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Holistic approach based on high resolution and multiple stage mass spectrometry to investigate ergot alkaloids in cereals

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ABSTRACT

A holistic approach based on high resolution and multiple stage mass spectrometry was developed for identification of less studied or novel ergot alkaloid derivatives. Initially, the fragmentation of nine known ergot alkaloids was studied to establish a strategy for the identification of novel ergot alkaloids. Ions with *m/z* 223 and *m/z* 251 were found to be common for all ergopeptines, ergoamides and ergopeptams. Subsequently, parent scan experiments using these ions were performed to screen grain samples for the presence of possible ergot alkaloid derivatives. Besides the six most common ergot alkaloids and their corresponding epimers (for which reference standards were available), eleven other ergot alkaloid derivatives were identified following the proposed strategy.

1. Introduction

Ergot alkaloids are secondary metabolites produced by *Claviceps* spp., which mostly infect grains and grasses [1,2]. Ingestion of food and feed contaminated with ergot alkaloids might cause adverse health effects in humans and animals (*e.g.*, ergotism). The poisoning is characterized by such symptoms as abdominal pain, vomiting, burning sensation of the skin, insomnia and hallucinations [3].

The majority of ergot alkaloids are commonly comprised of ergoamides, ergopeptines (or ergopeptides), also called cyclol ergot alkaloids, and the lactam ergot alkaloids, also named ergopeptams [1,4]. The ergoamides are D-lysergic acid amides (Fig. 1a), whereas the ergopeptines are D-lysergic acid peptides containing lysergic acid and three amino acids in their structure (Fig. 1b). The ergopeptams are tripeptidic non-cyclol ergot alkaloids (Fig. 1c). Their structure is similar to that of ergopeptines except that L-proline is exchanged by D-proline, and the tripeptide chain is a non-cyclol lactam [1].

To date, more than 40 ergot alkaloids are known. Several analytical techniques have been used to study the fragmentation of these compounds and attempts have been made to identify novel derivatives. In the last years, a number of new ergot alkaloids has been discovered [5–8]. Mohamed et al. [9] used triple quadrupole and multiple stage mass spectrometry (MS) to characterize six ergot alkaloids belonging to lysergic acid and peptide-type derivatives and could confirm the presence of ergosine in a rye flour extract at trace levels. Lehner et al. [7,10] using high-performance liquid chromatography (HPLC) coupled to tandem quadrupole MS and ion trap MS were able to establish the fragmentation patterns of eight ergot alkaloids and subsequently elucidate a new ergot alkaloid-related compound. Uhlig and Petersen [11] obtained structural information of four ergopeptams using LC-ion trap MS.

In the above-mentioned studies, fragment assignment was supported by hydrogen/deuterium exchange [9] and/or comparison of the fragmentation behavior of known ergot alkaloids, using unit mass resolution data acquired by triple quadrupole and ion trap instruments. Along with fragmentation trees, accurate mass measurement is a highly important feature for correct structure elucidation [12]. Orbitrap MS is becoming a more and more popular platform for identification purposes in natural product analysis [13]. Regarding the secondary fungal metabolites and more specifically ergot alkaloids, this type of MS has been utilized only in targeted analysis [14] or in pre-selected screening using a

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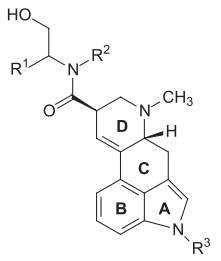
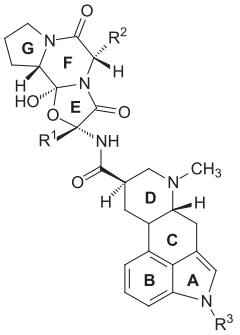
a

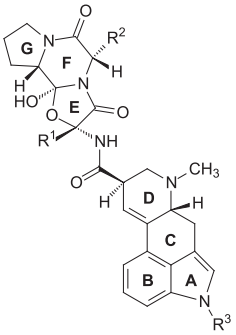


	R^1	R^2	R^3
Ergometrine (M= 325 Da)	$-CH_3$	-H	-H
Methylergometrine (M=339 Da)	$-CH_2CH_3$	-H	-H
Methysergide (M=353 Da)	$-CH_2CH_3$	-H	$-CH_3$



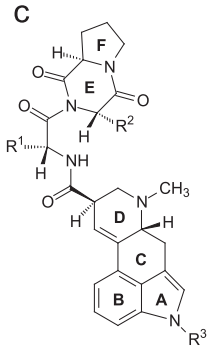
	R^1	R^2	R^3
Dihydroergotamine (M=583 Da)	$-CH_3$	$-CH_2Ph$	-H
Dihydroergocristine (M=611 Da)	$-CH(CH_3)_2$	$-CH_2Ph$	-H
Dihydroergocryptine (M=577 Da)	$-CH(CH_3)_2$	$-CH_2CH(CH_3)_2$	-H

b



	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

c



	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

Fig. 1. Common structures of ergoamides (a), ergopeptines (b) and ergopeptams (c) and representative ergot alkaloids for each class. *M*: molecular weight.

limited database [15]. The full-scan accurate mass capability of Orbitrap MS remains to be exploited in untargeted screening of ergot alkaloid derivatives.

In this work, a method based on high-resolution mass spectrometry (HRMS) and ion trap MS technology is proposed for the study of the fragmentation pattern of ergot alkaloids and the identification of less studied or novel ergot alkaloid derivatives. In particular, Orbitrap MS was used, which allows to achieve high mass resolution (up to 100,000 full width at half maximum (FWHM)) and high mass accuracy (mass error < 2 ppm), thereby leading to higher sensitivity, dynamic range and selectivity for the analysis of complex matrices [16]. The fragmentation of twelve ergot alkaloids, namely ergometrine, ergosine, ergotamine, ergocornine, α-ergokryptine, ergocristine, methylergometrine, methysergide, dihydroergotamine, ergocornam, ergocryptam and ergocristam was studied with the aim of establishing a simple strategy for identification of novel ergot alkaloid derivatives. Subsequently, this strategy was applied in screening of ergot alkaloid derivatives in a set of cereal samples.

2. Experimental

2.1. Standards

Fine film dried ergot alkaloid standards ergometrine, ergosine, ergotamine, ergocornine, α-ergokryptine, ergocristine, ergometrinine, ergosinine, ergotaminine, ergocorninine, α-ergokryptinine, ergocristinine, were purchased from Coring System Diagnostix GmbH (Gernsheim, Germany). The film-dried standards were, as indicated by the manufacturer, reconstituted in 5 mL of solvent (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 -inine isomers. Ergot alkaloids in solution readily undergo epimerization; therefore, from the freshly prepared standard solutions, frozen standard residues were prepared as follows: defined volumes of individual or mixed standard solutions were pipetted into dark brown or aluminum covered glass tubes, evaporated to dryness at 40 °C under a stream of nitrogen, and stored at −20 °C. Lauber et al. [17] reported that the ergot alkaloids stored under these conditions are stable for at least 1 year. The frozen standards were reconstituted in the required amount of solvent immediately before use. Methylergometrine (as methylergometrine maleate, purity: 98%), dihydroergotamine (as dihydroergotamine tartrate salt, purity: 99%) and methysergide (MeErgi) (as methysergide maleate) 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, methysergide) or in acetonitrile (dihydroergotamine) at a concentration of 1 mg/mL. These fresh solutions were used to prepare frozen standard residues as described above. The residues were reconstituted in the required amount of solvent immediately before use.

2.2. Reagents and materials

Methanol (MeOH) and acetonitrile (ACN) (both of LC–MS grade) were supplied by Biosolve (Valkenswaard, the Netherlands). Ammonium bicarbonate (NH_4HCO_3) was obtained from Sigma-Aldrich. Ammonium sulfate ($(NH_4)_2SO_4$) and ammonia (NH_3) (25%) were supplied by Merck (Darmstadt, Germany). ACN and MeOH (both of HPLC grade), and *n*-hexane were purchased from

VWR International (Zaventem, Belgium). Ethyl acetate (EtOAc) was obtained from Acros Organics (Geel, Belgium).

A Milli-Q purification system (Millipore, Brussels, Belgium) was used to purify demineralized water (H_2O). Ultrafree®-MC centrifugal filter units (0.22 μm) were purchased from Millipore (Darmstadt, Germany).

2.3. Sample preparation

Sample preparation procedure was as described by Diana Di Mavungu et al. [18]. Briefly, five gram of a rye 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). The sample extract was centrifuged and a phase separation was induced by adding 5 mL of a 0.2 M NH_4HCO_3 buffer pH 10 and 5 mL of a saturated solution of $(NH_4)_2SO_4$ to 15 mL of the extract. 5 mL of the EtOAc-phase was evaporated until 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 analyzed by LC–MS/MS.

2.4. LC–MS/MS analysis

The LC–MS/MS analyses were performed on an Alliance HPLC 2695 (Waters, Milford, MA, USA) platform coupled to a Micromass Quattro LC triple quadrupole mass spectrometer (Waters) equipped with a Z-spray ESI interface. Chromatographic separation was achieved using an XBridge MS C18 column (3.5 μm, 150 mm × 2.1 mm) with an XBridge Sentry guard column (3.5 μm, 10 mm × 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. The gradient elution was as follows: 0–30 min: 55% B; 30–35 min: 55–65% B; 35–90 min: 65% B; 90–100 min: 65–55% B; 100–120 min: 55% B. The injection volume was 10 μL. The mass spectrometer was operated in the positive electrospray ionization (ESI^+) mode. MS parameters for the analysis were as follows: ESI source block and desolvation temperatures: 150 °C and 300 °C, respectively; capillary voltage: 3.5 kV; argon collision gas: 1.2×10^{-3} mbar; probe nebuliser and desolvation gas flows: 100 and 830 L/h, respectively. Masslynx and Quanlynx software (Micromass, Manchester, UK) were used for data acquisition and processing.

2.5. LC–MS^n analysis

LC analyses were performed on a Surveyor Plus HPLC System (Thermo Fisher Scientific, San Jose, CA, USA). The mobile phase and the column were as described in section "LC–MS/MS analysis". Flow rate was 0.25 mL/min. The gradient elution profile was as follows: 0–10 min: 10–35% B; 10–30 min: 35% B; 30–45 min: 35–60% B; 45–50 min: 60–99% B; 50–55 min: 99% B; 55–57 min: 99–10% B; 57–65 min: 10% B. The temperature of the column was 40 °C and the injection volume was 5 μL.

Mass spectra were acquired using an LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific) equipped with an HESI source. The mass spectrometer was operated in the $HESI^+$ mode. The MS parameters were as follows: spray needle voltage 5 kV, capillary voltage 17 V, capillary temperature 200 °C, heater temperature 125 °C, nitrogen sheath gas flow 35 arbitrary units (a.u.), auxiliary gas flow 10 a.u. When performing MS^n experiments, the precursor ion was isolated in the ion trap with an isolation width of 3 Da and activated at different collision energy levels (CELs) to find the optimal conditions for distinct fragmentation. Xcalibur™ 2.0.7 software (Thermo Fisher Scientific) was used for instrument control, data acquisition and processing.

2.6. Ultra-high performance liquid chromatography (UHPLC)–HRMS analysis

LC analyses were performed on a Thermo Accela UHPLC system (Thermo Fisher Scientific). The mobile phases were as described in section "LC–MS/MS analysis". The column used was ZORBAX RRHD Eclipse Plus C18 (1.8 μm, 2.1 mm × 100 mm) from Agilent Technologies (Diegem, Belgium). The gradient elution program was set as follows: 0–1 min: 0% B; 1–5 min: 0–25% B; 5–15 min: 25–35% B; 15–25 min: 35–40% B; 25–40 min: 40–70% B; 40–47 min: 70–98% B; 47–50 min: 98% B; 50–51 min: 98–0% B; 51–55 min: 0% B. The mobile phase flow rate was 0.4 mL/min and the injection volume was 5 μL.

Accurate mass measurements of the precursor and product ions were carried out on an Orbitrap Exactive™ mass analyzer (Thermo Fisher Scientific) equipped with a HESI-II interface. The mass spectrometer was operated in the $HESI^+$ mode. The MS parameters were the following: spray voltage 4.5 kV, capillary temperature 250 °C, heater temperature 250 °C, sheath gas flow rate 45 a.u., auxiliary gas flow rate 10 a.u. The data were processed using Xcalibur™ 2.1 (Thermo Fisher Scientific). The instrument was operated in full scan mode with a resolution of 100,000 FWHM. The maximum injection time was 200 ms, AGC target was 500,000 and the number of microscans per scan was 1. Each full scan was followed by a same-polarity "all ion fragmentation" higher energy collisional dissociation (HCD) scan.

3. Results and discussion

3.1. Fragmentation pattern of ergot alkaloids

3.1.1. Ergopeptine and ergoamide alkaloids

The ergopeptines and ergoamides are structurally related compounds that share a common skeleton (see Fig. 1). This characteristic was exploited to establish a strategy for the screening and identification of unknown ergot alkaloid derivatives. This was achieved through a careful study of the fragmentation pattern of known derivatives, including six ergopeptines (ergosine, ergotamine, ergocornine, α-ergokryptine, ergocristine and dihydroergotamine), five ergopeptinines (ergosinine, ergotaminine, ergocorninine, α-ergokryptinine and ergocristinine) and three ergoamides (ergometrine, methylegometrine and methysergide).

MS^n fragmentation data of the ergopeptine alkaloids are summarized in Supplemental Table S1. It has to be mentioned that fragmentation behavior of ergopeptinines was identical to that of their corresponding ergopeptines and therefore is not discussed in the paper. It was observed that all the studied ergopeptines initially underwent a loss of a water molecule (−18 Da). Subsequently, in MS^3 and MS^4 experiments, neutral losses of 28 and 18 Da (corresponding to CO and H_2O) were observed. In further MS^5 experiments, losses of 91 Da for ergocristine, ergotamine and dihydroergotamine, 56 Da for ergosine and α-ergokryptine, and 42 Da for ergocornine occurred (Fig. 2). Taking into consideration the structural differences between the ergopeptines studied and the accurate mass data, these losses of 91, 56 and 42 Da were attributed to the radical R^2. The observed losses are consistent with a homolytic cleavage of the implied C–C bond. This would represent a violation of the even-electron fragmentation rule, however, such a phenomenon has been previously described [9]. While the loss of 91 Da (ergocristine, ergotamine and dihydroergotamine) could be inferred from the homolytic

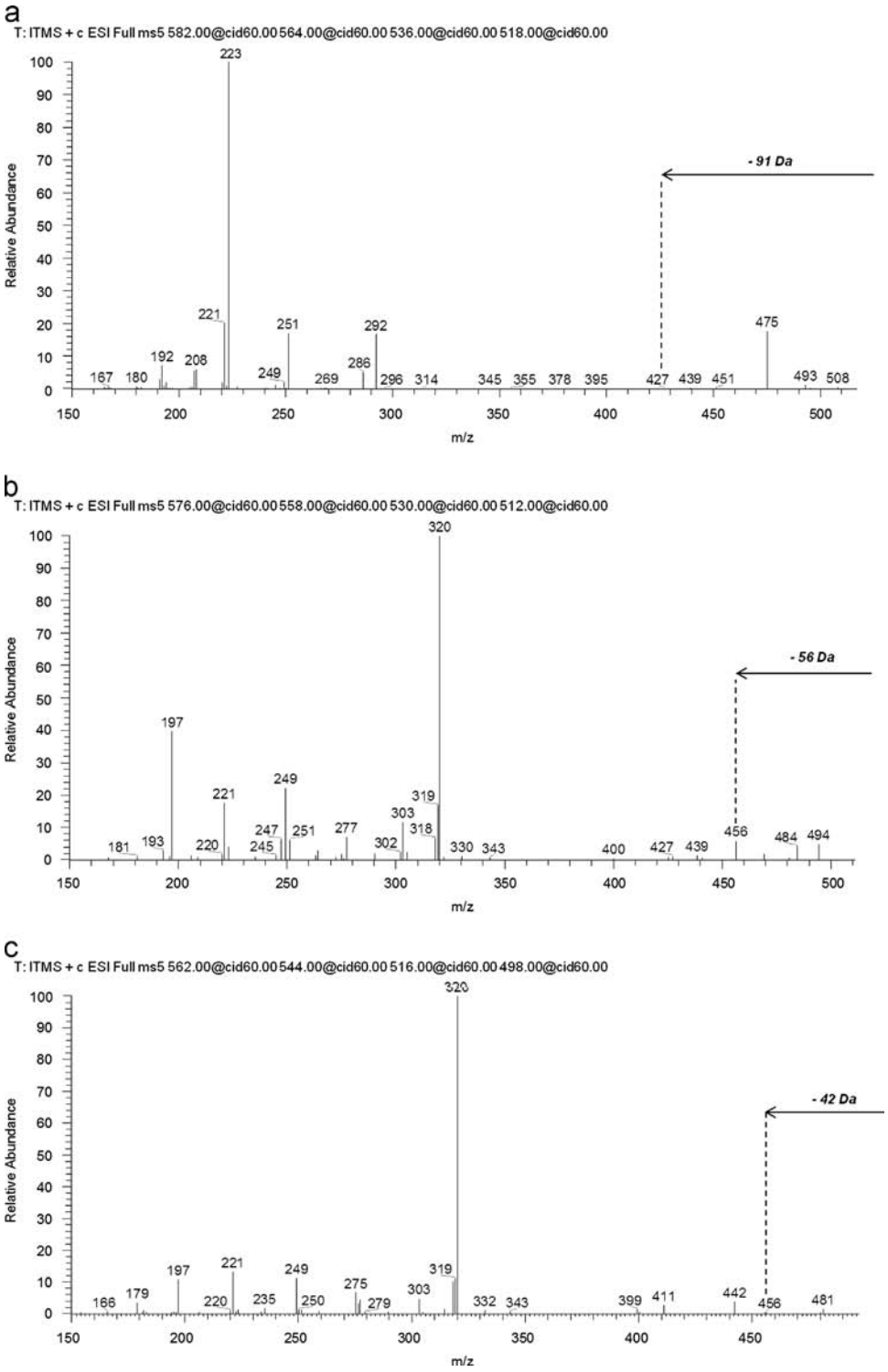


Fig. 2. Fourth-generation collisionally-induced dissociation (CID) spectra acquired for ergotamine (a), ergokryptine (b) and ergocornine (c) demonstrating the loss of the R^2 substituent from the main skeleton. R^2: $-CH_2Ph$ (for ergotamine), $-CH_2CH(CH_3)_2$ (for ergokryptine) and $-CH(CH_3)_2$ (for ergocornine).

cleavage in a straightforward fashion, the fragmentation mechanism for ergosine, α-ergokryptine and ergocornine implied the transfer of one hydrogen atom to the leaving fragment, leading to the formation of a double bond in the lost entity (Fig. 3).

Besides the loss of the R^2 radical, the ergopeptines underwent, subsequent to the losses of H_2O, CO and H_2O, a loss of most of the peptide ring system (Supplemental Fig. S1). For α-ergokryptine, ergocristine and ergocornine (where R^1 is an isopropyl radical), an ion with an *m/z* of 348.1698 Da was observed, corresponding to $C_{21}H_{22}N_3O_2$ (mass error: −2.4 ppm). The ergopeptines having a methyl group at the R^1 position (*i.e.* ergotamine, ergosine) produced a fragment with *m/z* 320.1388 Da ($C_{19}H_{18}N_3O_2$; −1.7 ppm). Dihydroergotamine (an ergotamine-derivative having a saturated C9–C10 bond) rather produced an ion at *m/z* 322.1549 assigned as $C_{19}H_{20}N_3O_2$ (−0.5 ppm). The resulting fragments further underwent a loss of CO (−28 Da), yielding *m/z* 320.1750 ($C_{20}H_{22}N_3O$, −2.2 ppm) and *m/z* 292.1439 ($C_{18}H_{18}N_3O$, −1.8 ppm) for the derivatives with an isopropyl and methyl R^1 radicals, respectively

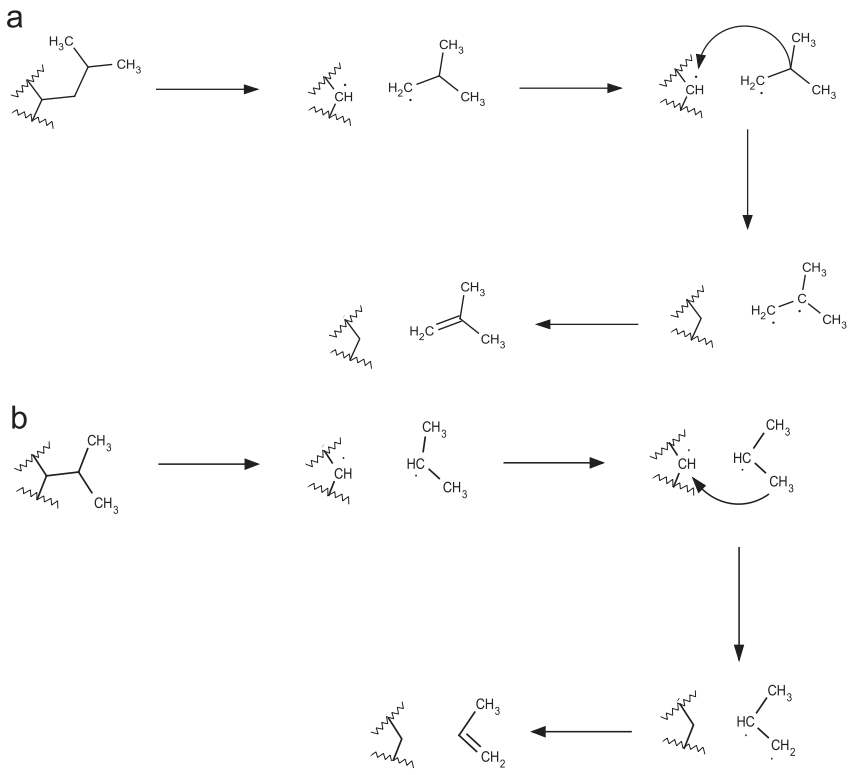


Fig. 3. Proposed fragmentation mechanism for the loss of the R^2 substituent from the main skeleton of ergosine and ergokryptine (a), and ergocornine (b).

(Supplemental Fig. S2). A fragment at *m/z* 294.1597 (assigned as $C_{18}H_{20}N_3O$; −0.4 ppm) was obtained for dihydroergotamine. Subsequent loss of the R^1–C–NH moiety gave an ion at *m/z* 251.1175 corresponding to $C_{16}H_{15}N_2O$ (−1.5 ppm) for all ergopeptines, except for dihydroergotamine, for which the corresponding ion at *m/z* 253.1332 ($C_{16}H_{17}N_2O$; −0.3 ppm) was obtained. Fragmentation of the ion at *m/z* 251.1175 yielded, among others, an ion at *m/z* 223.1227 ($C_{15}H_{15}N_2$; −1.4 ppm) through the loss of CO (−28 Da). The corresponding ion for the dihydro-derivatives (*m/z* 253) yielded a fragment at *m/z* 225.1384 ($C_{15}H_{17}N_2$; −0.2 ppm). The product ion at *m/z* 223.1227 (*m/z* 225.1384 for dihydroergotamine) proved to be the most abundant common fragment of the different ergopeptines. The product ion spectrum (Supplemental Fig. S2) of this common ion indicated that subsequently, a homolytic cleavage of the N–CH_3 bond in the D ring took place, giving rise to the radical cation at *m/z* 208.0993 ($C_{14}H_{12}N_2$; −0.8 ppm) for the ergopeptines and *m/z* 210.1151 ($C_{14}H_{14}N_2$; −0.1 ppm) for the dihydroergopeptines, as also reported by Mohamed et al. [9].

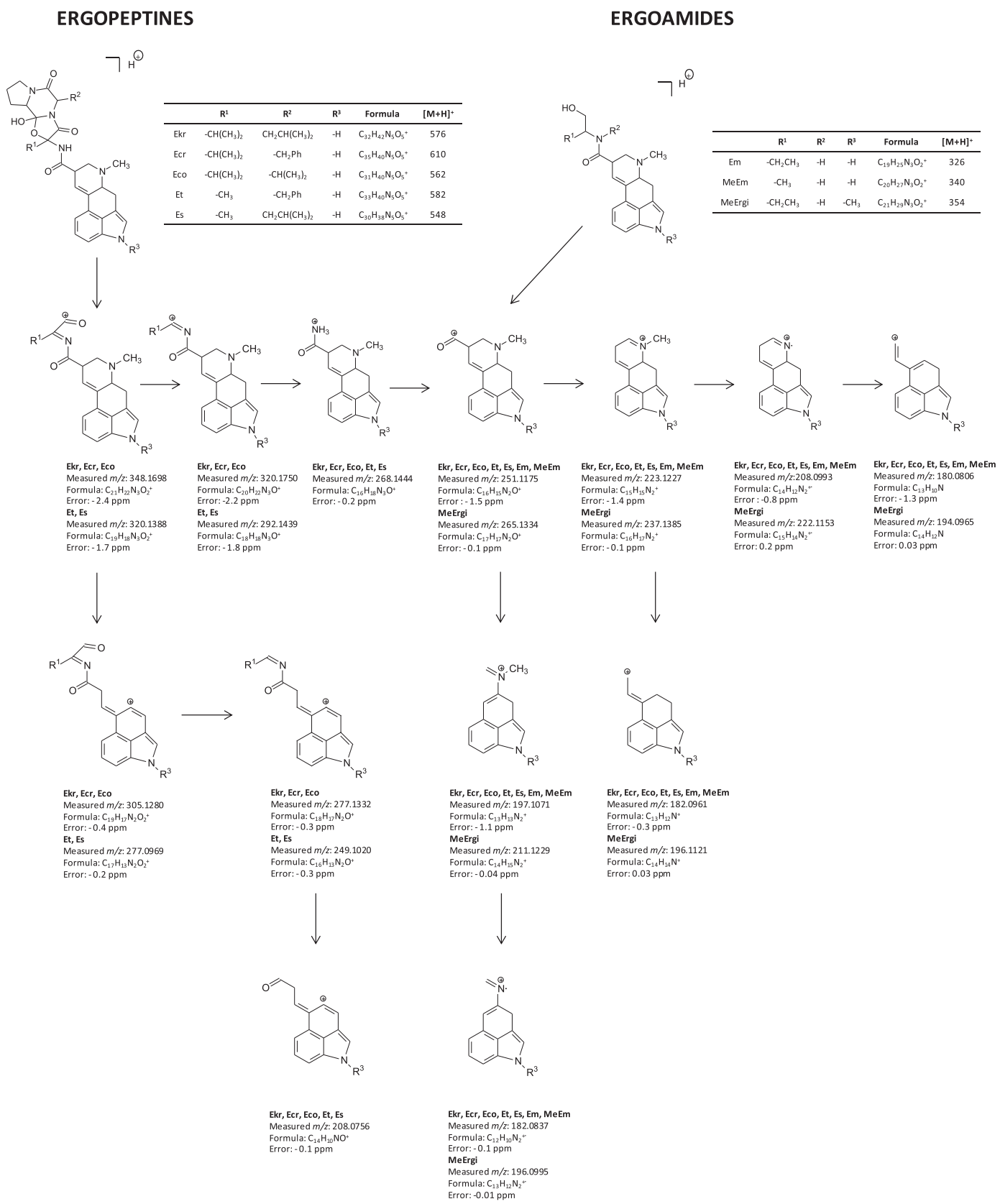
It was observed that the ions at *m/z* 348.1698 (α-ergokryptine, ergocristine) and *m/z* 320.1388 (ergocornine, ergotamine and ergosine), described above, followed an alternative fragmentation pathway. A fragment at *m/z* 305.1280 ($C_{19}H_{17}N_2O_2$; −0.4 ppm) or *m/z* 277.0969 ($C_{17}H_{13}N_2O_2$; −0.2 ppm) was obtained by cleavage within the lysergic D ring system for derivatives with isopropyl R^1 radical (α-ergokryptine, ergocristine) or methyl R^1 radical (ergocornine, ergotamine, ergosine), respectively. A subsequent loss of CO (−28 Da) gave *m/z* 277.1332 (α-ergokryptine, ergocristine) and *m/z* 249.1020 (ergocornine, ergotamine, ergosine) (assigned as $C_{18}H_{17}N_2O$; −0.3 ppm and $C_{16}H_{13}NO$; −0.3 ppm, respectively). A loss of the R^1–C(CO)–NH moiety from these fragments yielded a common ion at *m/z* 208.0756 ($C_{14}H_{10}NO$; −0.1 ppm).

Loss of the lysergic ring-$CONH_2$ moiety from the protonated moleculecular ion was also observed. The resulting fragment (Supplemental Fig. S3) subsequently lost a CO moiety or the radical R^2. Interestingly, the fragment obtained upon loss of R^2 confirmed the homolytic cleavage fragmentation mechanism described above for α-ergokryptine, ergosine and ergocornine, *i.e.*, the transfer of one hydrogen atom to the leaving fragment (Fig. 3).

MS^n fragmentation data of the ergoamide alkaloids are summarized in Supplemental Table S2. A fragmentation pattern similar to that of ergopeptines was observed for this group of ergot alkaloids. Initially, all the studied ergoamides lost a water molecule or –CH_3 by homolytic cleavage. The fragment at *m/z* 251.1175 (*m/z* 265.1334 for MeErgi) was obtained through a loss of R^1–CH(CH_2OH)–NH_2 from a protonated molecular ion $[M+H]^+$, and followed a common pathway with the ergopeptines (Fig. 4). The ergoamides also underwent a cleavage within the lysergic D ring as shown in Supplemental Fig. S4.

3.1.2. *Ergopeptam alkaloids*

Reference standards for the ergopeptams were not available during the course of this work. The study of their fragmentation pattern was achieved using a rye feed sample that was presumed to contain these ergot alkaloids based on the high levels and patterns of ergot alkaloids for which commercial standards were available. Using accurate mass measurements and fragmentation data, three known ergopeptams [11,19,20], namely ergocornam ($C_{31}H_{40}N_5O_4$, *m/z* 546.3072; −0.5 ppm), ergocryptam ($C_{32}H_{42}N_5O_4$, *m/z* 560.3234; 0.5 ppm) and ergocristam ($C_{35}H_{40}N_5O_4$, *m/z* 594.3078; 0.5 ppm) were detected in this sample. The fragmentation of these compounds is summarized in Supplemental Table S3. As expected from their structures, the initial loss of 18 Da (corresponding to the elimination of a water molecule) observed for ergopeptines did not occur for the ergopeptam derivatives. The studied compounds underwent a loss of the dipeptide ring system in MS^2 experiments of the protonated

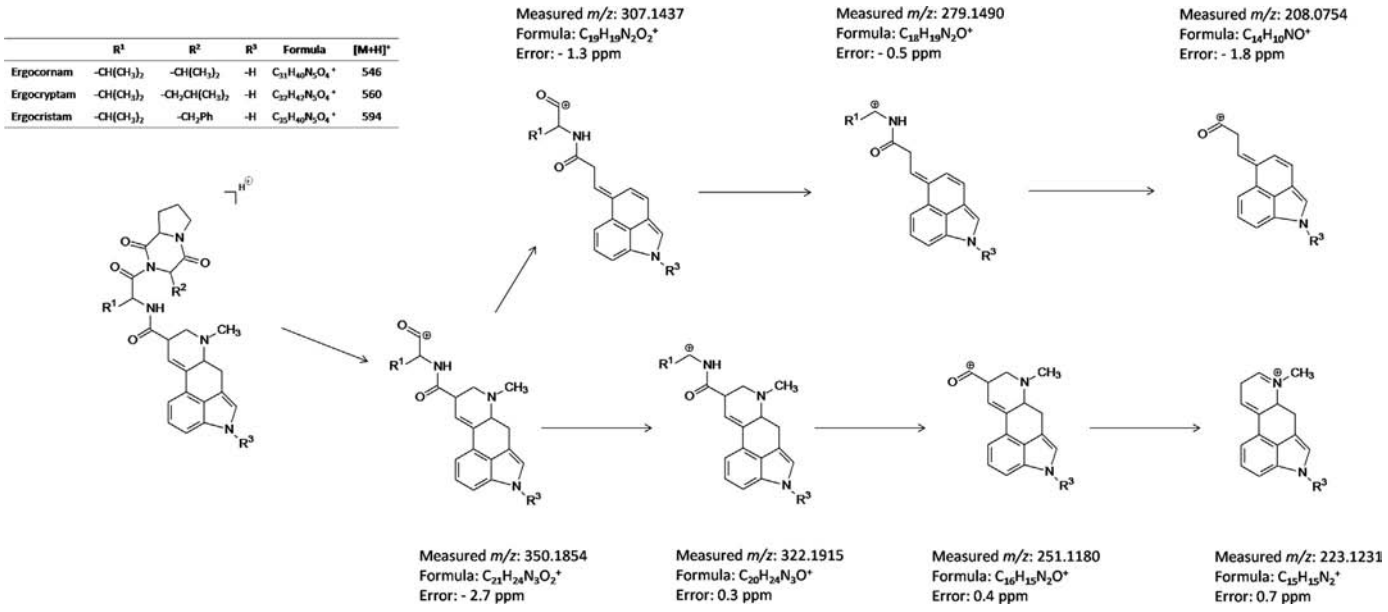


	R¹	R²	R³	Formula	[M+H]⁺
Ekr	-CH(CH₃)₂	CH₂CH(CH₃)₂	-H	$C_{32}H_{42}N_5O_5^+$	576
Ecr	-CH(CH₃)₂	-CH₂Ph	-H	$C_{35}H_{40}N_5O_5^+$	610
Eco	-CH(CH₃)₂	-CH(CH₃)₂	-H	$C_{31}H_{40}N_5O_5^+$	562
Et	-CH₃	-CH₂Ph	-H	$C_{33}H_{40}N_5O_5^+$	582
Es	-CH₃	CH₂CH(CH₃)₂	-H	$C_{30}H_{38}N_5O_5^+$	548

	R¹	R²	R³	Formula	[M+H]⁺
Em	-CH₂CH₃	-H	-H	$C_{19}H_{25}N_3O_2^+$	326
MeEm	-CH₃	-H	-H	$C_{20}H_{27}N_3O_2^+$	340
MeErgi	-CH₂CH₃	-H	-CH₃	$C_{21}H_{29}N_3O_2^+$	354

Fig. 4. Proposed common fragmentation pathway of ergopeptines and ergoamides.

molecular ions, resulting in a common and abundant fragment at *m/z* 350.1854 (Supplemental Fig. S5). This ion at *m/z* 350.1854 was attributed to the fragment depicted in Fig. 5, where the radical R^1, in accordance with the structure of the studied compounds, is –CH $(CH_3)_2$. A loss of CO from this fragment yielded an ion at *m/z* 322.1915. A subsequent fragmentation yielded the ion at *m/z* 251.1180, identical to that obtained for ergopeptines (Supplemental Fig. S2), as revealed by the further fragmentation. Based on the



	R¹	R²	R³	Formula	[M+H]⁺
Ergocornam	$-CH(CH_3)_2$	$-CH(CH_3)_2$	-H	$C_{31}H_{40}N_5O_5^+$	546
Ergocryptam	$-CH(CH_3)_2$	$-CH_2CH(CH_3)_2$	-H	$C_{32}H_{42}N_5O_5^+$	560
Ergocristam	$-CH(CH_3)_2$	$-CH_2Ph$	-H	$C_{35}H_{40}N_5O_5^+$	594

Fig. 5. Proposed fragmentation pathway of ergopeptam ergot alkaloids. The fragment at *m/z* 223 was identical to that shown in Supplemental Fig. S2, thereby following the same pathway.

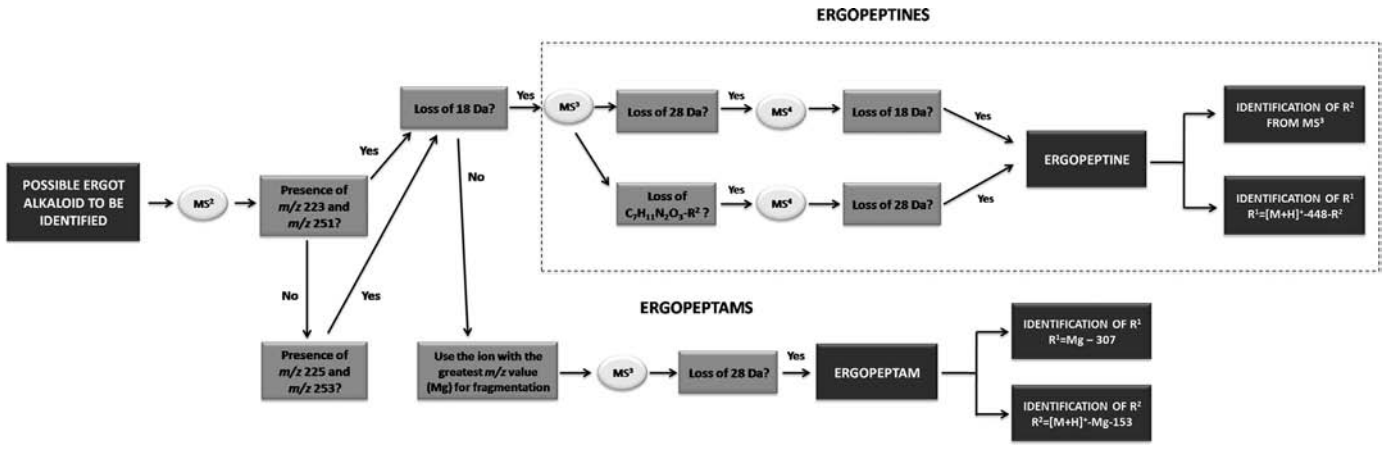


Fig. 6. Proposed strategy for identification of novel ergopeptines and ergopeptams.

above-mentioned data, a fragmentation pathway of the ergopeptams was proposed (Fig. 5).

3.2. Strategy for identification of novel ergopeptams and/or ergopeptines

A strategy for the identification of novel or less studied ergot alkaloids was proposed (Fig. 6). Based on fragmentation pathways described in section "Fragmentation pattern of ergot alkaloids", the ions at *m/z* 223 and *m/z* 251 were found to be common for all the ergopeptines, ergoamides and ergopeptams, while for the dihydroergopeptines, ions *m/z* 225 and *m/z* 253 were characteristic. Therefore, it was proposed to monitor these ions as a first step to screen for ergot alkaloids. Initially, the samples with possible ergot alkaloid derivatives were analyzed using HRMS in "all ion fragmentation" HCD mode to screen for the fragment at *m/z* 223.1230 ($C_{15}H_{15}N_2$) or *m/z* 225.1386 ($C_{15}H_{17}N_2$). In practice, this screening can be performed by applying a parent scan of the ions at *m/z* 223 and *m/z* 225. The fragmentation of the possible ergot alkaloid derivatives was further studied by LC-MS^n. Compound identification was performed according to the scheme indicated in Fig. 6, and supported by accurate mass data.

If the ions at *m/z* 223 or *m/z* 225 were observed in the spectrum, the next step is to check whether the molecule of interest looses water upon fragmentation of the protonated molecular ion. Formation of a dehydrate will indicate that the studied compound belongs to the group of ergopeptines or ergoamides. Noteworthy, if the cations at *m/z* 223 or *m/z* 225 were formed upon HRMS analysis, but the loss of H_2O from the protonated molecular ion did not occur, the compound can be a possible ergopeptam.

After confirmation of the presence of ergot alkaloids (Fig. 6), assignment of R^1 and R^2 radicals can be performed as described below. For ergopeptines, R^2 can be calculated according to Eq. (1), where Mg corresponds to the prominent ion with the greatest *m/z* value originating from the fragmentation of the $[M+H-18]^+$ ion. Subsequently, R^1 can be determined using Eq. (2).

$$R^2 = [M+H]^+ - \mathrm{Mg} - 171 \quad (1)$$

where 171 corresponds to the most of the peptide ring system of ergopeptines without R^2; Mg is the prominent greatest fragment

$$R^1 = [M+H]^+ - 448 - R^2 \quad (2)$$

where 448 corresponds to the ergopeptine structure without radicals

For ergopeptams, the ion with the greatest *m/z* value (Mg), originating from MS^2 of the protonated molecular ion, should be further fragmented. At this stage, a loss of 28 Da should be noted. Considering that the fragment at *m/z* 251 is obtained from consecutive losses of a 28 Da-moiety and NH–CH–R^1 from the greatest fragment, R^1 can be calculated as follows (Eq. (3)). R^2 is in turn calculated according to Eq. (4).

$$R^1 = \mathrm{Mg} - 307 \quad (3)$$

where Mg is the greatest fragment

$$R^2 = [M+H]^+ - \mathrm{Mg} - 153 \quad (4)$$

where 153 corresponds to the ergopeptam dipeptide ring without R^2.

3.3. Screening and identification of ergot alkaloid derivatives in grain samples

The fragmentation study of ergot alkaloids standards showed that the ion at *m/z* 223 was common for all the ergopeptines, ergoamides and ergopeptams. This observation has been previously described [9,10]. Therefore, detection of MS signals using *m/z* 223-parent scan experiments could point to possible occurrence of ergot alkaloids in real samples. The proposed identification strategy was applied for the screening of ergot alkaloid derivatives in grain samples. Parent scan monitoring of the *m/z* 223 ion revealed the presence of possible ergot alkaloid derivatives in several feed samples. Among 19 analyzed samples, four were free of ergot alkaloids. Ergometrine, ergosine, ergotamine, ergocornine, α-ergokryptine, ergocristine and their corresponding -inine epimers were identified in the remaining 15 samples by comparison with reference standards. Other presumed ergot alkaloid derivatives could not be identified at this stage. These unknowns were studied by fitting their mass spectral data into the proposed fragmentation pathway (described in section "Fragmentation pattern of ergot alkaloids"). Firstly, the authenticity of the ion at *m/z* 223 was checked through accurate mass measurements. Then, the ion trap fragmentation study was carried out for each unknown ergot alkaloid derivative. The obtained information was coupled with the exact mass data obtained with the Orbitrap instrument.

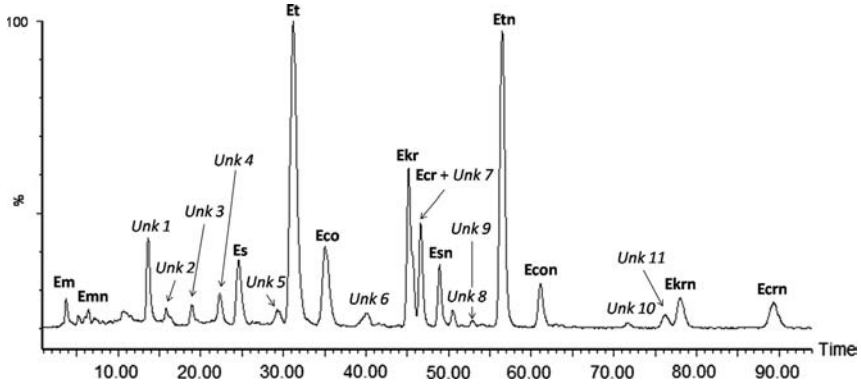


Fig. 7. Parent scan total ion chromatogram of a rye feed sample. Em: ergometrine; Emn: ergometrinine; Es: ergosine; Esn: ergosinine; Et: ergotamine; Etn: ergotaminine; Eco: ergocornine; Econ: ergocorninine; Ekr: ergokryptine; Ekrn: ergokryptinine; Ecr: ergocristine; Ecrn: ergocristinine; Unk: unknown.

Table 1
Ergot alkaloid derivatives identified in grain samples (only positive samples are shown).

Metabolite	Elemental composition	Measured *m/z*	Calculated *m/z*	Error (ppm)	Sample[a] 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Ergometrine	$C_{19}H_{24}N_3O_2$	326.1858	326.1863	−1.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ergometrinine	$C_{19}H_{24}N_3O_2$	326.1857	326.1863	−1.8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ergosine	$C_{30}H_{38}N_5O_5$	548.2863	548.2867	−0.7	+	+	−	+	−	+	+	+	+	+	+	+	+	+	+
Ergosinine	$C_{30}H_{38}N_5O_5$	548.2862	548.2867	−0.9	+	+	−	+	−	+	+	+	+	+	+	+	+	+	+
Ergotamine	$C_{33}H_{36}N_5O_5$	582.2702	582.2711	−1.5	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+
Ergotaminine	$C_{33}H_{36}N_5O_5$	582.2702	582.2711	−1.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ergocornine	$C_{31}H_{40}N_5O_5$	562.3019	562.3024	−0.9	+	+	−	+	−	+	+	+	+	+	+	+	+	+	+
Ergocorninine	$C_{31}H_{40}N_5O_5$	562.3019	562.3024	−0.9	+	−	−	+	−	+	+	+	+	+	+	+	+	+	+
α-Ergokryptine	$C_{32}H_{42}N_5O_5$	576.3174	576.3180	−1.0	+	+	−	+	−	+	+	+	+	+	+	+	−	+	+
Ergokryptinine	$C_{32}H_{42}N_5O_5$	576.3175	576.3180	−0.9	+	+	−	+	−	+	+	+	+	+	+	+	−	+	+
Ergocristine	$C_{35}H_{40}N_5O_5$	610.3016	610.3024	−1.3	+	+	−	+	−	+	+	+	+	+	+	+	−	+	+
Ergocristinine	$C_{35}H_{40}N_5O_5$	610.3017	610.3024	−1.1	+	+	−	+	−	+	+	+	+	+	+	+	−	+	+
Ergocornam (=Unk[b] 1)	$C_{31}H_{40}N_5O_4$	546.3072	546.3075	−0.5	+	−	−	+	−	+	+	+	+	+	+	−	−	−	+
Ergovaline (=Unk 2)	$C_{29}H_{36}N_5O_5$	534.2708	534.2716	−1.5	+	−	−	+	−	−	+	+	+	+	+	−	−	+	+
Ergocryptam (=Unk 3)	$C_{32}H_{42}N_5O_4$	560.3233	560.3231	0.4	+	−	−	−	−	−	+	+	+	+	+	−	−	−	+
Ergocryptam isomer (=Unk 4)	$C_{32}H_{42}N_5O_4$	560.3231	560.3231	0.0	+	−	−	−	−	−	+	+	+	+	+	−	−	−	+
Ergocristam (=Unk 5)	$C_{35}H_{40}N_5O_4$	594.3078	594.3075	0.5	+	−	−	+	−	−	+	+	+	+	−	−	−	−	+
Ergostine (=Unk 6)	$C_{34}H_{38}N_5O_5$	596.2886	596.2873	2.2	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+
β-Ergokryptine (=Unk 7)	$C_{32}H_{42}N_5O_5$	576.3176	576.3180	−0.7	+	−	−	−	−	−	+	+	+	+	+	+	−	+	+
Ergoptine (=Unk 8)	$C_{31}H_{40}N_5O_5$	562.3036	562.3029	1.2	+	−	−	+	−	−	+	+	+	+	+	+	−	+	+
Ergogaline (=Unk 9)	$C_{33}H_{44}N_5O_5$	590.3331	590.3342	−1.9	+	−	−	+	−	−	+	−	+	+	+	−	−	−	+
Ergostinine (=Unk 10)	$C_{34}H_{38}N_5O_5$	596.2888	596.2873	2.5	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Ergokryptine isomer (=Unk 11)[c]	$C_{32}H_{42}N_5O_5$	576.3195	576.3180	2.6	+	−	−	−	−	−	+	+	+	+	+	+	−	+	+

[a] '+': detected; '−': not detected.
[b] Assignment of compounds as Unk 1 to Unk 11 refers to Fig. 7.
[c] Most likely β-ergokryptinine [21].

A total ion chromatogram of a rye feed sample (sample 1) is given as example (Fig. 7). Besides the known ergot alkaloids, eleven putative ergot alkaloid derivatives were detected in this sample. Among these unknown derivatives, four (**Unk**s **1**, **3**, **4** and **5**) followed the same fragmentation pathway as observed for the ergopeptams, while the others, **Unk**s **2**, **6**–**11**, showed similarity with the fragmentation pathway of ergopeptines. Eventually, these compounds were identified as described in Table 1. These compounds, namely ergocornam, ergovaline, ergocryptam, ergocristam, ergostine, ergoptine and ergogaline, have been previously reported in grain and grass samples [6,11,22]. An overview of the ergot alkaloid derivatives identified in other grain samples is presented in Table 1.

Among the identified ergot alkaloids, some were accompanied by their respective epimers. Epimerization, with respect to the center of symmetry at C8, is a characteristic feature of ergot alkaloids that have a double bond between C9 and C10, resulting in rotating (C8-(S) configuration) isomers [1,3]. Since both forms are found together in naturally contaminated samples [1], this feature was used as additional confirmation of the identification. Elution order was used to distinguish the two ergot alkaloid forms as they have different physico-chemical properties; the epimers eluted after their corresponding main ergot alkaloids under the applied chromatographic conditions.

4. Conclusions

A simple approach based on high resolution and multiple stage mass spectrometry was proposed for identification of less studied or novel ergot alkaloids in cereals. Initially, the common fragmentation pathways of the main classes of ergot alkaloids, namely ergopeptines, ergoamides and ergopeptams, were elucidated. The identification strategy consisted of a few steps which made it quick to draw preliminary conclusions regarding the presence of ergot alkaloids in a sample or to directly identify an ergot alkaloid derivative. The subsequent application of the strategy in the screening of grain samples was successful and allowed identification of eleven metabolites for which commercial standards were not available.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.10.002.

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