ionization techniques to overcome the MEs strongly depends on the given analyte-matrix combination. For some combinations the use of APPI reduced the occurrence of the MEs.

As a high variability in the extent of the absolute MEs were observed as concern the same analyte-complex matrix combination under different extraction, chromatographic and ionization conditions, it is necessary to take into account all individual steps of the analytical procedure during the method development. The extent of the MEs should be investigated and the entire analytical method should be optimised not only towards the best possible sensitivity but also to achieve reasonable low suppression/enhancement for the intended analyte-matrix combinations.

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Supplementary material

Supplementary material may be found online at http://dx.doi.org/10.3920/QAS2012.0213.

Table S1. Sample set description and applied sample preparation procedures for assessment of relative matrix effects.

Table S2. Overview of LC-MS instruments and LC-MS conditions used for assessment of relative matrix effects.

Table S3. Overview of LC-MS instruments and LC-MS conditions used for the establishment of the 'reference' level of absolute matrix effects in the respective laboratory.

Table S4. Overview of used 'in-house' validated sample preparation protocols.

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