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Abstract:

Ergot alkaloids (EAs) are mycotoxins which are undesirable contaminants of cereal products, particularly rye. A method was developed employing clean-up by cation-exchange solid phase extraction, separation by HPLC under alkaline conditions and fluorescence detection. It is capable of separating and quantifying both C8-isomers of ergocornine, a-ergocryptine, ergocristine, ergonovine, and ergotamine. The average recovery was 61±10 % with limits of detection from 0.2 to 1.1 µg kg-1. Therty-four unknown rye flour samples from Danish mills contained on average 46 µg kg-1 with a maximum content of 234 µg kg-1. The most common ergot alkaloids were ergotamine and a-ergocryptine including their C8-isomers. 54 % of the ergot alkaloids were detected as C(8)-S isomers.

Keywords:- ergot alkaloids, HPLC, cereals, rye flour, survey

Introduction

Ergot alkaloids (EAs) are mycotoxins produced by fungi of the genus *Claviceps*. The fungus infects flowers of cereals and grasses and replaces the developing grain or seed with an alkaloid containing a wintering body, known as the ergot body or sclerotium. The sclerotia are harvested with grain, seed or grass and may result in EA contamination of cereal products and feedstuffs causing ergotism in humans and animals. Rye is the most susceptible species (Lorenz 1979) but *C. purpurea* attacks more than 400 monocotyledonous hosts (Bové 1970). Ergot cannot be controlled with fungicides (Tenberger 1999).

Epidemics of ergotism were common in Europe in the Middle Ages where it was known as "Holy Fire" or "St. Anthony's fire" (Barger 1931). A case of ergotism involving hundreds of people of the town of Pont St. Esprit, France occurred in 1951 (Gabbai *et al.* 1951;Fuller 1969). More recently an outbreak of gangrenous ergotism was observed in Ethiopia in 2001 (Urga *et al.* 2002). Symptoms of EA intoxication include abdominal pains, vomiting, burning sensations of the skin, insomnia, hallucinations, and in severe cases convulsions, cardiovascular collapse and gangrene of extremities (Barger 1931;Gabbai *et al.* 1951). Today effective cleaning techniques at the mills make it possible to remove a large proportion of ergots from grain (Shuey *et al.* 1973;Dexter *et al.* 1991;Posner & Hibbs 1997). Nonetheless EAs have been detected in surveys of Swiss, German, Danish, and Canadian cereals and cereal based products in concentrations up to 4000 µg kg^{-1} (Baumann *et al.* 1985;Klug *et al.* 1988;Scott *et al.* 1992;Scott & Lawrence 1980;Scott & Lawrence 1983;Lombaert *et al.* 2003;Rasmussen 1991).

In the EU no regulatory limits apply to grain for human consumption (Danish Food Directorate 1996;Egmond & Jonker 2004). A maximum content of 500 mg ergot bodies kg^{-1} in grain (0.05 % (w/w)) applies to cereals taken-over by intervention agencies (EU Commission 2000) and a threshold limit of 1000 mg ergot bodies kg^{-1} (0.10 % (w/w)) applies to feed products containing unground cereals (EU Parliament 2002). No limits apply to ground cereals. The presence of ergot alkaloids in feed, their effects on animal health and the possibility of carry-over to food of animal origin has recently been reviewed by the European Food Safety Authority (EFSA 2005). They attend the inaccuracy of expressing the rate of contamination by a percentage of sclerotia, as the alkaloid content and composition may vary with fungal strain, host plant, growth conditions and even within fields and cereal ears (Németh 1999;Young 1981a). Furthermore they recommend that more data is supplied on the variability of ergot alkaloids patterns in European feed materials.

There are few published articles on the content of ergot alkaloids in European cereal products. With the recent attention on the subject some survey have been conducted e.g. by Lauber (Lauber

et al. 2005). The latest Danish survey was conducted in 1987-88 (Rasmussen 1991) on 55 samples of cereal products including 15 rye flour samples. Rye flour is particularly interesting in Denmark, because rye is the most susceptible cereal (Lorenz 1979) and Danes consume on average 72 g of rye per day, which is more than in any other European country (Miraglia & Brera 2002). Other surveys on cereals and cereal based products are from 1980 (Scott & Lawrence 1980), 1983 (Scott & Lawrence 1983), 1985 (Baumann *et al.* 1985), 1988 (Klug *et al.* 1988), 1992 (Scott *et al.* 1992) and 2003 (Lombaert *et al.* 2003), the latest being concerned only with cereal based infant foods.

[Insert figure 1]

More than 100 EAs are known (Flieger *et al.* 1997). The most commonly found EAs in rye flour are ergotamine and ergocristine, with ergocryptine, ergocornine, ergosine, and ergonovine present in smaller amounts (Scott & Lawrence 1980;Scott & Lawrence 1983;Baumann *et al.* 1985;Scott *et al.* 1992). These EAs are very similar differing only in substituents on C8 (Figure 1). An important feature of EAs containing a C9=C10 double bond is the rapid isomerisation at C8, especially in aqueous acidic or alkaline solutions (Hofmann 1964;Komarova & Tolkachev 2001b). The C8-(R) isomers are designated the suffix *–ine* (e.g. ergotamine) while the C8-(S) isomers have the suffix *–inine* (e.g. ergotaminine). C8 isomers differ in physicochemical and biological properties. pK_a-values differ within 0.7 unit (Stoll *et al.* 1954;Maulding & Zoglio 1970), the epimers are readily separated by high-pressure liquid chromatography (HPLC) (Scott & Lawrence 1980;Szepesi *et al.* 1980;Baumann *et al.* 1985;Ware *et al.* 1986;Klug *et al.* 1988), and C8-(R) isomers are physiologically active, while the C8-(S) isomers are inactive (Berde & Stürmer 1978;Pierri *et al.* 1982). However, this does not mean that the C8-(S) isomers may be disregarded. C8-isomerisation may occur during the extraction and clean-up procedure which imply acidic or alkaline solutions (Stoll *et al.* 1949). It is thus not possible to say how much of the EA was in the C8-(R) and the C8-(S) form prior to sample treatment.

Another important characteristic of EAs is the alkalinity of N6 with pK_a values of the protonised EAs ranging from 5-7.4 (Hofmann 1964;Stoll *et al.* 1954). Thus EAs are positively charged in acidic solutions and neutral at higher pH values. EAs are also light sensitive and degrade upon UV-radiation (Rutschmann & Stadler 1978;Komarova & Tolkachev 2001b). Many EAs, including the most common, are naturally fluorescent (Komarova & Tolkachev 2001b;Flieger *et al.* 1997).

Analysis for EAs is most commonly conducted by Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) combined with UV or fluorescence detection (Komarova & Tolkachev 2001a;Flieger *et al.* 1997) or by LC-MS (Mohamed *et al.* 2006). In most known and validated methods for qualitative and quantitative determination of EAs in cereal products extraction is done either with non-polar organic solvents under alkaline conditions (Scott & Lawrence 1980;Baumann

et al. 1985;Ware *et al.* 1986;Klug *et al.* 1988;Rottinghaus *et al.* 1993;Fajardo *et al.* 1995; Lauber *et al.* 2005) or with polar solvents under acidic conditions (Ware *et al.* 2000), thus exploiting the acid/base properties of N6. Clean-up is done either by liquid/liquid partitioning at alternating acidic and alkaline conditions (Scott & Lawrence 1980;Baumann *et al.* 1985;Ware *et al.* 1986;Klug *et al.* 1988;Scott *et al.* 1992; Lauber *et al.* 2005) or by solid phase extraction (SPE) (Rottinghaus *et al.* 1993;Fajardo *et al.* 1995;Ware *et al.* 2000). The use of health and environmentally damaging organic solvents is undesirable and liquid/liquid partitioning is time consuming, so SPE is preferred. Separation of the cleaned extract is done by RP-HPLC with mobile phases of acetonitrile (ACN) mixed with either aqueous base (Scott & Lawrence 1980;Baumann *et al.* 1985;Ware *et al.* 1986;Klug *et al.* 1988;Rottinghaus *et al.* 1993; Lauber *et al.* 2005) or acid (Scott *et al.* 1992;Fajardo *et al.* 1995;Ware *et al.* 2000) solutions. Acidic mobile phases are often preferred because silica based HPLC columns are degraded at high pH, but none of the methods employing acidic mobile phases report on the detection of both C8-isomers of EAs.

In this paper we report a method developed for detecting and quantifying both C8-isomers of the most common EAs in rye flour without employing hazardous organic solvents and time-consuming liquid/liquid partitioning. The method was subsequently used to determine the EA content and specific ergot alkaloid distribution of rye flour containing known amounts of ergot sclerotia and unknown rye flour samples from Danish mills.

Experimental

Chemicals and reagents

The same brands of materials were used throughout the experimental period. Chemicals were of analytical reagent grade. Solvents were of analytical reagent grade or of HPLC grade when in contact with the HPLC system. De-ionized water was cleaned on a Milli-Q water purification system (Millipore Corp., Bedford, MA). The standards employed were ergonovine, α-ergocryptine, and ergocristine (Sigma-Aldrich), ergotamine hemitartrate (Tocris), and ergocornine (ICN Biomedicals Inc.). Each standard was dissolved in methanol to a concentration of 500 µg ml^{-1} of the pure standard. Ergocornine was insoluble in pure methanol and was dissolved in methanol:dichloromethane (1:1 v/v). These standard solutions were stored at –20°C and used for calibration and for spiking of flour samples. Prior to analysis dilution was performed with mobile phase A (acetonitrile:aq. 0.01 M ammonium carbamate (1:4 v/v)). To avoid light-induced degradation of the EAs all procedures were performed under subdued light. Furthermore glassware was either amber or wrapped in aluminium foil.

Extraction and clean-up

The extraction and clean-up method was modified after (Ware *et al.* 2000). 25.0 g of rye flour was weighed in a 250 ml Erlenmeyer flask. 100 ml of extraction solution (methanol:0.013 M aq.

phosphoric acid (70:30 v/v) was added and the EAs extracted for 30 min. at room temperature on a horizontal shaker (Heto, Birkerød, Denmark). Extracts were transferred to 100 ml glass centrifuge tubes and centrifuged for 10 min. at room temperature (3500 rpm, i.e. 2630 RCF). The supernatant was filtered through a filter paper (Macherey-Nagel, 15 cm Ø, filter paper circles) into Erlenmeyer flasks. 2.0 ml of the extract was mixed with 2.0 ml 0.013 M aq. phosphoric acid and filtered through a Sartorius Minisart RC15 (0.20 µm) filter. The SPE columns (SPEC PLUS 3ML SCX, Ansys Diagnostics Inc., USA) were mounted in a vacuum manifold (Vac Elut 20, Varian) and conditioned with 1.0 ml methanol followed by 1.0 ml 0.013 M aq. phosphoric acid. 3.0 ml of the diluted extract was then applied to the SPE columns followed by 1.0 ml rinsing solution (methanol:0.007 M aq. phosphoric acid (70:130 v/v) to rinse off unwanted matrix compounds. The SPE discs were not allowed to dry out until after the rinsing; when they were dried under vacuum for 1 min. Up to this point all eluates were discarded. Elution of EAs was done into a test tube with 2.5 ml elution solution (methanol:0.01 M ammonium acetate adjusted to pH 10.2 with 1 M sodium hydroxide (60:40 v/v)). A flow of approximately 2 ml/min was maintained throughout the SPE clean-up. Eluted samples were evaporated to dryness in a thermostated waterbath (max. 40 °C) under a gentle stream of N_2. Prior to HPLC analysis samples were reconstituted in 1.0 ml mobile phase A (acetonitrile:aq. 0.01 M ammonium carbamate (1:4 v/v)).

HPLC protocol

The chromatographic analysis was performed on a Hewlett Packard Series 1100 HPLC systems equipped with the corresponding HP 1100 series fluorescence detector. The column was an X-Terra MS C_{18} (250 mm x 3.0 mm; 5 µm) (Waters) protected by a 3.0 x 20 mm guard column of the same material. 20.0 µl of sample was injected and separated by gradient elution employing the following gradient (time, mobile phase B):0.0 min, 0 %; 7.0 min, 0 %; 7.1min, 1 %; 10.0 min, 16 %; 14.0 min, 16 %;16.0 min, 32 %; 24.0 min, 32 %; 33.0 min,75 %; 38.0 min, 75 %; 40.0 min, 0 %; 50.0 min, 0 %. The flow was 0.7 ml min^{-1} for the first 7 minutes and 1.0 ml min^{-1} from 7-50 minutes. Mobile phase A consisted of acetonitrile: aq. 0.01 M ammonium carbamate adjusted to pH 9.6 with 0.5 M sodium hydroxide (1:4 v/v) and mobile phase B of acetonitrile. Column temperature was maintained at 25°C and fluorescence detection was performed with excitation at 240 nm and emission at 410 nm.

Data processing

Peaks from ergocornine, ergocristine, α-ergocryptine, ergonovine and ergotamine and their C8-isomers were identified based on comparison with retention times of pure standards (Figure 2). The rapid C8-isomerisation was unavoidable in samples and standards. Therefore quantification was done on the assumption of mass conservation and equal response factors for both C8-isomers by relating the summed peak area of two C8-isomers to the known initial concentration of

standard. For convenience five compound groups were defined, each group comprising the two C8-isomers of a standard: ErNo = ergonovine + ergonovinine; ErTm = ergotamine + ergotaminine; ErCo = ergocornine + ergocorninine; ErCp = α-ergocryptine + α-ergocryptinine; ErCs = ergocristine + ergocristinine.

Statistical comparisons were performed as one- or two-way analysis of variance utilising the ANOVA or GLM procedure of the SAS system (SAS Institute 2001). Linear regressions were also performed with the GLM procedure. A significance level of 0.05 applies to all statistical calculations.

Validation

The storage stability of standards (-20 °C and 5 °C), evaporated samples (5 °C) and standards and reconstituted samples during a HPLC sequence was tested by comparing HPLC response of standards and samples repeatedly over time.

Recoveries were determined with two series of recovery experiments, each with unspiked flour samples and 4 spiking levels. Four repetitions were performed for each level in each series, resulting in 8 repetitions at each level and 40 samples in total. A batch of organic rye flour with a very low EA content was used and spiked with standard solution diluted with methanol. 100 µl of solutions containing 100, 25, 13, 6.3 or 0 µg ml^{-1} were added to each portion of flour to give concentrations of 400, 100, 50, 25 or 0 µg kg^{-1}, respectively. The standard solutions were added to the flour and mixed by shaking 5-10 min. before extraction solution was added. For calibration curves two serial dilutions of standard solutions were performed with mobile phase A on separate days.

Background levels of EAs could be detected in the flour used for spiking experiments. The concentrations were however below the LOQs for many standards, so determination of EA background concentrations was done by the method of standard addition performed on the results of the recovery experiment. The recovered amounts of ergot alkaloids from spiked samples were corrected for the background concentrations in the rye flour before calculation of recoveries.

Ergot alkaloids in rye flour

To test the method and evaluate the variability in EA content and composition, rye flour samples with known amounts of ergot sclerotia from four locations (I-IV) were produced and analysed. Sclerotia and grain were collected at different farms immediately after harvest in 2003 (I, II, and ergot free grain) and approximately 1 month after harvest in 2004 (III and IV). Sclerotia and grain were stored at –20 °C until analysis. Rye flour containing 5000 mg ergot sclerotia kg^{-1} (0.50 %

(w/w)) was produced for each location by grinding 1000 mg of sclerotia with 199 g ergot free grain from a fifth location on a centrifugal mill with a 0.50 mm filter. Flour samples containing 500 and 2500 mg ergot sclerotia kg^{-1} (0.05 and 0.25 % (w/w)) were produced by mixing the ergot containing flour with ergot free flour produced from hand sorted grain milled on the same mill. The samples were analysed for their content of the EAs identifiable by the method.

To evaluate the EA content of Danish rye flour in general 34 rye flour samples collected at mills all over Denmark between 2000 and 2005 were analysed for their content of the EAs detected by the developed method. The sampling procedure followed the official EU method for control of ochratoxin A (EU Commission 2002) e.g. sampling of 100 subsamples of approximately 100 g from lots of 50 to 300 tons followed by thorough homogenisation. Flour samples were stored at –20°C. The determined recovery rates were used for recovery correction of the concentrations found in rye flour both naturally contaminated and samples with known amounts of sclerotia.

Results and discussion

Method development

Stepwise development of the method was performed by varying one or two factors at a time. For the extraction, different proportions of methanol to 0.013 M aqueous phosphoric acid (40:60, 50:50, 60:40 and 70:30 v/v) were tested and a 70:30 mixture was found to be the most efficient. The elution solution of 0.05 M phosphate buffer in methanol (40:60) (Ware *et al.* 2000) was tested against 0.01 M aqueous ammonium acetate in methanol (40:60) and no significant difference was found in recoveries. 0.01 M ammonium acetate was chosen because it is volatile, so samples eluted with this buffer could be evaporated to dryness thereby prolonging the storage time. Reconstitution was done in the mobile phase of the HPLC system minimising the risk of precipitation and immiscibility in the chromatographic system. The optimal pH of the elution solution was 10.2. Optimal conditioning and elution volumes were determined within the ranges 0.5-2.0 ml and 1.0-2.5 ml, respectively. The original rinsing solution consisting of extraction solution was discovered to remove not only matrix compounds but also 16 % of added ErTm standard (n=3). As the sample extracts were diluted with 0.013 M aq. phosphoric acid prior to the SPE clean-up the extraction solution used for rinsing actually contained more methanol than the sample solution. A 2x2 factorial experiment with phosphoric acid concentration (0.013 and 0.007 M) and methanol:phosphoric acid-ratio (70:30 and 70:130 v/v) as independent variables showed that the mixing ratio had a significant effect (P=0.003) on the ErTm concentration in the eluent. With the rinsing solution of methanol:0.007 M phosphoric acid (70:130 v/v) the loss of ergotamine with rinsing solution was reduced to 2 % (n=3).

[Insert figure 2]

To minimise the damaging effect of alkaline mobile phases on silica based HPLC columns a hybrid column containing both inorganic (silica) and organic (organosiloxane) components was employed for the chromatographic separation. The chosen X-Terra column was stable over time. After more than 500 runs over a period of a year there were only minor differences in performance between the old column and a newly acquired one. The chromatographic conditions were carefully adjusted for optimal resolution. The interference of matrix peaks X with EAs was found to be highly dependent on the pH of the mobile phase. pH values of 8.8, 9.3 and 9.8 in pure mobile phase buffer gave retention times (Rt) for peak X of 17.9, 20.0, and 20.8 respectively, while Rt for e.g. ergocristine differed only 0.2 min. With a pH value of 9.6 in the ammonium carbamate buffer used for mobile phase A, X eluted between ergocristine and ergotaminine, where the risk of interference with standards was minimal (Figure 2). Equilibration of the X-terra column is slow after rinsing with unbuffered solutions of water and ACN. The problem was solved by introducing an equilibration sequence of 3-4 runs with a test sample prior to resuming analysis after the cleaning procedure.

Validation

Standard solutions stored at –20 ℃ for two months showed no significant reduction in response for the standard compound groups in calibration samples. Standard solution could be stored at 5 ℃ for 8 days without significant reduction in the response for the standard groups in calibration samples. Evaporated samples could be stored at 5 ℃ for 1 day without significant degradation ($P>0.05$, $n=3$).

[Insert Figure 3]

Repeated analysis of a cleaned-up extract of spiked flour and a calibration sample documented the C8-isomerisation occurring during execution of a HPLC sequence (Figure 3). Isomerisation was particularly fast for ergocornine and very slow for ergonovine. No significant effect of time on compound group concentrations in the extracts was found during 66 hours at room temperature. It is thus apparent that recoveries and repeatability could only be determined for EA groups, and not for the individual C8-isomers.

The calibration curves for the method were examined in the range 6.7-530 µg kg^{-1} ($2.5 \cdot 10^{-3}$ - 0.200 µg ml^{-1}) with duplicate preparations of each of the six serial standard dilutions. Linear regressions were performed and residual plots examined. The calibration curves were linear in the range 0-400 µg kg^{-1}. If the highest level of 530 µg kg^{-1} was included in the linear regression the residual plots gave evidence of a negative curvature. The intercepts of the 0-400 µg kg^{-1} calibration curves were

not significantly different from 0. Therefore the calibration curves were forced through origin. Correlation coefficients were in the range 0.9990 to 0.9999.

Limits of detection (LOD) and limits of quantification (LOQ) were determined for all standards and their C8-isomers as, respectively 3 and 10 times the average signal noise in 10 runs of an EA free rye flour sample made from hand-sorted rye kernels. Noise was determined as the standard deviation of a linear regression on the signal in noise intervals of approximately 1 min around the retention times of standards. The LODs were in the range 0.2 – 1.1 μg kg^{-1} for ergocryptine and ergonovine, respectively. This is low and comparable to previous methods (Klug *et al.* 1988;Scott *et al.* 1992;Ware *et al.* 2000).

[Insert Table I]

The recoveries determined for spiked rye flour samples were on average 61 % with a standard deviation of 10 %. The lowest recoveries are obtained for ErNo, but the difference between EA groups is small (Table I). The recoveries are low compared to recoveries of previous method of 77 % (Scott *et al.* 1992), 93 % (Rottinghaus *et al.* 1993) and 88 % (Ware *et al.* 2000). In these studies the procedure for the recovery determination is not described in details, and the disparity may be due to differences in the applied spiking procedures. In the present study standards were mixed with dry flour 5-10 min prior to extraction. Experiments performed with flour extract spiked after centrifugation and filtration gave recoveries of 85-94 % (n=6). The application of standard solutions to dry flour was considered a more realistic scenario for the evaluation of extraction efficiency from a naturally EA contaminated flour sample. However, the low recoveries indicate that extraction of EAs from whole flour is the critical step.

Ergot alkaloids in rye flour.

The EA content and composition of the grain samples with added sclerotia (Table II) vary between locations. The sclerotia from one location (III) contained none of the five EAs identifiable by this method. Sclerotia from two locations contained only ErCo and ErCp while the sclerotia from another location contained all five standard EAs.

[Insert Table II]

The high variability in EA composition is consistent with the findings of a previous investigation of Canadian rye sclerotia (Young 1981a). Based on the results within the linear range of the calibration curves the average contents of ergot alkaloids in the sclerotia from the four locations were calculated to 270, 130, non detectable and 50 μg g^{-1}, respectively. These values exhibit a large variation between locations (coefficient of variation > 100 %) and are low compared to the

average contents measured by Osborne & Watson (Osborne & Watson 1980), Young (Young 1981b;Young 1981a), Young *et al.* (Young *et al.* 1983), and Porter *et al.* (Porter *et al.* 1987) for rye and wheat, where average concentrations in tested sclerotia were between 1100 and 3600 μg g^{-1}. However, the existence of virtually EA free ergot strains has also been reported (Ruokola 1961). Large variations in EA content is also observed in Canadian ergot sclerotia (Young 1981b;Young 1981a). It cannot be excluded that some degradation of EAs has occurred during the storage of collected samples, but the deterioration of EAs measured by percent and activity by bioassay is reported to proceed very slowly in sclerotia stored dry at room temperature (Barger 1931). The EA content of 20-25 year old sclerotia has also been determined to 1460 μg g^{-1} (0.146 %) (Frach & Blaschke 1998).

Of the 34 rye flour samples from Danish mills 32 contained ergot alkaloids in concentrations above LOQ (Table III). The distribution between individual EAs was highly variable with some samples containing all five types and their epimers, while other contained only a few types, thus consistent with the variations between ergot sclerotia from different locations. The most common EAs were ErCp and ErTm. Other surveys report ErTm and ErCs to be the most common EAs in rye flour (Scott & Lawrence 1980;Young 1981a;Scott & Lawrence 1983;Baumann *et al.* 1985;Scott *et al.* 1992). The overall mean content of EA was 46 μg kg^{-1} with a maximum content of 234 μg kg^{-1}, which is comparable to the findings of the latest Danish survey (Rasmussen 1991).

[Insert Table III]

With the measured EA content of 270 μg g^{-1} in sclerotia and a sclerotia content of 500 mg kg^{-1} in grain, corresponding to the maximum recommendation, the EA concentration in flour would be 135 μg kg^{-1}. The maximum concentration of 234 μg kg^{-1} observed in one sample from a Danish mill can therefore only be obtained with sclerotia of higher EA content or by sclerotia contents in grain exceeding 500 mg kg^{-1}. With an EA content in sclerotia of 0.25 % (2500 μg g^{-1}) as determined by Young (Young 1981a) the EA concentration would be 1250 μg kg^{-1}. Taking into account, that ergot sclerotia have different milling properties to grain (Shuey *et al.* 1973;Dexter *et al.* 1991;Fajardo *et al.* 1995), the EA concentrations in commercial flour are expected to be lower than the EA concentrations in grain before milling. About 25 % of the ergot alkaloids present in wheat before milling was retained in straight-grade flours of a pilot mill (Fajardo *et al.* 1995). It is thus reasonable to assume that ergot alkaloid concentrations in Danish ergot sclerotia can be higher than the 270 μg g^{-1} found in the examined sclerotia. With large variations in EA content of sclerotia (Young 1981a;Young 1981b;Osborne & Watson 1980) a threshold limit based on percent of ergot bodies in grain is of limited relevance.

Both conventionally and organically grown rye flour samples were examined. In organic rye flour samples examined by Rasmussen (Rasmussen 1991) the average EA content was 205 µg kg^{-1} (n=2) compared to 3 µg kg^{-1} (n=13) for conventional rye flour. No such difference between organic and conventional rye flour was found by Klug (Klug *et al.* 1988) and an inverse relationship was seen by Lauber (Lauber *et al.* 2005). The differences in total EA content between the 17 organic and 17 conventional rye flour samples of this investigation are not significant. Differences in EA content in rye flour are more likely to relate to the particle size of cereal products. Ergot sclerotia have different milling properties from wheat and ends up mainly in the coarsest milling fractions like bran and germ (Shuey *et al.* 1973;Dexter *et al.* 1991;Fajardo *et al.* 1995). A similar partitioning is expected for rye.

Finally, the C8-(S) isomers account for on average 54 % of the determined EAs in the 34 analysed flour samples, ranging from 0 % ergonovinine in the ErNo group to 73 % ergocryptinine in the ErCp group. It is thus apparent, that determination of the C8-(S) isomers is of vital importance for the overall assessment of EA levels in rye flour, since the distribution between isomers prior to sample processing is unknown.

Conclusion

The HPLC method reported here performed well for the detection of both C8-isomers of the most common ergot alkaloids: ergocornine, α-ergocryptine, ergocristine, ergonovine and ergotamine. The clean-up by cation exchange SPE combined with HPLC separation under alkaline conditions retains and separates both C8-isomers without the use of hazardous organic solvents. C8-isomerisation took place during the HPLC analysis which emphasizes the importance of determining both isomers, as the C8(R) to C8(S) ratio prior to extraction is unknown. The C8-(S) isomers accounted for 54 % of the total EA content of the 34 tested samples and there was no significant difference in EA content between organic and conventional rye flour. 32 samples were positive and the most common ergot alkaloids were ergotamine and α-ergocryptine including their C8-isomers. Large variations were observed in the EA content of sclerotia from different locations emphasizing the inaccuracy of the maximum recommended content of 500 mg kg^{-1} (0.05 % (w/w)) ergot bodies in grain for human consumption. The method provides chemical determination of both C8-isomers of the most common EAs and gives a more accurate measure of EA content in grain, than the common weight percentages or methods measuring only the C(8)-R isomers.

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Legends to figures

Figure 1: Chemical structure and CAS no. of common ergot alkaloids.

Figure 2: Chromatogrammes of I: standard solution (0.025 µg ml^{-1}) and II: rye flour sample naturally contaminated with ergot alkaloids. The numbered EAs are: Ergonovine (1), ergonovinine (2), ergotamine (3), ergocornine (4), α-ergocryptine (5), ergocristine (6), ergotaminine (7), ergocorninine (8), α-ergocryptinine (9) and ergocristinine (10). X designates the matrix peak named X.

Figure 3: C8-isomerisation of ergocornine to ergocorninine (I) and ergonovine to ergonovinine (II) in reconstituted sample during storage in the HPLC autosampler. The amount of each isomer is expressed as the percent of the total amount.

Table I: Recoveries of EAs ± standard deviations at four spiking levels (n=8). The values are corrected for background levels calculated by the method of standard addition. Repeatabilities (n=32) are standard deviations for recoveries at all concentration levels, measured in two series on two separate days.

EA[a]	Amount added (µg kg^{-1})				Mean rec. (%)	Repeatability (%)
	25.0	50.0	100.0	400.0		
ErCo	60 ± 11	62 ± 11	61 ± 15	61 ± 7.3	61	12
ErCp	61 ± 15	61 ± 12	60 ± 15	60 ± 7.6	61	13
ErCs	62 ± 8.8	67 ± 10	65 ± 13	65 ± 6.8	65	9.9
ErNo	55 ± 7.7	62 ± 7.7	57 ± 12	58 ± 6.3	58	8.8
ErTm	61 ± 8.0	63 ±7.2	63 ± 10	63 ± 7.7	62	8.4

[a]ErCo: Ergocornine+ergoconinine; ErCp: α-ergocryptine+α-ergocryptinine; ErCs: ergocristine+ergocristinine; ErNo: ergonovine+ergonovinine; ErTm: ergotamine+ergotaminine.

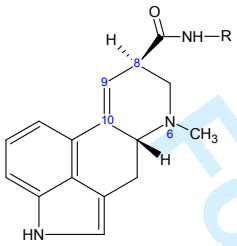
	Location			
EA	I	II	III	IV
ErCo	430	450	nd	17
ErCp	390	260	2.9	30
ErCs	nd	nd	nd	100
ErNo	nd	nd	nd	39
ErTm	4.6	nd	nd	56
Total	830	710	2.9	240

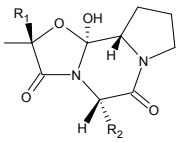
Table II: Ergot alkaloid content of rye flour with ergot sclerotia from different locations added, to sclerotia contents of 5000 mg kg^{-1}. For compound abbreviations see Table I. A discrepancy between the sum of subtotals and the stated total is due to rounding of errors.

Table III: Number of positive samples ($n_{positive}$), average concentrations (mean, $mean_{positive}$), and maximum concentrations (max.) of 34 conventional and organic rye flour samples collected between 2000 to 2005. All concentrations are in µg kg^{-1} and corrected for recovery percentages. The average percentages of the alkaloids found as C8-(S) isomers (% C8-(S)) are shown in the bottom line. For compound abbreviations see table I.

		ErCo	ErCp	ErCs	ErNo	ErTm	Total
Conventional	$n_{positive}$	10	14	11	3	9	15
(n=17)	Mean	9.1	19	12	1.7	18	60
	$Mean_{positive}$	15	23	19	9.4	35	68
	Maximum	27	73	58	17	83	230
Organic	$n_{positive}$	13	15	10	0	6	15
(n=17)	Mean	6.6	16	2.5	0	6.2	32
	$Mean_{positive}$	8.7	18	4.3	0	17	36
	Maximum	27	62	15	0	38	100
All	% C8-(S)	56	73	68	0	30	54

Figure 1



Ergot alkaloid	CAS no.	R	R_1	R_2
Ergonovine	60-79-7	$CH(CH_3)CH_2OH$		
Ergocornine	564-36-3		isopropyl	isopropyl
α-ergocryptine	511-09-1		isopropyl	isobutyl
Ergocristine	511-08-0		isopropyl	benzyl
Ergotamine	113-15-5		CH_3	benzyl

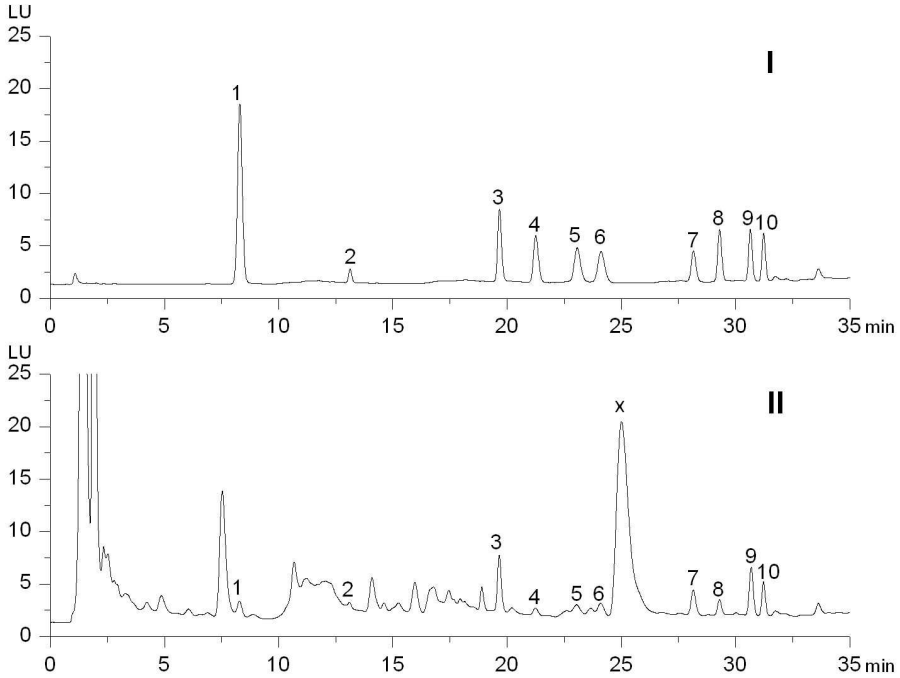


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Figure 3