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RESEARCH PAPER

A systematic assessment of the variability of matrix effects in LC-MS/MS analysis of ergot alkaloids in cereals and evaluation of method robustness

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Abstract The study presents for the first time a systematic investigation of matrix effects in the LC-MS/MS analysis of ergot alkaloids in cereals. In order to assure the accuracy of the results, several approaches to minimize/eliminate matrix effects were investigated including variation of ionization techniques, chromatography and sample preparation on different grain types and grain varieties. It was revealed that the use of UPLC and careful choice of sample preparation might reduce signal suppression/enhancement. In general, ergometrine was found to be the most susceptible among the ergot alkaloids studied, but none of the used approaches suggested a total elimination of matrix effects; only less than half of its MS signal could be recovered. The late-eluting compounds were less affected by matrix components in all conditions tested. Further, the robustness of the applied LC-MS method was checked by means of a fractional factorial design. The results indicate that small changes to the sample preparation parameters, namely pH and concentration of extraction buffer, shaking time, drying temperature and extraction volumes, did not significantly (α=0.05) affect the recoveries of ergot alkaloids.

Keywords Ergot alkaloids · Matrix effect · Robustness · LC-MS/MS

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Introduction

Ergot alkaloids are mycotoxins produced by fungi of the *Claviceps* genus, mainly by *Claviceps purpurea* [1], and are known to cause adverse health effects in humans and animals [2–4]. These substances have been mostly detected in cereals and cereal products [5–8].

A combination of liquid chromatography (LC) with electrospray ionization mass spectrometry (ESI-MS) is preferred for the quantitative analysis of ergot alkaloids [7]. However, matrix effects are a major drawback of this technique. Matrix effect (or signal suppression/enhancement (SSE)) is a change in MS signal of an analyte due to co-eluting matrix. The analyte signal can be enhanced or suppressed resulting in inaccurate performance characteristics of the method. Due to possible over- or underestimation of the analyte concentration, matrix effect is a parameter of concern during method development and/or validation.

One of the approaches to deal with the occurrence of matrix effects in quantitative LC-MS analyses is the use of isotopically labelled standards [9]. Since these compounds have the same chemical properties and therefore the same retention times as the non-labelled substances, exact correction to the signal suppression or enhancement can be achieved. However, to date, no isotopically labelled standards of ergot alkaloids are commercially available. Hence, in various liquid chromatography tandem mass spectrometry (LC-MS/MS) methods,

matrix-matched calibration (i.e., preparing the standards in a blank extract of the targeted matrix) is applied to compensate for matrix effects [10–12]. The closeness of match between the matrix to be used for the calibration and the samples to be investigated is of great importance in achieving reliable and accurate results when this approach is applied. Matuszewski et al. [13] demonstrated a high variability of matrix effect among different lots of plasma and highlighted the need to investigate beside the absolute matrix effect, the relative matrix effect which can reveal differences in response among various lots of the same matrix. Cereals which are complex matrices might also result in variability of matrix effects between and within grain types and grain variety.

On the other hand, matrix effects can be minimized by a careful selection of sample preparation, as well as chromatographic and ionization conditions. Krska et al. [11] proposed primary secondary amine (PSA) as a fast and effective technique for the analysis of ergot alkaloids in different cereals and cereal-based products. A tendency toward underestimation of the analyte concentration was observed for wheat and malted-milk biscuits. SSE values in the range of 80–86 % were obtained for seven ergot alkaloids including ergometrine, ergosine and ergocornine [11]. Importance of a clean-up step in sample preparation of ergot alkaloids was also shown by Mohamed et al. [10]. The use of C18 solid-phase extraction (SPE) cartridges demonstrated lower matrix effects compared to a simple extraction with acetonitrile. Lenain et al. [14] developed a class-selective molecularly imprinted solid-phase extraction (MISPE) with the prospect of improved efficiency in minimizing matrix effects as compared to conventional SPE.

Kokkonen and Jestoi [15] described the application of MycoSep®150 Ergot SPE push-through clean-up columns. Ultra-high performance liquid chromatography (UPLC) was applied in the analysis of ergot alkaloids, which provides high separation efficiency and might play a significant role in occurrence of matrix effects. However, the influence of this separation technique on matrix effects was not investigated.

The ion interface can also affect matrix effect as physicochemical processes of ion formation vary depending on the ionization technique. ESI, atmospheric pressure chemical ionization (APCI) and atmospheric pressure photo ionization are widely used in mycotoxin LC-MS analysis [9]. For the determination of ergot alkaloids in particular, ESI was mostly applied in daily analysis. A comparison of matrix effect with other modes has not been reported yet, likewise in-depth studies on matrix effects in the analysis of ergot alkaloids.

The aim of this study was therefore a systematic assessment of signal suppression/enhancement in LC-MS/MS analysis of ergot alkaloids in grains, including different grain types and grain varieties using a previously developed LC-MS/MS method. The effect of the sample preparation procedure, the chromatographic separation and the ionization technique on the matrix effect was also investigated.

Furthermore, the applied LC-MS/MS was tested for robustness for determination of six major ergot alkaloids along with their epimers in grains.

Materials and methods

Standards

Fine film dried ergot alkaloid standards of ergometrine, ergosine, ergotamine, ergocornine, ergokryptine, ergocristine, ergometrinine, ergosinine, ergotaminine, ergocorninine, ergokryptinine and ergocristinine, were purchased from Coring System Diagnostix GmbH (Gernsheim, Germany). The film dried standards were reconstituted in 5 mL acetonitrile, to give concentrations of 100.0 μg/mL (uncertainty, ±5.0 μg/mL) and of 25.0 μg/mL (uncertainty, ±1.5 μg/mL), respectively for the main ergot alkaloids and for the epimers. To minimize epimerization of the analytes, from the freshly prepared standard solutions, deep frozen standard residues were prepared as follows: defined volumes of standard solutions were pipetted into dark brown or aluminium covered glass tubes, evaporated to dryness at 40 °C under a stream of nitrogen, and deep frozen at −20 °C. Under these conditions, the ergot alkaloids are stable for at least 1 year [16]. The deep frozen standards were reconstituted in the required amount of solvent immediately before use. Methylergometrine (as methylergometrine maleate, purity: 98 %) and dihydroergotamine (as dihydroergotamine tartrate salt, purity: 99 %), used as internal standards, were purchased from Sigma-Aldrich (Bornem, Belgium). From the crystalline standards, individual stock solutions were prepared respectively in methanol/acetonitrile (10/90, *v*/*v*) (methylergometrine) or in acetonitrile (dihydroergotamine) at a concentration of 1 mg/mL. These fresh solutions were used to prepare deep frozen standard residues as described above. The residues were reconstituted in the required amount of solvent immediately before use.

Reagents and materials

Methanol (MeOH) and acetonitrile (ACN) of HPLC-grade were both supplied from VWR International (Zaventem, Belgium). *n*-Hexane, anhydrous disodium hydrogen phosphate (Na_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) were also obtained from VWR International. LC-MS-grade MeOH and ACN were bought from Biosolve (Valkenswaard, The Netherlands). Ethyl acetate (EtOAc) and triethylamine (Et_3N) were purchased from Acros Organics (Geel, Belgium). For purification of demineralized

water (H_2O) a Milli-Q purification system (Millipore, Brussels, Belgium) was used. Hydrochloric acid (HCl), sodium hydroxide (NaOH), ammonium carbonate ($(NH_4)_2CO_3$) and ammonium sulphate $(NH_4)_2SO_4$ were bought from Merck (Darmstadt, Germany). Sigma-Aldrich was a supplier of ammonium bicarbonate (NH_4HCO_3) and citric acid. Concentrated ammonia (NH_3) (25 %) was purchased from Vel (Leuven, Belgium). Ultrafree®-MC centrifugal filter devices (0.22 μm) from Millipore (Bedford, MA, USA) were used. MycoSep®150 SPE multifunctional columns were provided by Romer Labs® (Tulln, Austria). Discovery® DSC-SCX SPE columns (500 mg, vol. 6 mL) were purchased from Sigma-Aldrich. Bondesil PSA bulk sorbent (40 μm) from Agilent Technologies (Diegem, Belgium) was used for dispersive solid-phase extraction. Paper filters (grade 3 hw, 65 g/m^2) were supplied from Egilabo NV (Kontich, Belgium). Bond Elut empty SPE cartridges (3 mL) and polypropylene frits (diameter 9.5 mm; 20 μm) were provided by Agilent Technologies.

Spiking

For evaluation of matrix effects, the ergot alkaloids were spiked in cleaned-up extracts of blank grain samples and in the injection solvent free of matrix at four concentrations (ranging between 5 and 100 μg/kg for ESI mode and between 25 and 500 μg/kg for APCI mode). The samples were prepared and analyzed in triplicate on two different days.

For evaluation of extraction recovery as well as for robustness testing, the ergot alkaloids were fortified in blank grain samples which were then subjected to the corresponding extraction procedure. Liquid–liquid extraction (LLE) (see section "Liquid–liquid extraction") was used in the robustness study.

For evaluation of epimerization, the ergot alkaloids (main compounds and epimers spiked separately) were spiked at 100 μg/kg in blank grain samples, which were then subjected to the corresponding extraction procedure. A mixture of main compounds or epimers prepared in injection solvent was also analyzed to provide a reference.

Sample preparation

Liquid–liquid extraction

The LLE procedure exploited in this work was previously developed and optimized by Diana Di Mavungu et al. [12] for analysis of ergot alkaloids in grains and grain products. Briefly, 5 g of fine-ground grain sample were extracted with 40 mL EtOAc/MeOH/0.2 M NH_4HCO_3 pH 8.5 (62.5/25/12.5, *v*/*v*/*v*) during 30 min on an Agitelec overhead shaker (J. Toulemonde & Cie, Paris, France) [Step 1]. The sample extract was centrifuged and a phase separation was induced by adding 10 mL of a saturated solution of $(NH_4)_2SO_4$ and 10 mL of a 0.2 M NH_4HCO_3 pH 10 to 30 mL of the extract [Step 2]. Five millilitres of the ethyl acetate phase was evaporated to dryness, and the residue was reconstituted in 200 μL of MeOH/ACN/H_2O (20/40/40, *v*/*v*/*v*). Subsequently, 200 μL of *n*-hexane were added and the resulting mixture was vortexed and centrifuged in an Ultrafree®-MC centrifugal device for 10 min at 14,000×*g*. The *n*-hexane was discarded and the aqueous phase was then used to redissolve the dried residues of ergot alkaloid standards.

Solid-phase extraction using primary secondary amine

The sample preparation procedure using PSA was adapted from [11]. Five grams of fine-ground grain sample were extracted with 25 mL of ACN/$(NH_4)_2CO_3$ (200 mg/L) (84/16, *v*/*v*) during 30 min on an Agitelec overhead shaker (J. Toulemonde & Cie). The extract was filtered through a paper filter (grade 3 hw, 65 g/m^2, Egilabo NV). Consequent dispersive solid-phase extraction was done by vortex-mixing 4 mL of the extract in a glass tube containing 200 g PSA for 45 s. Then, the mixture was filtered through a paper filter and 2 mL were dried under a stream of nitrogen at 40 °C. The residue was redissolved in 200 μL of injection solvent MeOH/ACN/water (20/40/40, *v*/*v*/*v*), and 100 μL of it was used to redissolve the dried residues of ergot alkaloid standards.

SPE using strong cation exchange columns

Five gram of fine-ground grain sample were extracted with 40 mL of ACN/NH_4HCO_3 (16 g/L) pH 8.5 (85/15, *v*/*v*) during 30 min on an Agitelec overhead shaker (J. Toulemonde & Cie). The extract was centrifuged for 10 min at 4,000×*g*, 5 mL of supernatant were brought into a test tube and evaporated till dryness at 40 °C under a stream of nitrogen. The residue was reconstituted with 5 mL of absorption buffer [8 mM citric acid containing 4 mM Na_2HPO_4 pH 4, adjusted with NaOH]. Five millilitres of *n*-hexane were added to the solution, vortexed and centrifuged for 10 min at 4,000×*g*. The *n*-hexane phase was discarded and 4 mL of the lower phase was brought onto a preconditioned strong cation exchange (SCX) SPE column. The SPE column was conditioned with 6 mL of 3.2 mM HCl, followed by 3 mL H_2O and 2×3 mL absorption buffer. After sample loading, the column was washed with 1 mL H_2O. The ergot alkaloids were eluted with 3 mL MeOH/NH_3 (80/20, *v*/*v*) [the solvent composition was optimized by means of an experimental design using Modde 9.1 statistical software (Umetrics, Umea, Sweden) (Electronic Supplementary Material Fig. S1)].

The eluate was collected into a glass tube and evaporated till dryness at 40 °C under a stream of nitrogen. The residue was redissolved in 200 μL of injection solvent MeOH/ACN/H_2O (20/40/40, *v*/*v*/*v*) and centrifuged in an Ultrafree®-MC

centrifugal device for 10 min at 14,000×*g*. This solution was then used to redissolve the dried residues of ergot alkaloid standards.

SPE using MycoSep® Ergot multifunctional columns

The sample preparation procedure using MycoSep®150 Ergot SPE clean-up columns was according to the instructions given by the manufacturer (Romer Labs®) with only slight adaptations. Five grams of fine-ground grain sample were extracted with 25 mL of ACN/$(NH_4)_2CO_3$ (200 mg/L) (84/16, *v*/*v*) during 30 min on an Agitelec overhead shaker (J. Toulemonde & Cie). The extract was centrifuged for 10 min at 4,000×*g* and 4 mL of supernatant were brought into the provided glass tube. The extract was cleaned-up by pushing MycoSep® column through the extract till the bottom of the tube. Afterwards, 1 mL of the purified extract was evaporated till dryness at 40 °C under a stream of nitrogen. The dried-down residue was redissolved in 100 μL of injection solvent MeOH/ACN/H_2O (20/40/40, *v*/*v*/*v*). This solution was then used to redissolve the dried residues of ergot alkaloid standards.

SPE using MIP

Five grams of ground sample was extracted with 40 mL ACN/NH_4HCO_3 (16 g/L) pH 8.5 (85/15, *v*/*v*) for 30 min. Afterwards, the extract was centrifuged for 10 min at 4,000×*g* and 10 mL of supernatant was evaporated at 40 °C till dryness under a stream of nitrogen. The residue was redissolved with 250 μL ACN/buffer KH_2PO_4–Na_2HPO_4 pH=7 (80/20, *v*/*v*) (pH of a 0.1 M Na_2HPO_4 solution was adjusted to pH 7 using a 0.1 M KH_2PO_4 solution). A MISPE column was prepared by packing 50 mg MIP between two frits in an empty SPE cartridge. The MIP preparation and extraction procedure were according to Lenain et al. [14]. The packed column was firstly conditioned by passing 2 mL ACN. The sample extract (250 μL) was then loaded on the SPE column. Two millilitres of H_2O was used in the following washing step. Elution was finally achieved by passing 3 mL MeOH/Et_3N (95/5, *v*/*v*) through the column. The collected fraction was dried under a stream of nitrogen and reconstituted in 500 μL injection solvent MeOH/ACN/H_2O (20/40/40, *v*/*v*/*v*). This solution was then used to redissolve the dried residues of ergot alkaloid standards.

HPLC-MS/MS analysis

The HPLC-MS/MS analyses were performed on an Alliance HPLC 2695 (Waters, Milford, MA, USA) platform coupled to a Micromass Quattro triple quadrupole mass spectrometer (Waters) equipped with Z-spray ESI and APCI interfaces. Chromatographic separation was achieved using an XBridge MS C18 column (3.5 μm, 150×2.1 mm) with an XBridge Sentry guard column (3.5 μm, 10×2.1 mm i.d.) both supplied by Waters. The column temperature was set at 30 °C. A mobile phase consisting of eluents A [H_2O/0.2 M NH_4HCO_3 pH 10/MeOH (85/5/10, *v*/*v*/*v*)] and B [H_2O/0.2 M NH_4HCO_3 pH 10/MeOH (5/5/90, *v*/*v*/*v*)] was used at a flow rate of 0.15 mL/min. A gradient elution in HPLC-ESI-MS/MS analyses was applied as follows: 0–3 min, 30–15 % A; 3–7 min, 15 % A; 7–10 min, 15–0 % A; 10–13 min, 0 % A; 13–14 min, 0–30 % A; 14–23 min, 30 % A. In HPLC-APCI-MS/MS analyses the flow rate was set at 0.15 mL/min. The injection volume was 10 μL.

The mass spectrometer was operated in ESI and APCI modes, both in positive and negative polarities. During tuning experiments, a 10 μg/mL solution of individual ergot alkaloid standards was infused into the MS using a syringe pump. The MS and MS/MS spectra were recorded. A precursor ion was selected for each analyte, then its product ions were obtained with an optimized combination of cone voltages and collision energies. For increased sensitivity and selectivity, data acquisition was performed in selected reaction monitoring (SRM) mode. Two SRM transitions were selected for each of the analytes.

MS parameters for the ESI analysis were as follows: ESI source block and desolvation temperatures: 120 °C and 300 °C (400 °C in ESI^-), respectively; capillary voltage: 3.5 kV in ESI^+ and 3.8 kV in ESI^-; argon collision gas: 1.2×10^{-3} mbar; cone nitrogen and desolvation gas flows: 100 and 830 L/h, respectively. The monitored SRM transitions as well as the optimized cone voltages and collision energies in ESI^+ are described in [12], while the ESI^- parameter settings can be found in Electronic Supplementary Material Table S1.

MS parameters for the APCI analysis were as follows: APCI source block temperature, 150 °C; probe temperature, 550 °C in $APCI^+$ and 500 °C in $APCI^-$; corona voltage, 4 kV in both $APCI^+$ and $APCI^-$; argon collision gas, 1.2×10^{-3} mbar; cone nitrogen and desolvation gas flows, 100 and 830 L/h, respectively. The monitored SRM transitions as well as the optimized cone voltages and collision energies are presented in Electronic Supplementary Material Tables S2 and S3.

UPLC-MS/MS analysis

The UPLC-MS/MS analyses were performed on a Waters ACQUITY UPLC system coupled to a Micromass Quatro Micro triple quadrupole mass spectrometer (Waters) equipped with a Z-spray ESI. Chromatographic separation was achieved using an ACQUITY UPLC BEH C18 column (1.7 μm, 100×2.1 mm i.d.) with an ACQUITY BEH C18 VanGuard pre-column (1.7 μm, 5×2.1 mm i.d.), both supplied by Waters, or ZORBAX RRHD Eclipse plus C18 column (1.8 μm, 100 mm×2.1 mm i.d.), supplied by

Agilent Technologies. The column temperature was set at 30 °C. A mobile phase consisting of eluents A [H_2O/0.2 M NH_4HCO_3 pH 10/MeOH (85/5/10, *v*/*v*/*v*)] and B [H_2O/0.2 M NH_4HCO_3 pH 10/MeOH (5/5/90, *v*/*v*/*v*)] was used at a flow rate of 0.3 mL/min. A gradient elution was applied as follows: 0–1 min, 50–40 % A; 1–2.5 min, 40–35 % A; 2.5–6.5 min, 35 % A; 6.5–7 min, 35–30 % A; 7–9 min, 30 % A; 9–12 min, 30–10 % A; 12–13 min, 10 % A; 13–14 min, 10–50 % A; 14–17 min, 50 % A. The injection volume was 5 μL.

The mass spectrometer was operated in ESI positive mode. MS parameters for the ESI analysis were as follows: ESI source block and desolvation temperatures: 120 and 300 °C, respectively; capillary voltage: 3.5 kV; cone nitrogen and desolvation gas flows: 20 and 500 L/h, respectively. The monitored SRM transitions as well as the optimized cone voltages and collision energies are shown in Electronic Supplementary Material Table S4.

Robustness

The assessment of method robustness using LLE ("Liquid–liquid extraction" section) was based on evaluation of extraction recoveries of ergot alkaloids varying the following method parameters: pH of the extraction buffers, buffer concentration, extraction volume, shaking time and drying temperature. These factors were selected as potential sources of variability when performing the analysis. The intervals of the factors were set at values which slightly exceed the variations which can be expected when a method is transferred from one laboratory to another. The influence of these factors on the response was examined by means of two-level fractional factorial design using Modde 9.1 statistical software (Umetrics, Umeå, Sweden). Having three replicates of the centre-point and eight factors, the total number of runs was 19. The experimental design set-up is presented in Electronic Supplementary Material Table S5.

Matrix effects

For evaluation of matrix effects, the ergot alkaloids were spiked in cleaned-up extracts of blank grain samples and in the injection solvent free of matrix at four concentrations, and analyzed by LC-MS/MS. A linear function of the calibrants spiked in cleaned-up extract was compared to that of calibrants spiked in matrix-free solvent. Matrix effects were expressed as SSE and calculated according to Eq. 1.

$$\mathrm{SSE}\ (\%) = 100 \times \text{slope spiked extract} / \text{slope calibrants in matrix}-\text{free solvent} \quad (1)$$

An SSE value of 100 % indicates no matrix effect, while a value above 100 % designates signal enhancement and an SSE below 100 % points to signal suppression due to presence of matrix.

Epimerisation of ergot alkaloids, which might have considerable impact on SSE results, was minimized by maintaining the autosampler at 4 °C and immediate analysis of the samples.

For the data treatment, a statistical software, IBM SPSS Statistics 20 (IBM Corporation, New York, USA), was used.

Method sensitivity

To verify the sensitivity of the method, limits of detection (LODs) and limits of quantification (LOQs) were calculated. The LODs and LOQs were defined as the minimum concentration of analyte in the artificially fortified blank samples inducing ergot alkaloid peaks with a signal-to-noise ratio of 3 and 10, respectively.

Results and discussion

Evaluation of matrix effects

Grain type and grain variety

As common cereal grains can be infected with *Claviceps* spp. [7], it was the intention of the study to investigate at first stage matrix effects when analyzing different grain types. Five cereals were chosen for the experiment, namely rye, wheat, triticale, oat and barley. The samples were subjected to LLE and analyzed by HPLC-MS/MS as described in the sections "Liquid–liquid extraction" and "HPLC-MS/MS analysis". The SSE data are visualized in Fig. 1. A difference in matrix effects between the grain types was observed with up to 80 and 90 % decrease of signal for ergometrine in barley and oat, respectively. The late-eluting ergokryptinine and ergocristinine demonstrated nearly no signal suppression/enhancement in rye, wheat and triticale, whereas in oat and barley the SSE were below or equal to 50 %. An ANOVA test showed that SSE means for the different grain types were significantly different (p value<0.001). A Tukey multiple comparison test (Electronic Supplementary Material Table S6) revealed that the mean SSE values of the different combinations of grain types were significantly different (p values<0.001) except for the combinations of rye–wheat (p value=0.078) and wheat–triticale (p value=0.124).

Considering the fact that different grain varieties are cultivated all over the world, at the next stage, an assessment of SSE for different varieties within one grain type was done using rye as an example. The difference in matrix effects between the three varieties of rye investigated (Askari, Conduct and Evolo) was significant (ANOVA p value=0.001). Tukey multiple comparison test revealed a significant difference in SSE

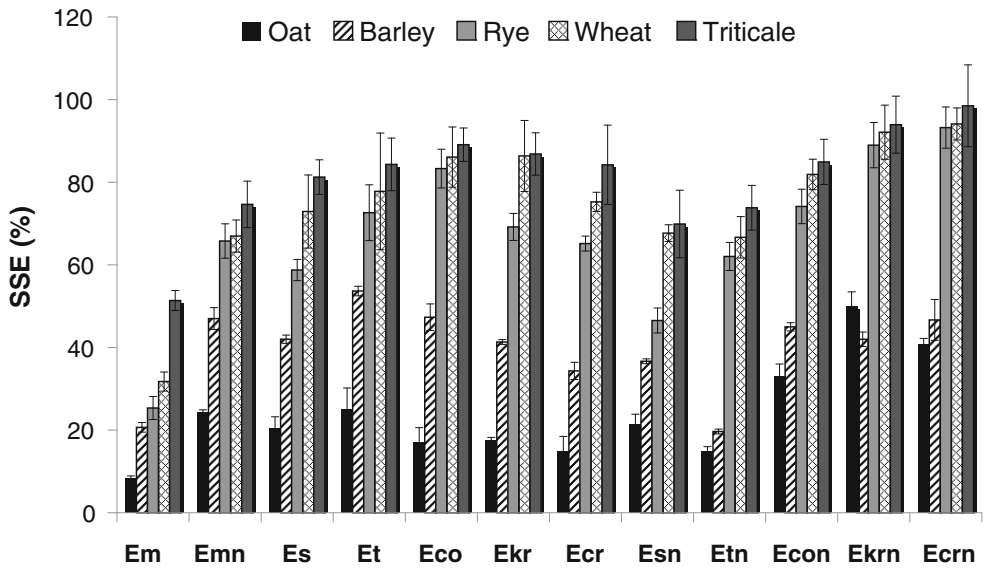

Fig. 1 SSE (%) for the different grain types

between Askari and Conduct (p values=0.001) (Electronic Supplementary Material Table S6 and Fig. S2), while no significant difference could be demonstrated for the combinations Askari–Evolo (p value=0.271) and Conduct–Evolo (p value=0.063). Overall, it can be stated that the differences in matrix effects between grain varieties were moderate (Electronic Supplementary Material Fig. S2), as compared to grain types. However, the results of this investigation clearly highlight the need for a correct choice of the blank matrix when matrix-matched calibration has to be performed.

Comparison of different ion sources and ionization modes

The commonly used ionization techniques in the LC-MS/MS analysis of mycotoxins are ESI and APCI in either positive or negative mode [17, 18]. Due to their different ionization mechanisms, the efficiency of formation of the targeted ions in the presence of the same co-eluting compounds can vary depending on the utilized interface. Considering this fact, matrix effects in the LC-MS/MS analysis of ergot alkaloids using different ion sources (as well as different ionization modes) were studied under identical sample preparation and chromatographic conditions. Rye samples were subjected to LLE and analyzed by HPLC-MS/MS as described in sections "Liquid–liquid extraction" and "HPLC-MS/MS analysis".

It was demonstrated that the type of ion source can have a relevant effect on analyte MS signal (Fig. 2; Electronic Supplementary Material Table S7). A suppressive matrix effect was observed in the ESI mode for almost all analytes with ergometrine being the most susceptible. Less signal suppression occurred using the ESI interface in the positive instead of negative ionization mode; the MS signal of the ergot alkaloids dissolved in matrix-free solvent was ten times higher in ESI^+ than in ESI^-. Using ESI^+, a huge signal suppression for ergometrine in rye and wheat was also noticed by Kokkonen and Jestoi [15]. The use of APCI instead of ESI resulted in a tremendous signal enhancement for most of the ergot alkaloids (SSE around 200 % for ergometrine) leading to an overestimated ergot alkaloid content in cereal samples.

An ANOVA test demonstrated that the mean SSE values applying different ionization modes significantly differ (p value<0.001). By further Tukey multiple comparison analysis, it was shown that significant differences were observed between ESI and APCI (p values<0.001) (Electronic Supplementary Material Table S7). The SSE means between polarity modes, namely, ESI^+ and ESI^- (p value=0.204), and $APCI^+$ and $APCI^-$ (p value=0.065), were not significantly different (Electronic Supplementary Material Table S7).

When choosing ion source and ionization mode for the LC-MS analysis of ergot alkaloids, it should be considered that these compounds give a much higher signal in ESI^+ than in other modes, as was also observed by other authors [11, 12]. On the other hand, notably high standard deviations of SSE values observed in APCI do not give preference to this ionization mode (Fig. 2).

Influence of chromatography

To minimize matrix effects, a good chromatographic separation of an analyte from matrix components can play an important role. For this purpose, UPLC (instead of HPLC) can be applied, since the use of a column with smaller particle size leads to sharper peaks and therefore to an improved chromatographic resolution [15]. Consequently, a better separation between analytes and matrix components can be expected, leading to reduced matrix effects.

The rye samples fortified with ergot alkaloids underwent the LLE ("Liquid–liquid extraction" section) and were analyzed either by HPLC-MS/MS using a C18 XBridge column or by UPLC-MS/MS using C18 ACQUITY BEH and C18

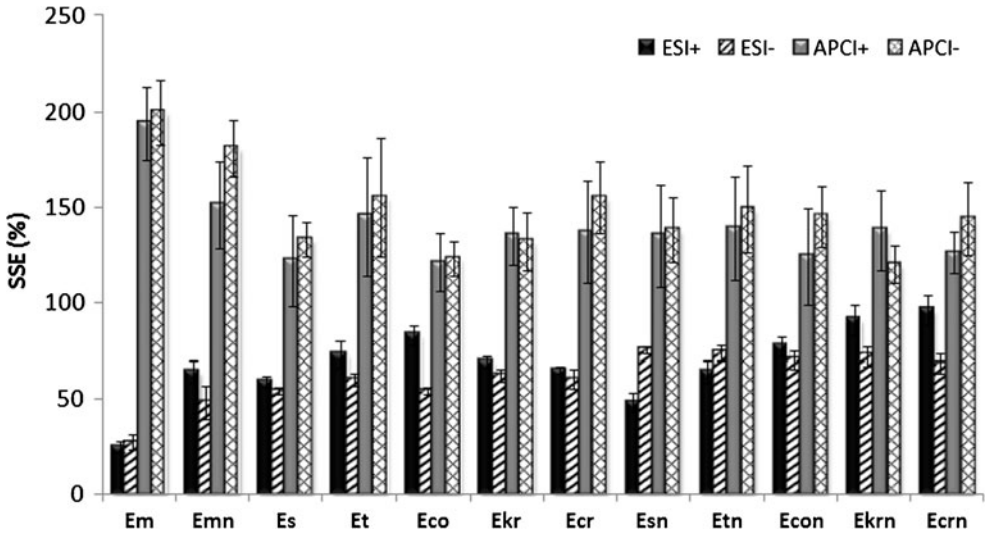

Fig. 2 SSE (%) for the different ion sources and ionization modes

Zorbax RRHD Eclipse columns ("HPLC-MS/MS analysis" and "UPLC-MS/MS analysis" sections).

In the conditions applied, a significant difference between the chromatographic systems was found (ANOVA, p value= 0.002). The Tukey multiple comparison test showed that HPLC and UPLC using ACQUITY BEH (p value=0.009), or HPLC and UPLC using Zorbax column (p value=0.012) had significantly different SSE profiles; whereas both UPLC columns demonstrated similar SSE means (p value=0.994) (Electronic Supplementary Material Table S8). Compared to Zorbax, the ACQUITY BEH column provided higher signal suppression for ergometrinine (about 20 % difference) and ergocorninine (30 %) (Electronic Supplementary Material Fig. S3). In turn, the Zorbax column accounted for lower SSE values for ergokryptine (about 40 % difference) and ergosinine (about 20 %). Regarding the early eluting ergometrine, improvement in matrix effect using UPLC was not achieved; the SSE values were still below 45 %.

In general, UPLC was shown to be a suitable option for minimizing matrix effects and would be preferred in routine ergot alkaloid analysis.

Comparison of different sample preparation procedures

As matrix effect is caused by interference of matrix components with the analyte peak, it can be reduced by applying a suitable sample preparation. This step in the analysis is the most critical, since grain is considered as a complex matrix. In this study, different sample preparation procedures, namely LLE and SPE using PSA, MIP, MycoSep and SCX columns ("Sample preparation" section) were evaluated with regard to matrix effects (Fig. 3).

A great difference in matrix effects was observed for the different sample preparation procedures tested and the ergot alkaloids studied. Overall, the highest signal suppression was observed for ergometrine and ergometrinine. Applying LLE, the signal suppression observed for these compounds was less pronounced as compared to the other sample preparation procedures. The use of a relatively less polar solvent (ethyl acetate) could account for these observations.

The use of SCX and multifunctional SPE cartridges resulted in almost no signal suppression or signal enhancement for the later eluting ergot alkaloids. Owing to easy handling and elimination of matrix interferences for the majority of ergot alkaloids, MycoSep® multifunctional columns could be preferred for the clean-up with the only limitation that recovery of ergometrine was below 15 % (data not shown) combined with about 90 % of signal suppression. The use of MIP and PSA as solid sorbents for clean-up was less effective with regards to matrix effects compared to the two above-mentioned SPE columns, but SSE values still exceeded 60 % for the later eluting analytes.

Additionally to matrix effects, the influence of the sample preparation procedure on epimerization rate of the ergot alkaloids was also taken into consideration as it can affect the results of analysis. Ergot alkaloids, having asymmetric carbon at the C8-position, form two epimeric configurations which possess different bioactivity [19, 20]. It is known that bidirectional epimerization is observed under both acidic and alkaline conditions [1]. Figure 4 presents the epimerization rates of the investigated ergot alkaloids applying the different extraction protocols. LLE procedure overall induced lesser epimerization (or no epimerization as in the case of ergometrine and ergometrinine) compared to the other extraction protocols. The composition of the extraction solvent and cautions in the extraction steps could account for this. Although carbonate buffers pH 8.5 and 10 (required for optimal extraction) were applied, these were not allowed to interact with the ergot alkaloids for an extended time. In another study, application of LLE for extraction of ergot alkaloids in acidic conditions caused their epimerization with equilibrium shifted towards the epimers [17]. Other data showed that almost no epimerization

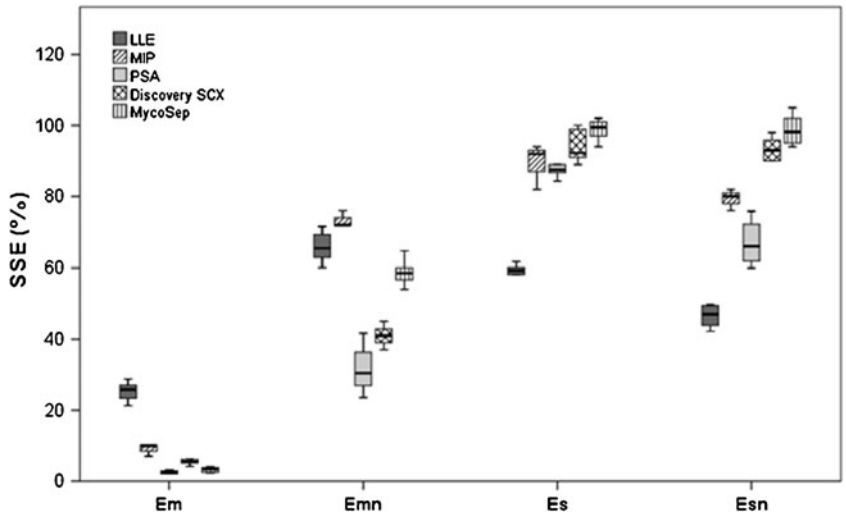

Fig. 3 SSE (%) applying different sample preparation procedures (for reasons of clarity only ergot alkaloids with the most pronounced matrix effects are shown)

occurred using Oasis® HLB SPE cartridges, however, using Oasis® MCX SPE cartridges, designed for bases, the epimerization (up to 27 % ergosinine) was promoted more strongly due to presence of 5 % NH_3 in the elution solvent [11]. Current results demonstrated higher degree of epimerization using SCX for clean-up step; the highest rate was noted for ergotaminine (57 %). This can be attributed to elution of ergot alkaloids with a solvent containing a higher concentration of NH_3 (20 %). The same reasoning can be applied for epimerization rates up to 70 % using MIP; here, triethylamine was applied as a component of the elution mixture. It has been previously reported that in alkaline conditions tendency for ergot alkaloid conversion increases [12, 20]. Krska et al. [11] reported non-significant epimerization employing PSA in the ergot alkaloid sample preparation. Data of the present study showed elevated epimerization rates, up to 40 %. This can be ascribed to the introduction of a drying step in the present experiment set-up. The concentration of extract was required to increase sensitivity; however, temperature has been shown to cause epimerization in other studies [12, 20, 21].

Generally, the two lysergic acid derivatives, ergometrine and ergometrinine, were more stable with regards to epimerization compared to other ergot alkaloids included in this work. The interconversion of the epimers into their corresponding main ergot alkaloids was observed for all extractions applied and all ergot alkaloids investigated. The exception was ergometrinine, which epimerized to its main compound only using MIP in sample preparation.

Robustness study

Besides matrix effects, robustness of the method, which plays an important role in ensuring correct quantification, was also investigated. The influence of small changes of the sample preparation parameters on the recovery of ergot alkaloids was evaluated. The samples were extracted following the usually applied LLE procedure ("Liquid–liquid extraction" section). Parameters such as pH and concentration of extraction buffer, shaking time, drying temperature and extraction volumes were included in the investigation (Electronic Supplementary

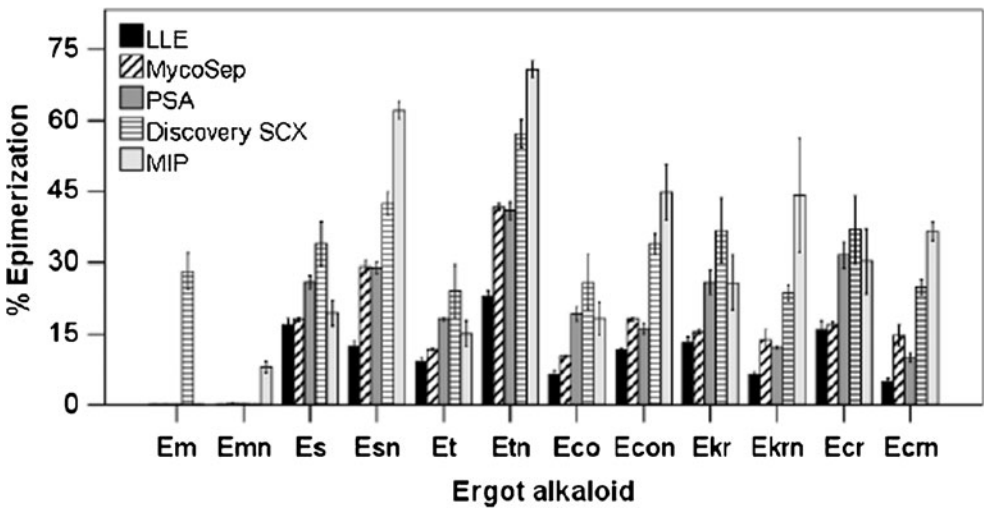

Fig. 4 Epimerization rate (%) of ergot alkaloids applying different sample preparation procedures (difference in MS signal of the main compound and the epimer as well as epimerization in standard mixture was taken into account). *Em* ergometrine, *Emn* ergometrinine, *Es* ergosine, *Esn* ergosinine, *Et* ergotamine, *Etn* ergotaminine, *Eco* ergocornine, *Econ* ergocorninine, *Ekr* ergokryptine, *Ekrn* ergokryptinine, *Ecr* ergocristine, *Ecrn* ergocristinine

Material Fig. S4). A fractional factorial design was used. The central values were those specified in the method and the extreme values (low and high) were computed from the nominal ones taking into account possible uncertainty (Electronic Supplementary Material Table S5). It was observed that in the range examined, changes in the above-mentioned parameters did not significantly affect the recoveries of ergot alkaloids (α=0.05); the recovery values for all compounds were within the 95 % confidence interval. Therefore, the applied LC-MS/MS method for determination of ergot alkaloids was considered to be robust and is expected to be easily transferred from one operator/laboratory to another.

Application of the method to grain samples

Considering the above-given information, a complete elimination of matrix effects in the LC-MS/MS analysis of ergot alkaloids in grains could not be achieved. However, application of the LLE procedure in combination with UPLC-ESI^+-MS/MS analysis could be suggested as a good experimental condition for reduction of matrix effects (especially for the earlier eluting compounds). The method was characterized by LODs ranging from 0.3 to 1.0 μg/kg and LOQs ranging from 0.8 to 3.1 μg/kg. To test practical applicability of the recommended conditions, a set of 46 commercial rye grain samples was analyzed for the presence of the six major ergot alkaloids and their corresponding epimers (Electronic Supplementary Material Table S9). The total ergot alkaloid content ranged from <LOQ to 1,267 μg/kg with mean value of 127 μg/kg and the median of 17 μg/kg. The most frequently occurring main ergot alkaloids were ergokryptine, ergosine followed by ergocornine. Among the epimers, ergosinine was detected most frequently. The highest levels were noted for ergosine (796 μg/kg); two- to three times lower maximum concentration values were measured for ergosinine (387 μg/kg), ergocornine (287 μg/kg), ergokryptine (278 μg/kg) and ergotamine (245 μg/kg). In majority of samples, ergot alkaloids occurred simultaneously with their corresponding epimers.

Conclusions

Matrix effects can lead to inaccurate quantification. With the goal of ensuring accuracy and robustness in LC-MS/MS analysis of ergot alkaloids, matrix effects and different ways to minimize/eliminate them were systematically investigated. Initially, it was observed that signal suppression or enhancement for ergot alkaloids varied not only between grain types, with the most pronounced effect in oat, but also to a lesser extent within one grain type. These findings should be definitely considered when preparing/searching an appropriate blank grain sample for preparation of a matrix-matched calibration.

The ionization technique was also found to have an influence on matrix effect occurrence. Generally, suppressive effects were characteristic for ESI. The use of APCI instead could not be a good option, since it showed a tremendous signal enhancement for most ergot alkaloids leading to overestimation of concentration in a sample. The differences between ionization modes were not significant with regard to matrix effect.

In general, ergometrine was the most susceptible ergot alkaloid to matrix effect which can be attributed to its early and thereby simultaneous elution with matrix interferences in the HPLC system. Application of UPLC in this case did not solve the problem of signal suppression, whereas considering the later eluting compounds, this technique was preferred.

The observed matrix effects were affected by the sample preparation procedure. In this work, an LLE procedure using ethyl acetate was compared to SPE performed on different sorbents. For all the procedures tested, no clear signal enhancement was noted. A more effective clean-up which could be expected from MycoSep® and MIP columns developed especially for ergot alkaloids did not demonstrate improvements for ergometrine. For the later eluting ergot alkaloids, MycoSep® and SCX cartridges were useful in minimization of signal suppression.

The "dilute-and-shoot" approach, another way of eliminating matrix effect, was not included in the study, since it necessitates the use of a highly sensitive LC-MS instrument to achieve low limits of detection.

Another important method parameter—robustness—was also investigated in the present study. The influence of small changes of the sample preparation parameters on the recovery of ergot alkaloids was evaluated by experimental design. The eight most critical factors were considered. It was finally observed that the changes in sample preparation parameters did not significantly affect the recoveries (α=0.05). Hence, the applied LC-MS/MS method for determination of ergot alkaloids in grains proved to be robust and can therefore be used in a different laboratory or operated by a different analyst still providing accurate measurements.

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