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An integrated targeted and untargeted approach for the analysis of ergot alkaloids in cereals using UHPLC – hybrid quadrupole time-of-flight MS

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Abstract

An ultra-high performance liquid chromatography (UHPLC) hybrid quadrupole – time of flight (Q-TOF) mass spectrometry (MS) method is described for the simultaneous quantitative determination of common ergot alkaloids and the screening, detection and identification of unexpected (less studied or novel) members of this class of toxic fungal secondary metabolites. The employed analytical strategy involves an untargeted data acquisition (consisting of full scan TOF MS survey and information dependent acquisition (IDA) MS/MS scans) and the processing of data using both targeted and untargeted approaches. Method performance characteristics for the quantitative analysis of 6 common ergot alkaloids i.e. ergometrine, ergosine, ergotamine, ergocornine, ergocristine, ergokryptine and their corresponding epimers in rye were comparable to those previously reported for triple-quadrupole (QqQ) MS/MS. The method limits of quantification (LOQ's) were in the range from 3 to 19 µg/kg, and good linearity was observed for the different ergot alkaloids in the range from LOQ to 1000 µg/kg. Furthermore, the method demonstrated good precision (RSD's at 50 µg/kg not higher than 14.6% and 16.2% for the intra-day and inter-day precision, respectively), and the trueness values at different concentration levels were all between 89 and 115 %. The method was applied for the analysis of a set of 17 rye samples and demonstrated the presence of these ergot alkaloids in the range from < LOQ to 2811 µg/kg. Further mining of the same data based on a 'non-targeted peak finding' algorithm and the use of full MS and MS/MS accurate mass data allowed the detection and identification of 19 ergot alkaloids that are commonly not included in most analytical methods using QqQ instruments. Some of these alkaloids are reported for the first time in naturally contaminated samples.

Keywords: Ergot alkaloid identification, targeted and untargeted analysis, TOF-MS - IDA MS/MS, TripleTOF

1. Introduction

Ergot alkaloids (Figure 1) represent a class of indole metabolites produced by grain and grass pathogens such as *Claviceps spp*., mainly by *Claviceps purpurea* (Komarova and Tolkachev, 2001a; Komarova and Tolkachev, 2001b; Naudè *et al*., 2005). Among the cereal species, rye and triticale are especially susceptible, but wheat, barley, oats are also potential fungal hosts (Flieger *et al*., 1997; Krska and Crews, 2008). The fungus infects the host by replacing the developing grain or seed with specialized fungal structures known as sclerotia or ergot, which contains poisonous substances (the ergot alkaloids). Generally it is possible to remove up to 82 % of ergot by mechanical means with conventional grain cleaning equipment such as sieves and separators used during the harvesting process. But despite these cleaning procedures, different surveys have demonstrated that ergot alkaloids can still be present in cereal-based food and feed, sometimes in excessive amounts (Crews *et al*., 2009; Diana Di Mavungu *et al*., 2011; Malysheva *et al*., 2014; Lombaert *et al*., 2003; Müller *et al*., 2009; Reinhold and Reinhardt, 2011).

More than 40 different ergot alkaloids have been reported. However, survey on the occurrence of ergot alkaloids commonly focus on only a few major compounds for which standards are available. Evaluation of the exposure and actual risk due to consumption of contaminated food and feedstuffs requires suitable analytical strategies. Analytical methods for the determination of ergot alkaloids include thin layer chromatography (TLC) (Salvat and Godoy, 2001), capillary electrophoresis (CE) (Franch and Blaschke, 1998), enzyme linked immunosorbent assay (ELISA) (Hill *et al*., 2001), gas chromatography (GC) with electron capture detection (ECD) (Barrow and Quigley, 1975), liquid chromatography (LC) with ultraviolet (Veress, 1993), fluorescence (Komarova and Tolkachev, 2001a; Storm *et al*., 2008) or mass spectrometric (MS) (Diana di Mavungu *et al*., 2012; Burk *et al*., 2006; Kokkonen and Jestoi, 2010; Krska *et al*., 2008a; Mohamed *et al*., 2006b) detection. Over the last decade, LC–MS has become a dominant tool for mycotoxin determination, and has provided an unequivocal identification of ergot alkaloids in various matrices (Diana di Mavungu *et al*., 2012; Friedrich *et al*., 2004; Kokkonen and Jestoi, 2009; Krska *et al*., 2008; Lehner *et al*., 2005; Mohamed *et al*., 2006a; Mohamed *et al*., 2006b). Due to its simple operation and low cost, ergot alkaloid analysis is currently mainly conducted using a triple-quadrupole (QqQ) MS system. The system demonstrates high sensitivity, selectivity, speed and a wide dynamic

range operating in selected reaction monitoring (SRM) mode. The main limitation of this technique is the inability to investigate compounds that have not been previously optimized and included in the analytical method. We have previously demonstrated the usefulness of high resolution mass spectrometry (HRMS) to enable the detection and identification of less studied and novel ergot alkaloids (Arroyo-Manzanares *et al*., 2014). Although HRMS has demonstrated great value for qualitative analysis, this technique is commonly not used for quantitative analysis because it is considered to be inferior to QqQ MS/MS in terms of sensitivity, robustness and linear dynamic range. In recent years, new and improved HRMS instruments together with very powerful data-acquisition and data-mining software have become available, offering new possibilities for qualitative and quantitative analysis.

The aim of this study was therefore to investigate the suitability of a modern hybrid quadrupole – time-of-flight (Q-TOF) HRMS instrument based on the TripleTOF® technology to provide simultaneously a quantitative analysis of common ergot alkaloids and the screening, detection and identification of unexpected and less studied or novel ergot alkaloids. The selected workflow involves an untargeted data acquisition (consisting of full TOF-MS survey scan and information dependent acquisition (IDA) MS/MS scans) and the subsequent processing of data using both targeted and untargeted approaches.

2. Experimental

2.1. Standards

Fine film-dried ergot alkaloid standards ergometrine (Em), ergosine (Es), ergotamine (Et), ergocornine (Eco), ergokryptine (Ekr), ergocristine (Ecr), and the corresponding epimers, ergometrinine (Emn), ergosinine (Esn), ergotaminine (Etn), ergocorninine (Econ), ergokryptinine (Ekrn) and ergocristinine (Ecrn), were purchased from Coring System Diagnostix GmbH (Gernsheim, Germany). The film-dried standards were, as indicated by the manufacturer, reconstituted in 5 mL of acetonitrile (MeCN), to give concentrations of 100.0 µg/mL (uncertainty: ± 5.0 µg/mL) for the main ergot alkaloids and of 25.0 µg/mL (uncertainty: ± 1.5 µg/mL) for the epimers. Because of the rapid epimerization of ergot alkaloids in solution, dried standard residues were made of the freshly prepared standard solutions as follows: defined volumes of individual or mixed 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. Ergot alkaloids stored under these conditions

are stable for at least one year (Lauber *et al*., 2005). Immediately prior to use the deep frozen standards were redissolved in the required amount of solvent. Methylergometrine (MeEm, as methylergometrine maleate, VWR International, Zaventem, Belgium) was used as internal standard (IS) for Em, while the IS for the other compounds was dihydroergotamine (DhEt, as dihydroergotamine tartrate, Sigma Aldrich, Bornem, Belgium). From stock solutions at 1 mg/mL were prepared in methanol (MeOH): MeCN (5:5, v/v) and in MeCN, respectively. 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.

2.2. Reagents and materials

MeOH and MeCN (both of LC-MS grade) were supplied by Biosolve (Valkenswaard, the Netherlands). Ethyl acetate (EtOAc) was obtained from Acros Organics (Geel, Belgium) and ammonium bicarbonate and ammonium formate from Sigma-Aldrich. MeCN and MeOH (both of HPLC grade) used for sample treatment, and *n*-hexane were purchased from VWR International. Ammonium sulphate, ammonium acetate, ammonium carbonate and ammonia (25%) were supplied by Merck (Darmstadt, Germany).

The buffers i.e. ammonium formate (0.2 M) pH 9, ammonium acetate (0.2 M) pH 9 and ammonium carbonate (0.2 M) pH 10 were prepared by dissolving the necessary amount of ammonium formate, ammonium acetate and ammonium bicarbonate, respectively, in milli-Q water, and subsequently adjusting to the required pH with ammonia 25 %.

Mycosep® 150 Ergot SPE clean-up columns used for sample treatment were supplied by Romer Labs® (Tulln, Austria). A Milli-Q purification system (Millipore, Brussels, Belgium) was used to purify demineralized water.

2.3. Samples

The cereal samples analyzed consisted of 17 rye samples obtained from feed producers in France. Method optimization and method validation as well as the calibration curves for the quantitative analyses were performed using sample material that was tested free from ergot alkaloids. The absence of ergot alkaloids was confirmed as follows: a portion of sample was analyzed as such and another portion was spiked with the target analytes prior to analysis. By comparing with a solution of standards, no peaks corresponding to the target analytes were found in the non-spiked sample, whereas they were found in the spiked sample. The moisture

content of the samples was determined using a Mettler Toledo LP16 infrared dryer **(**Zaventem, Belgium).

2.4. LC-MS/MS analysis

The experiments were carried out using a hybrid Q-TOF MS instrument, the AB SCIEX TripleTOF® 4600 (Concord, Ontario, Canada), equipped with a DuoSpray™ and coupled to an Eksigent ekspert™ ultraLC 100-XL system. The DuoSpray™ ion source (consisting of both electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) probes) was operated in the positive ESI mode (ESI^+). The atmospheric pressure chemical ionization (APCI) probe was used for automated mass calibration using the Calibrant Delivery System (CDS). The CDS injects a calibration solution matching polarity of ionization and calibrate the mass axis of the TripleTOF® system in all scan functions used (MS and/or MS/MS).

The Q-TOF HRMS method consisted of full scan TOF survey (dwell time 100 ms, 100-1600 Da) and a maximum number of eight IDA MS/MS scans (dwell time 50 ms). The MS parameters were as follows: curtain gas (CUR) 25 psi, nebulizer gas (GS 1) 50 psi, heated gas (GS 2) 60 psi, ion spray voltage (ISVF) 5.5 kV, interface heater temperature (TEM) 500 ºC, collision energy (CE) 10 V and declustering potential (DP) 70 V. For the IDA MS/MS experiments, a CE of 35 V was applied with a collision energy spread (CES) of 15 V.

Chromatographic separation was achieved using an ACQUITY UPLC BEH C18 column (1.7 μm, 100 x 2.1 mm i.d) (Waters, Milford, MA, USA) with a pre-column (1.7 μm, 5 x 2.1 mm i.d.) of the same material. In the final method, a mobile phase consisting of solvent A [H_2O:0.2 M ammonium formate buffer pH 9:MeOH (85:5:10, v/v/v)] and solvent B [H_2O:0.2 M ammonium formate buffer pH 9:MeOH (5:5:90, v/v/v)] was used at flow rate of 0.4 mL/min. The eluent gradient profile was as follows: 0-4 min: 0% B; 4-10 min: 0-75% B; 10-12 min: 75% B; 12-13 min: 75-0% B; 13-16 min: 0% B. The column temperature was set at 40 °C and the injection volume was 10 μL. To minimize epimerization during the LC-MS/MS analysis, the autosampler temperature was maintained at 4 °C and the sample sequence limited to a maximum of 24 hours (Diana Di Mavungu, *et al*., 2012). Data were acquired using Analyst® TF 1.6 Software, while data processing was performed using MultiQuant™ software version 3.0.1. and PeakView® software version 2.1 with MasterView™ version 1.0. For correct quantification, the matrix-matched calibration approach was applied, i.e. the

calibrants for the standard curves were prepared in an analyte free matrix. Calculations were performed using peak areas after applying internal standard corrections.

2.5. Sample preparation

2.5.1. Liquid-Liquid extraction

Samples were prepared following the method described by Diana Di Mavungu *et al*. (2012). Five gram of ground grain sample was extracted with 40 mL EtOAc:MeOH:0.2 M ammonium bicarbonate pH 8.5 (62.5:25:12.5, v/v/v) during 30 min on an Agitelec overhead shaker and the sample extract was centrifuged (10 min, 4000 g). After centrifugation, a phase separation was induced by adding 5 mL of saturated solution of ammonium sulfate and 5 mL of 0.2 M ammonium carbonate buffer pH 10 to 15 mL of the supernatant, previously transferred to a falcon tube. Following 5 min of shaking and 10 min of centrifuging (4000 g), 5 mL of the EtOAc-phase was evaporated to dryness at 40°C under a stream of nitrogen. The residue was reconstituted in 200 µL of MeOH:MeCN: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 14000 g. The *n*-hexane was removed and the aqueous phase was analysed by UHPLC-HRMS.

2.5.2. Solid Phase Extraction (SPE) using MycoSep® 150 Ergot multifunctional columns

The extraction procedure using Mycosep® 150 Ergot SPE clean-up columns was adapted from manufacturer instructions. Five gram of ground grain sample was extracted with 25 mL of MeCN: ammonium carbonate (200 mg/mL) (84:16, v/v) during 30 minutes on an Agitelec overhead shaker. After centrifugation (10 minutes, 4000 g), 4 mL was transferred to a provided test tube. Subsequently, the extract was purified by pushing the Mycosep® column through the extract till the bottom of the test tube. Then, 1 mL of the cleaned-up extract was evaporated to dryness at 40°C under a stream of nitrogen and reconstituted in 200 µL of MeOH:MeCN:H_2O (20:40:40, v/v/v) prior to UHPLC-HRMS analysis.

2.6. Matrix effect

To investigate the influence of matrix components on the MS signal, ergot alkaloids were spiked in analyte-free matrix and in pure solution (i.e. injection solvent). Samples were prepared in triplicate at three concentration levels (20, 50 and 200 µg/kg) and analyzed using the UHPLC-HRMS method. Matrix effects were expressed as signal

suppression/enhancement (SSE) and calculated by comparing the linear function of the ergot alkaloids spiked in cleaned-up extract to that of the ergot alkaloids spiked in matrix-free injection solvent (Eq. 1). An SSE of 100 % indicates no effect of the matrix on the signal, while a value above 100 % indicates signal enhancement and a value below 100 % means suppression of the signal due to the presence of the matrix.

SSE (%) = 100 x (slope spiked cleaned-up extract/ slope spiked matrix-free injection solvent) **(Eq. 1)**

2.6. Validation of the method

Method linearity, limits of detection (LODs) and limits of quantification (LOQs), system and method precision and method trueness in terms of recovery were evaluated by spiking experiments using rye samples that were tested free from ergot alkaloids. The LODs and LOQs were determined as the minimum concentration of analyte in the spiked blank samples inducing an extracted ion chromatogram with a signal-to-noise ratio of 3 and 10, respectively. To assess the linearity of the method, the blank samples were spiked with the different ergot alkaloids over a concentration range of 1–1000 µg/kg. In total, eight different concentrations were included and the analytical procedure was performed in triplicate at each concentration. A linear regression was applied. The trueness, referred to as apparent recovery (Thorburn *et al.*, 2002), was evaluated by recovery experiments. Since a suitable sample with certified concentrations of ergot alkaloids was not available, artificially fortified ergot alkaloid-free samples were analysed and the percent analyte recovery assessed. In detail, samples that were spiked with the different ergot alkaloids over a concentration range of 50, 200 and 400 µg/kg were analysed. The observed signal was plotted against the actual concentration. The measured concentration was determined using the obtained calibration curves, and the recovery was calculated as follows (Eq. 2):

% Recovery = 100 × measured concentration/ actual (added) concentration (**Eq. 2)**

The precision of the method was studied by repeated analysis of spiked samples. The experiments were carried out at different concentrations of the analyte in the sample on the same day (intra-day precision) and on 3 consecutive days (inter-days precision). The precision was calculated as relative standard deviation (RSD) of replicate measurements. An estimate of the relative standard uncertainty (u_c) (Hund *et al.*, 2003) associated with the results was gained for each alkaloid by combining the precision under reproducibility conditions (S_R) with the

uncertainty associated with the purity of standards ($U(C_{ref})$) as well as the uncertainty associated with the mean recovery (S_{bias}) as follows (Eq. 3):

$$u_C = \sqrt{S_R^2 + U(C_{ref})^2 + S_{bias}^2} \qquad \textbf{(Eq. 3)}$$

An estimate of the expanded uncertainty (U) corresponding to a confidence interval of approximately 95% was obtained by multiplying the combined uncertainty by a coverage factor of 2, i.e. (Eq. 4).

$U = 2 \times \mu_c$ **(Eq. 4)**

3. Results and discussion

3.1. Optimization and verification of the UHPLC HRMS (TOF-MS – IDA-MS/MS) method

Selection of the LC conditions was based on a previously developed method for ergot alkaloid determination in cereals and cereal products using a QqQ LC-MS system (Diana Di Mavungu *et al.*, 2012). This method employed an XBridge C18 stationary phase and a mobile phase containing an ammonium carbonate buffer at pH 10. The basic pH ensures good chromatography of the ergot alkaloids (pKa values between 4.8 and 6.2). The use of a basic mobile phase also resulted in very good MS signal for the ergot alkaloids in ESI^+ (Diana Di Mavungu *et al*., 2012). The signal in ESI^+ was in general 10 times higher than that in negative ESI mode. Considering the instability of ammonium carbonate based mobile phase and the resulting retention time reproducibility issue, the use of ammonium acetate and ammonium formate buffers was considered in this study. The ammonium formate buffer resulted in an improved MS signal and was therefore selected for further development. As stationary phase, the ACQUITY UPLC BEH C18 column was selected because of its stability over a broad pH range (1-12) and its suitability for the analysis of a wide polarity range of analytes (Romero-González *et al.*, 2011). A generic gradient was applied as described under section 2.4. Though not crucial, a good separation of the most commonly analysed ergot alkaloids namely Em, Et, Es, Eco, Ecr and Ekr was achieved. Under the selected chromatographic conditions, the required separation of the main compounds from their corresponding epimers was straightforward.

Optimization of the MS parameters demonstrated that the manufacturer's suggested settings were sufficient for the intended generic method. The accumulation time for the TOF MS survey scan was set at 100 ms. Investigation of the accumulation time for the IDA MS/MS scans indicated that a dwell time of 50 ms was required for high quality fragmentation spectra. The actual mass accuracy and mass resolution attainable under 'real life' experimental conditions were assessed. The accuracy data were satisfactory with mass errors below 3 ppm for the different ergot alkaloids, thereby increasing the confidence in the use of accurate mass for automatic dereplication of data. The observed low mass errors also offered the possibility to use a narrow ion extraction window for analyte detection, resulting in the reduction of noise (better signal-to-noise ratio) and consequently, a more accurate quantification. Furthermore, mass measurements in MS and MS/MS modes with low mass error should simplify the determination of elemental composition of molecular and fragment ions, and facilitate their structural elucidation. Overall, the resolution was well above 30000 full width at half maximum (FWHM), which was considered to be sufficient for the purpose of this study. As part of method optimization, the suitability of a clean-up strategy using MycoSep® 150 Ergot multifunctional commercial SPE columns was investigated as compared to the liquid-liquid extraction (LLE) approach applied in the original QqQ LC-MS method (Diana Di Mavungu *et al.*, 2012). Overall, the use of the LLE procedure favored the recovery of the late eluting compounds, while MycoSep® SPE cartridges proved to be more effective for early eluting analytes (Supplemental Figure S1). This was attributed to the difference in extraction solvents: a relatively more apolar solvent mixture was required for the LLE procedure (62.5:25:12.5 EtOAc:MeOH:0.2 M NH_4HCO_3) as compared to the solvent prescribed for the MycoSep® protocol (MeCN:$(NH_4)_2CO_3$ 84:16). The LLE approach, which already demonstrated good performance in previous studies (Diana Di Mavungu *et al.*, 2012; Malysheva *et al.*, 2014), was chosen.

3.2. Validation of the method

With the prospect of its use in quantitative determination of ergot alkaloids, ~~the generic~~ HRMS (TOF MS – IDA MS/MS) method was validated for twelve ergot alkaloids (six major compounds and their corresponding epimers) in rye, according to the Commission Regulation No. 401/2006. The method validation data are summarized in Table 1. LODs and LOQs comparable to those commonly reported using QqQ instruments (Kokkonen and Jestoi, 2009; Mohamed, *et al.*, 2006a) were achieved for the different ergot alkaloids examined. Although no regulatory limits have been set for the amount of ergot alkaloids in food and feed, these

results indicate that the proposed method is appropriate for the detection and the quantification of ergot alkaloids at low µg/kg level. Compared to the initial method developed using a dedicated QqQ-MRM approach (Diana di Mavungu, *et al.*, 2012), the proposed TOF MS - IDA MS/MS method is somewhat less sensitive. However, it has the major advantage of allowing to obtain additional useful information on non-targeted analytes, while providing sufficiently high-quality data for the quantitative analysis of targeted ergot alkaloids. Good linearity was observed for all compounds in the range from LOQ to 1000 µg/kg with determination coefficients (R^2) above 0.96. The trueness values (in terms of apparent recovery) were within the 80-110 % range for all analytes at the high concentrations (200 and 400 µg/kg) and were therefore in good agreement with the Commission Regulation 401/2006 performance criteria for quantitative methods of analysis. At the low concentration (50 µg/kg), the trueness values were also between 80 and 110 % for most of ergot alkaloids, except for Emn (115 %) and Ecr (112 %). According to Commission Regulation 401/2006, precision values for the repeated analysis of fortified material, under repeatability (RSD_r) and reproducibility (RSD_R) conditions, shall not exceed the level calculated by the Horwitz Equation (5 and 6). Using this equation for the mass fraction of 50 µg/kg resulted in an unreasonably high acceptance limit for both RSD_r and RSD_R. Therefore, in this case, as low as possible RSD_r and RSD_R values were desired. Data in Table 1 demonstrate an overall good precision of the HRMS method, except for the RSD_r values for Emn and Ecrn at 400 µg/kg, which are slightly above the calculated acceptance limit of 12 %. The uncertainty was not higher than 35 % for the different ergot alkaloids at all concentration levels studied.

$$RSD_r = 2/3(2^{[1-0.5 \log C]}) \qquad \textbf{(Eq. 5)}$$

$$RSD_R = 2^{[1-0.5 \log C]} \qquad \textbf{(Eq. 6)}$$

where c is the concentration ratio (i.e. 1 = 100g/100g, 0.001 = 1 000 mg/kg)

As part of the evaluation of the HRMS method for quantitative determination of ergot alkaloids, the influence of matrix interferences on method performance was investigated. Different level of the matrix effect was observed depending on the ergot alkaloids (Figure 2). Considerable signal was observed for compounds such as Ecrn, Etn, Econ and Ekrn. Therefore, the matrix-matched calibration approach was chosen to perform correct quantitative analysis. Furthermore, by spiking the standards in the matrix prior to the

extraction and clean-up steps, the resulting data were automatically corrected for losses due to the sample preparation.

3.3. Application of the TOF MS – IDA MS/MS for targeted and untargeted analysis of ergot alkaloids in cereal samples

3.3.1. Quantitative analysis of common ergot alkaloids

Data acquired using the TOF MS – IDA MS/MS method were processed in a targeted way to investigate the presence of 6 common ergot alkaloids (i.e. Em, Es, Et, Eco, Ekr, Ecr) and their corresponding epimers in rye samples. Multiple points of selectivity were considered for analyte identification in samples: accurate mass, retention time, isotope distribution, MS/MS spectra library searching and the confirmation of the formula by combining MS and MS/MS data. The combination of these criteria and in particular the inclusion of MS/MS spectra library searching ensured a more confident compound identification than the use of accurate mass alone (non-hybrid HRMS instruments) or the monitoring of a maximum of two MRM transitions (QqQ instruments). One or more ergot alkaloids were detected and confirmed in 15 out of 17 samples analyzed. For quantification, the full scan TOF MS data unbiasedly acquired for each analyte were used. Though a maximum of eight IDA MS/MS scan were acquired together with the TOF MS survey scan, the instrument scan speed permitted to collect sufficient data points, ensuring a good description of peaks and consequently a correct and robust quantification. Quantitative data for the 12 target analytes are summarized in Table 2. The content of individual ergot alkaloids varied from <LOQ to 2811 µg/kg in the samples analyzed. Em and its epimer Emn were found to be the most frequently occurring ergot alkaloids, while the highest levels of contamination were observed with Et. In general the main derivative co-occurred with its corresponding epimer. In positive samples, the total ergot alkaloid contents were in the range of 5- 12600 µg/kg.

3.3.2. Screening and identification of unknown and less studied ergot alkaloids

In an effort to provide a more comprehensive insight on the occurrence of ergot alkaloids in the samples described above, the same data were further investigated for the presence of unexpected ergot metabolites (not included in the list of the 12 target compounds and for which reference standards were not available). Hence a non-targeted processing of these data

was performed. The 'Non-Targeted Peak Finding' algorithm of Masterview™ software was applied to automatically detect peaks in samples. A 'sample-control' comparison was performed to eliminate the endogenous components that are the same in both types of samples, thereby allowing to retain and focus only on more relevant peaks. Examination of the MS/MS spectra of the retained unknown compounds allowed to further narrow down to only those peaks that showed the typical ergot alkaloid fragmentation pattern. Indeed, the fragmentation pattern of ergot alkaloids was previously studied (Arroyo-Manzanares *et al.*; 2014; Mohamed *et al*., 2006b) and indicated that all derivatives give rise mainly to fragment ions with *m/z* 223 and 208 corresponding to the four-ring system of the lysergic moiety and its demethylated counterpart, respectively. In total, 19 different compounds demonstrated a fragmentation pattern that pointed to ergot alkaloids. A typical example is given in Figure 3. Potential empirical formulas for each of these unknown analytes were generated using the exact mass of the molecular ion and confirmed using fragmentation data (acquired with high mass accuracy) and isotopic pattern. To automatically identify putative structures that match a given formula, the data were linked to the ChemSpider database. The proposed structures were confirmed by comparing their theoretical fragmentation to the actual MS/MS spectra acquired for the unknown analyte (illustrated in Figure 4 for an unexpected ergot alkaloid with *m/z* 534.2711). In this manner, empirical formulas could be established for 13 unknown compounds that were not present in the control (blank) and which showed a characteristic ergot alkaloid fragmentation pattern. Assessing the acquired MS/MS data in light of the simulated fragmentation spectra, the structure of these unexpected ergot alkaloids could be assigned. These included ergocornam, ergovaline, ergocryptam, ergocryptam isomer, ergocristam, ergostine, ergoptine, ergogaline, ergostinine, ergoptinine, ergogalinine, ergokryptine isomer and hydroxyergotamine (Table 3). These compounds have been previously reported in grain and grass samples (Cvak *et al*., 1994; Cvak *et al*., 2005; Duringer *et al*., 2007; Lehner *et al*., 2005).

For 6 other compounds (namely *m/z* 340.1651 at 7.6 min (M1), 340.1651 at 10.9 min (M2), 368.1964 at 8.1 (M3), 368.1964 at 10.4 (M4), 382.2118 at 10.6 min (M5) and 382.2118 at 11.9 min (M6)) that showed a typical ergot alkaloid fragmentation pattern, putative structures could not be proposed by searching the ChemSpider database, using initially generated empirical formulas. The molecular formula for M1 and M2, M3 and M4, M5 and M6 were indeed assigned as $C_{19}H_{22}N_3O_3^+$, $C_{21}H_{26}N_3O_3^+$ and $C_{22}H_{28}N_3O_3^+$, respectively. This pattern of identical *m/z* values for couples of peaks with different retention times pointed to the existence of these compounds as epimers. To elucidate the structure of these compounds, a

detailed fragmentation pattern study was performed in light of the ergot alkaloid identification strategy we proposed previously (Arroyo-Manzanares *et al*. 2014). The mass range of each of the 3 compounds and corresponding epimers (*m/z* of 368, 340 and 382) suggested that they could be ergoamides. As previously demonstrated (Arroyo-Manzanares *et al*. 2014), initially, ergoamides undergo a loss of H_2O (18 Da) or CH_3 by homolytic cleavage (15 Da). In the spectrum of compounds M1, M3 and M5 (Figure 5) the loss corresponding to the homolytic cleavage of the CH_3 moiety could be observed (fragments with *m/z* 325, 353 and 367 for M1, M3 and M5 respectively); however, the initial loss of 18 Da (corresponding to the elimination of a water molecule) was not seen. The ergoamides also undergo a cleavage within the lysergic D ring, losing 43 Da ($-C_2H_5N$); this loss was observed in respective spectra. Subsequent to the loss of a C_2H_5N moiety, the model ergoamide Em undergoes a loss of H_2O. Such a loss was not observed for compounds M1 and M3. Instead, a loss of 46 Da was seen, indicating that the difference between these two unexpected ergot alkaloids and Em was at the level of the R_2 group, while the difference between M1 and M3 was due to R_1 (Figure 6). M5 underwent a loss of 60 Da instead 46 Da, giving the ion with *m/z* 279, the same as that obtained for M3. This suggested that M3 and M5 share the same R_2 but differ at the level of R_1. Data described above are consistent with the substitution of the alaninol moiety of Em with alanine in M1. M3 corresponds to the substitution of alaninol (Em) with valine (M3), while M5 would be its methylester derivative. Hence, M1, M3 and M5 were identified as lysergyl alanine, lysergyl valine (ergoval) and N-(d-lysergyl)-l-valine methylester, respectively. The structures and the proposed fragmentation pathway for these compounds are shown in Figure 6. N-(d-lysergyl)-l-valine methylester has been reported to be a degradation product of ergocristam by methanolysis (Olsovská *et al*., 2008). Whether such methanolysis occurred under the applied analytical conditions is uncertain. Lysergyl valine has been rarely found in food samples (Urga *et al*., 2002); however there is no evidence that lysergyl alanine has been detected in naturally contaminated food samples. To the best of our knowledge, this is the first time epimers of lysergyl alanine and lysergyl valine have been reported in food samples.

4. Conclusion

An integrated qualitative and quantitative UHPLC – HRMS strategy based on TripleTOF MS technology is proposed for the analysis of ergot alkaloids in cereals. The usefulness and applicability of this methodology was demonstrated by its performance characteristics for quantitative analysis of 6 common ergot alkaloids and their corresponding epimers: method

LODs and LOQs were comparable to those previously achieved with QqQ instruments. Furthermore, the method demonstrated good linearity, precision and trueness. The employed TOF MS - IDA MS/MS acquisition method allowed quantitative analysis of targeted analytes (ie Em, Es, Et, Eco, Ecr, Ekr and epimers) and screening, detection and identification of unexpected ergot alkaloids in rye samples in a single UHPLC-HRMS run. This has become possible owing to the availability of modern hybrid quadrupole – TOF MS instruments that combine the quantitative speed, sensitivity, linear dynamic range and robustness of triple quadrupole with the qualitative analysis power through high resolution and accurate mass measurement offered by HRMS technology. Also improvements in data processing software play and will continue to play an important role in the extraction of the untapped information generated by these instruments. Compared to the commonly used QqQ MRM approach, the proposed TOF MS - IDA MS/MS methodology is easy to set up (dedicated compound optimization is not required) and has the merit of achieving a comprehensive screening of ergot alkaloids, including those for which no analytical standards are available. In addition, the availability of accurate mass MS and MS/MS data for an unlimited number of analytes should allow posterior mining of these data to provide answers to new/emerging research questions.

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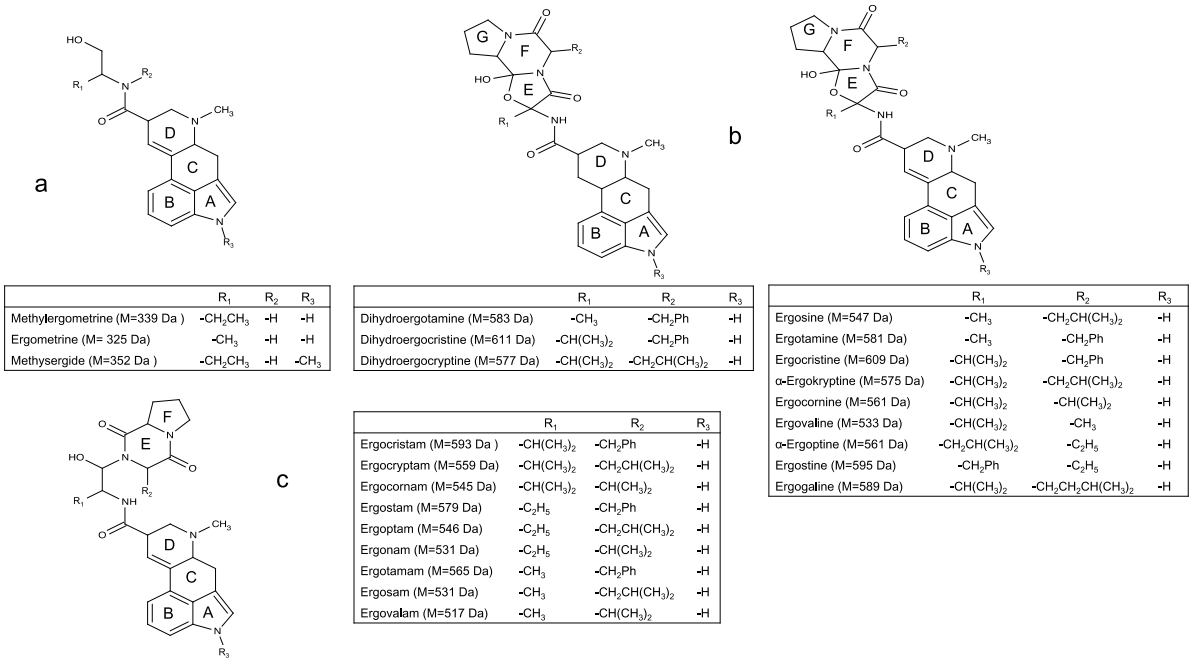


	R_1	R_2	R_3
Methylergometrine (M=339 Da)	$-CH_2CH_3$	-H	-H
Ergometrine (M= 325 Da)	$-CH_3$	-H	-H
Methysergide (M=352 Da)	$-CH_2CH_3$	-H	$-CH_3$

	R_1	R_2	R_3
Dihydroergotamine (M=583 Da)	$-CH_3$	$-CH_2Ph$	-H
Dihydroergocristine (M=611 Da)	$-CH(CH_3)_2$	$-CH_2Ph$	-H
Dihydroergocryptine (M=577 Da)	$-CH(CH_3)_2$	$-CH_2CH(CH_3)_2$	-H

	R_1	R_2	R_3
Ergosine (M=547 Da)	$-CH_3$	$-CH_2CH(CH_3)_2$	-H
Ergotamine (M=581 Da)	$-CH_3$	$-CH_2Ph$	-H
Ergocristine (M=609 Da)	$-CH(CH_3)_2$	$-CH_2Ph$	-H
α-Ergokryptine (M=575 Da)	$-CH(CH_3)_2$	$-CH_2CH(CH_3)_2$	-H
Ergocornine (M=561 Da)	$-CH(CH_3)_2$	$-CH(CH_3)_2$	-H
Ergovaline (M=533 Da)	$-CH(CH_3)_2$	$-CH_3$	-H
α-Ergoptine (M=561 Da)	$-CH_2CH(CH_3)_2$	$-C_2H_5$	-H
Ergostine (M=595 Da)	$-CH_2Ph$	$-C_2H_5$	-H
Ergogaline (M=589 Da)	$-CH(CH_3)_2$	$-CH_2CH_2CH(CH_3)_2$	-H

	R_1	R_2	R_3
Ergocristam (M=593 Da)	$-CH(CH_3)_2$	$-CH_2Ph$	-H
Ergocryptam (M=559 Da)	$-CH(CH_3)_2$	$-CH_2CH(CH_3)_2$	-H
Ergocornam (M=545 Da)	$-CH(CH_3)_2$	$-CH(CH_3)_2$	-H
Ergostam (M=579 Da)	$-C_2H_5$	$-CH_2Ph$	-H
Ergoptam (M=546 Da)	$-C_2H_5$	$-CH_2CH(CH_3)_2$	-H
Ergonam (M=531 Da)	$-C_2H_5$	$-CH(CH_3)_2$	-H
Ergotamam (M=565 Da)	$-CH_3$	$-CH_2Ph$	-H
Ergosam (M=531 Da)	$-CH_3$	$-CH_2CH(CH_3)_2$	-H
Ergovalam (M=517 Da)	$-CH_3$	$-CH(CH_3)_2$	-H

Figure 1. General structures of ergoamides (a), ergopeptines (b) and ergopeptams (c) and representative ergot alkaloids for each class.

M: molecular weight.

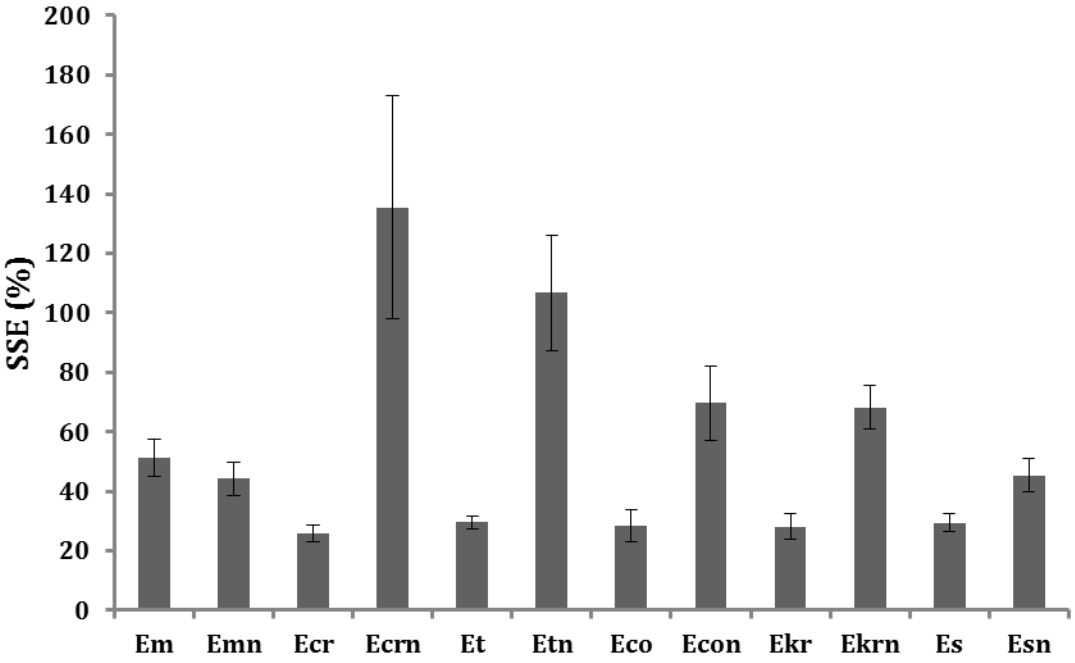


Figure2. Matrix effects calculated for the different ergot alkaloids.

The bars depict the mean values of signal suppression/enhancement (SSE), while the error lines represent the standard deviations for 3 measurements.

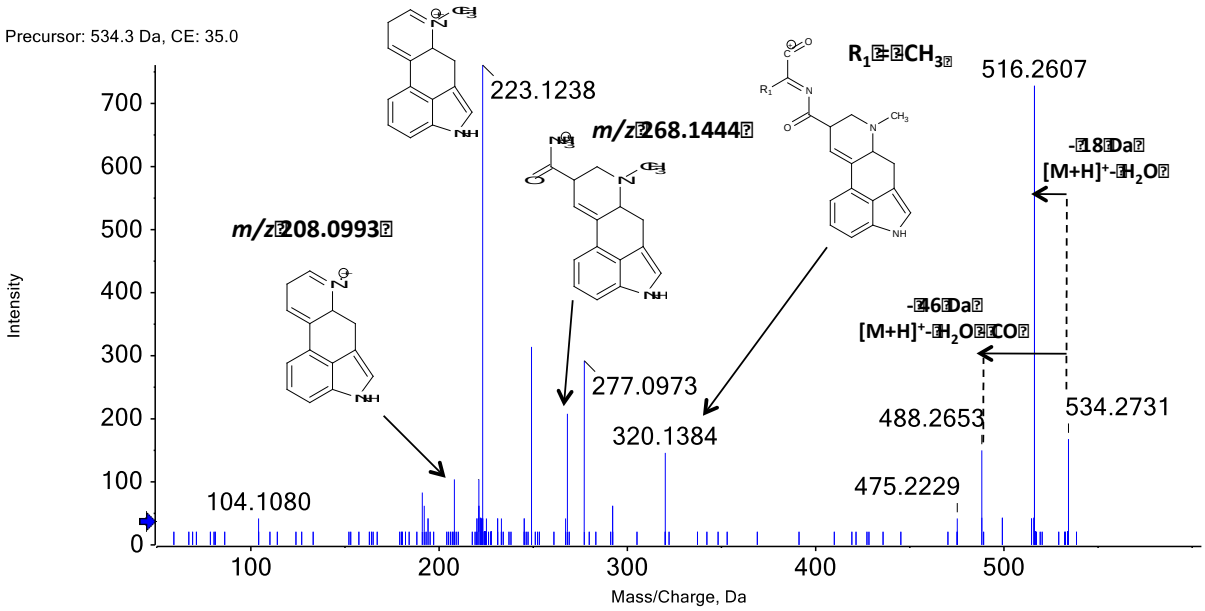


Figure 3. Ergovaline TOF MS^2 spectrum revealing characteristic ergot alkaloid fragmentation

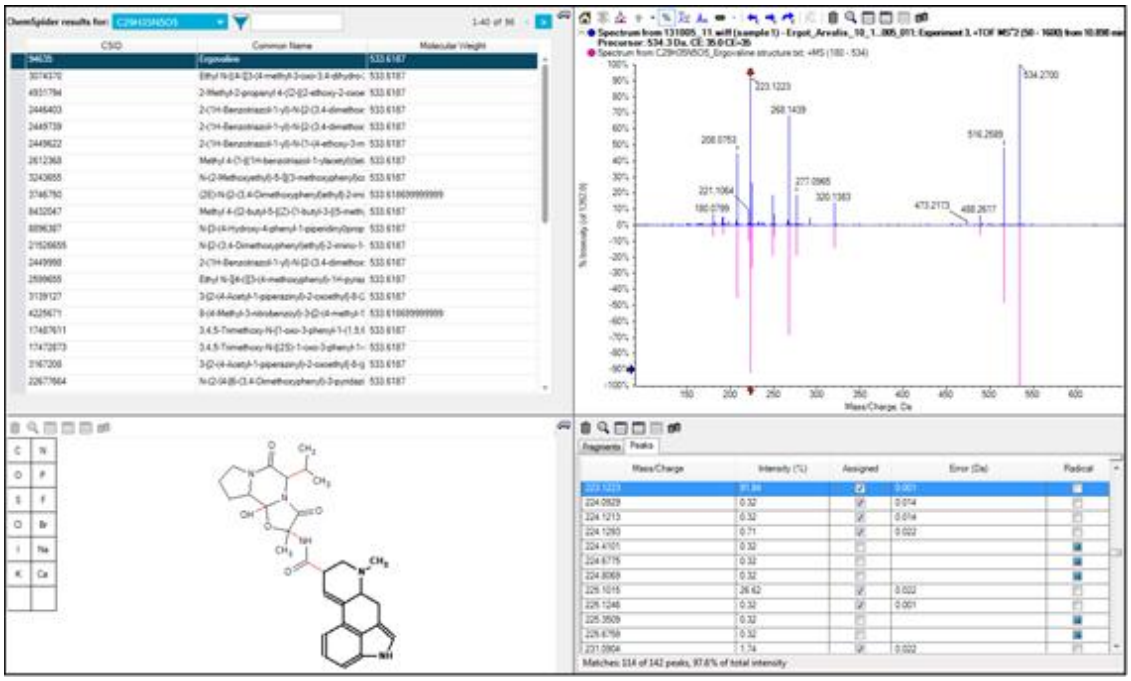


Figure 4. Identification of the unexpected ergot alkaloid with *m/z* 534.3 by linking MS and MS/MS data to ChemSpider database.

The upper left pane lists putative compounds for the empirical formula $C_{29}H_{35}N_5O_5$ generated using MS and MS/MS accurate mass data. The structure for the selected hit (ergovaline) is displayed in the lower left pane. In the upper right pane a perfect match can be seen between the acquired MS/MS spectrum for m/z 534.3 (spectrum in blue) and the one that was simulated for the selected structure (spectrum in red displayed as inverted overlay). By selecting a fragment (eg m/z 223.1223) on the MS/MS spectrum, the corresponding part of the structure is highlighted (in bold) in lower left pane. The lower right pane gives the fragmentation details.

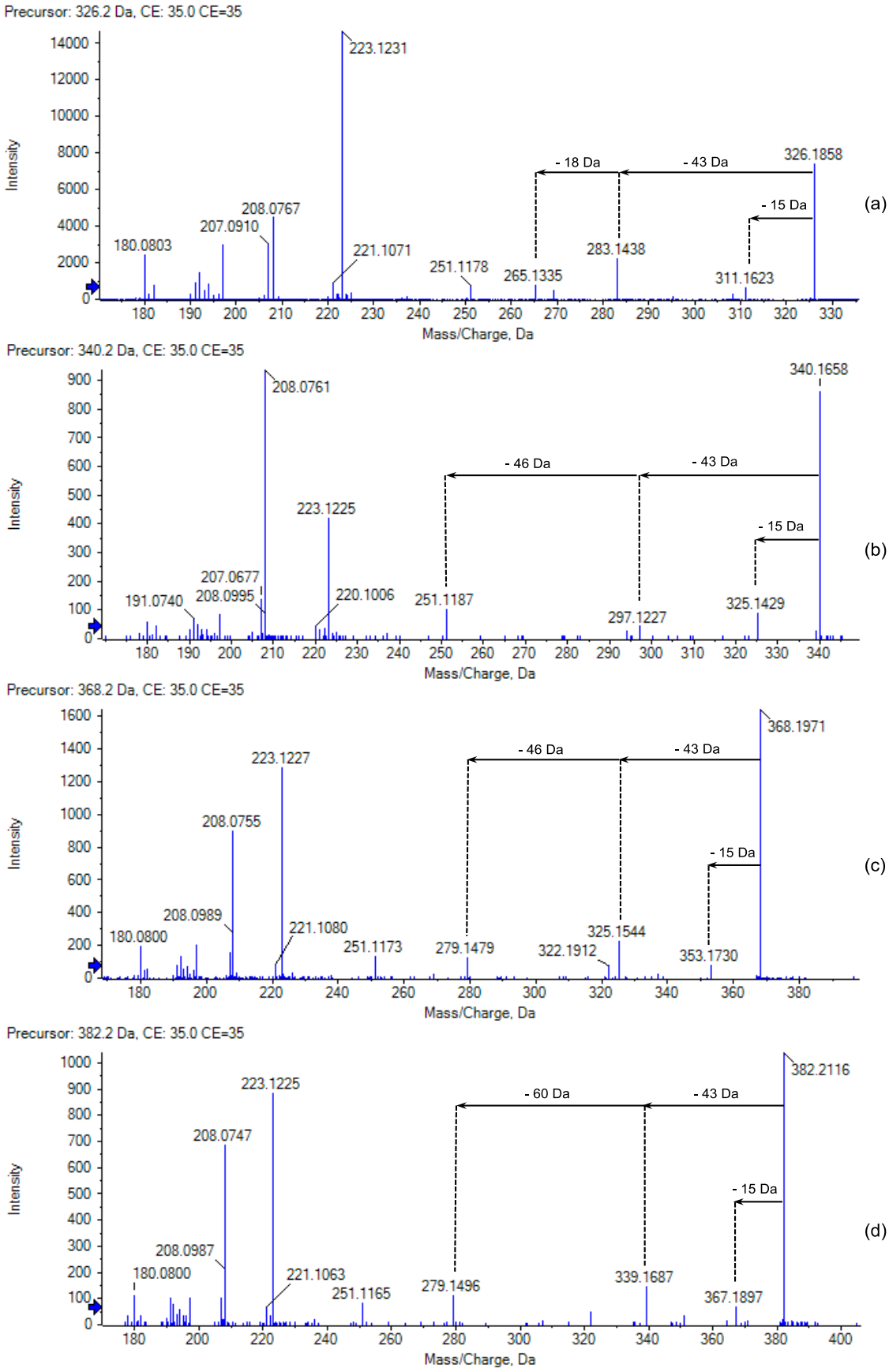
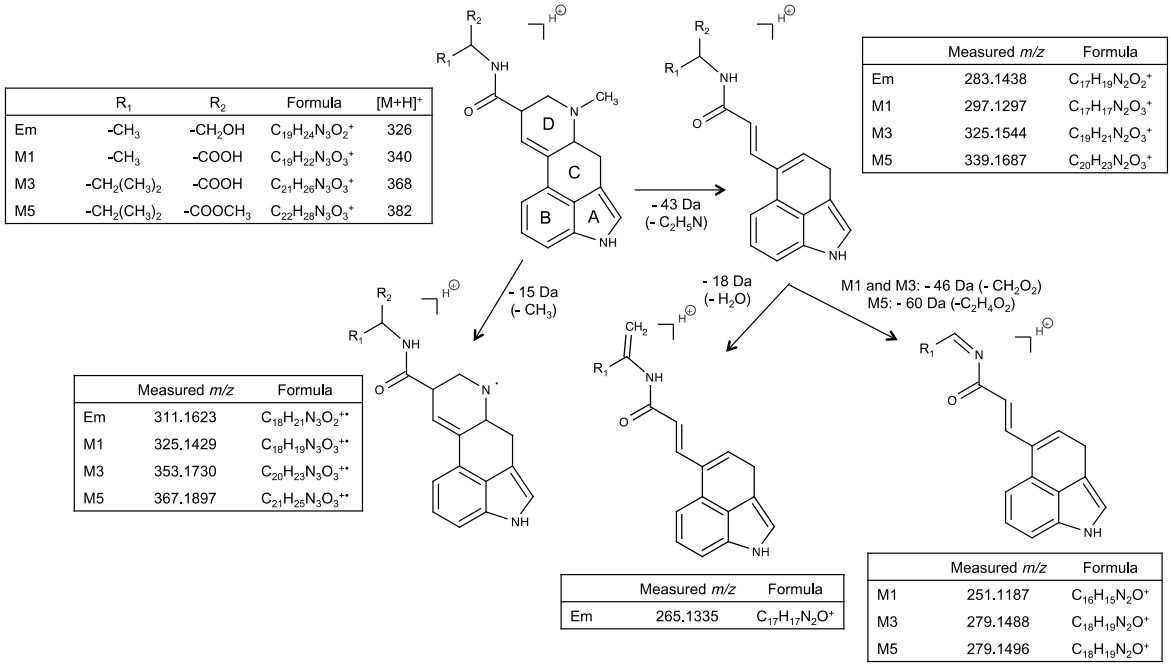


Figure 5. TOF MS/MS spectra acquired for (a) Em, (b) M1, (c) M3 and (d) M5



	R_1	R_2	Formula	$[M+H]^+$
Em	$-CH_3$	$-CH_2OH$	$C_{19}H_{24}N_3O_2^+$	326
M1	$-CH_3$	-COOH	$C_{19}H_{22}N_3O_3^+$	340
M3	$-CH_2(CH_3)_2$	-COOH	$C_{21}H_{26}N_3O_3^+$	368
M5	$-CH_2(CH_3)_2$	$-COOCH_3$	$C_{22}H_{28}N_3O_3^+$	382

	Measured *m/z*	Formula
Em	283.1438	$C_{17}H_{19}N_2O_2^+$
M1	297.1297	$C_{17}H_{17}N_2O_3^+$
M3	325.1544	$C_{19}H_{21}N_2O_3^+$
M5	339.1687	$C_{20}H_{23}N_2O_3^+$

	Measured *m/z*	Formula
Em	311.1623	$C_{18}H_{21}N_3O_2^{+\bullet}$
M1	325.1429	$C_{18}H_{19}N_3O_3^{+\bullet}$
M3	353.1730	$C_{20}H_{23}N_3O_3^{+\bullet}$
M5	367.1897	$C_{21}H_{25}N_3O_3^{+\bullet}$

	Measured *m/z*	Formula
Em	265.1335	$C_{17}H_{17}N_2O^+$

	Measured *m/z*	Formula
M1	251.1187	$C_{16}H_{15}N_2O^+$
M3	279.1488	$C_{18}H_{19}N_2O^+$
M5	279.1496	$C_{18}H_{19}N_2O^+$

Figure 6. Proposed structures for M1, M3 and M5 and fragmentation pathway

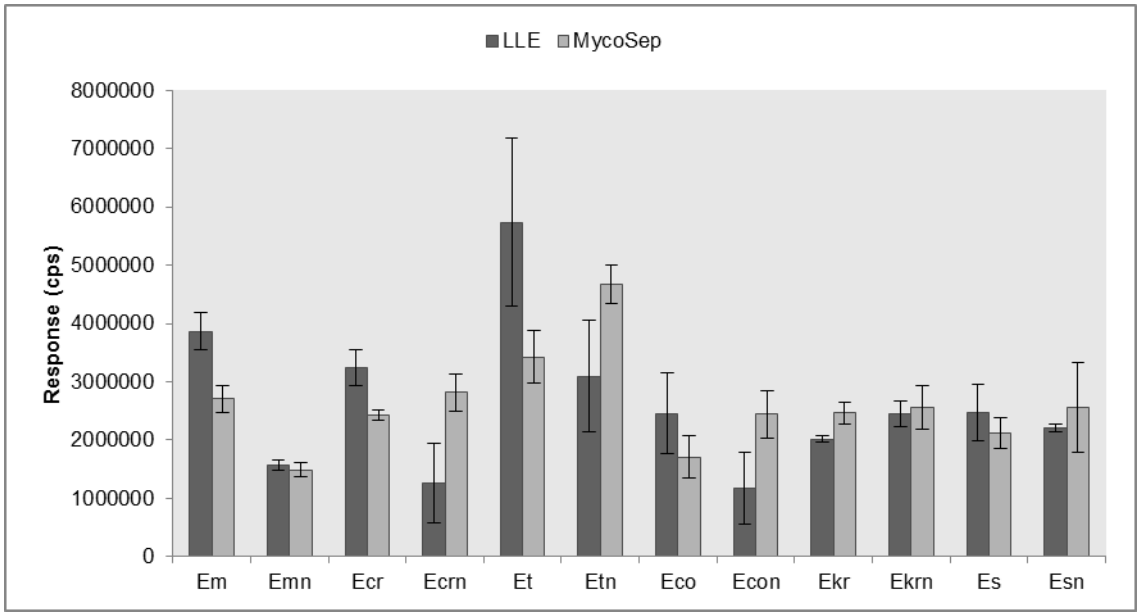


Figure S1. Comparison of LLE and SPE using MycoSep® multifunctional columns

Tables

Table 1. Method validation data for ergot alkaloid determination in rye matrix

Ergot alkaloid	Equation	Linear range (µg/kg)	Linearity R^2
Em	Y = 0.00491 x + 0.01033	5 – 1000	0.9913
Emn	Y = 0.00472 x + 0.00943	3 – 1000	0.9885
Ecr	Y = 0.00054 x + 0.00049	19 – 1000	0.9861
Ecrn	Y = 0.00240 x – 0.00102	6 – 1000	0.9914
Et	Y = 0.00055 x – 0.00082	19 – 1000	0.9766
Etn	Y = 0.00150 x – 0.00124	4 – 1000	0.9863
Eco	Y = 0.00080 x + 0.00101	17 – 1000	0.9903
Econ	Y = 0.00233 x + 0.00111	9 – 1000	0.9829
Ekr	Y = 0.00074 x + 0.00210	19 – 1000	0.9841
Ekrn	Y = 0.00294 x – 0.00107	10 – 1000	0.9907
Es	Y = 0.00060 x + 0.00183	15 – 1000	0.9682
Esn	Y = 0.00141 x – 0.00012	10 – 1000	0.9873

Ergot alkaloid	LOD (µg/kg)	LOQ (µg/kg)	Intra-day precision, RSD_r (%)			Inter-day precision, RSD_R (%)		
			50 µg/kg	200 µg/kg	400 µg/kg	50 µg/kg	200 µg/kg	400 µg/kg
Em	1.5	5.1	10.4	10.0	10.9	12.4	10.0	10.9
Emn	1.0	3.4	12.6	10.0	12.7	12.9	10.0	12.6
Ecr	5.6	18.6	13.2	6.1	7.0	13.2	6.9	8.8
Ecrn	1.8	6.0	8.3	10.3	14.3	9.6	10.5	14.3
Et	5.8	19.4	14.6	4.7	3.6	16.2	6.9	5.0
Etn	1.3	4.4	8.4	6.4	8.0	9.8	9.9	10.7
Eco	5.1	17.0	9.3	4.8	5.6	9.3	5.0	5.6
Econ	2.6	8.7	9.9	8.0	5.7	11.6	10.9	5.7
Ekr	5.9	19.7	12.9	6.4	8.6	12.9	6.4	10.7
Ekrn	3.0	10.1	12.2	10.6	10.5	12.2	13.3	10.4
Es	4.6	15.4	12.6	5.8	4.4	15.1	5.9	9.4
Esn	3.1	10.4	5.1	5.8	4.4	6.0	5.9	9.3

Ergot alkaloid	Trueness %			Expanded measurement uncertainty %		
	50 µg/kg	200 µg/kg	400 µg/kg	50 µg/kg	200 µg/kg	400 µg/kg
Em	109	104	90	27	21	23
Emn	115	102	89	23	21	27
Ecr	112	98	101	28	15	19
Ecrn	105	104	105	21	22	30
Et	110	99	101	35	15	11
Etn	109	105	105	21	22	24
Eco	106	99	99	19	11	12
Econ	106	103	103	25	24	12
Ekr	104	98	105	27	13	23
Ekrn	110	106	103	26	29	22
Es	109	99	102	33	12	21
Esn	104	101	98	13	12	21

Table 2. Occurrence data for 6 common ergot alkaloids and their epimers

Ergot alkaloid	Formula	*m/z*	Concentration (µg/kg)*															
			1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
			10.3%**	11.4%	10.2%	8.6%	9.1%	10.1%	9.2%	8.9%	8.2%	9.3%	10%	8.7%	11.1%	9.5%	9.7%	9.3%	11.7%
Em	$C_{19}H_{23}N_3O_2$	326.1863	ND***	68	46	ND	29	249	8	73	76	692	76	71	308	325	12	< LOQ	89
Emn	$C_{19}H_{23}N_3O_2$	326.1863	ND	24	18	ND	16	167	5	44	51	434	51	46	197	232	8	< LOQ	67
Ecr	$C_{35}H_{39}N_5O_5$	610.3024	ND	298	285	ND	67	587	< LOQ	162	168	1671	168	188	1363	852	25	< LOQ	ND
Ecrn	$C_{35}H_{39}N_5O_5$	610.3024	ND	80	66	ND	22	187	8	62	42	558	42	52	272	247	7	< LOQ	ND
Et	$C_{33}H_{35}N_5O_5$	582.2711	ND	< LOQ	< LOQ	ND	< LOQ	94	< LOQ	101	333	2811	333	423	1762	1797	87	< LOQ	ND
Etn	$C_{33}H_{35}N_5O_5$	582.2711	ND	< LOQ	ND	ND	9	56	< LOQ	55	154	1342	154	197	844	847	40	5	ND
Eco	$C_{31}H_{39}N_5O_5$	562.3024	ND	40	30	ND	28	225	< LOQ	62	54	654	54	83	365	323	< LOQ	< LOQ	ND
Econ	$C_{31}H_{39}N_5O_5$	562.3024	ND	19	15	ND	19	158	10	54	37	543	37	46	225	233	< LOQ	< LOQ	ND
Ekr	$C_{32}H_{41}N_5O_5$	576.3181	ND	113	84	ND	43	429	< LOQ	125	97	1362	97	138	919	583	< LOQ	< LOQ	ND
Ekrn	$C_{32}H_{41}N_5O_5$	576.3181	ND	12	< LOQ	ND	< LOQ	40	< LOQ	14	< LOQ	146	< LOQ	11	52	52	< LOQ	ND	ND
Es	$C_{30}H_{37}N_5O_5$	548.2868	ND	282	177	ND	51	430	36	136	151	1276	151	203	717	689	21	< LOQ	ND
Esn	$C_{30}H_{37}N_5O_5$	548.2868	ND	< LOQ	ND	ND	47	294	40	101	112	1112	112	163	786	648	18	< LOQ	ND
Total ergot alkaloid content (µg/kg)			ND	936	721	ND	332	2918	107	989	1274	12600	1274	1623	7809	6828	218	5	157

* Quantitative data are of undried material, the moisture content is given; ** moisture content (%); *** ND: Not Detected.

Table 3. Occurrence data for unexpected ergot alkaloids

Metabolite	Formula	*m/z*	Sample															
			1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
			10.3%*	11.4%	10.2%	8.6%	9.1%	10.1%	9.2%	8.9%	8.2%	9.3%	10%	8.7%	11.1%	9.5%	9.7%	9.3%	11.7%
Ergocornam	$C_{31}H_{39}N_5O_4$	546.3075	+	+	+	ND	+	++	ND	+	+	+++	+	+	++	++	ND	ND	ND
Ergovaline	$C_{29}H_{35}N_5O_5$	534.2711	ND **	+	+	ND	+	++	ND	+	+	++	+	+	++	++	ND	ND	ND
Ergocryptam	$C_{32}H_{41}N_5O_4$	560.3231	+	+	+	ND	+	+	ND	+	+	++	+	+	++	+	ND	ND	ND
Ergocryptamisomer	$C_{32}H_{41}N_5O_4$	560.3231	ND	+	+	ND	+	++	ND	+	+	++	+	+	++	++	ND	ND	ND
Ergocristam	$C_{35}H_{39}N_5O_4$	594.3075	ND	+	+	ND	+	++	ND	+	+	+++	+	+	+++	++	ND	ND	ND
Ergostine	$C_{34}H_{37}N_5O_5$	596.2868	ND	+	+	ND	+	+	ND	+	+	++	+	+	++	++	ND	ND	ND
Ergoptine	$C_{31}H_{39}N_5O_5$	562.3024	ND	++	++	++	++	++++	+	++	++	+++++	++	+++	+++++	++++	+	ND	ND
Ergoptinine	$C_{31}H_{39}N_5O_5$	562.3024	ND	++	+	++	++	++++	+	++	++	+++++	++	++	+++++	++++	+	ND	ND
Ergogaline	$C_{33}H_{43}N_5O_5$	590.3337	ND	+	+	ND	+	++	ND	+	+	++	+	+	++	++	+	ND	ND
Ergostinine	$C_{34}H_{37}N_5O_5$	596.2868	ND	+	+	ND	+	+	ND	+	+	++	+	+	+	+	+	ND	ND
Ergogalinine	$C_{33}H_{43}N_5O_5$	590.3337	ND	ND	ND	ND	ND	+	ND	ND	ND	++	ND	ND	ND	ND	ND	ND	ND
Ergokryptine isomer	$C_{32}H_{41}N_5O_5$	576.3181	ND	++	+	ND	+	+++	ND	++	++	++++	++	++	+++++	+++	+	ND	ND
Hydroxyergotamine	$C_{33}H_{35}N_5O_6$	598.2668	ND	+	+	++	++	++++	+	++	++	+++++	++	++	+++++	+++++	+	ND	ND

Lysergyl alanine	$C_{19}H_{21}N_3O_3$	340.1651	ND	ND	ND	ND	ND	++	ND	+	ND	+++	ND	+	++	++	ND	ND	ND
Lysergyl alanine isomer	$C_{19}H_{21}N_3O_3$	340.1651	ND	ND	ND	ND	ND	+	ND	ND	ND	++	ND	ND	+	+	ND	ND	ND
Lysergylvaline	$C_{21}H_{25}N_3O_3$	368.1964	ND	+	+	ND	ND	++	ND	+	+	+++	+	+	++	+++	ND	ND	ND
Lysergylvalineisomer	$C_{21}H_{25}N_3O_3$	368.1964	ND	+	+	+	+	++	ND	++	+	++++	+	++	++	++	ND	ND	ND
N-(d-lysergyl)-l-valinemethyester	$C_{22}H_{27}N_3O_3$	382.2119	ND	++	+	ND	ND	++	ND	+	+	++	+	+	+++	++	ND	ND	ND
N-(d-lysergyl)-l-valinemethyesterisomer	$C_{22}H_{27}N_3O_3$	382.2119	ND	ND	ND	ND	ND	+	ND	ND	ND	++	ND	ND	+	+	ND	ND	ND

'+': area ≤ 10*3; '++': 10*3 < area ≤ 5·10*3; '+++': 5·10*3 < area ≤ 10*4; '++++': 10*4 < area ≤ 2·10*4; '+++++': area > 2·10*4;

* moisture content (%); ** ND: Not Detected.