HPLC/MS/MS METHOD FOR THE DETERMINATION OF ERGOT ALKALOIDS IN CEREALS

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GLOSSARY

ACN Acetonitrile

amu Atomic mass unit. The weight of a compound or part of a compound as measured by a mass spectrometer.

Background. The non-analyte 'baseline' signal seen in a chromatogram that is due to small responses derived from minor unidentified compounds extracted from a sample and/or from impurities in the solvents used and/or from the column material and contaminants from prior samples.

C18 A material for chromatography based on the 18 carbon compound octadecylsilane

C8 A material for chromatography based on the equivalent 8 carbon compound

Chromatogram. A picture showing the signal produced by an LC-MS or LC-MS/MS system over a period of time. When a compound elutes from an HPLC column it produces a response in the detector that is plotted against time.

DCM Dichloromethane

EFSA European Food Safety Authority

ELISA Enzyme-linked immunosorbent assay. An alternative specific detection method based on antibody interaction.

ES Electrospray ionisation. An operating mode in LC-MS in which ionised particles are produced.

ESI-MS Electrospray ionisation mass spectrometry. Usually operated in the positive mode ESI(+).

FLD Fluorescence detection. A means of detecting fluorescent compounds used in HPLC as an alternative to MS.

GC Gas chromatography. An instrumental technique for separating relatively volatile compounds.

HLB Hydrophilic–lipophilic balance

HPLC (LC) High performance liquid chromatography. An instrumental technique for separating relatively non-volatile compounds with high efficiency.

HPLC-UV-MS HPLC with ultraviolet absorption detection combined with mass spectrometric detection.

LC Liquid chromatography. An instrumental technique for separating relatively non-volatile compounds. Sometimes a low pressure system but more frequently an abbreviation of HPLC.

LC-MS (HP)LC coupled to mass spectrometry as a detector.

LC-MS/MS (HP)LC coupled to a mass spectrometer having a second fragmentation stage in tandem. An ion produced in the first stage can selected and fragmented by collision with gas molecules to produce a mass spectrum derived solely from that ion. Selected monitoring of the production of one or more ions from that spectrum (SRM) provides very high selectivity which in turn gives a low background signal and hence high sensitivity.

LOD The limit of detection. Defined as the lowest concentration that will be detected with a defined probability. In simple terms the lowest amount that can be distinguished from the background signal.

LOQ The limit of quantification. The smallest amount of analyte that can be quantitatively determined with suitable precision and accuracy. In simple terms the lowest amount that can be quantified with an acceptable degree of certainty and hence normally used as a reporting limit.

m/z The mass to charge ratio of an ion produced in a mass spectrometer, usually the same as amu.

$(M+H)^+$ The protonated molecular ion. In LC-MS and LC-MS/MS the ion produced by addition of H^+ to the unfragmented analyte molecule. This can give an indication of the analyte's molecular weight and to confirm the identity of a response (peak) in the chromatogram. Fragmentation of the protonated molecular ion is commonly used in LC-MS/MS.

Mass spectrometer. An instrument for measuring and identifying the output from and HPLC system (LC-MS). It fragments molecules emerging from the HPLC column into charged particles (ions) that are separated and measured in terms of mass (=identity) and intensity (=concentration).

MS Mass spectrometry. Detection technique where analytes (usually separated by chromatography) are ionised to produce fragments that can be separated and characterised as a mass spectrum and quantified.

OTA Ochratoxin A, a non-ergot mycotoxin.

ppm Parts per million, milligrams per kilogram or litre, micrograms per gram or millilitre (mg per kg, mg per litre, µg/g, µg/ml).

PSA Primary secondary amine. A sorbent material with properties of separating impurities from sample extracts

PTFE Polytetrafluoroethylene. Used as an inert material to make fine filters to remove particles from sample extracts that might damage LC-MS systems RSD The relative standard deviation

RSU The relative standard uncertainty associated with results of a measurement.

S/N The signal-to-noise-ratio. A measure of the intensity of a signal derived egg from LC-MS compared to the background signal.

s0 standard deviation

SOP Standard Operating Procedure

SPE Solid-Phase Extraction

Spectrum A mass spectrum

SRM Selected Reaction Monitoring

SSE signal suppression/enhancement

TLC Thin layer chromatography, a (usually qualitative) separation technique.

TOF Time-Of-Flight. An ion separation system in mass spectrometry that provides highly accurate molecular weight information.

U Expanded measurement uncertainty

u0 standard uncertainty at low concentrations

SUMMARY

A method has been developed and validated which enables the quantification of the six major ergot alkaloids in a number of cereals and cereal based food products. The six ergot alkaloids studied (ergometrine, ergotamine, ergosine, ergocristine, ergocryptine and ergocornine) have been defined by EFSA as of the major importance. In addition the method can determine the epimer isomers (-*inines*) corresponding to the major ergot alkaloids. This is of considerable importance in terms of the differences in toxicity of the isomeric forms.

The method was developed and validated using 10 different cereal and food samples. The extraction and clean-up procedure is simple and the analysis time short. The limits of quantification were 0.17 to 2.78 µg/kg depending on the analyte and matrix. Recovery values for the 12 ergot alkaloids spiked into 10 different matrices at levels of 5, 50 and 100 µg/kg were between 70 and 105% for 85 of 90 recovery measurements made over six days.

Measurement uncertainty values were highly satisfactory. At a concentration level of 5 µg/kg the expanded measurement uncertainty ranged from ± 0.56 to ± 1.49 µg/kg, at a concentration level of 100 µg/kg the expanded measurement uncertainty ranged from ± 8.9 to ± 20 µg/kg. Both LOQs and measurement uncertainties were dependent on the analyte but almost independent of the matrix.

The method performance was satisfactory when tested in a mini-intercomparison study with two other laboratories that used alternative methods.

1. Introduction

1.1. General

Ergot alkaloids are mycotoxins produced by fungi of all species of the *Claviceps* genus, most notably by *C. purpurea* which parasitise the seed heads of living plants at the time of flowering. Although fungi are the primary source of ergot alkaloids they are also synthesized by some plants, mainly of the morning glory family (Wilkinson, 1987). Fungal infections are most prevalent in rye and triticale that have open florets but also wheat and other small grains are potential hosts of these fungal species (Lorenz, 1979; Kobel and Sanglier, 1986; Řeháček and Sajdl, 1990; Flieger *et al*., 1997).

Other important sources of these ergot alkaloids are grasses infected with endophytes, for example, tall fescue (*Festuca arundinacea*) infected with *Claviceps* spp. or *Acremonium*

coenophialum (Powell and Petroski, 1992). The fungus replaces the developing grain or seed with the alkaloid containing wintering body, known as ergot, ergot body or sclerotium. Recently, also ergot contamination on sorghum has been discovered (Bandyopadhyay *et al.,* 1998) which is caused by *Claviceps africana.* Sorghum species, principally *Sorghum bicolor,* are an important food and fodder crop in Africa, Central America, and South Asia. In sorghum *Claviceps* spores germinate and grow into the unfertilised seed producing a sclerotia. *Claviceps africana* produces primarily dihydroergosine with lesser amounts of dihydroelymoclavine and festuclavine.

Ergot is derived from the old French word argot, meaning the cock´s spur (van Dongen and de Groot, 1995). Before or during harvest time the usually violet or black sclerotia fall on the ground and remain intact during winter and during storage of grains. Mature sclerotia may vary in number and size from a few millimetres to more than 4 centimetres according to the host plant (Meyer, 1999, Kamphues and Drochner, 1991), and differ in mass from a few grams to 25 grams per 100 sclerotia. The ergot sclerotium contains up to 40% of fatty oils (Komarova and Tolkachev, 2001a).

In addition, sclerotia show significant differences in their total alkaloid content that varies between 0.01 to 0.5% (w/w) (Schoch and Schlatter, 1985; Lorenz, 1979; Wolff, 1989) and show large differences in the patterns of alkaloids produced that are determined by the individual fungal strain in a geographical region and the host plant. The sclerotia are harvested together with the cereals or grass and can thus lead to contamination of cereal based food and feed products with ergot alkaloids, ingestion of which can cause ergotism in humans and animals.

Ergot is ubiquitous, yet the prevalence of the species is dependent on climatic conditions and is especially pronounced in seasons with heavy rainfall and wet soils (Craig and Hignight, 1991). Investigations in Germany indicate an increase in the occurrence of Claviceps purpurea infections in the last 10 years. This increase seems to be associated with the more extensive use of hybrid varieties of rye and perennial rye breeds (Amelung, 1999; Engelkes, 2002). However, today effective cleaning techniques at the mills enable the removal of up to 82% of ergot sclerotia from grain (Posner and Hibbs, 1997).

Cleaning procedures become less reliable when the intact ergot sclerotia break into smaller fragments during transport or when dry climatic conditions produce fungal sclerotia which are similar in size to the grain (Lauber *et al*., 2005). Despite effective cleaning procedures, ergot alkaloids have been detected in surveys of Swiss, Canadian, Danish and German

cereals and cereal products at total levels of up to 7255 µg/kg in German rye flours (Dusemund *et al*, 2006; EFSA, 2005; Baumann *et al*., 1985; Scott *et al*., 1992; Scott and Lawrence, 1980; Lombaert *et al*., 2003).

1.2. Toxicity

Intoxications induced by *Claviceps purpurea* have been known in Europe for many centuries. The most severe effects of ergot contaminated grains are described in the medieval literature as St. Anthony´s Fire or Holy Fire, with respect to the intense pain resulting from vasoconstriction and subsequent gangrene with loss of fingers, hands, feet and even entire limbs. Other symptoms of ergot alkaloid intoxication include abdominal pains, vomiting, burning sensations of the skin, insomnia and hallucinations (Gabbai, 1951). The epidemics decreased due to changes in farming practices including deep ploughing which resulted in the sclerotia being buried. In addition, wheat replaced rye as the major grain crop and this was much less susceptible to ergot infection.

Human poisoning from ergot has also occurred in more recent times in France (Fuller, 1968), India (Bhat *et al*., 1976; Krishnamachari and Bhat, 1976) and Ethiopia (Demeke *et al*., 1979). Recently, a severe outbreak of gangrenous ergotism was again reported in Ethiopia (Urga *et al*, 2002). Despite these reports ergotism has nowadays practically been eliminated as a human disease but remains an important veterinary problem, particularly in cattle, horses, sheep, pigs and chicken (Bennet and Klich, 2003). There are numerous reports of poisoning of farm animals by ergot contaminated feed (e. g. Hogg, 1991) and by endophyte-infected grasses (Porter, 1995; Miles *et al*., 1996). Recently, gangrenous ergotism has also been reported among free-living moose and roe deer in Norway (Handeland and Vikoren 2005; Uhlig *et al*., 2007).

There is little information available on the metabolism of ergot alkaloids. However, the peptide alkaloids (below) are the most physiologically active. They disappear rapidly from blood and tissues with a high first-pass clearance by the liver (Moubarak *et al*., 1996**)**. In contrast, their physiological effects persist for lengthy periods of time. Their mode of action is largely mediated via inhibitory effects on prolaction secretion by the pituitary, by activating the D2-dopamine receptors in pituitary lactotrophs. This is due to the structural similarities of the lysergic acid derivatives with the noradrenaline transmitters dopamine and serotonin.

Based on the biological effects described, ergot alkaloids have been used for medical purposes since the beginning of the 19th century. Their beneficial pharmacological effects

stimulated research into ergot compounds and natural and semi synthetic drugs have been produced from them (Flieger *et al.*, 1997). The long list of pharmacological effects includes prolactin inhibition, treatment of Parkinsonism, and uterine stimulation (Berde and Schild, 1978). Ergotamine was first used in the treatment of migraine in 1926 and is still used by patients. The semi synthetic psychotomimetic drug diethyllysergamide (LSD-25) (Stoll and Hofmann, 1943) was originally produced for experimental use as a treatment for schizophrenia but eventually became popular as drug of abuse due to its hallucinogenic effects.

1.3. Chemistry

In 1920 ergotamine was the first alkaloid isolated from ergot reported in the literature and used for therapeutic purposes e.g. in gynaecology to control postpartum haemorrhage (Stoll, 1920; Favretto *et al.*, 2007). Since then about 50 different ergot alkaloids (also denoted ergolines) have been found and determined with different methods (Flieger *et al.*, 1997).

The common structural feature of ergot alkaloids is the ergoline ring which is methylated on the N-6 nitrogen atom, substituted on C-8 and possesses a C-8,C-9 or C-9,C-10 double bond (Flieger *et al.*, 1997). The main groups of natural ergot alkaloids are simple lysergic acid derivatives such as ergometrine (ergonovine), the peptide alkaloids or ergopeptines (e.g. ergotamine, ergocornine) with an additional peptide moiety linked to the basic tetracyclic ergoline (see Figure 1), the clavine alkaloids (which are hydroxyl- and dehydro- derivatives of 6,8-dimethylergoline, e.g. agroclavine) and the lactam ergot alkaloids (e.g. ergocristam) (Flieger *et al.*, 1997).

The main ergot alkaloids produced by *Claviceps* species which are contained in the sclerotia are ergometrine, ergotamine, ergosine, ergocristine, ergocryptine and ergocornine and the group of agroclavines, the latter being less toxic (EFSA, 2005). These ergot alkaloids are very similar, differing only in substituents on C-8 (Figure 1). The amount and pattern of ergot alkaloids vary between fungal strains, depending on the host plant and the geographical region.

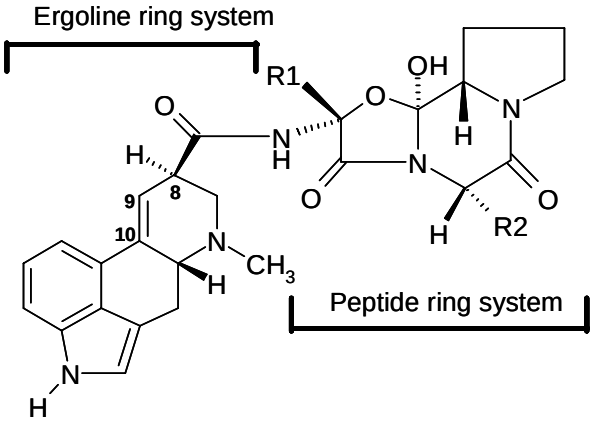


Figure 1 Structure of ergopeptines.

See Table 1 for substituents of the common ergot toxins. The chiral carbon atom C-8 is responsible for the epimerisation

Table 1 Substituents of the major ergot alkaloid toxins

			Substituent 2 R2
			-
			$CH(CH_3)_2$
			$CH(CH_3)_2$
			CH_3
			CH_3
			$CH_3CHCH_2CH_3$

The various peptide-type alkaloids differ by the presence of two substituents in the C2´(methyl or isopropyl) and C-5´ (isopropyl, isobutyl or benzyl) positions. These alkaloids possess molecular weights relatively higher than those of the lysergic acid derivatives (ergometrine) ranging from 548 to 609 Da.

Ergot alkaloids appear as colourless crystals that are readily soluble in various organic solvents, but insoluble or only slightly soluble in water. Ergot alkaloids containing C9=C10 double bond (=ergolenes) readily exhibit epimerisation, especially in the presence of alkalis, with respect to the centre of symmetry at C-8 (see Figure 2) (Lehner *et al*., 2005a). This forms a series of right-hand rotation (S)-isomers representing isolysergic

acid (*iso*-LA) derivatives (Komarova and Tolkachev, 2001a). According to international classification the left-hand rotation isomers of ergot alkaloids representing LA derivatives (C-8-(R) configuration) are termed ergopeptines (e.g. ergotamine) and ergopeptames while the right-hand rotation diastereomers representing isolysergic acid (*iso*-LA) derivatives (C-8-(S) configuration) are termed ergopeptinines (e.g. ergotaminine). In nature, ergopeptinines always accompany ergopeptines. Considerable amounts of ergopeptinines may form during storage of raw materials over prolonged time or in improper conditions, or during extraction of ergot alkaloids from cereals.

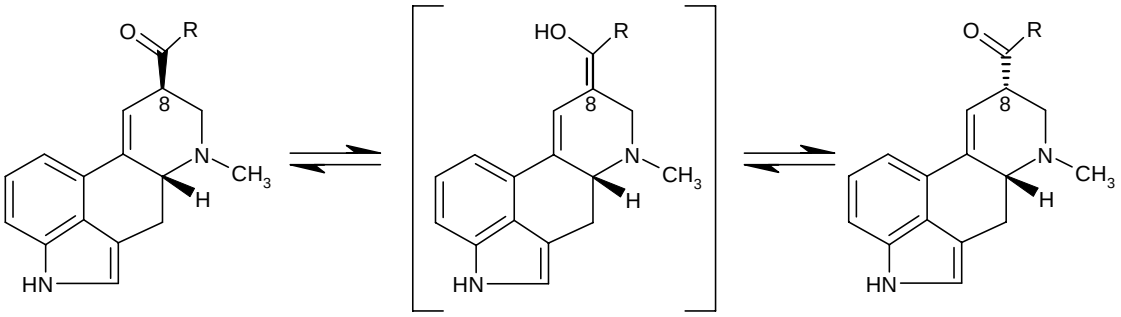


Figure 2 Epimerisation of ergot alkaloids containing a C9=C10 double bond.

The C-8 epimers differ in biological and physicochemical properties. pK_a-values of the *-ines* vary from 5.5 (ergocristine) to 6.0 (ergometrine) and for the *–ininess* from 4.8 (ergocorninine) to 6.2 (ergometrinine) (Stoll *et al*., 1954; Maulding and Zoglio, 1970). Ergot alkaloids are therefore positively charged at N-6 in acidic solutions and neutral at higher pH values. C-8-(R) isomers (*-ines*) are biologically active, while the C-8-(S) isomers (*-ininess*) are inactive (Berde and Stürmer, 1978; Pierri *et al.*, 1982).

The conversion of *–ines* to *–ininess* is rapid, especially in aqueous acidic or alkaline solutions (Hofmann, 1964; Komarova and Tolkachev, 2001a) and has therefore to be taken into careful consideration during the extraction and clean-up procedures to avoid conversion (Lampen and Klaffke, 2006). It was found that ergopeptinines can also convert back into the *–ine* form e.g. in methanol, aqueous organic solvents and acids (Buchta and Cvak, 1999). Therefore, both epimers have to be taken into consideration when the contamination level of cereals with these ergot alkaloids is determined.

Another important characteristic of ergot alkaloids is their sensitivity to light which leads to both isomerisation and degradation (Rutschmann and Stadler, 1978; Komarova and Tolkachev, 2001a).

Ergot alkaloids were quite stable during the processing of flour into pasta and oriental noodles (Fajardo *et al*., 1995). Processing flour into pan bread also had only minimal effect on ergot alkaloid levels. However, Scott and Lawrence (1982) reported up to 100% losses of ergot alkaloids during bread baking from wheat flour. A 25% decrease in ergot alkaloid content during baking of a rye roll was recently observed (Bürk *et al*., 2006). Obviously, the degree of degradation of the ergot alkaloids during baking is dependent several factors, including heat transfer, interaction of ergot alkaloids with dough components and the initial alkaloid level.

1.4. Statutory limits

1.4.1. Limits for ergot bodies

In the EU no regulatory limits apply to ergots in grain for human consumption (Egmond and Jonker, 2004). A maximum value of 500 mg ergot bodies per kg in grain (0.05% w/w) has been set for interventional grain (EU Commission, 2000) but not for grain for consumption. (The EU has established an intervention system, in order to stabilise the markets and ensure a fair standard of living for the agricultural community in the cereals sector (EU Council Regulation, 2003). Through this system the EU dictates certain standards in grain for interventional affairs within the common market, which individual countries can accept also for consumption grain (Bürk *et al*., 2006)). A maximum limit of 1000 mg ergot bodies per kg (0.10% (w/w)) has been introduced for feed products containing unground cereals (EU 2002). The maximum permissible level in the US and Canada is 300 mg ergot per kg grain. Feed materials exceeding this limit are labelled ergoty rye or ergoty wheat and are discarded or mixed with non-contaminated batches (Weipert, 1996). Besides the guideline limits mentioned the ergot content is to some extent controlled by Good Agricultural Practice, including segregation of sclerotia by cleaning machinery.

1.4.2. Limits for ergot alkaloids

The EU Scientific Panel on Contaminants in the Food Chain of the European Food Safety Authority (EFSA) has reviewed the ergot issue recently (EFSA, 2005). EFSA concluded that the alkaloid concentrations are very variable and a consistent relationship between the amount of sclerotia and the total ergot alkaloid (ergoline) concentration cannot be established. Since the total ergot alkaloid content within each single ergot shows

significant variations, between 0.01 to 0.5% (w/w) (Schoch and Schlatter, 1985), no limiting value for maximum ergot alkaloid level can be derived from the maximum value for ergot bodies (Bürk *et al*., 2006). However, assuming an average alkaloid content of 0.2% (w/w) in the ergot, a level of 0.05% ergot equals a total ergot alkaloid content of 1000 µg/kg (Wolff *et al*., 1988). Based on this consideration and available toxicological data, guideline limits have recently been discussed for ergot alkaloids in cereals for human consumption of 400-500 µg/kg and 100 µg/kg in Germany and Switzerland, respectively, (Lampen and Klaffke, 2006; Bürk *et al*., 2006). Guideline limits for the total ergot alkaloid content in feed exist only in Canada and Uruguay, where they vary from 450 to 9000 µg/kg depending on the animal (Egmond and Jonker, 2004). No country has yet set limits for individual ergot alkaloids in food or feed.

1.5. Determination of ergot alkaloids

1.5.1. Calibrants

Analyses of agricultural commodities and foods for ergopeptide alkaloids should be carried out in subdued light to minimize formation of "lumiergopeptines", which are water addition products (Stoll and Schlientz, 1955). Epimerisation to ergopeptinines may occur in solution, particularly at room temperature, leading to equilibrium mixtures (Smith and Shappell, 2002). The degree of epimerisation depends on the solvent, so stock standard solutions should be prepared in aprotic solvents such as chloroform and stored at $<0^{o}$C in amber vials (Scott, 2007). Calibrants should be freshly prepared or immediately evaporated to dryness after preparation, stored deep frozen at -18°C and reconstituted just before use (Lauber *et al*., 2005). Ware *et al*. (2000) suggests dissolving the ergot alkaloids in a stabilizing solution consisting of ethylene glycol (100 g), 1,2-propanediol (100 g) and tartaric acid (1.0 g) diluted to 1 L with ethanol-water (25:75, v/v). Ergot alkaloid standards are less readily available than other common mycotoxins. Some sources are given by Lombaert (2001). Currently, the major ergot alkaloids ergometrine, ergotamine, ergosine, ergocristine, ergocryptine and ergocornine are all commercially available as naturally occurring α-isomers. Ergocryptine and ergocryptinine, may, however, also occur as β-isomers which are not yet available commercially.

1.5.2. Sampling

There has been no research on sampling plans for grains or grain products to be analysed for ergot alkaloids. However, for determining ergot bodies as a percentage of the net weight of a grain sample (e.g. wheat, rye or barley), the minimum representative portion set in Canada is 500 g and the optimum is 1000 g (Canadian Grain Commission, 2004;

Scott, 2007). Due to the inhomogeneous distribution of ergots in the grain, a sample size of 1000 – 5000 g is recommended for optical assessment of the presence of ergot bodies (Lampen and Klaffke, 2006).

1.5.3. Extraction and clean-up

In most methods for the qualitative and quantitative determination of ergot alkaloids in cereals, extraction has either been performed with non-polar organic solvents under alkaline conditions or with polar solvents under acidic conditions. A mixture of dichloromethane, ethyl acetate, methanol and 25% ammonium hydroxide (50:25:5:1, v/v) was used by Scott *et al*., (1992) and Müller *et al.* (2006) whereas methanol-0.25% conc. phosphoric acid (40:60, v/v) was used by Ware *et al*. (2000).

Clean-up is carried out either by liquid-liquid partitioning by exploiting the acid/base properties of N-6 (Scott and Lawrence, 1980) or more recently by similar acid-base partition on solid phase extraction columns (SPE) (Ware *et al*., 1986; Fajardo *et al*., 1995). Other clean-up procedures include partition using Extrelut columns (Baumann *et al*., 1985), use of strong cation exchange particle-loaded membrane extraction disks (Ware *et al*., 2000) and silica gel columns (Rottinghaus *et al*., 1993), which conveniently did not retain the inactive –inine isomers. All those methods gave satisfactory recoveries of individual ergot alkaloids from spiked matrices (Scott, 2007).

1.5.4. TLC and other analytical methods

A wide variety of methods has been explored for the final determination of ergot alkaloids in grains, grasses, feeds and grain foods. They include simple detection procedures – colorimetry (Robbers *et al*., 1975; Young, 1981a), thin layer chromatography (TLC) (Agurell, 1965; Lobo *et al*. 1981) and immunoassays (ELISA) (Shelby and Kelley, 1990 and 1992) - or instrumental procedures such as capillary zone electrophoresis (Frach and Blaschke, 1998) or gas chromatography (GC), usually with mass spectrometric (MS) detection (Scott, 1993; Klug et al., 1993). A review of available analytical methods for the determination of ergot alkaloids including the most frequently employed LC-methods was published by Scott (1995) and Komarova and Tolkachev (2001b) and has recently been updated by the former author (Scott, 2007).

ELISA techniques seem to be an attractive option for screening of ergot alkaloids in agricultural crops and grain flour but it is difficult to identify a marker toxin for monitoring the extent of the contamination. Cross reactivity may be high for one group of ergot alkaloids, and low for another (Schnitzius *et al*., 2001**)**.

GC is not very useful for determination of ergopeptide alkaloids (Scott, 1993) as they decompose in a hot injector. The resulting peptide fragments can be separated by capillary GC with MS identification, but this procedure only identifies the peptide portion of the molecule and epimers such as ergotamine and ergotaminine are not differentiated (Scott, 2007).

The main criterion of usefulness of TLC for determining ergot alkaloids in agricultural products is that the solvent system should separate the alkaloids of interest. Comprehensive studies by Agurell (1965), Röder *et al*. (1969), Fowler *et al*. (1972) and Lobo *et al*. (1981) using silica gel and alumina thin layers and several solvent systems illustrated the difficulty in achieving this objective. Of the 12 main alkaloids usually found in rye ergots, i.e. ergometrin(in)e, ergosin(in)e, ergotamin(in)e, ergocornin(in)e, ergocryptin(in)e and ergocristin(in)e, ergocryptine and ergocristine were particularly inseparable even with 2-dimensional TLC. TLC could be used in developing countries, preferably using extraction and clean-up procedures developed for LC methods (Scott, 2007).

1.5.5. LC-FLD and LC-MS/MS methods

TLC screening methods have mostly been replaced by high performance liquid chromatographic (HPLC) procedures with reversed-phase columns and subsequent UV and mainly fluorescence detection (FLD) under UV light (Komarova and Tolkachev, 2001a, Scott *et al*. 1992; Ware *et al*., 2000; Lombaert *et al*., 2003). Ergot alkaloids frequently analyzed together by HPLC include ergometrine, ergotamine, ergocornine, ergocryptine, ergocrystine, ergosine and their respective -*inine* isomers; the sum of the ergot alkaloids determined is often referred to as the total alkaloid content (Mainka *et al*., 2005).

A simple means of confirmation of the identity of ergot alkaloids is to heat a portion of the extract in 2% acetic acid and observe the appearance of the ergopeptine/–inine isomers by LC with fluorescence detection (Rottinghaus *et al*., 1993). Excitation wavelengths in the range 235-250 nm have been used (Scott and Lawrence, 1980; Ware *et al*., 2000). The response factor for ergometrine was about twice that of the other five grain ergot alkaloids in a study by Young (1981b). Reported detection limits for individual ergot alkaloids in grains and grain foods were of the order of 0.01 µg/kg to 0.5 µg/kg (Müller *et al*., 2006) or 1-2 µg/kg (Scott *et al*. 1992; Ware *et al*., 2000); the limit of quantitation reported by Lombaert *et al*. (2003) for infant cereals was 4 µg/kg. However, the papers do not clearly

describe how the reported limits of detection and quantitation have been obtained and may not be well comparable.

C18 (Mohamed *et al*., 2006a) and Phenomenex Gemini C18 column materials (Lehner *et al*., 2005a,b) have mainly been employed for the LC separation of ergot alkaloids. Various isocratic mobile phases and gradient systems were used for reversed phase LC of ergot alkaloids (Scott, 2007). Acetonitrile mixed with either aqueous base (Scott and Lawrence, 1980; Baumann *et al*., 1985) or acidic solutions (Ware *et al*., 2000) has often been used.

Acidic mobile phases are often preferred because many silica based LC phases are degraded at high pH. In addition, it is common practice to employ volatile weak acids for enhancing the ionisation of basic compounds in mass spectrometry operated in electrospray positive mode (Peng *et al*., 2007). On the other hand those methods employing acidic phases do not report the detection of both epimers (-*ines* and –*inines*). Typical LC run times for the separation of ergometrine, ergotamine, ergosine, ergocristine, ergocryptine and ergocornine and their corresponding epimers are around 45 min (Lauber *et al*., 2005; Müller *et al*, 2006).

LC coupled to mass spectrometry (LC-MS) and LC tandem MS (LC-MS/MS), usually with electrospray ionisation operated in the positive mode ESI(+) has been employed for the quantification of ergot alkaloids as an alternative to FLD. The use of this technique provides in addition an unequivocal identification of the alkaloids. Shelby *et al.* (1997) used LC-positive ion electrospray ionization mass spectrometry (MS) to identify ergot alkaloids including ergine, ergovaline, ergosine and ergonine in endophyte-infected tall fescue; ergonovine and ergotamine were not found.

More recently, Stahl and Naegele (2004) have reported nano-LC-MS/MS analysis of fungal extracts, with ion trap detection enabling MS^n experiments which enabled the identification of three unknown ergot alkaloid derivatives. Mohamed *et al*. (2006b) studied the fragmentation mechanism of six major ergot alkaloids by triple quadrupole and ion trap mass spectrometers operated in ESI(+). Characteristic product ions at *m/z* 223 and 208 were observed for peptide-type and lysergic acid derivatives. As a result precursor ion scanning of the most abundant *m/z* 223 ion was employed for survey studies of rye samples.

Lehner *et al*. (2005a) demonstrated the facility of using ESI(+) mass spectrometry with selected reaction monitoring (SRM) for screening grass and forage samples for novel ergot alkaloids. The same authors (Lehner *et al*., 2005b) have made a thorough study of

the fragmentation patterns of selected ergot alkaloids by LC-MS/MS which allows the prediction of mass spectra of related compounds for which standards are not readily available.

Mohamed *et al*. (2006a) used LC-MS/MS with SRM after C18 clean-up for the quantification of five ergot alkaloids (ergocristine, ergotamine, ergonovine, ergocornine and α-ergocryptine) in rye flour and obtained recoveries from 24% (ergonovine) to 92% (α-ergocryptine) and limits of quantification of 11 – 37 µg/kg. Bürk *et al*. (2006) reported an LC-MS/MS method capable of quantifying five ergot alkaloids down to 0.1 – 1 µg/kg (LOQ) with mean recoveries from 65 to 82% without the need for any clean-up. These methods do not determine both -*ines* and –*inines*, possibly because of the lack of available standards, and some (Bürk *et al*. 2006) are unfortunate in using the undesirable chlorinated solvent dichloromethane as part of the extraction mixture (Scott, *et al*., 1992).

Apart from LC methods the performance characteristics of most methods are not well known. None of the methods mentioned, including LC-methods, has been validated by interlaboratory study and there are no certified matrix reference materials or proficiency studies available for the determination of ergot alkaloids. Recently, the European Food Safety Authority (EFSA, 2005) concluded that validated analytical methods for the quantification of ergot alkaloids in feed materials are needed as a prerequisite for a survey on the occurrence of ergot alkaloids in feed materials in Europe. Analytical techniques should aim to detect the major ergot alkaloids as well as their corresponding biologically active metabolites formed in exposed animals.

2. Project objectives

The major goal of this project was to develop an analytical method using HPLC-MS/MS for the rapid and simultaneous determination of the six major ergot alkaloids defined by EFSA (2005), namely ergometrine, ergotamine, ergosine, ergocristine, ergocryptine and ergocornine and their corresponding epimers (-*inines*), with an LOQ of 1 µg/kg for each of the ergot alkaloids analysed. The method should be validated in-house through the analysis of four raw cereals (wheat, barley, oats and rye) and six processed cereal products spiked at different contamination levels on six separate days.

The method development should be achieved through the optimisation of the extraction and clean-up procedures using spiked and naturally contaminated samples. The matrix effects of naturally contaminated raw cereal and processed cereal food samples should be thoroughly investigated. The validation studies should reveal the precision, recovery, LOD and LOQ and the measurement uncertainty of the method should also be calculated. Owing to the lack of appropriate ergot alkaloid reference materials for this project a mini-comparison study should be carried out between three laboratories to check for systematic errors and to reveal the comparability of the developed method. Finally, an SOP should be produced.

A validated method covering the major ergot alkaloids found in grains will enable further research and study of the ergot problem, providing the means for the acquisition of data on the contamination levels of feed and food and characterising ergot toxins. The method will be another step towards the development of officially recognised procedures and the certification of reference materials for ergot alkaloids in foods. In the long term it is likely to help in the development of a method suitable for use by official control laboratories.

3. Experimental

3.1. Calibrants

Crystalline ergot alkaloids were purchased from Prof. Miroslav Flieger, Laboratory of Physiology and Genetics of Fungi of the Institute of Microbiology, Academy of Sciences of the Czech Republic (flieger@kav.cas.cz). Acetonitrile and water (fluorescence grade) were supplied by Fisher Scientific (UK). Ammonium carbonate (for HPLC) was obtained from Fluka (UK).

Individual stock solutions of six ergot alkaloids, ergocristine, ergotamine, ergocornine, ergosine, α-ergocryptine, ergometrine (as ergometrine hydrogenmaleate) and their corresponding six epimers ergocristinine, ergotaminine, ergocorninine, ergosinine, α-ergocryptinine and ergometrinine were prepared in acetonitrile at levels of 50-200 µg/mL. From these individual stock solutions mixed calibrants of all 12 ergot alkaloids were prepared through dilution with acetonitrile at a level of 100 ng/mL. All solutions were stored in dark brown glass vials in a freezer at -24°C in darkness to prevent any isomerisation problems. Diluted standard solutions were freshly prepared in acetonitrile before use. Since minor precipitation was observed in the ergometrine calibrants at a level of 200 µg/mL after 4 weeks, immediate dilution of the stock solutions is recommended. Alternatively, stock solutions at lower levels or the use of a more polar solvent is possible.

3.2. Optimisation of HPLC Conditions

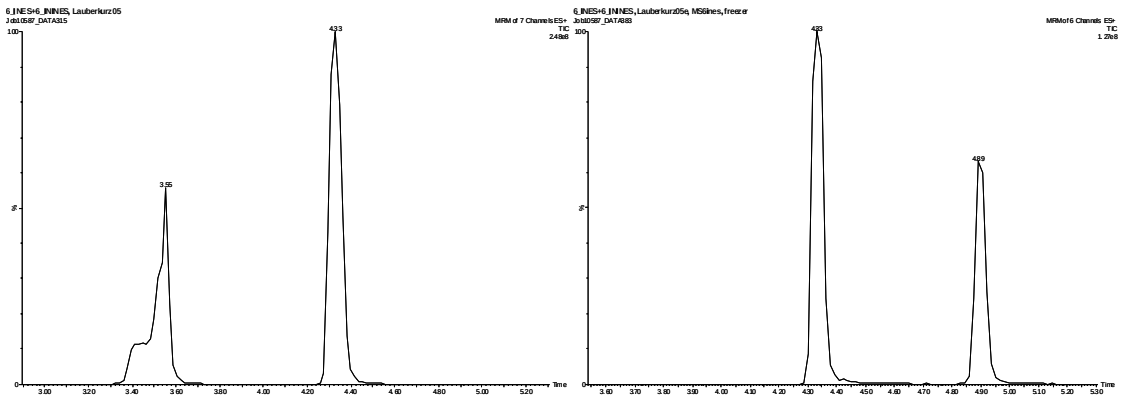
Objective: To find the HPLC conditions that will separate the six ergot alkaloid standards under investigation.

Due to possible protonation of the basic nitrogen-containing ergot alkaloids poor HPLC separation can be expected for these compounds. Therefore, 3mM ammonium carbonate buffer and acetonitrile were used as solvents to avoid protonation and to improve separation. HPLC-(ESI(+)-MS/MS analysis of ergot alkaloid standards was carried out on a Waters Alliance 2695 HPLC equipped with a Quattro Ultima and a Quattro Ultima Platinum tandem quadrupole instrument respectively, and a Phenomenex Gemini, C18 column, 2 mm x 150 mm x 5 µm particle size, including a 4 mm x 2mm phenylpropyl guard column. Both columns show good stability at high pH. Elution proceeded by means of a gradient with 0.5 mL/min flow rate using solvent C= ammonium carbonate (3.03 mM), D= acetonitrile as shown in Table 2.

Table 2 LC-gradient used for the separation of the selected ergot alkaloids

Time (min)	C (%)	D (%)	Flow (ml/min)	Rate
0.0	5.0	95.0	0.50	immediate
1.0	17.0	83.0	0.50	linear
2.0	47.0	53.0	0.50	linear
10.0	54.0	46.0	0.50	linear
15.0	80.0	20.0	0.50	linear
16.0	5.0	95.0	0.50	linear
21.0	5.0	95.0	0.50	immediate

The LC column temperature was 30°C. The autosampler temperature was kept at 15°C to minimise epimerisation. The injection volume was 10 µL. In order to avoid peak-fronting at the initial LC-conditions of 17% acetonitrile / 83% buffer when 10 µL acetonitrile were injected, the run was started with 5% acetonitrile for the first minute. The positive effect of this measure in separating the first eluting compound (ergometrine) from the second (ergometrine) is shown in Figure 3. The peak fronting was clearly eliminated and the abundance of the ergometrine peak increased considerably. With the optimized elution gradient the six most prevalent ergot alkaloids ergometrine, ergotamine, ergosine, ergocristine, ergocryptine and ergocornine (EFSA, 2005) and their corresponding epimers could be baseline separated within less than 15 min (Figure 4a).



a) b)

Figure 3 Effect of changing solvent in the first minute of the chromatographic run.
From 17% acetonitrile / 83% buffer (a) to 5% acetonitrile (b).

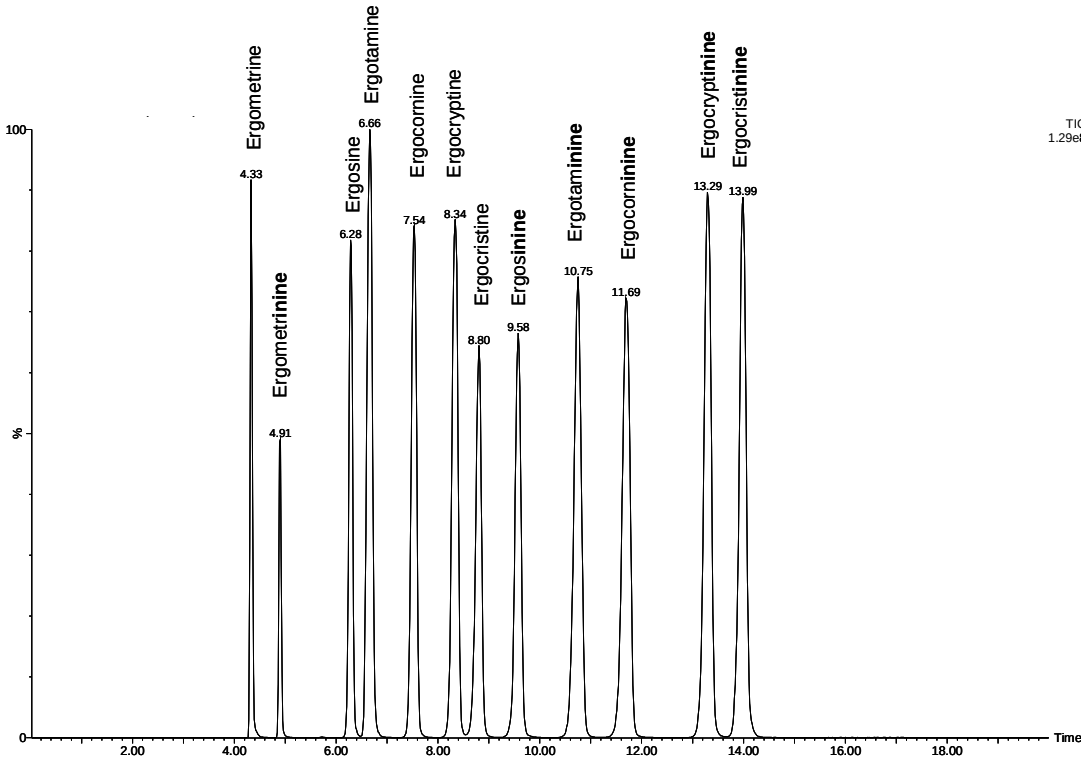


Figure 4a. Optimised ESI(+)LC-MS/MS TIC chromatogram of ergot alkaloids. Ergocristine (0.97 µg/mL), ergotamine (2.92 µg/mL), ergocornine (0.48 µg/mL), ergosine (0.49 µg/mL), ergocryptine (0.97 µg/mL), ergometrine (as ergometrine hydrogenmaleate) (0.49 µg/mL) and their corresponding six epimers ergocristinine (1.46 µg/mL), ergotaminine (1.46 µg/mL), ergocorninine (0.97 µg/mL), ergosinine (0.49 µg/mL) and ergocryptinine (0.97 µg/mL).

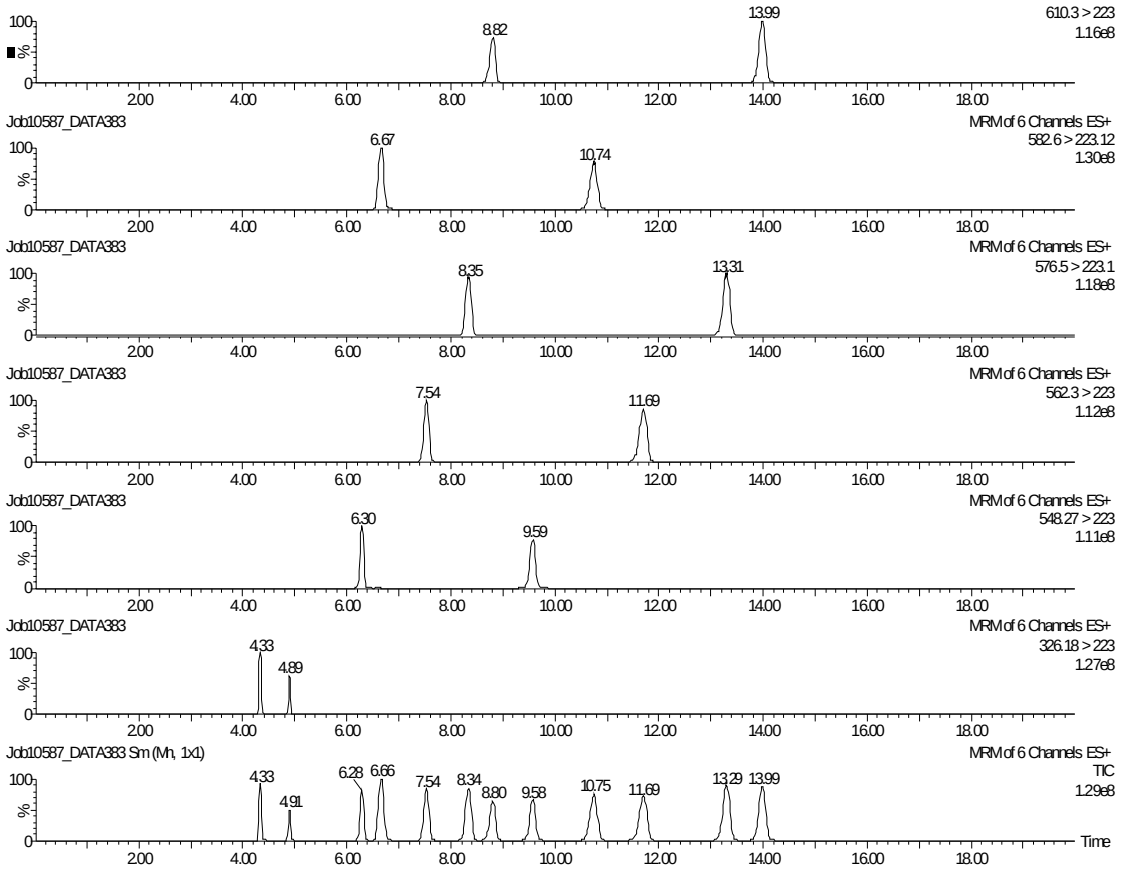


Figure 4b. Optimised SRM chromatogram of ergot alkaloids. Ergocristine (0.97 µg/mL), ergotamine (2.92 µg/mL), ergocornine (0.48 µg/mL), ergosine (0.49 µg/mL), ergocryptine (0.97 µg/mL), ergometrine (as ergoemetrine hydrogenmaleate) (0.49 µg/mL) and their corresponding six epimers ergocristinine (1.46 µg/mL), ergotaminine (1.46 µg/mL), ergocorninine (0.97 µg/mL), ergosinine (0.49 µg/mL) and ergocryptinine (0.97 µg/mL).

Figure 4 Optimised ESI(+)LC-MS/MS TIC and SRM chromatogram of ergot alkaloids

3.3. Optimisation of HPLC-MS/MS Parameters

Objective: To obtain a suitable quantitative method of analysis for the ergot alkaloid standards using HPLC-MS/MS with an LOQ of 1 µg/kg or better for each of the individual ergot alkaloids.

3.3.1. Instrumentation

Analyses were performed on a Micromass (Waters, UK) Quattro Ultima and a Quattro Ultima Platinum tandem quadrupole instrument, respectively. MS/MS detection was realised in positive electrospray ionisation using selected reaction monitoring (SRM) acquisition mode. Nitrogen was used as nebulizer and collision gas. Resultant data were smoothed with Micromass MassLynx version 4.0 software.

Typical ESI-MS instrument parameters for the detection of the ergot alkaloids are shown in Tables 3 and 4.

Table 3 MS Instrument Parameters:

Parameter	Value
Polarity	ES+
Capillary (kV)	3.00
Cone (V)	35
RF Lens 1	20.0
Aperture (V)	0.0
RF Lens 2	0.2
Source Temperature (°C)	120
Desolvation Temperature (°C)	350
Cone Gas Flow (L/Hr)	98
Desolvation Gas Flow (L/Hr)	704
LM 1 Resolution	13.5
HM 1 Resolution	13.5
Ion Energy 1	0.5
Entrance	0
Collision (ev) -see Table 5	55
Exit	0
LM 2 Resolution	15.0
HM 2 Resolution	15.0
Ion Energy 2	0.5
Multiplier (V)	650
Collision Cell Pressure(mbar)	1.08e-3

Table 4 SRM-Settings:

Parameter	Value
Scans in function:	1322
Cycle time (secs):	0.900
Inter Scan Delay (secs):	0.05
Retention window (mins):	0.000 to 20.000
Ionization mode:	ES+
Data type:	SIR or SRM data
Function type:	SRM of 6 channels

3.3.2. Optimisation of the Selected Reaction Monitoring (SRM) parameters

Each toxin's mass spectrometric selected reaction monitoring (SRM) parameters (mode, collision energy and cone voltage) were optimized using syringe pump infusion. Ergot

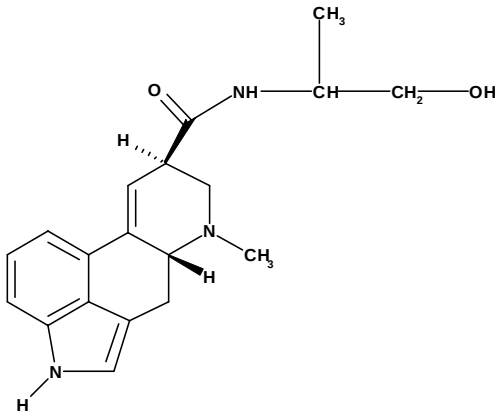
alkaloid calibrants (0.1 µg/mL) were prepared for direct infusion ESI (+) MS analysis by dilution 1:100 of 10 µg/mL stock solution with ammonium carbonate buffer : acetonitrile, 1:1 and infused with a syringe pump (Harvard apparatus, pump 11). In positive mode, [M+H]+ ions yielded the strongest signal compared to [M+NH4]+ and [M+Na]+. The negative mode did not result in useful signal intensities.

In the following sections the six ergot alkaloids and their corresponding epimers, their accurate mass, molecular formula and structure together with the individually optimised SRM detection parameters are discussed.

The individual MS spectra obtained from product ion scans performed at different collision energies are given in Annex 1.

3.3.2.1. Ergometrine and Ergometrinine

Accurate mass: 325.1790



Molecular formula: $C_{19}H_{23}N_3O_2$

Figure 5 Structure of ergometrine

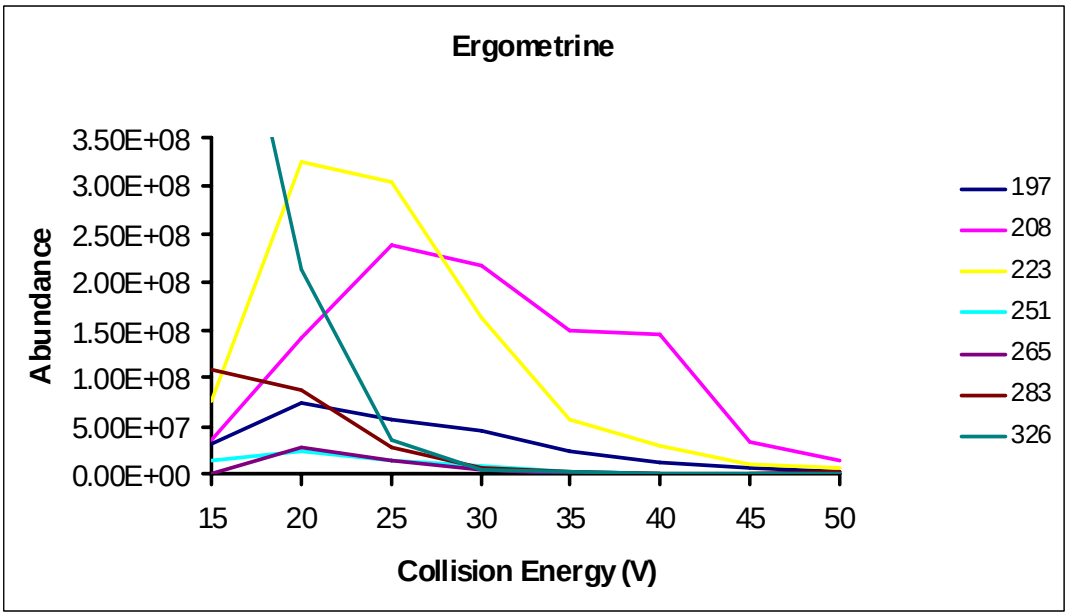


Figure 6 Abundance of ergometrine product ions with varying collision energies.
Order of most abundant transitions *m/z* 326 $(M+H)^+$ => 208; 326 => 223; 326 => 197; 326 => 265.

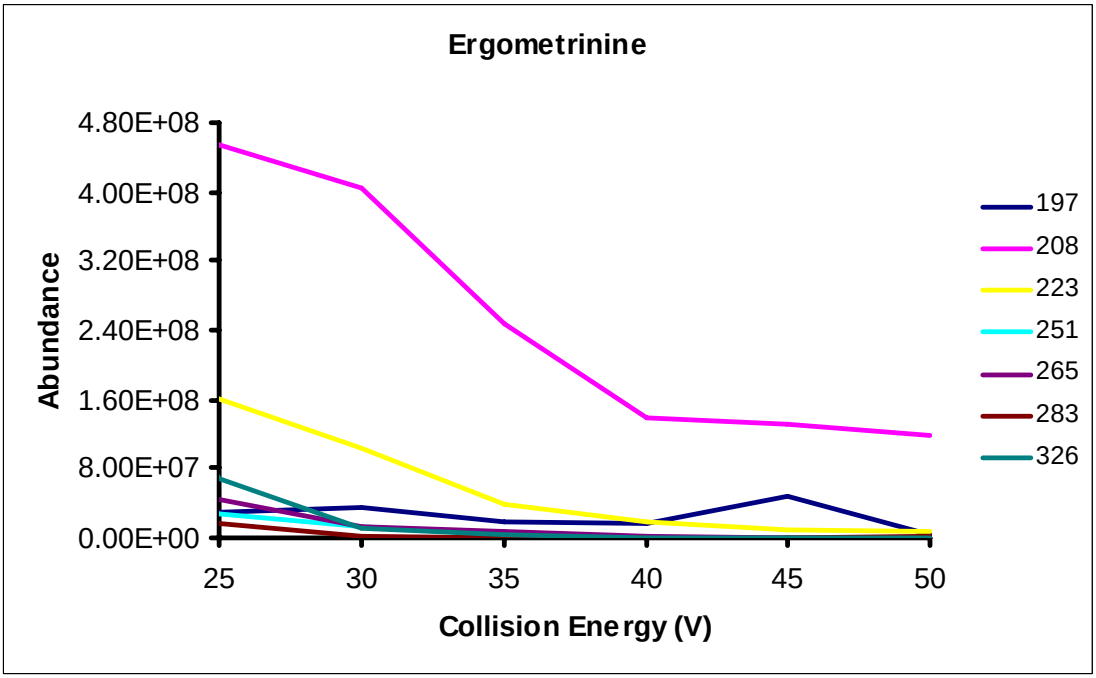


Figure 7 Abundance of ergometrine product ions with varying collision energies.
The order of most abundant transitions is *m/z* 326 $(M+H)^+$ => 223; 326 => 208; 326 => 265; 326 => 197

3.3.2.2. ***Ergosine*** *and* ***Ergosinine***

Accurate mass: 547.2795

Molecular formula: $C_{30}H_{37}N_5O_5$

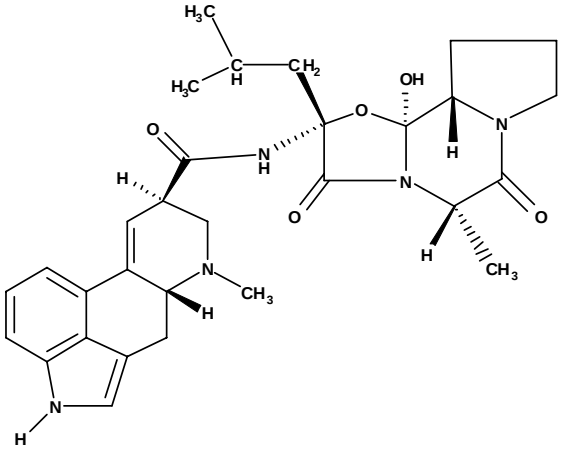


Figure 8 Structure of ergosine

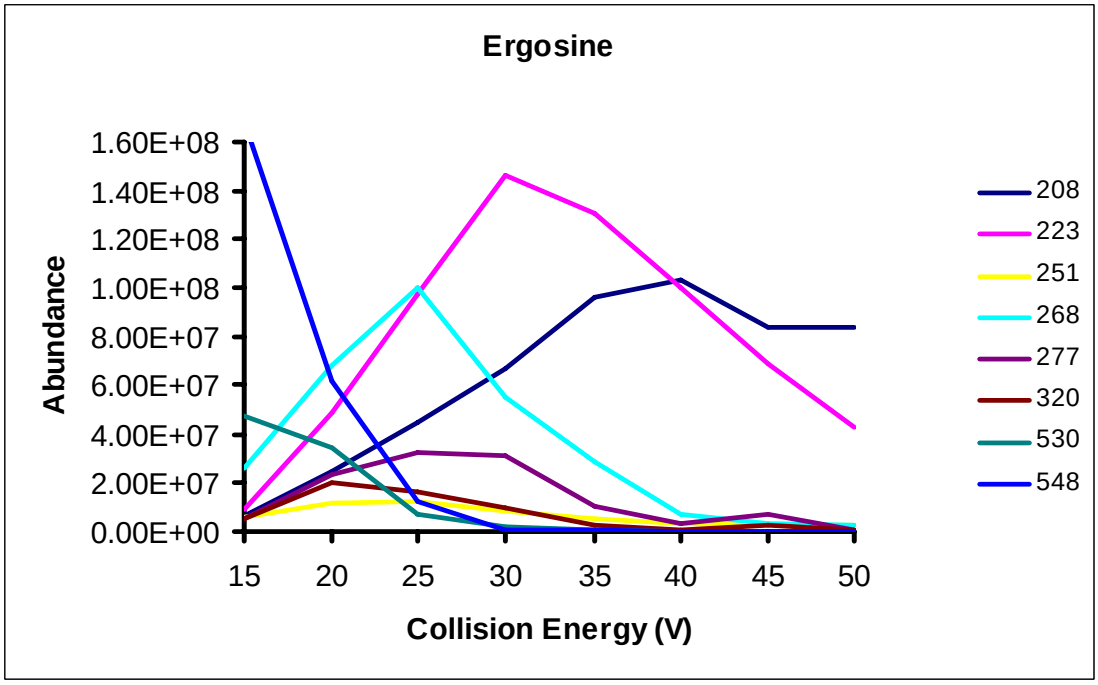


Figure 9 Abundance of ergosine product ions with varying collision energies.

Order of most abundant transitions *m/z* 548 $(M+H)^+$=> 223; 548 => 208; 548 => 268; 548 => 530

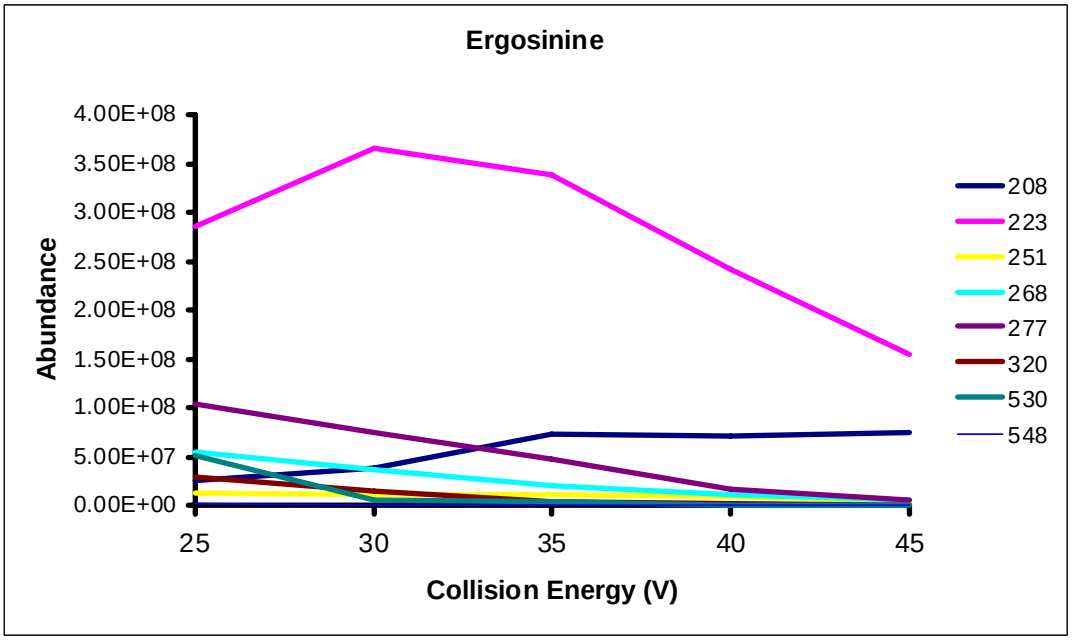
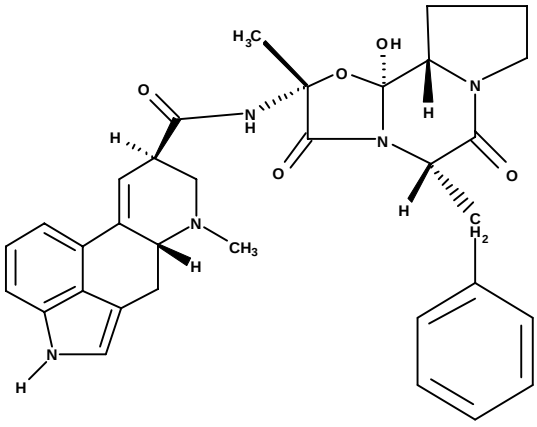


Figure 10 Abundance of ergosinine product ions with varying collision energies.

Order of most abundant transitions *m/z* 548 $(M+H)^+$=> 223; 548 => 277; 548 => 208; 548 => 268

3.3.2.3. ***Ergotamine*** *and* ***Ergotaminine***

Accurate mass: 581.2638



Molecular formula: $C_{33}H_{35}N_5O_5$

Figure 11 Structure of ergotamine

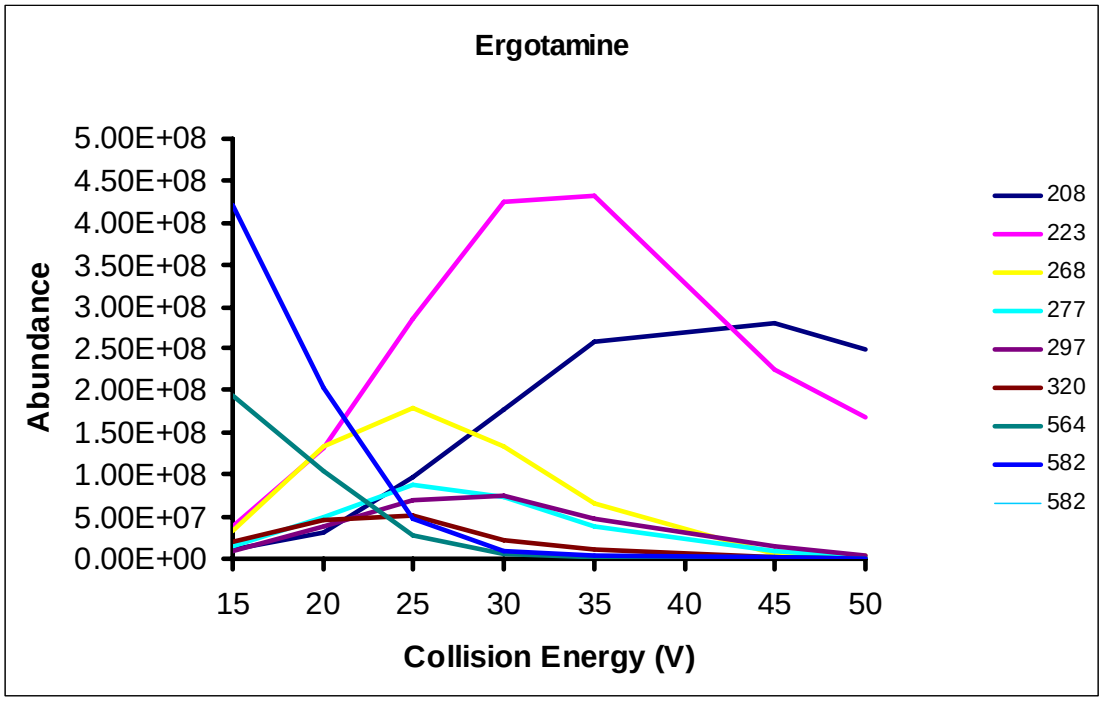


Figure 12 Abundance of ergotamine product ions with varying collision energies.

Order of most abundant transitions: *m/z* 582 $(M+H)^+$ => 223; 582 =>208; 582 => 268; 582 => 277.

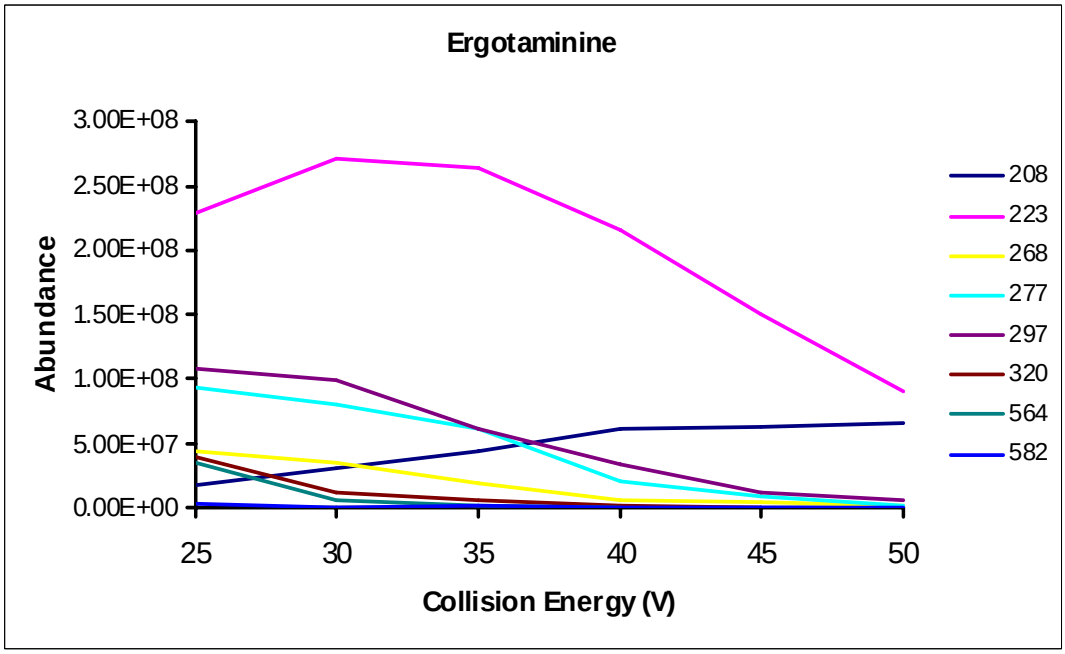


Figure 13 Abundance of ergotaminine product ions with varying collision energies.

Order of most abundant transitions *m/z* 582 => 223; 582 =>297; 582 => 277; 582 => 208

3.3.2.4. ***Ergocornine*** *and* ***Ergocorninine***

Accurate mass: 561.2951

Molecular formula: $C_{31}H_{39}N_5O_5$

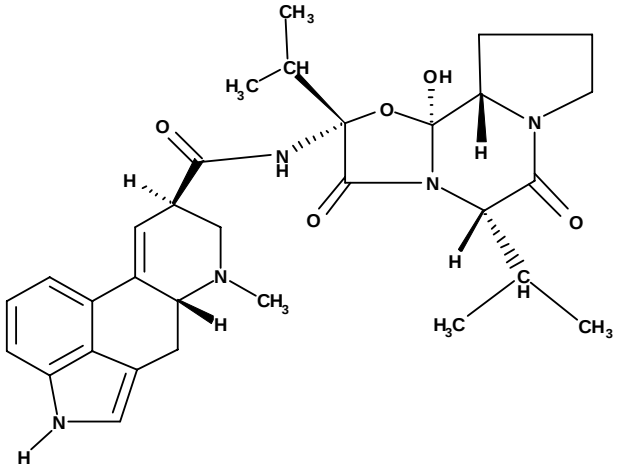


Figure 14 Structure of ergocornine

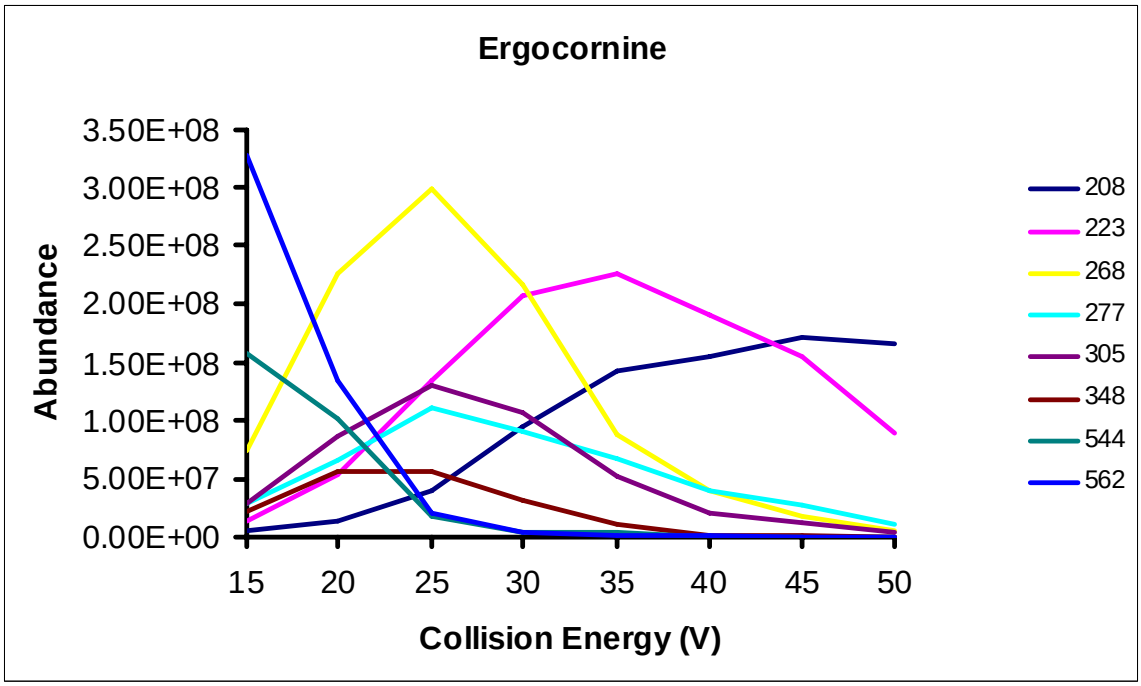


Figure 15 Abundance of Ergocornine product ions with varying collision energies.

Order of most abundant transitions *m/z* 562 $(M+H)^+$=> 268; 562 => 223; 562 => 208; 562 => 305.

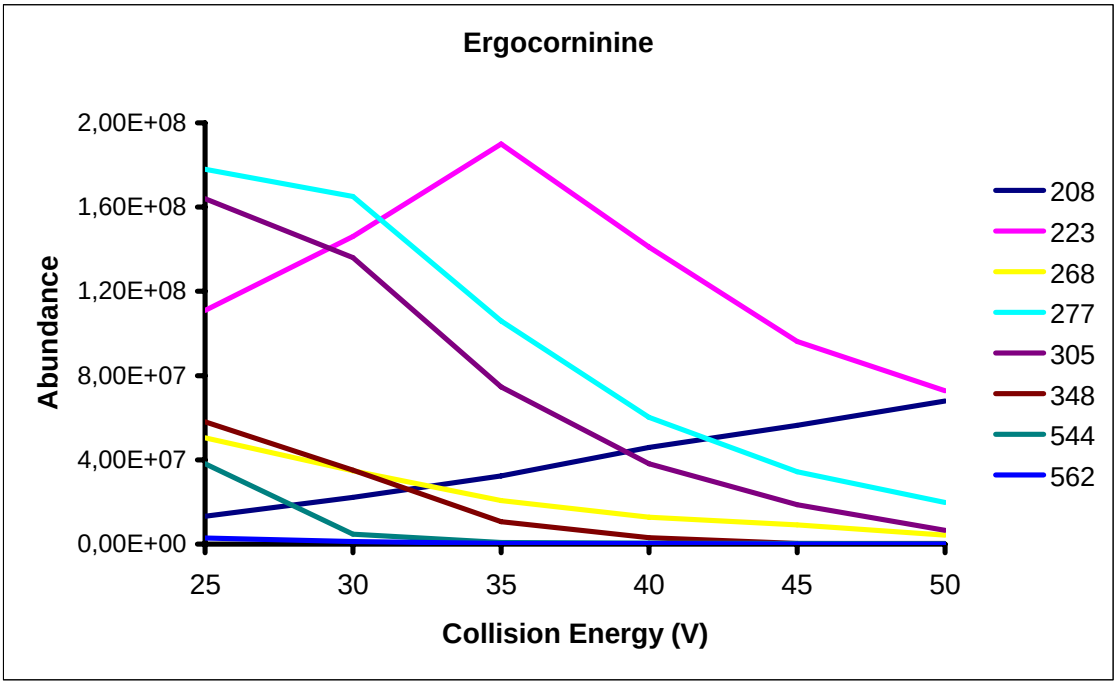
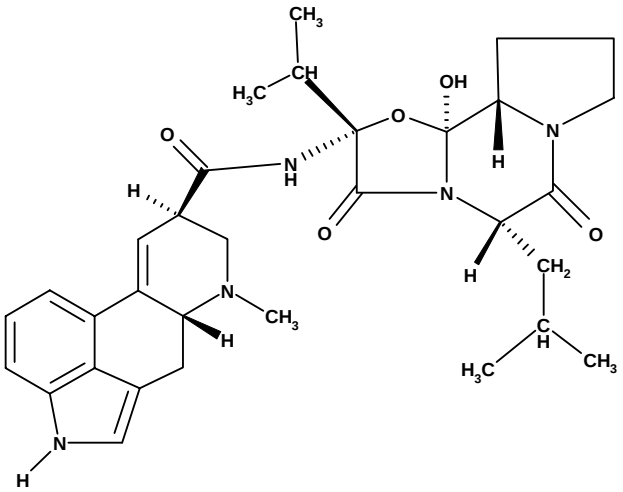


Figure 16 Abundance of ergocorninine product ions with varying collision energies. Order of most abundant transitions *m/z* 562 $(M+H)^+$=> 223; 562 => 277; 562 => 305; 562 => 208

3.3.2.5. ***Ergocryptine*** *and* ***Ergocryptinine***

Accurate mass: 575.3108



Molecular formula: $C_{32}H_{41}N_5O_5$

Figure 17 Structure of Ergocryptine

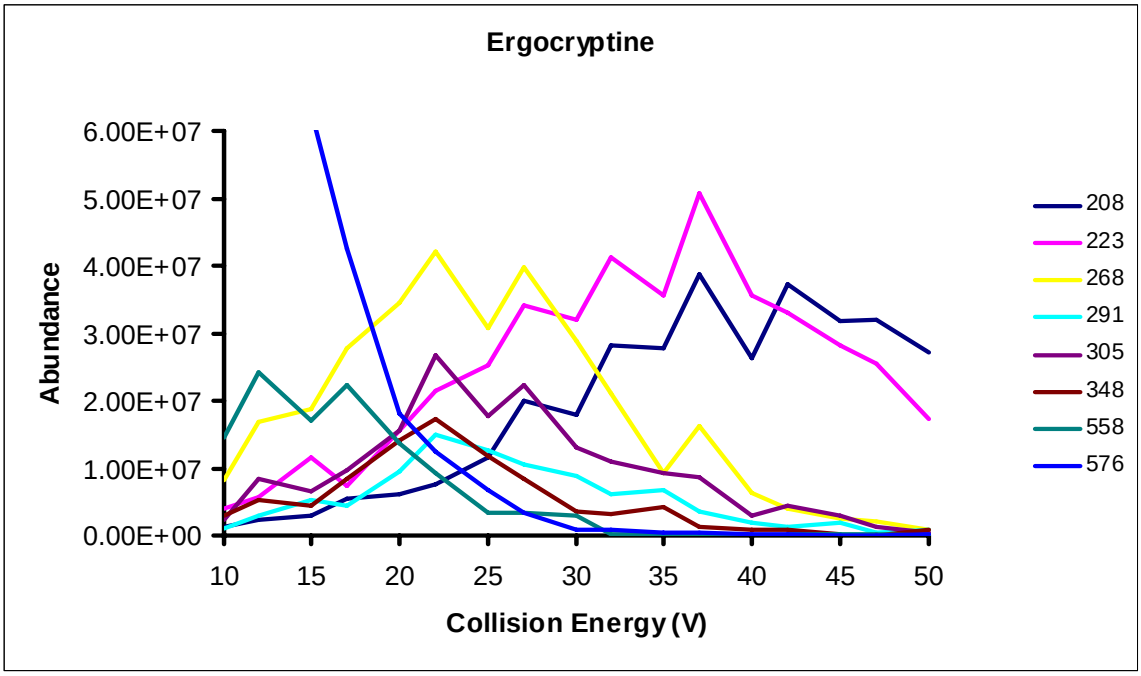


Figure 18 Abundance of ergocryptine product ions with varying collision energies.

Order of most abundant transitions: *m/z* 576 $(M+H)^+$ => 223; 576 =>268; 576 => 208; 576 => 305.

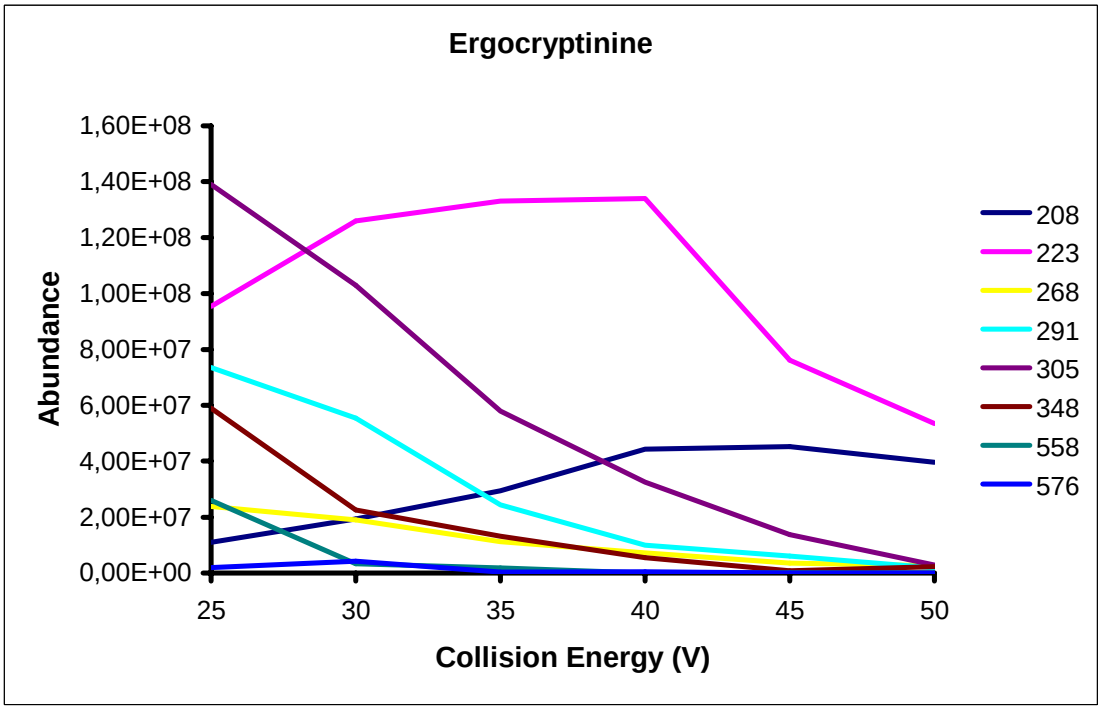


Figure 19 Abundance of ergocryptinine product ions with varying collision energies.

Order of most abundant transitions m/z 576 => 223; 576 =>291; 576 => 208; 576 => 305

3.3.2.6. ***Ergocristine*** *and* ***Ergocristinine***

Accurate mass: 609.2951

Molecular formula: $C_{35}H_{39}N_5O_5$

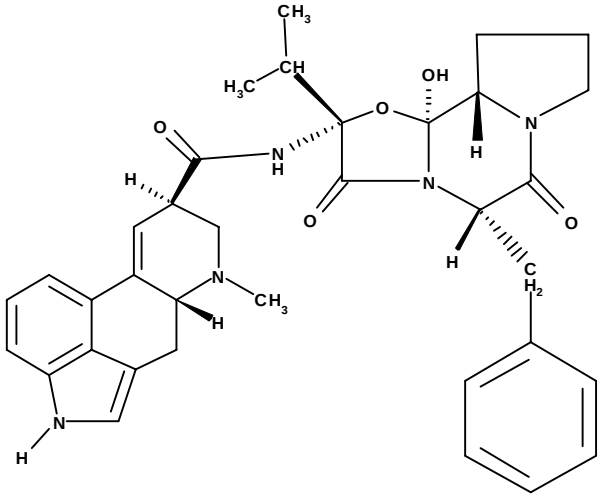


Figure 20 Structure of ergocristine

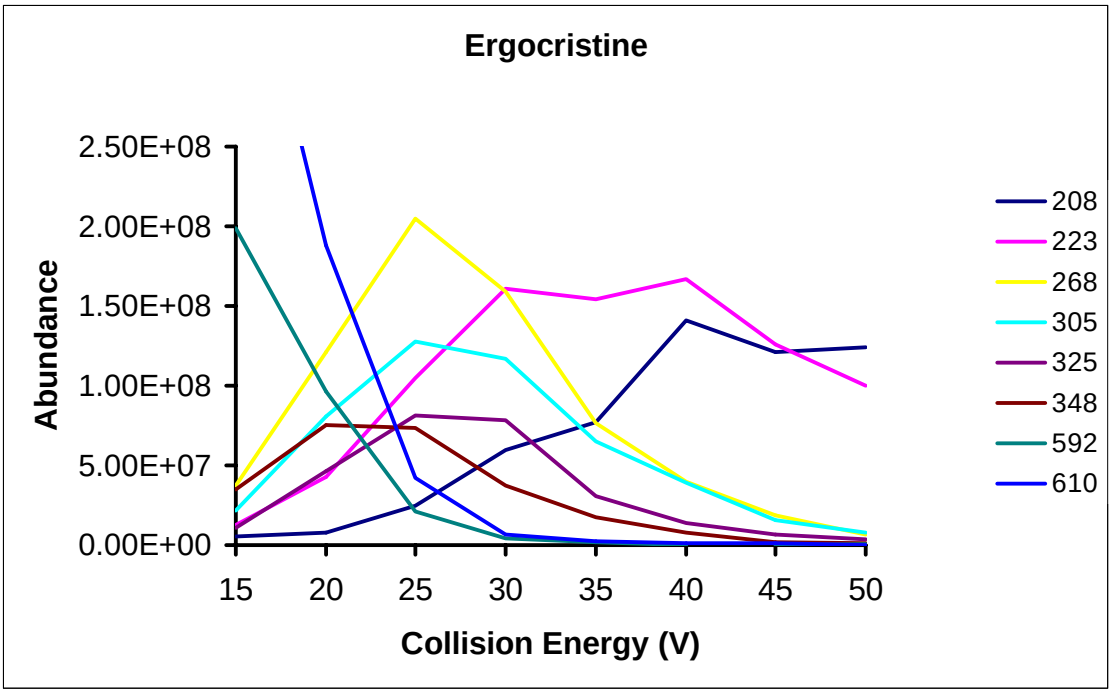


Figure 21 Abundance of ergocristine product ions with varying collision energies.

Order of most abundant transitions *m/z* 610 $(M+H)^+$=> 268; 610 => 223; 610 => 208; 610 => 305

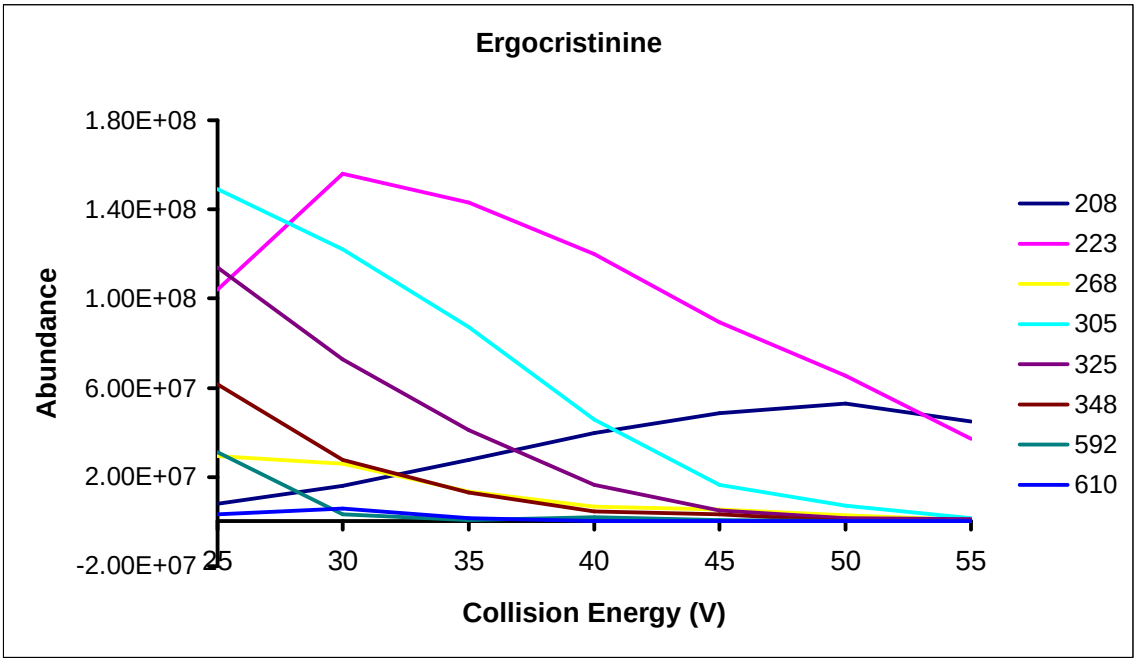


Figure 22 Abundance of ergocristinine product ions with varying collision energies.
Order of most abundant transitions *m/z* 610 $(M+H)^+$=> 223; 610 => 305; 610 => 325; 610 => 348

3.3.3. Discussion of results

As shown in Figures 6 to 22 the most abundant SRM transition of the ergot alkaloids investigated is that to the product ion *m/z* 223. Cleavages involved in the release of the m/z 223 fragment and its demethylated counterpart at m/z 208 have been studied by Lehner *et al*. (2004). Exceptions were ergocornine and ergocristine with the most abundant transition to *m/z* 268, however, with a smaller range of maximum abundance. For ergometrinine the most abundant transition was to *m/z* 208.

The figures also show that the fragmentation behaviours of the -*ines* and –*inines* can differ significantly. Fragmentation mainly occurs as a result of the loss of the peptide side-chain and its associated NH_2-HC=O linkage as described by Lehner *et al*. (2004). SRM detection was carried out in a two-stage process with time windows from 2.0 to 5.5 minutes monitoring ergometrine and ergometrinine, and from 5.2 to 16.0 minutes monitoring the remaining alkaloids. As a compromise and in favour of the more important –*ines*, the transitions and optimised collision energies given in Table 5 were employed.

The optimised MS-parameters found for the –*ines* were hence also employed for detection of the –*inines.* For all 12 ergot alkaloids tested the transitions from the protonated precursor ion $(M+H^+)$ to *m/z* 223 was employed as quantifier ion. The transitions to *m/z*

208 served as qualifier ion for ergometrine, ergosine, ergocornine and ergotamine, the transition to *m/z* 268 as qualified for ergocryptine and ergocristine.

Table 5 Collected transitions with optimised conditions.

Ergot alkaloid	Time window *	Precursor ion (m/z) $(M+H)^+$	Product ion (m/z)	Dwell (s)	Cone (V)	Collision (eV)	Dwell (s)
Ergometrine and ergometrinine	1	326.18	223.00	0.10	35.0	15.0	0.05
	1	326.18	208.00	0.10	35.0	20.0	0.05
Ergosine and Ergosinine	2	548.27	223.00	0.05	40.0	25.0	0.05
	2	548.27	208.00	0.05	40.0	35.0	0.05
Ergocornine and Ergocorninine	2	562.30	223.00	0.05	35.0	30.0	0.05
	2	562.30	208.00	0.05	35.0	40.0	0.05
Ergotamine and Ergotaminine	2	582.60	223.00	0.05	45.0	30.0	0.05
	2	582.60	208.00	0.05	45.0	40.0	0.05
Ergocryptine and Ergocryptinine	2	576.50	223.00	0.05	35.0	32.0	0.05
	2	576.50	268.00	0.05	35.0	18.0	0.05
Ergocristine and Ergocristinine	2	610.30	223.00	0.05	40.0	35.0	0.05
	2	610.30	268.00	0.05	40.0	20.0	0.05

*- Time window function 1 is from 2.00 to 5.50 minutes, and function 2 is from 5.2 to 16.00 minutes.

3.3.3.1. Limit of detection in solvent

The limit of detection (LOD) of each analyte in acetonitrile using the Quattro Ultima Platinum instrument was estimated from the signal-to-noise-ratio (S/N) obtained when measuring concentration levels ranging from 0.024 ng/mL (ergometrinine) to 0.292 ng/mL (ergotamine) (see Table 6) and subsequent calculation of concentration (=LOD) at a S/N of 3. An example is given in Figure 23, which shows the detection of ergocristine at a concentration of 0.097 ng/mL with a S/N of 12.65. According to this calculation, LODs ranging from 0.007 ng/mL (ergosine) to 0.043 ng/mL (ergometrinine) were obtained. Based on the initial assumption of a 5 g grain sample containing 1 µg/kg alkaloids extracted with 30 mL solvent and 2 mL further purified and obtained from a clean-up column this corresponds to a concentration of 0.167 ng/mL in the solution finally injected. According to Table 6 the LODs of the ergot alkaloids investigated was well below a

concentration of 0.167 ng/mL. As a consequence it was concluded that also an LOQ of 1 µg/kg should be achievable when analysing real world samples.

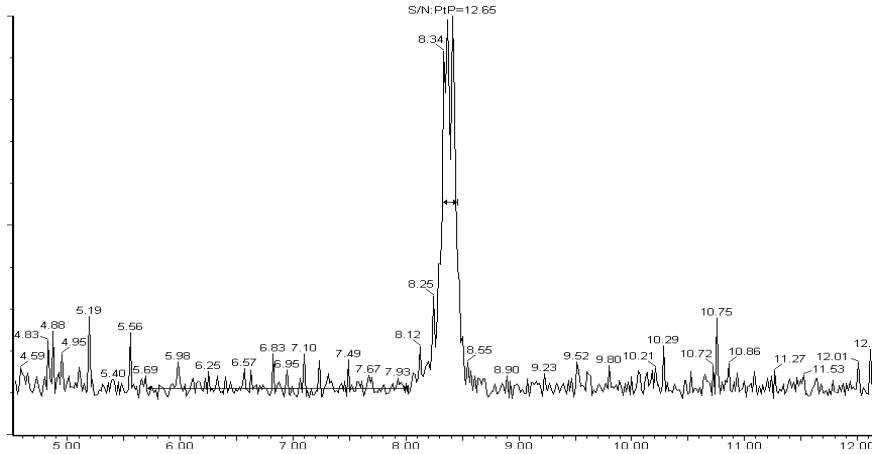


Figure 23 SRM of Ergocristine (610 => 223) at 0.097 ng/mL in acetonitrile (S/N = 12.65)

Table 6 LODs for ergot alkaloids analysed using the Quattro Ultima Platinum

Compound	SRM transition	S/N	Concentration measured (ng/mL)	Estimated LOD (ng/ml solution / µg/kg in grain)
Ergometrine	326 => 223	5.75	0.049	0.025 / 0.125
Ergometrinine	326 => 223	1.67	0.024	0.043 / 0.215
Ergosine	548 => 223	19.74	0.049	0.007 / 0.035
Ergotamine	582 => 223	28.72	0.292	0.031 / 0.155
Ergocornine	562 => 223	11.65	0.097	0.025 / 0.125
Ergocryptine	576 => 223	15.75	0.097	0.018 / 0.09
Ergocristine	610 => 223	12.65	0.097	0.022 / 0.09
Ergosinine	548 => 223	17.71	0.049	0.008 / 0.04
Ergotaminine	582 => 223	17.6	0.146	0.025 / 0.125
Ergocorninine	562 => 223	10.86	0.048	0.013 / 0.065
Ergocryptinine	576 => 223	21.34	0.097	0.014 / 0.07
Ergocristinine	610 => 223	21.99	0.146	0.020 / 0.1

Figure 24 shows the chromatograms for all 12 ergot alkaloids studied at low concentrations (0.024 ng/mL (ergometrinine) to 0.292 ng/mL (ergotamine)).

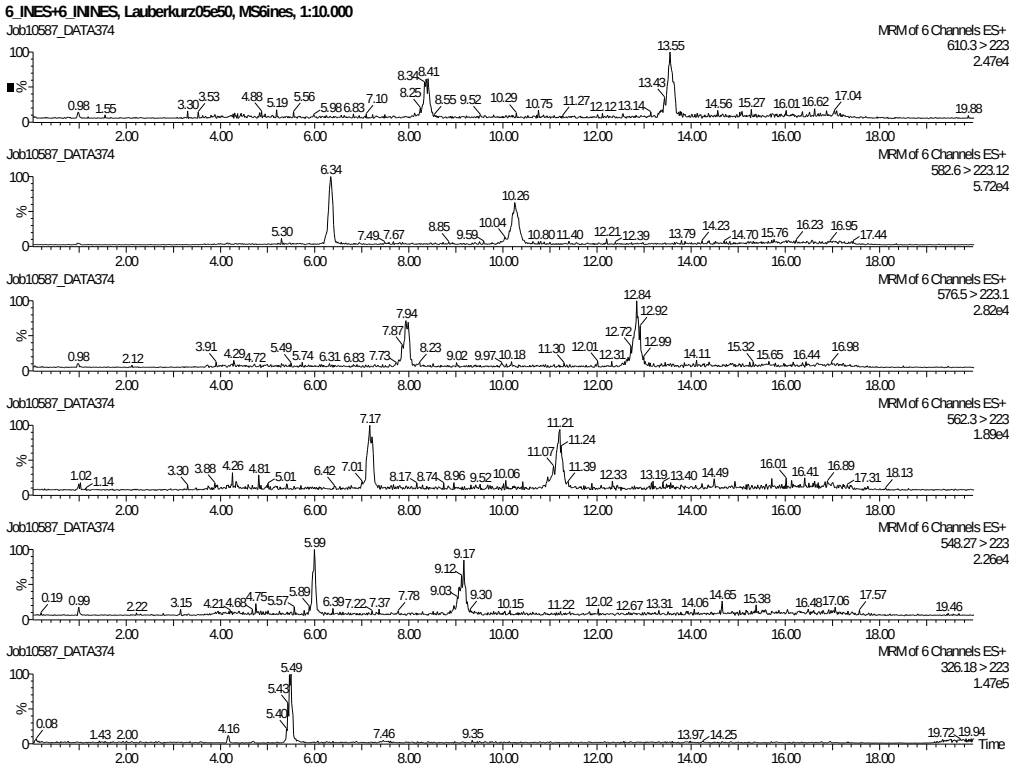
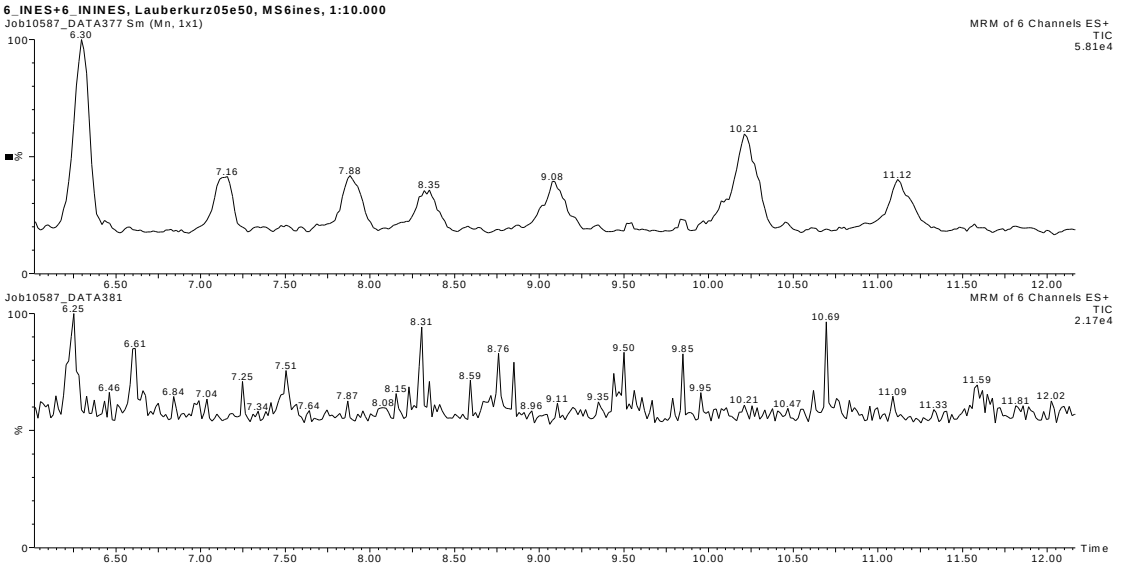


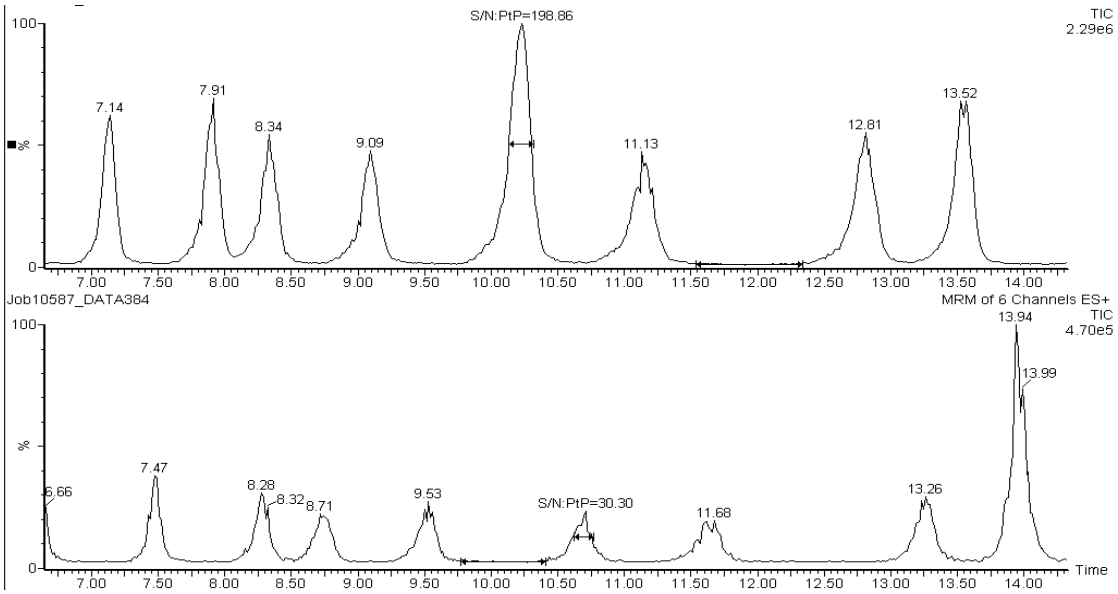
Figure 24 SRM chromatograms of 12 ergot alkaloids at 0.024 to 0.292 ng/ml.
For concentrations see Table 6.

3.3.3.2. Quattro Ultima vs Quattro Ultima Platinum: Comparison of sensitivity

Based on comparison measurements using the Quattro Ultima *Platinum* and the Quattro Ultima instrument respectively, a sensitivity improvement of a factor 6-7 can be expected when measuring samples with the more sophisticated *Platinum* instrument. This factor can be calculated from Figure 25b, where a S/N-ratio of 199 was obtained with the Platinum compared to a ratio of 30 with Quattro Ultima. Still, based on the figures given in Table 6, an LOD of 1 µg/kg ergot alkaloid in grain, corresponding to a concentration of 0.2 ng/mL in the standard solution should also be achievable with the latter instrument, Only for ergometrinine might the LOD be slightly higher.



a) SRM TIC of eight ergot alkaloids at concentration given in Table 6 * from 0.024 ng/mL (ergometrinine) to 0.292 ng/mL (ergotamine) obtained with the Quattro Ultima Platinum (upper trace) and the Quattro Ultima (lower trace).



b) SRM TIC of eight ergot alkaloids at levels 100 times higher than given in Table 6. Obtained with the Quattro Ultima Platinum (upper trace) and the Quattro Ultima (lower trace).

Figure 25 a and b: Comparison of sensitivities of Quattro Ultima and Ultima Platinum.

3.3.4. Conclusions

Optimised LC and MS parameters have been obtained for the determination of six ergot alkaloids and their corresponding epimers. These included: a) the optimum LC-gradient which enabled chromatographic baseline separation of the 12 compounds within 14 min with a total LC-run time of 21 min, which is about half the time required in previously published LC methods for these ergot alkaloids (Lauber *et al*., 2005), b) selection of most appropriate SRM transitions, c) optimised cone-voltages and collision-energies for the selected transitions. The optimised conditions enabled the detection of the ergot alkaloids down to 0.007 ng/mL (ergosine) and 0.043 ng/mL (ergometrinine) respectively. Based on the measurements of the ergot alkaloids in pure acetonitrile, the corresponding limits of quantification (LOQs) for the ergot alkaloids investigated in grain would be below the required level of 1 µg/kg.

3.4. Determination of purity of calibrants

Specific Objective: To assess the stability over time of ergot alkaloids in solution and in both raw cereal and cereal based processed food extracts. The purity of the crystalline ergot alkaloids was assessed and later considered for the calculation of the measurement uncertainties (see 3.8.).

3.4.1. Purity of the crystalline ergot alkaloids

The purity of the standards obtained from Prof. M. Flieger (see 3.1.) was investigated in cooperation with the Technical University of Denmark, Biocentrum, Center for Microbial Biotechnology (Prof. Kristian F. Nielsen) and the IFA-Tulln (Dr. Franz Berthiller) using LC-DAD-ESI-TOF-MS (HPLC-UV-MS).

About 1mg of ergocornine (0.802mg), ergocristine (0.580mg), ergocryptine (1.228mg), ergometrine (as hydrogenmaleate, 1.432mg), ergosine (0.912mg) and ergotamine (1.672mg) were carefully weighted into 8ml glass vials using a microbalance. 1ml pure acetonitrile was added to the standards and vortexed vigorously for 1min. 30µl of the solutions were transferred into HPLC microvials already containing 270µl acetonitrile to produce solutions of approx. 100µg/ml.

Isomeric forms of the higher ergots (ergocorninine, ergocristinine, ergocryptinine, ergometrinine, ergosinine and ergotaminine) and 18 other ergots alkaloids or derivatives (agroclavine, chanoclavine, dihydroergine, dihydroergosine methylsulfonate, dihydroergotamine, dihydrolysergic acid, dihydrolysergol, elymoclavine, elymoclavine monofructoside, ergine, erginine, festuclavine, iso-dihydrolysergol, lysergic acid, lysergol,

methysergide, ox-elymoclavine, ox-luol) were also obtained from Prof. M. Flieger as qualitative reference standards in acetonitrile.

The samples were measured with an HPLC-DAD (Agilent 1100) connected to a Micromass LCT-TOF-MS. Separation was achieved using a Phenomenex Gemini C6-Phenyl column (50 x 2mm, 3µm particle size) at 40°C. An aqueous solution of 10mM ammonium formate and 20mM formic acid was used as eluent A, acetonitrile containing 20mM formic acid was used as eluent B. The linear gradient started at 5%B and reached 70%B after 18min. Afterwards, the column was washed with 100%B till 25min and re-equilibrated with 5%B till the end of the run at 36min. 3µl of the ergot solutions in acetonitrile were injected into a flow of 300µl/min. DAD spectra were acquired from 200nm to 700nm with a range interval of 2nm. Electrospray positive spectra were acquired in the *m/z* range of 100 to 900. Other MS parameters were as follows: capillary 3000V, sample cone 15V, RF lens 200V, extraction cone 5V, desolvation temperature 450°C, source temperature 120°C, acceleration 200V, MCP detector 2800V, pusher frequency 20000, ion energy 42V, tube lens 3V, TOF flight tube 4600V, reflectron 1780V.

Retention times and high-resolution molecular masses were used as identification points for impurities and the standards. Antibase 2005, a fungal compounds database from Wiley with about 30,000 entries, was searched for other *Claviceps* metabolites in case unknown compounds were present, with a search window of 0.02amu. Peaks were integrated using the UV trace only to estimate the concentration of the impurities, assuming similar UV absorbance.

3.4.2. Results of purity measurements

Predominately $[M+H]^+$ ions were formed from the ergots and high-resolution mass spectrometry additionally proved the identity of the compounds. Masses measured fitted the theoretical masses of the ergots extremely well (within 7 ppm). For clarity, in the following paragraphs the standards are written in bold and the impurities are underlined.

Ergocristine (impurity at 10.56 min, 610.3082 amu, 485 mAU·min = 0.2% ergocristinine), **ergocryptine** (impurity at 10.06 min, 576.3200 amu, 1540 mAU·min = 0.4% ergocryptinine) and **ergometrine** (impurity at 4.53 min, 326.1876 amu, 1080 mAU·min = 0.2% ergometrinine) were very pure standards (>99.6%) and just showed minor impurities corresponding to the mass and retention time of their respective isomers in the chromatograms. While it cannot be ruled out that the isomers were formed in solution

rather then being impurities in the solid standards, this seems unlikely as in the other three measured ergots (ergocornine, ergosine and ergotamine) no isomers were found.

In the **ergocornine** standard both 0.8% ergocryptine (impurity at 9.64 min, 576.3128 amu, 2230 mAU·min) as well as 0.6% ergocryptinine (impurity at 9.88 min, 576.3183 amu, 1440 mAU·min) were found, resulting in a total purity estimate of about 98.6%.

The **ergosine** standard showed one of the highest amounts of impurities, also containing 2.7% ergotamine (impurity at 9.00 min, 582.2724 amu, 8480 mAU·min) and 0.9% ergocryptine (impurity at 9.58 min, 576.3191 amu, 3020 mAU·min), resulting in just 96.4% purity. A chromatogram of the ergosine standard, also showing the mass spectra of the major peaks is shown in Figure 26.

The **ergotamine** standard contains two more polar impurities at 7.90 min (598.2698 amu, 1140 mAU·min = 0.2%) and at 8.80 min (598.2642 amu, 3070 mAU·min = 0.6%). While none of the measured 30 ergots show matching mass, a search in Antibase 2005 resulted in both 8α-hydroxyergotamine and 8β-hydroxyergotamine as very likely hits. The total purity was estimated at 99.1%.

Ergocorninine was contaminated with 0.5% ergocryptinine (impurity at 10.08 min, 576.3191 amu, 8860 mAU·min) and with 0.1% ergocryptine (impurity at 9.94 min, 576.3242 amu, 1100 mAU·min), it therefore had a purity of 99.5%.

The **ergocristinine** standard was the most contaminated of all of the ergots analysed with an estimated purity of just 94.2%. Impurities comprised 1.7% ergocristine (impurity at 9.76 min, 610.3031 amu, 50900 mAU·min), 0.8% ergocorninine (impurity at 9.46 min, 562.3026 amu, 24100 mAU·min) and 3.3% ergocryptinine (impurity at 9.94 min, 576.3161 amu, 97000 mAU·min).

The **ergocryptinine** standard also contained 0.1% ergocryptine (9.42 min, 576.3191 amu, 1410 mAU·min) and a less polar compound 14 amu heavier than ergocryptinine, which is characteristic for methylation (impurity at 10.66 min, 590.3300 amu, 4520 mAU·min = 0.4%).

According to Antibase 2005, the most likely identities are O-12'-Methyl-α-ergocryptine, ergogaline or ergoheptine. The purity of the ergocryptinine standard was therefore estimated as 99.4%.

Ergometrinine was extremely pure (99.9%), the sole less polar impurity being at 5.74 min (340.1993 amu, 419 mAU·min = 0.1%) was not described in Antibase 2005 and thus

was potentially a novel ergot alkaloid. The mass shift of 14 amu hints at a methylation of ergometrine or ergometrinine. The substitution of the sole methyl group with an ethyl group would also yield the same molecular mass and this substitution is known from other ergots. MS/MS experiments could be performed to elucidate the structure of this minor impurity, but the very low amount of the substance in the standard might render these tests difficult.

In the **ergosinine** standard both 2.0% ergosine (impurity at 8.50 min, 548.2866 amu, 18700 mAU·min) and 2.5% ergotaminine (impurity at 8.74 min, 582.2731 amu, 23100 mAU·min) were found, giving a purity of 95.5% for the standard.

Ergotaminine (99.7% purity) showed a single minor impurity at 9.42 min (596.2913 amu, 4030 mAU·min = 0.3%). According to the Antibase database the impurity could be ergostine, ergostinine or the MW595 ethyl ergoxin group substituent described by Lehner et al. (2005).

An overview of the results obtained from the characterisation of the ergot standards is given in Table 7. This shows the formulae, monoisotopic masses (amu) for the neutral compounds, retention times (min), UV areas (mAU·min), measured *m/z* values of the protonated compounds (amu), calculated mass errors (ppm) and total purity (assuming similar UV absorbance of impurities, including isomeric forms).

Table 7 Characteristics of the neutral and protonated ergot standards.

Standard	**Formula**	**mass (amu) (neutral molecule)**	**Ret time (min)**	**UV area**	***m/z***	**Error (ppm)**	**Purity (UV, %)**
ergometrine	$C_{19}H_{23}N_3O_2$	325.1790	3.95	5.15E+05	326.1846	-6.9	99.8
ergometrinine	$C_{19}H_{23}N_3O_2$	325.1790	4.91	6.85E+05	326.1866	-0.8	99.9
ergosine	$C_{30}H_{37}N_5O_5$	547.2795	8.62	3.06E+05	548.2892	3.5	96.4
ergosinine	$C_{30}H_{37}N_5O_5$	547.2795	8.26	8.82E+05	548.2866	-1.2	95.5
ergocornine	$C_{31}H_{39}N_5O_5$	561.2951	9.06	2.60E+05	562.3011	-3.2	98.6
ergocorninine	$C_{31}H_{39}N_5O_5$	561.2951	9.42	1.91E+06	562.3015	-2.5	99.5
ergocryptine	$C_{32}H_{41}N_5O_5$	575.3108	9.58	4.18E+05	576.3172	-2.4	99.6
ergocryptinine	$C_{32}H_{41}N_5O_5$	575.3108	9.90	1.02E+06	576.3197	1.9	99.4
ergotamine	$C_{33}H_{35}N5O_5$	581.2638	8.98	4.78E+05	582.2722	1.0	99.1
ergotaminine	$C_{33}H_{35}N_5O_5$	581.2638	8.74	1.43E+06	582.2711	-0.8	99.7
ergocristine	$C_{35}H_{39}N_5O_5$	609.2951	9.90	1.95E+05	610.3025	-0.7	99.8
ergocristinine	$C_{35}H_{39}N_5O_5$	609.2951	10.38	2.80E+06	610.3044	2.5	94.2

All of the standards investigated showed purity levels considerably above 96% apart from ergocristinine (94.2%), ergosine (96.4%) and ergosinine (95.5%). The purity of the ergot alkaloid standards was considered satisfactory, particularly in view of the limited sources and numbers of ergot alkaloid standards available and the purity of other commercial mycotoxin standards, which are usually between 95 and 99%.

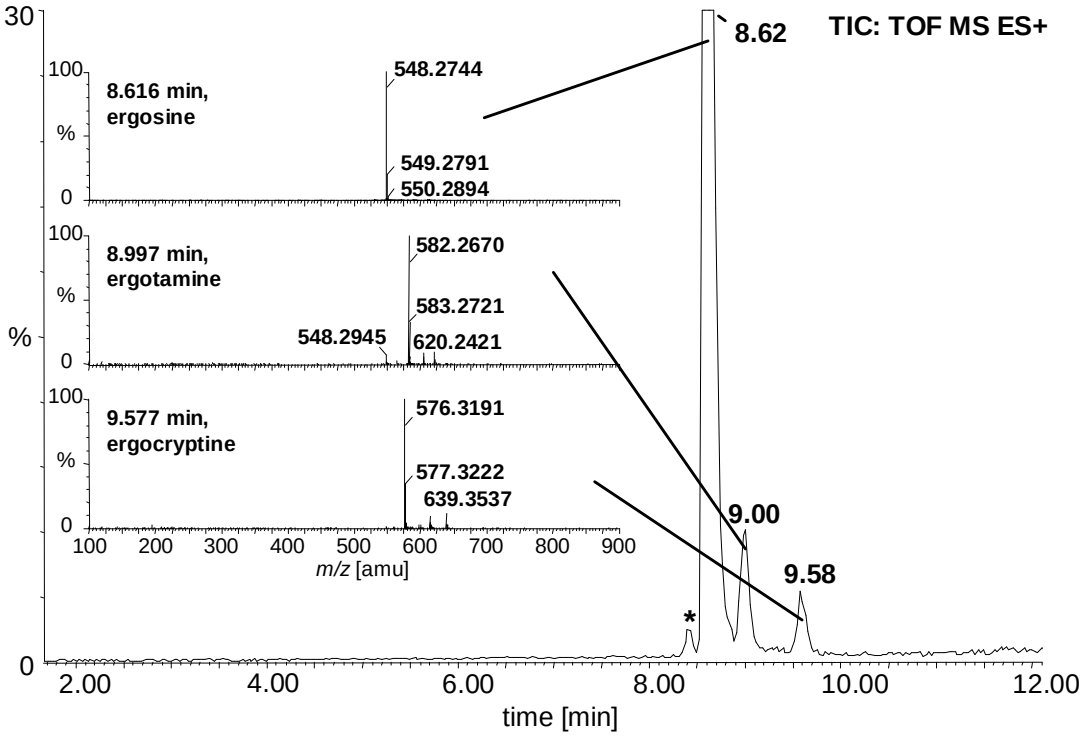


Figure 26 Total ion (ES+) chromatogram of the ergosine standard.

In the chromatogram of the ergosine standard shown in Figure 26 the impurities at 9.00min and 9.58min were identified as ergotamine and ergocryptine respectively. The peak at 8.44min labelled with * is system related and appears also in a blank. Mass spectra of the peaks are also shown at the time of the highest intensity. Masses are lower at the peak Total Ion (ES+) chromatogram of the ergosine standard.

3.5. Determination of epimerisation and stability of calibrants

Specific Objective: To assess the stability of ergot alkaloids in solution and in both raw cereal and cereal based processed food extracts over time.

The degree of epimerisation of *–ines* to *–ininess* and the stability of the ergot alkaloids were tested in a) seven different solvents during a 6 weeks storage study at three different

temperatures (-20°C, +4°C, +20°C); b) during the course of a typical HPLC run sequence at room temperature and c) in extracts of rye, wheat, barley and oats in acetonitrile:ammonium carbonate buffer (200 mg/L) 84+16 at three different temperatures (-20°C, +4°C and 20°C). A reference temperature of -80°C was used as it can be expected that the ergot alkaloids are completely stable at this low temperature. Epimerisation from -*inine* to -*ine* was not observed during any of the preliminary experiments, and as the focus was on the physiologically active form of the toxin this aspect was not studied.

3.5.1. Six-weeks storage study in seven solvents

The degree of epimerisation of ergometrine, ergocornine, ergocristine, α-ergocryptine, ergosine and ergotamine to their corresponding –*inines* forms and the stability of these ergot alkaloids were tested in seven different solvents and extraction mixtures, respectively which are typically used for storage or extraction of ergot alkaloids (see Table 8). Storage time was six weeks in total, with samples taken after one and three weeks.

Table 8 Overview of the seven solvents used for six-week stability testing

	Composition
A	Acetonitrile
B	Chloroform
C	Methanol/dichloromethane 50/50
D	Stabilising solution - 1,2-ethanediol (100g), 1,2-propanediol (100g), tartaric acid (1g), ethanol (750 ml) water (250 ml)
E	Extraction mixture - Ammonia 25% (1), methanol (5), ethyl acetate (25),dichloromethane (50)
F	Methanol/phosphoric acid 0.25% 40/60
G	Acetonitrile/ammonium carbonate buffer (200mg/L pH 9) 80/20

Mixed solutions containing 100 ng/mL of ergometrine, ergocornine, ergocristine, ergocryptine, ergosine and ergotamine in the seven solvents to be tested (Table 8) were

prepared and 22 aliquots of 1 mL filled into HPLC vials. These were stored at the chosen storage temperature (-80°C = reference temperature, -20°C, +4°C and 20°C). The stability studies were carried out as isochronous measurements (Lamberty et al, 1998). Briefly, samples are stored for different times at various storage conditions. Shorter-time samples are kept in preserving conditions (-80°C) until the last storage time has passed. This enables the measurement of all samples together under repeatability conditions, by avoiding the potential fluctuations due to day-to-day variations.

For this study, vials were removed from storage one, three and six weeks after preparation of the solutions and were immediately transferred to -80°C (chosen as reference storage temperature). See Table 9 for the number of vials per storage condition and storage time.

Table 9 Distribution of sample vials stored under different conditions

Conditions	Storage time (weeks)			
	0	1	3	6
-80°C	6	-	-	-
-20°C	-	-	-	2
+ 4°C	-	2	2	2
+ 20°C dark	-	2	2	2
+ 20°C daylight	-	2	-	-

Before measurement, vials were taken from –80°C and placed in the dark to reach room temperature. After removing 200 µL for measurement, the storage vials were recapped and again placed at -80°C to preserve them for further use. The sample preparation for the 200 µL aliquot was dependent on the solvent:

Volatile solvents (acetonitrile; chloroform; methanol/dichloromethane; ammonia/ methanol/ethyl acetate/dichloromethane) were evaporated and restored in 1000 µL acetonitrile/aqueous ammonium carbonate buffer (90+10) before measurement. The final concentration of these samples was 20 ng/mL.

Samples in solvents with non-volatile components (stabilizing solution with ethylene glycol and methanol/phosphoric acid) were diluted by adding 800 µL acetonitrile/aqueous ammonium carbonate buffer (80+20) to 100 µL stored solution. The theoretical concentration

of the measurement solution of these samples was 10 ng/mL per alkaloid. Samples in acetonitrile/aqueous ammonium carbonate buffer (80+20) were diluted with 200 µL acetonitrile before measurement, resulting in a concentration of 50 ng/mL for each alkaloid.

An Agilent 1100 HPLC system with a Phenomenex Gemini column (5 µm particle diameter, 150 x 4.6 mm) coupled to an Applied Biosystems QTrap 4000 with an ESI-interface in multireaction monitoring mode was used to separate and detect the toxins. Gradient elution, with the ratio of Solvent A (200 mg ammonium carbonate /L water) to Solvent B (pure acetonitrile) varying between 70/30 and 20/80, was performed.

The peak areas of the transitions from the protonated molecule ion to the product ion with *m/z* 223 were considered for the data evaluation. A direct comparison of the detector responses of epimer pairs was not meaningful as the S-forms' signals were much stronger than the R-forms' signals at the same concentration. This effect depended on the source temperature and seems to correlate with the volatility of the injected HPLC effluent. For all measurements of solvents used for the six week storage study, the source temperature was 150°C (for the rest of the project's measurements 350°C was used).

The *–inine* (R-form) signal was therefore corrected by multiplication with the response factors listed in Table 10 to enable comparison of the peak areas obtained for *–ines* and *-inines*. Response factors were calculated by measuring both epimers at the same concentrations and dividing the detector response of the *-inine* by that of the *–ine*. The average factor was calculated for 6 calibrants at concentrations of 1 to 50 ng/mL each measured twice. For each pair of *-inine* and *-ine* the ratio was determined, and the mean calculated. The results are shown in Table 10.

Table 10 Response factors for the *–inine* peaks for measurements at 150°C.

Standard	**factor**	**RSD%**
Ergine/Erginine	1.84	11.1
Ergometrine/ Ergometrinine	1.65	14.3
Ergosine/ Ergosinine	6.22	24.7
Ergocornine/ Ergocorninine	5.45	22.2
Ergocryptine/ Ergocryptinine	5.32	14.5
Ergotamine/ Ergotaminine	5.82	30.0
Ergocristine/ Ergocristinine	4.17	12.0

3.5.1.1. Results of stability studies

During storage, the toxins could also undergo other transformation/degradation processes other than epimerisation of *–ines* to *–ininess* (see 3.5.1.2.), as e.g. epimerisation of the peptide moiety to aci-forms, addition of water to lumi-ergot alkaloids etc. To investigate losses owing to these potential stability problems, the sum of the concentrations measured for *-ines* and *-ininess* (the response of the latter corrected by the factors listed in Table 10) after six weeks at -20°C, +4°C and +20°C was related to the concentrations of the *–ines* found after storage at -80°C. The ratios obtained for ergometrine/inine are depicted in Figure 27 for all solvents tested, in which 100% recovery refers to a ratio of 1, i.e. no degradation of the analyte. No statistically significant difference was found between the concentrations found for the simple lysergic acid derivative (ergometrine) at the three different storage temperatures and the Reference temperature of –80°C.

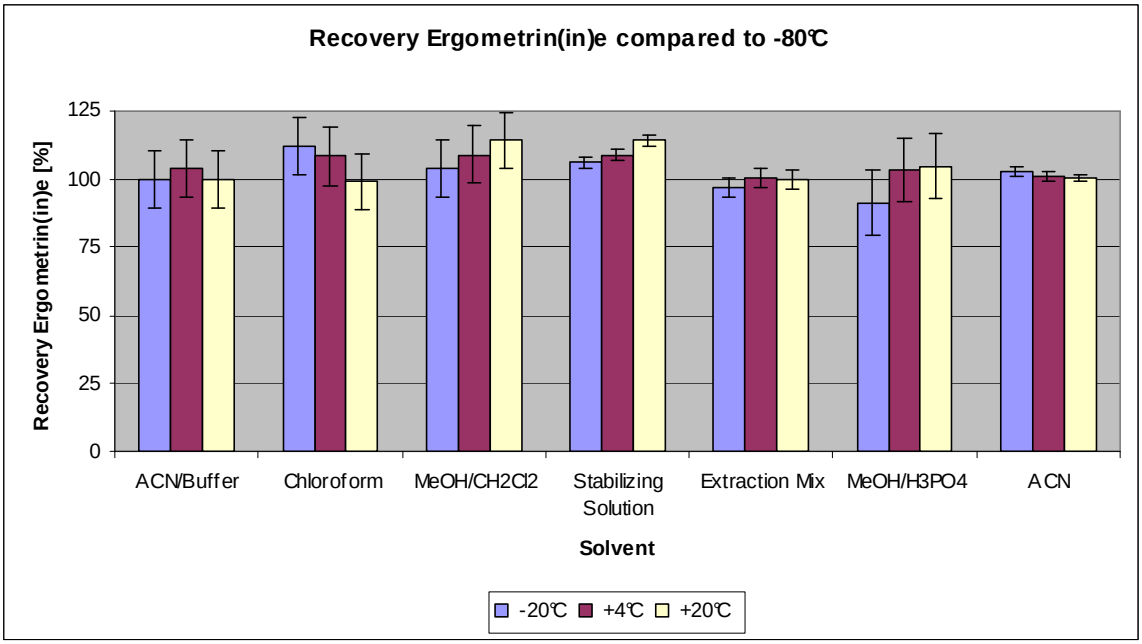


Figure 27 Recovery of ergometrin(in)e after six weeks' storage at -80°C.

Results are different for the ergopeptide-type toxins, e.g. ergotamine (Figure 28), which show a significant decrease for acetonitrile and acetonitrile/buffer and methanol/dichloromethane after six weeks storage at +4°C and +20°C. In the extraction mixture the concentration decreased to approximately 90% for all storage temperatures after six weeks. Storage in methanol/phosphoric acid resulted in an increase in the large standard deviation of the total of ergotamine(in)e, which might have been caused by the use of dilution instead of evaporation of the storage solvent mix. For all other ergopeptides the situation was similar. The details of results for the stability studies are provided in Annex 2.

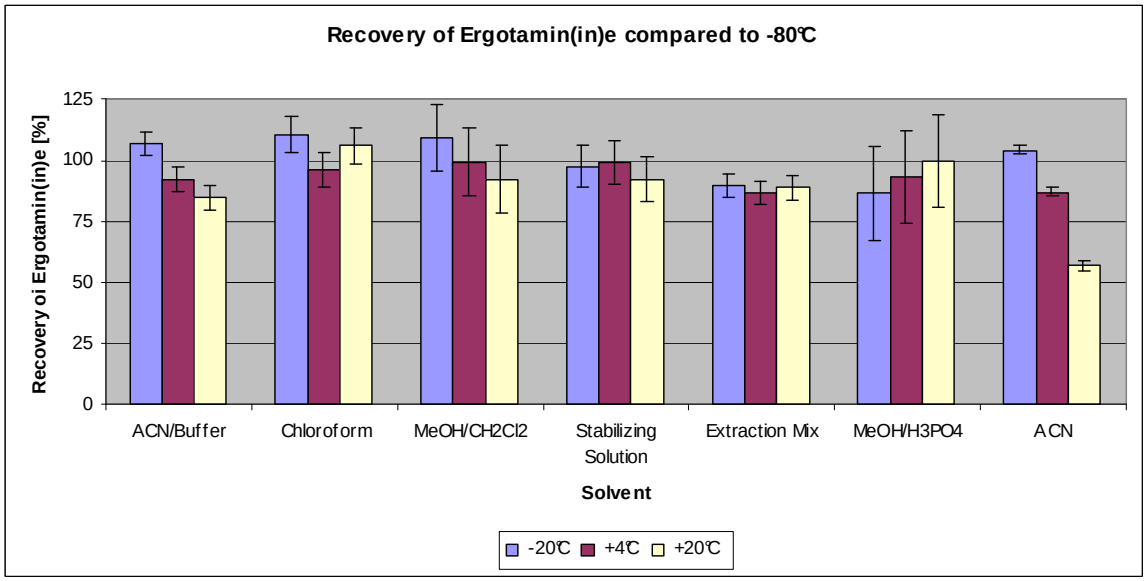


Figure 28 Recovery of ergotamin(in)e compared to the reference storage for six weeks at -80°C.

3.5.1.2. Comparison of storage at –20°C and -80°C

Six randomly chosen aliquots of each calibrant were placed at –80°C to serve as reference for the study. As –80°C storage is not readily available, the more common storage at -20°C was also included in this study. Figure 29 illustrates the results obtained from these samples, i.e. the recoveries of *–ines* + *-inines* with respect to the reference temperature at –80°C (=100%). No significant difference between storage at –20°C and –80°C could be found for all toxins tested.

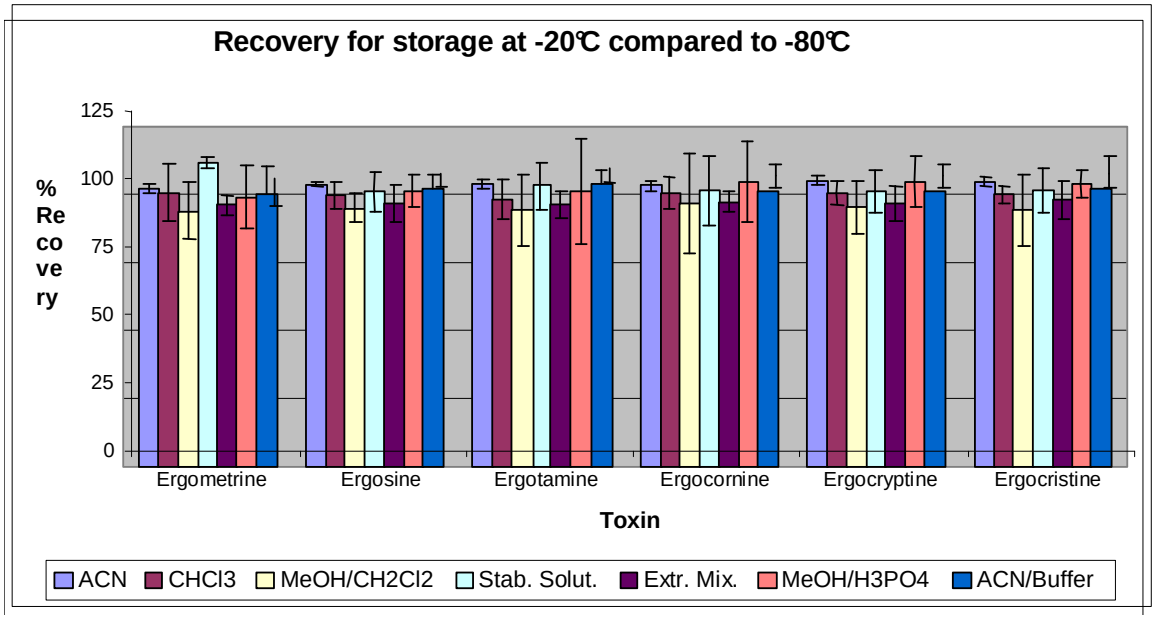


Figure 29 Recovery of toxins stored in seven solvents at -20°C compared to -80°C.

3.5.13. Results of epimerisation study

Of all solvents tested methanol/dichloromethane promoted epimerisation of all tested alkaloids the most strongly. More than 30% of the alkaloid present was in S-(-inine)-form after six weeks at room temperature, except for ergometrine (11%).

In general, the order of epimerisation-promotion was methanol/dichloromethane > acetonitrile/buffer > extraction mix > stabilizing solution > acetonitrile >> chloroform.

In all solvents tested, ergometrine showed the lowest tendency to epimerise, with ergosine having the greatest (except for acetonitrile, where ergotamine epimerised more strongly). The highest degree of epimerisation was reached with ergosine in methanol/dichloromethane at 46% (Table 11).

Table 11 High and low epimerisation alkaloid for each solvent after six weeks at 20°C.

Composition	Minimal epimerisation	Maximum epimerisation
Acetonitrile	Ergometrin(in)*ine* 0.2%	Ergotamin(in)*ine* 31%
Chloroform	Ergometrin(in)*ine* 0.3%	Ergosin(in)*ine* 5.8%
Methanol/dichloromethane	Ergometrin(in)*ine* 11%	Ergosin(in)*ine* 43%
Stabilising solution	Ergometrin(in)*ine* 0.5%	Ergosin(in)*ine* 9%
Extraction mixture	Ergometrin(in)*ine* 2.5%	Ergosin(in)*ine* 24%
Methanol/phosphoric acid	Ergometrin(in)*ine* 0.6%	Ergosin(in)*ine* 12%
Acetonitrile/buffer	Ergometrin(in)*ine* 5%	Ergosin(in)*ine* 26%

Storage temperature is of crucial importance, particularly to minimise the degree of epimerisation. At -20°C the epimer ratio after six weeks was comparable to the reference conditions of –80°C for all solvents. –20°C is thus a convenient storage temperature for all ergot alkaloids and all solvents tested.

3.5.1.4. Six-week storage – Degree of epimerisation for each toxin

Ergometrine/ergometrinine

At 20°C 11% ergometrinine were found in methanol/dichloromethane after six weeks (see Figure 30). For acetonitrile/buffer, 5% epimerisation occurred; the extraction mixture resulted in 2.5%. The other solvents had an ergometrinine level of less than 0.6% after six weeks at room temperature.

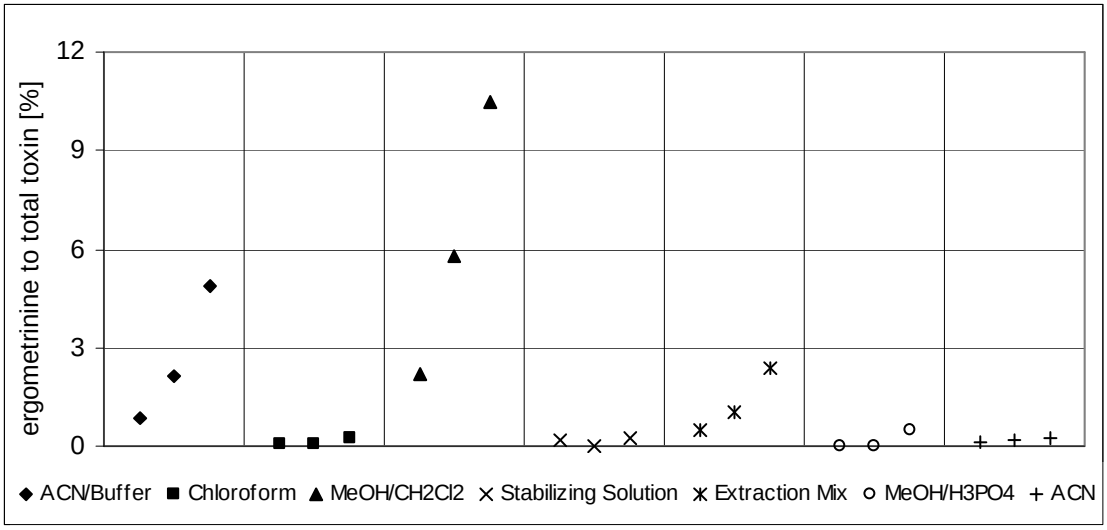


Figure 30 Stability of ergometrine in various solvents at +20°C.

The first data point indicates the result after one week, the second after three, the third after six weeks of storage for each toxin.

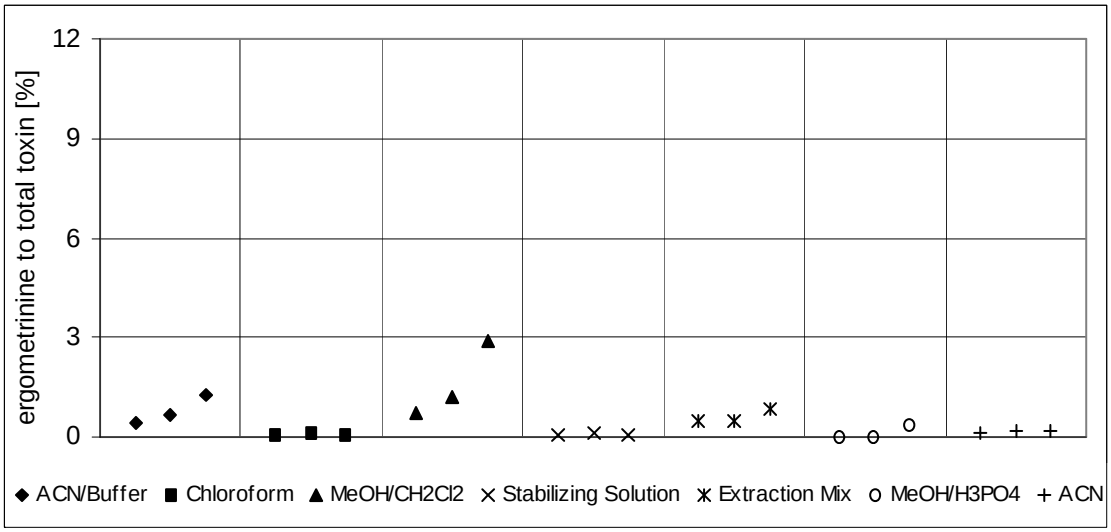


Figure 31 Stability of ergometrine in various solvents at +4°C.

The first data point indicates the result after one week, the second after three, the third after six weeks of storage for each toxin.

Similar to storage at +20°C, methanol/dichloromethane was also most favourable for epimerisation at 4°C, resulting in about 3% ergometrinine. All other solvents tested resulted in an ergometrinine level below 1.4% of the total toxin after six weeks. Less than 1% ergometrinine was measured in samples stored at –20°C.

Ergosine/ergosinine

Storage of ergosine in methanol/dichloromethane for six weeks at 20°C resulted in an ergosinine level of 43% (see Figure 32). In acetonitrile/buffer, 26% were measured after the same time and 24% for the extraction mixture as solute. In acetonitrile/buffer, 28% of the ergosine epimerised to ergosinine after six weeks. When dissolved in chloroform, the ergosinine level stayed at approximately 6% for six-week storage at 20°C, which was also the reference value measured for storage at –80°C.

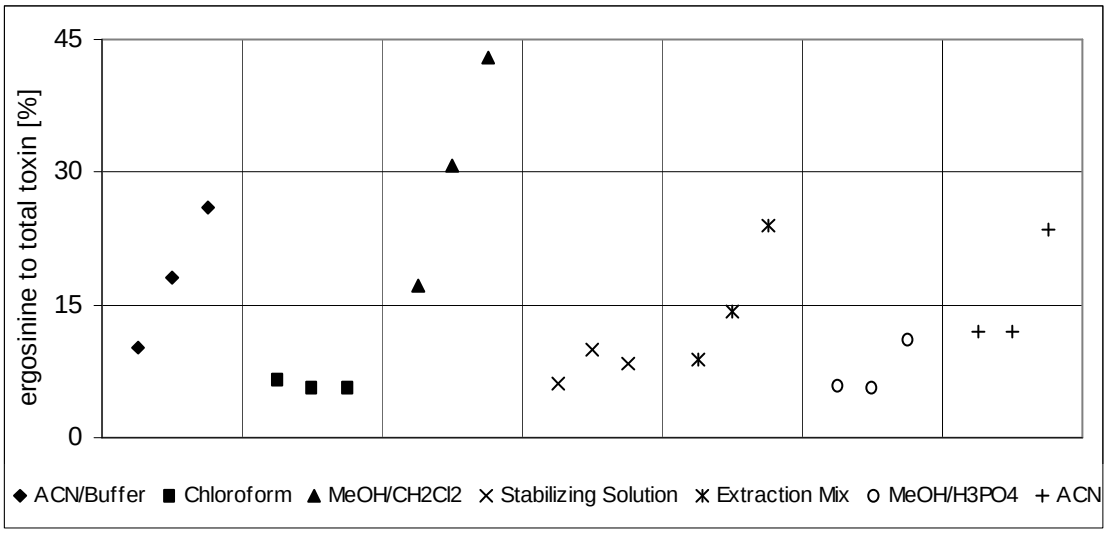


Figure 32 Stability of ergosine in various solvents at +20°C.
The first data point indicates the result after one week, the second after three, the third after six weeks of storage for each toxin.

Storage at 4°C (Figure 33) resulted in lower levels of ergosinine compared to 20°C. In methanol/dichloromethane, 23% of the added ergosine were present as ergosinine after six weeks, with approximately 12% for acetonitrile, acetonitrile/buffer and the extraction mix. When dissolved in the stabilizing solution or chloroform, only 5% ergosinine could be detected.

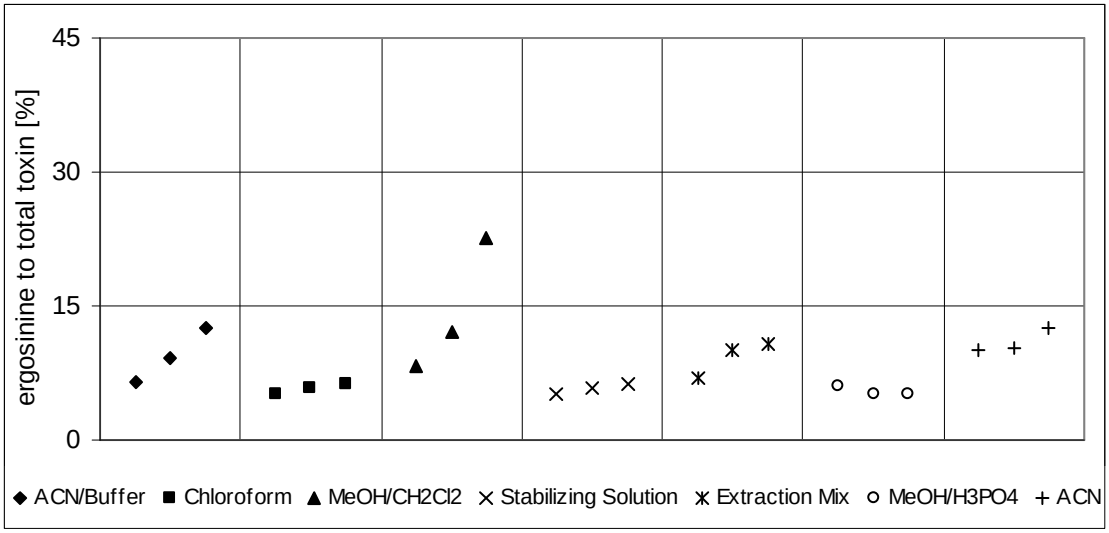


Figure 33 Percentage of ergosinine in various solvents at +4°C.

The first data point indicates the result after one week, the second after three, the third after six weeks of storage for each toxin.

Ergotamine/ergotaminine

Use of methanol/dichloromethane as solvent resulted in an ergotaminine content of 40% of the total alkaloid in acetonitrile/buffer after six weeks at 20°C (Figure 34). The average level of ergotaminine during storage in acetonitrile was comparable to acetonitrile/buffer, with individual samples varying between 18% and 32%. Chloroform preserved the ergotaminine-percentage for the whole six weeks at 20°C at the reference storage temperature of –80°C (~1.5%).

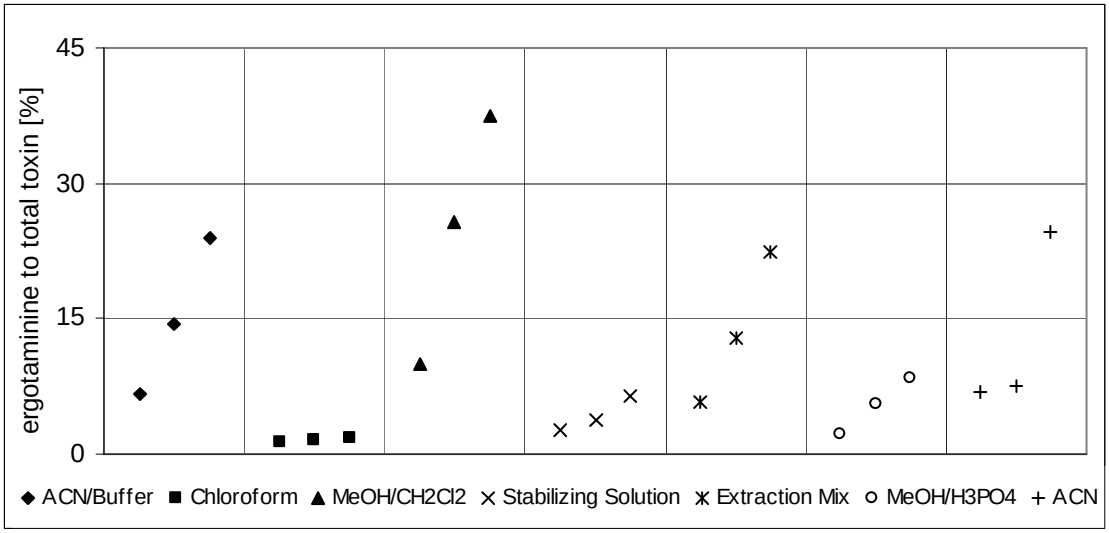


Figure 34 Percentage of ergotaminine in relation to total toxin content in various solvents at +20°C.

The first data point indicates the result after one week, the second after three, the third after six weeks of storage for each toxin.

The largest ratio of ergotaminine was obtained on keeping methanol/dichloromethane at 4°C for six weeks (Figure 35) The extraction mix, acetonitrile/phosphoric acid and acetonitrile/buffer (7-10%) produced lower levels. The other solvents tested resulted in ergotaminine levels below 4%, with chloroform resulting in 1.5%.

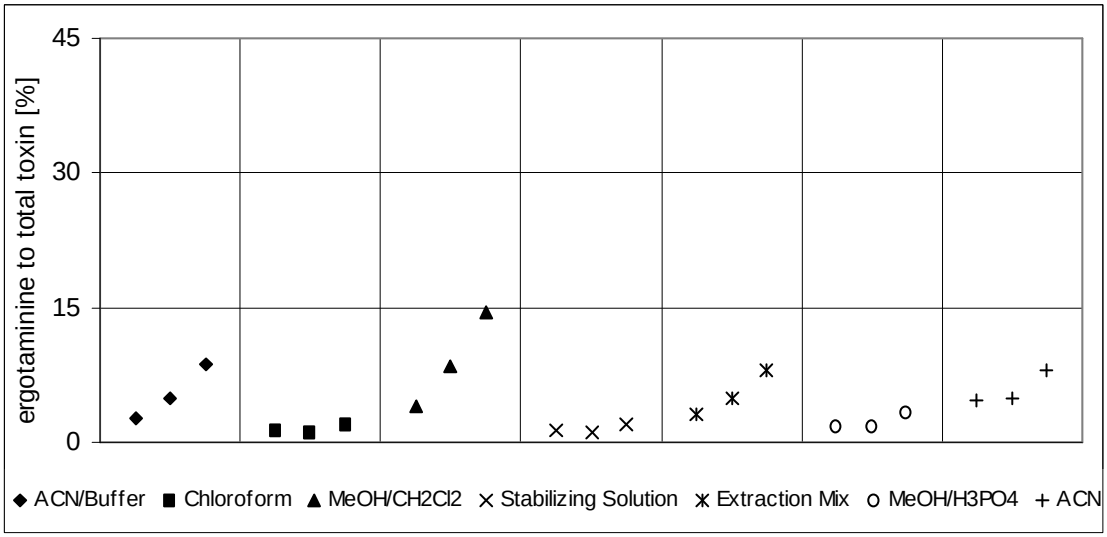


Figure 35 Ergotaminine in relation to total toxin content in various solvents at +4°C.

The first data point indicates the result after one week, the second after three, the third after six weeks of storage for each toxin.

Ergocornine/ergocorninine

The largest shift from ergocornine to ergocorninine was observed in samples stored in methanol/dichloromethane at 20°C (Figure 36). Dissolving the toxin in chloroform reduced the epimerisation process. After six weeks levels of ergocorninine varied between 3% and 36% for chloroform and methanol/dichloromethane, respectively. In acetonitrile/buffer, 23% of ergocornine was transformed to ergocorninine; in the stabilizing solution less than 10% of the toxin spiked was detected as ergocorninine after six weeks. The epimerisation in acetonitrile is comparable to storage in extraction mix or stabilizing solution.

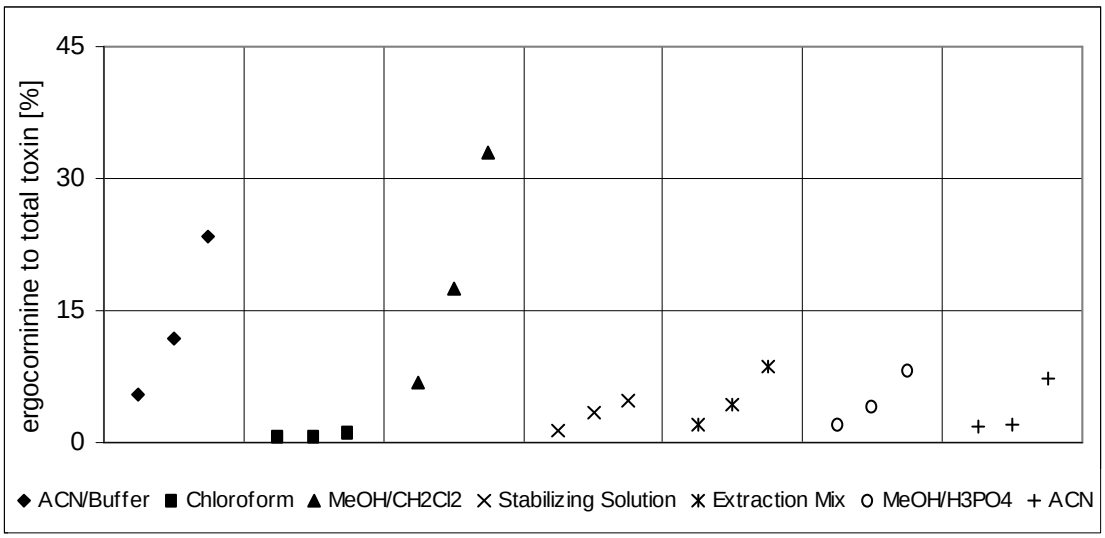


Figure 36 Ergocorninine in relation to total toxin content in various solvents at +20°C.
The first data point indicates the result after one week, the second after three, the third after six weeks of storage for each toxin.

Again, lower temperatures reduced the extent of epimerisation. Only 10% -*inines* were formed at 4°C within six weeks (Figure 37). The best result (lowest ergocorninine-content after six weeks) was obtained for chloroform at any temperature, or storage at -20°C for all solvents.

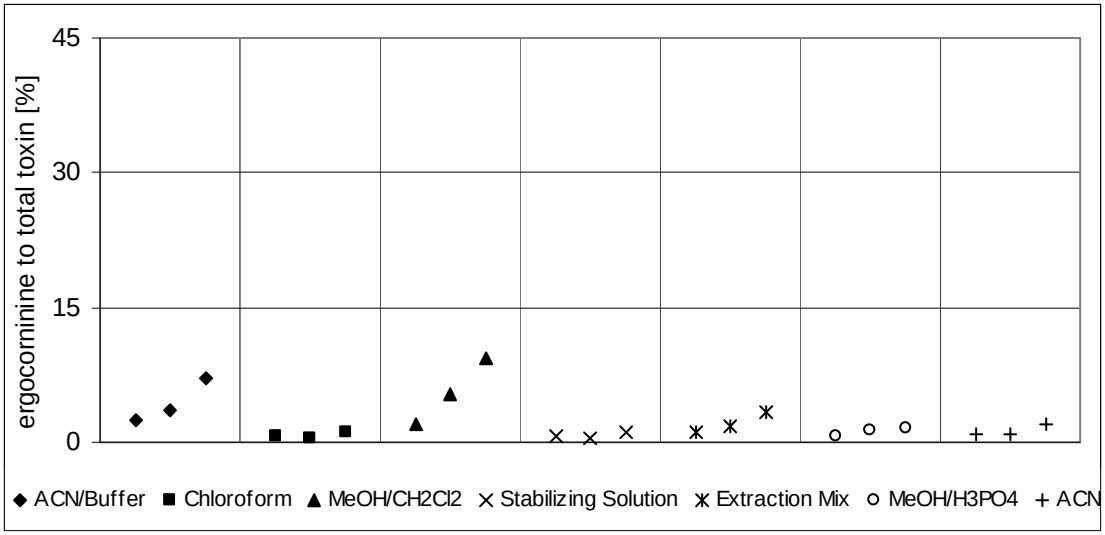


Figure 37 Ergocorninine in relation to total toxin content in various solvents at +4°C.

The first data point indicates the result after one week, the second after three, the third after six weeks of storage for each toxin.

After six weeks' storage at 20°C, the largest percentage of ergocryptinine was detected in methanol/dichloromethane (35%) as shown in Figure 38. Acetonitrile/buffer resulted in 23%, the extraction mix in 11%, pure acetonitrile 10% and the stabilizing solution in 6% ergocryptinine. For storage in chloroform, the percentage of ergocryptinine stayed constant at approximately 0.6% for all sampling times and 20°C.

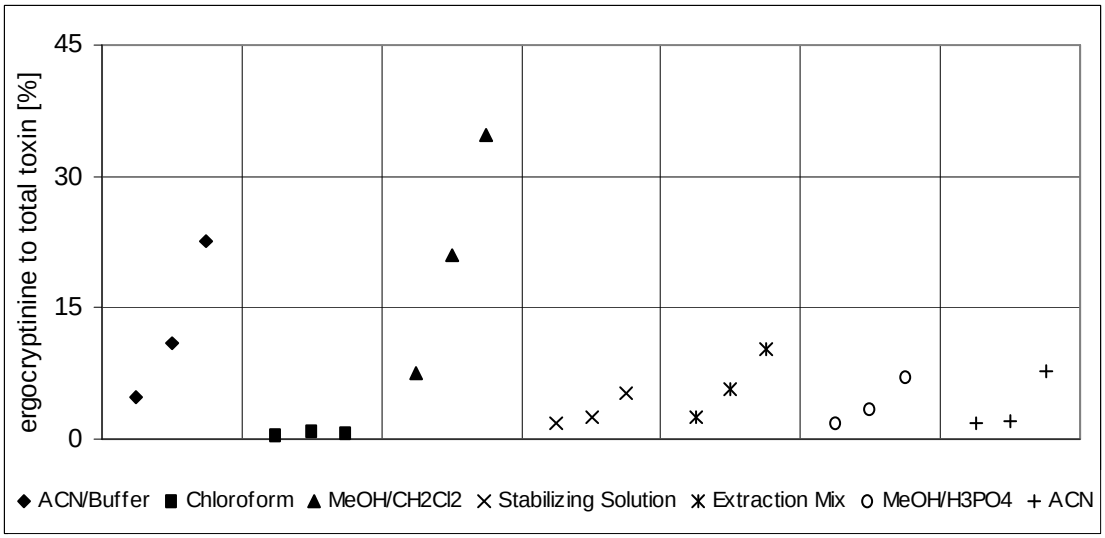


Figure 38 Ergocryptinine reached in relation to total toxin content in various solvents at +20°C.

The first data point indicates the result after one week, the second after three, the third after six weeks of storage for each toxin.

At 4°C, methanol/dichloromethane resulted in 14% ergocryptinine, acetonitrile/buffer gave 6% (Figure 39). The ergocryptinine-content in the other solvents was below 4% of the total toxin quantity.

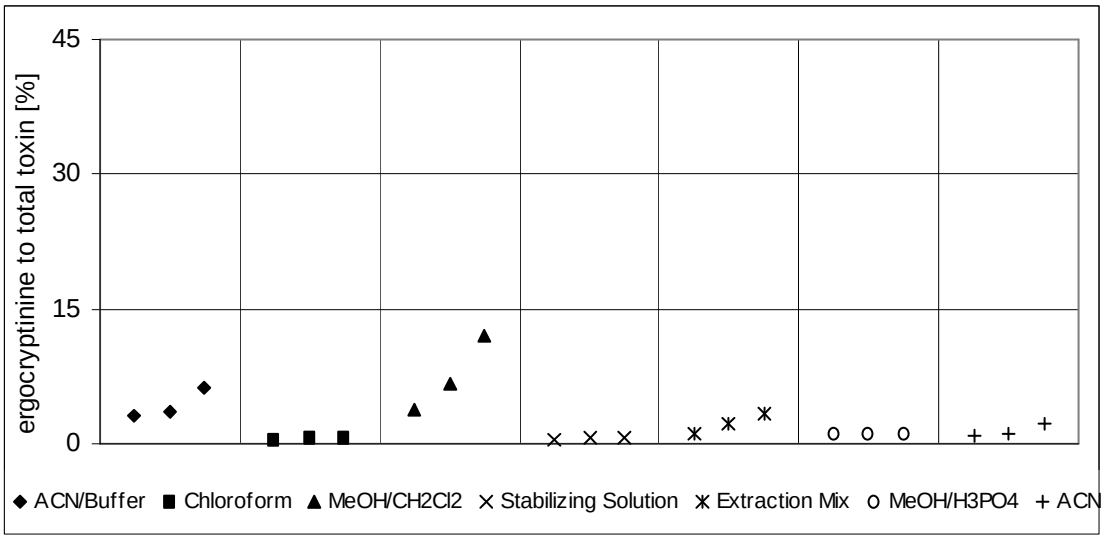


Figure 39 Ergocryptinine in relation to total toxin content in various solvents at +4°C.
The first data point indicates the result after one week, the second after three, the third after six weeks of storage for each toxin.

Ergocristine/Ergocristinine

The strongest epimerisation trend was observed in methanol/dichloromethane, resulting in nearly 40% ergocristinine after six weeks. In acetonitrile/buffer, approximately 25% were measured for the same storage time; in the other five solvents less than 15% ergocristinine was produced. After six weeks the lowest level of ergocristinine was observed for storage in chloroform (Figure 40).

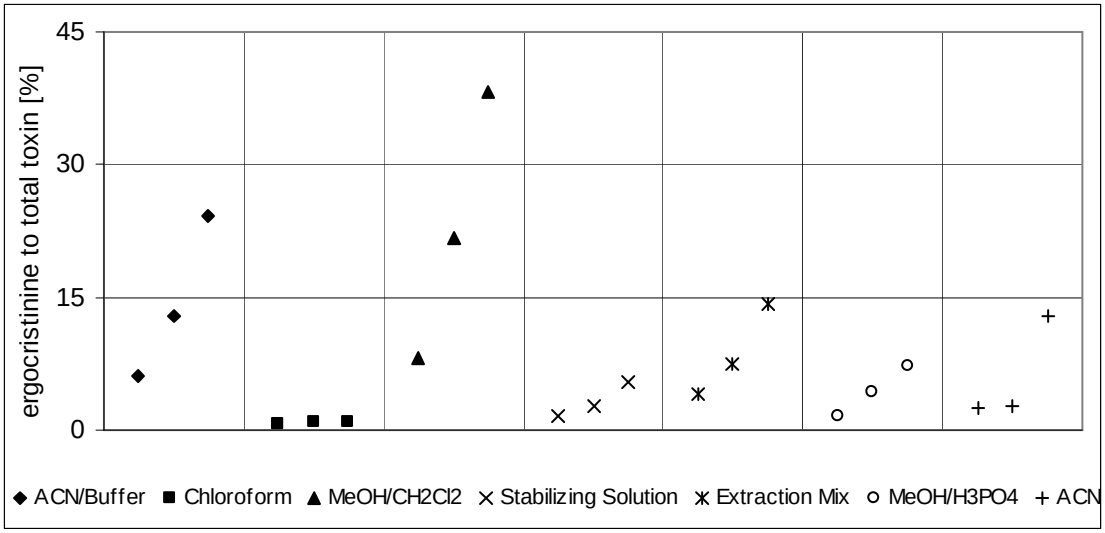


Figure 40 Ergocristinine in relation to total toxin content in various solvents at +20°C.
The first data point indicates the result after one week, the second after three, the third after six weeks of storage for each toxin.

After six weeks at 4°C the level of ergocristinine measured in methanol/dichloromethane was 13-14% and approximately 7% in the acetonitrile/buffer-mixture (Figure 41). Results for storage in stabilizing solution and chloroform were comparable with approximately 1.5% ergocristinine, acetonitrile was slightly worse at 4%. No increase was detectable for storage at -20°C compared to samples kept at -80°C.

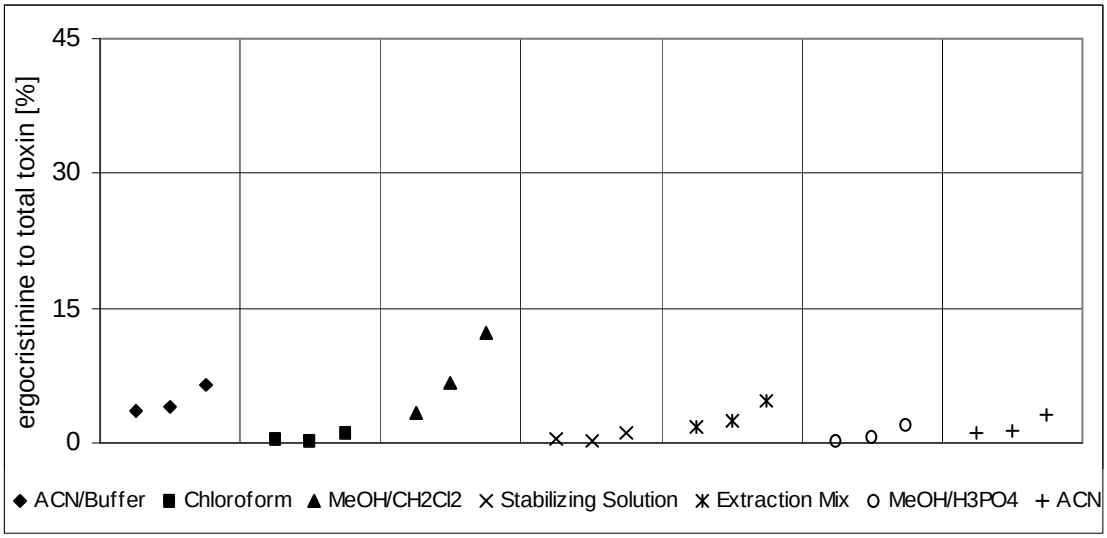


Figure 41 Ergocristinine in relation to total toxin content in various solvents at +4°C.

The first data point indicates the result after one week, the second after three, the third after six weeks of storage for each toxin.

3.5.2. Stability of calibrants during an HPLC-sequence

This part of the study tested the short-time stability of ergometrine, ergocornine, ergocristine, ergocryptine, ergosine and ergotamine during the course of a typical HPLC-MS/MS run sequence (18 hours) at 20°C. In this study five different solvents and solvent mixtures, respectively, were tested: pure acetonitrile, two ratios of acetonitrile/ammonium carbonate buffer (80+20 and 84+16), acetonitrile/ammonium-hydrogen carbonate buffer (84+16; 200 mg/L) and acetonitrile/ ammonium acetate buffer (10+20; 10mM).

The concentration of each toxin in the calibrants was 20 ng/mL. 12 aliquots of 400 µL of each solution were transferred into amber HPLC-vials and analysed sequentially within 18 hours to determine epimerisation during the measurement duration.

Figure 42 depicts the percentage of ergometrinine to total ergometrin(in)e over 18 hours at 20°C. The ergometrine ratio stayed constant (~5%) in all solvents tested (pure acetonitrile, acetonitrile/ aqueous ammonium carbonate buffer with a ratio of 80+20 and 84+16, acetonitrile/ammonium-hydrogen carbonate (84+16) and acetonitrile/ ammonium acetate buffer (10+20)), while the peptide ergot alkaloids' –*inine* form showed a slight increase from ~4% to ~6% for acetonitrile. Despite this small increase, a trend is clearly visible when compared to ergometrin(in)e.

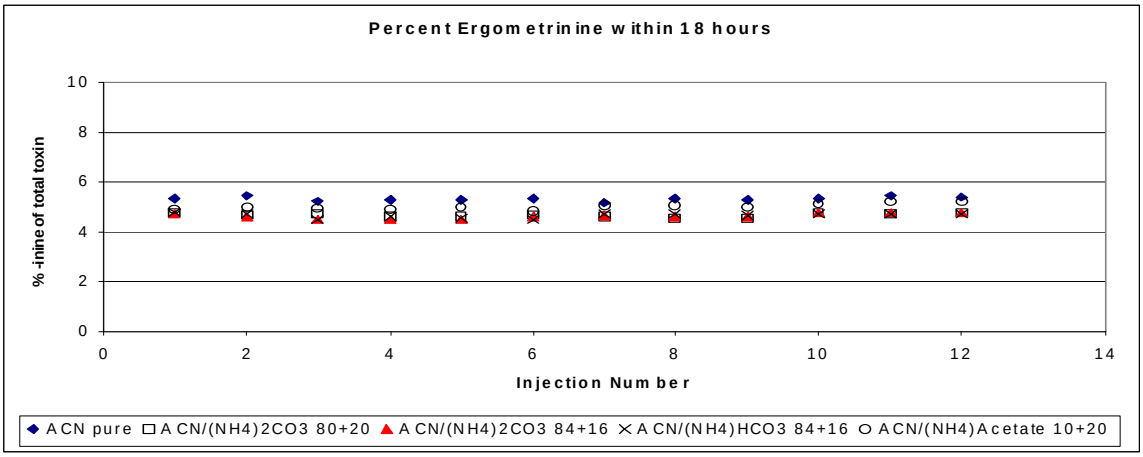


Figure 42 Change in the ergometrinine level compared to the total toxin content at 20°C during twelve repeat injections (18 hours).

A similar picture was obtained with ergosine/ergosinine (Figure 43).

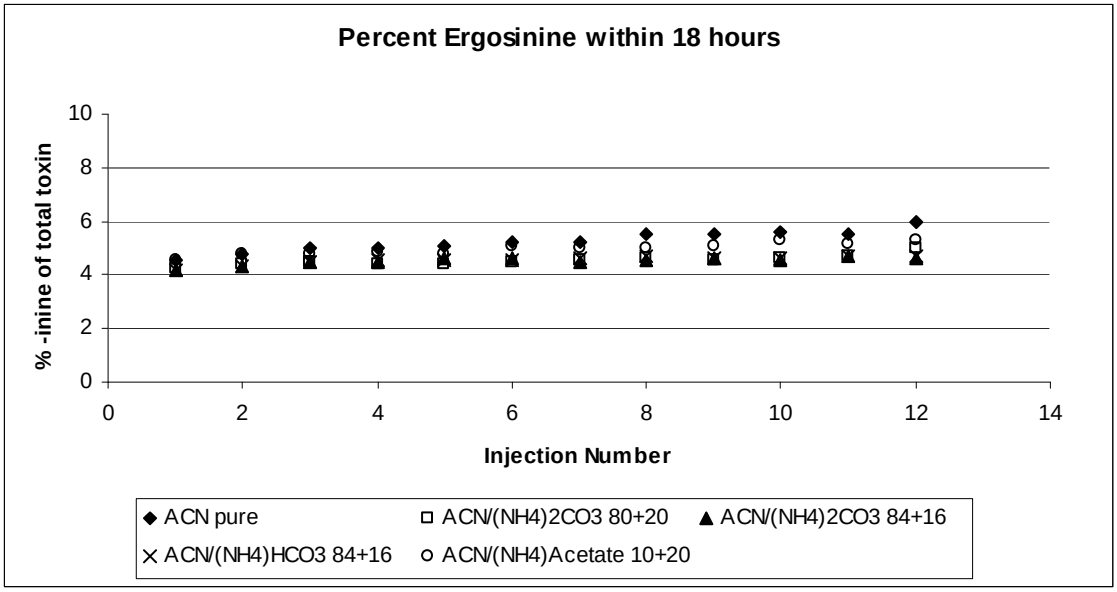


Figure 43 Change in proportion of ergosinine over 18 hours (twelve repeat injections).

The total amount of each ergopeptine/inine (R- and S-form combined) dropped in all solvents at 20°C over the 18 hours measurement time to approximately 86% of the first injection (83.2% for ergotamin(in)e). However, the simple lysergic acid derivatives stayed above 97% of the initial amount one after injection twelve, which is an indication that the decrease is not due to a sensitivity drift of the detector. This phenomenon did not occur during the validation study, for which a standard deviation of less than 2% was achieved the calibrants. However, the latter was carried out at a temperature of 15°C.

Overall it can be concluded that despite the minor trend observed for ergopeptides the ergot alkaloids investigated are reasonably stable during an 18 hour HPLC run at room temperature. However, the small drift of 4 to 6% in *-inine* increase should be considered during calculation of the method uncertainty. Moreover, cooled autosamplers are recommended for this kind of analysis.

3.5.3. Stability of ergot alkaloids in cereal extracts

3.5.3.1. Stability over 6 days at 20°C

Blank ground rye, wheat, barley and oats were extracted with the extraction solvent used in the validated method described later (3.8.), namely acetonitrile/ammonium carbonate buffer (200 mg/L; 84+16; 25 g / 100 mL) and each extract was spiked at a level of 10ng/mL for ergometrine, ergocornine, ergocristine, ergocryptine, ergosine, ergotamine and their epimers to simulate the presence of both forms in naturally contaminated samples. The ratio of the epimer ratio was monitored over 6 days. Sample aliquots were removed and stored at –20°C

immediately and after one and two days at 20°C. Six days after preparation, a final aliquot was removed, the frozen samples thawed and all analysed together by means of LC-MS/MS.

As illustrated in Figure 44 the lysergic acid derivative ergometrin(in)e is not sensitive to storage in cereal extract. No change in the ratio of *–inines* to *–ines* can be observed when stored at 20°C for six days.

The ergopeptides, on the other hand, show a severe epimerisation tendency, as shown for ergocornin(in)e in Figure 45. For all tested extracts (rye, wheat, barley, oat) a shift in the epimer ratio of ergocornine to ergocorninine can be observed.

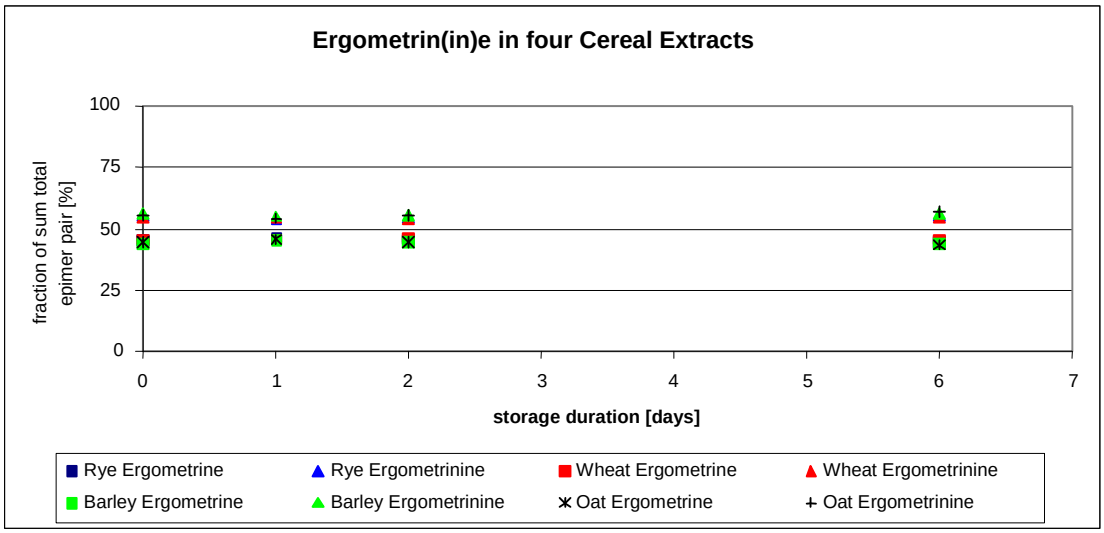


Figure 44 Ergometrin(in)e epimer ratio in four cereal extracts within six days.

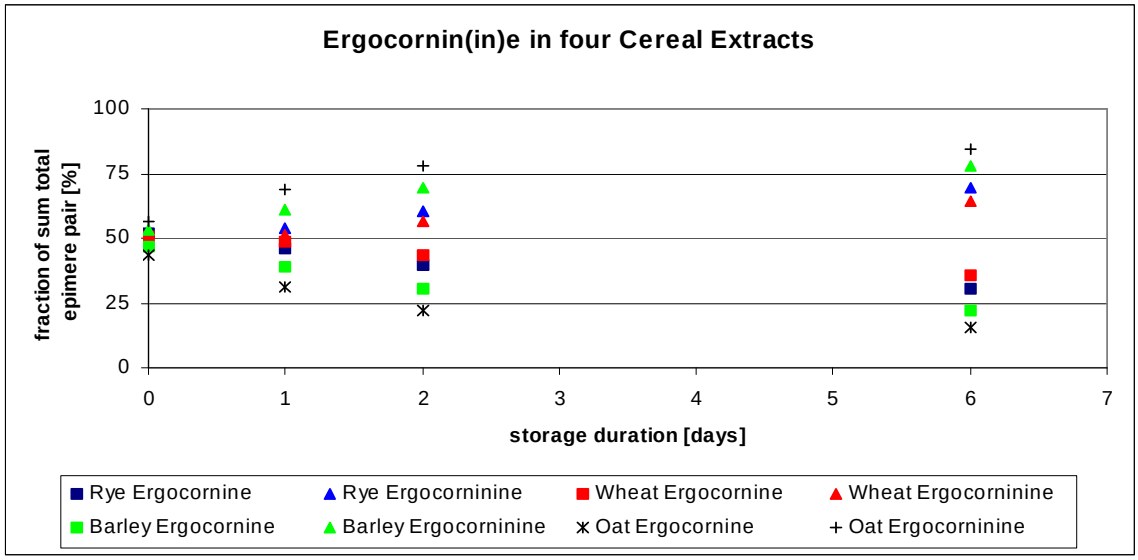


Figure 45 Ergocornin(in)e epimer ratio changes in four cereal extracts within six days.

3.5.3.2. Stability overnight at 4°C and 15 °C and over 14 days at 4°C

Finally, the stability of extracts of the six ergot alkaloids and their six epimers was tested on storage overnight at 4°C (fridge) at 15°C (HPLC autosampler, dark conditions) and over 14 days at 4°C. For that purpose acetonitrile/ammonium carbonate buffer (200 mg/L; 84+16; 25 g / 100 mL) extracts of barley and rye after clean-up with the PSA material (see 3.6.) were spiked at levels of 50 and 100 µg/kg, respectively and subsequently stored at the allocated temperatures.

As can be seen from Figure 46, the barley extracts could easily be kept overnight at 4 or 15°C (in the dark) without any significant epimerisation. However, when kept over a period of 2 weeks, significant epimerisation (about 10%) could be observed for ergocornine, ergocryptine and ergocristine, even at 4°C. As the –ines decreased there was an equivalent increase in the concentrations of the corresponding –ininies showing that there were no losses above and beyond those due to epimerisation. This can be seen for ergocristine/inine in Figure 46.

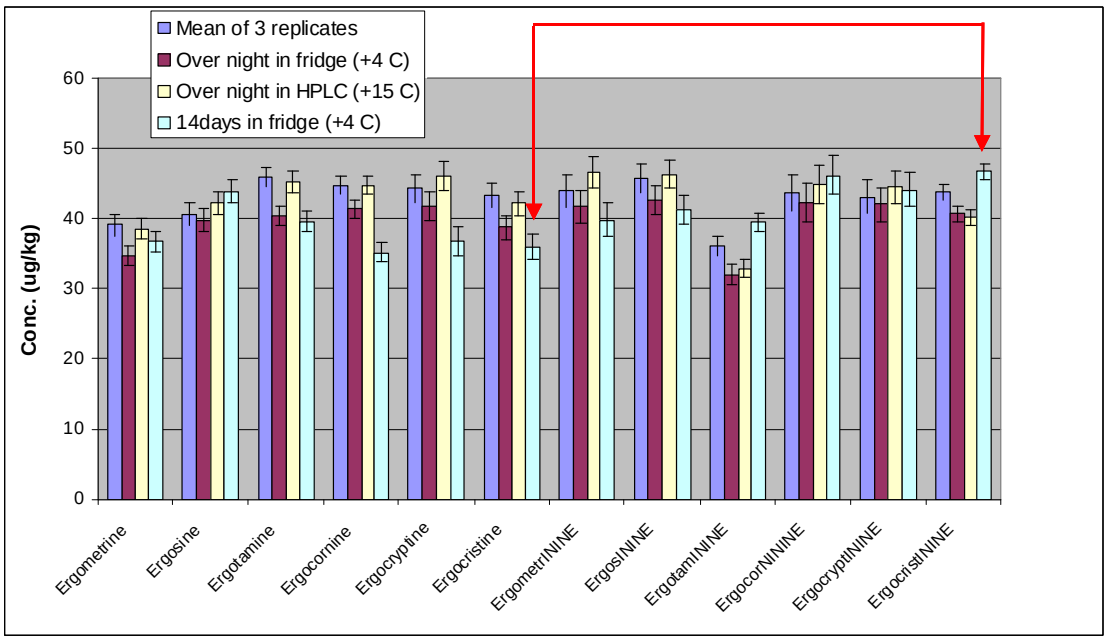


Figure 46 Stability of ergot alkaloids in barley extract after PSA clean-up.

A more pronounced but similar situation was observed for the rye extracts. As can be seen from Figure 47, the rye extracts could also be kept overnight at 4 or 15°C (in the dark) without any significant epimerisation. However, when kept over a period of 2 weeks at 4°C, severe epimerisation of more than 50% (ergocornine) was observed, but a high degree of epimerisation also occurred for ergotamine, ergocryptine and ergocristine. Similarly to the barley extracts, the decrease of *–ines* lead to an increase of the same extent of the concentrations of the corresponding *–inines* as shown in Figure 47 for ergocristine/inine.

According to these results the degree of epimerisation was obviously strongly dependent on the matrix. When extracts are stored more than one night they should be stored at -20°C.

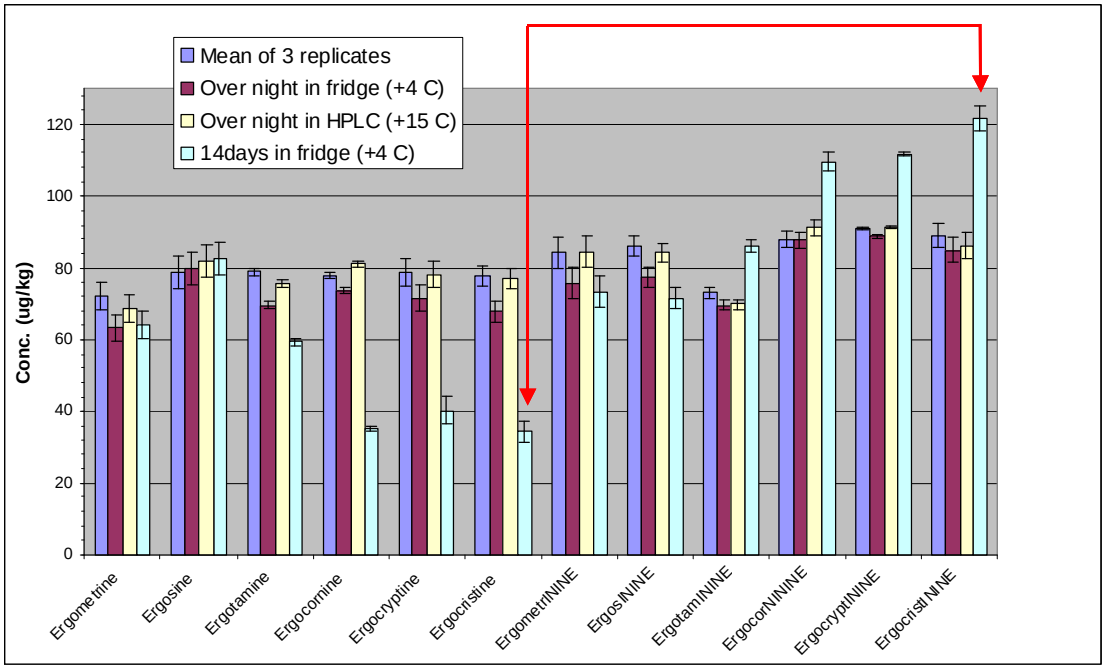


Figure 47 Stability of ergot alkaloids in rye extract after PSA clean-up.

3.5.4. Conclusions from the stability and epimerisation studies

A previous study by Smith and Shappel (2002) determined the epimerisation of α-ergocryptine and ergovaline in organic and aqueous solvents and mixtures. Their results show that aprotic solvents are more favourable for long-term stability of the toxins tested.

In this study the stability and degree of epimerisation of all six ergot alkaloids mentioned in the recent EFSA report (EFSA, 2005) was monitored at three different temperature levels (-20°C, +4°C and +20°C) in seven different solvents over periods of 18 hours and six weeks. Moreover, the stability of the ergot alkaloids was tested in different cereal extracts over night and over 6 and 14 days, respectively.

Of the toxins tested, the ergopeptides ergosine, ergotamine, ergocornine, ergocryptine and ergocristine showed comparable behaviour. However, the simple lysergic acid derivative was more stable and showed hardly any epimerisation to ergometrinine, with the sum of both epimers remaining constant in all seven solvents. The ergopeptides tested show variable epimerisation tendencies, and are also less stable which was revealed during the six weeks study at 20°C. Ergosine showed the highest degree of epimerisation (38% after 6 weeks at 20°C). With the exception of chloroform, epimerisation increased considerably with increasing temperature and storage time in all tested solvents. For example ergosine showed 0% epimerisation at –20°C, 23% at 4°C and 44% at 20°C after six weeks storage in methanol/dichloromethane. In general, the order of epimerisation-promotion was

methanol/dichloromethane > acetonitrile/buffer > extraction mix > stabilizing solution > acetonitrile >> chloroform.

We have only a tentative explanation of the reasons for the differences in epimerisation. Ergosine(ine) has the largest substituent at C2' (R1 in Figure 1), this *iso*-butyl group might positively influence the formation of conjugated systems when the free electron-pair of the nitrogen atom in the amide bond is taken into consideration. For all other ergot alkaloids R1 is smaller (*iso*-propyl or only methyl), and the "relative increase of the electron density is therefore smaller. It is obvious that in different solvents the presence of protons affects epimerisation. It is, however, quite puzzling that there is such a large difference between the effects of methanol/dichloromethane compared with methanol/phosphoric acid.

No equilibrium was achieved after three weeks as an increase up to the maximum storage time of six weeks was detected. The slopes indicate no equilibrium even after six weeks in all solvents except chloroform.

Long term-storage at room temperature can only be carried out in chloroform, which showed no epimerisation for all toxins even at 20°C and also kept the sum of R- and S-form constant, which indicates no formation of aci-epimers or other degradation products.

The most convenient solvent for use with HPLC is acetonitrile. To use this for storage of ergot alkaloids requires low temperatures (-20°C) for long term. Degradation and epimerisation were significant at +4°C and +20°C during the six weeks' study. No epimerisation, however, was observed at –20°C but repetitive thawing increased epimerisation up to 3%

Stable conditions were also found for the other solvents tested when kept at -20°C but these likewise showed various rates of epimerisation and degradation at higher temperatures. Their usage is therefore not recommended for long-term storage of standards above -20°C as they provide no advantage over acetonitrile.

Despite the minor trend observed for ergopeptides the constant epimer ratio of all ergot alkaloids in acetonitrile/ammonium carbonate buffer (200 mg/L; 92+8) during an HPLC analysis (18 hours) indicates that they are reasonably stable at room temperature. However, the small increase from 4 to 6% -*inine* suggests consideration during calculation of the overall uncertainty of the method. Moreover, cooled autosamplers are recommended for this kind of analysis.

Storage of crude extracts at 20°C cannot be recommended, as a change in the epimer ratio of ergopeptides was observed after a storage time of 24 hours. Extracts should thus be prepared and analysed the same day or stored at lower temperatures. Barley and rye extracts which were stored at 4 and 15°C after PSA clean-up proved to be stable over night. However, storage over a period of 14 days at 4°C resulted in significant epimerisation which was most pronounced in rye and particularly for ergocornine, ergocryptine and ergocristine.

3.6. Optimisation of extraction and clean-up procedures

Specific Objective: To optimise the extraction procedure for both raw cereals and processed cereal products. The extraction procedure should be optimised by investigating the composition and pH of the extraction solvent, the extraction time and the sample/solvent ratio.

3.6.1. Optimisation of the extraction

In most methods for the qualitative and quantitative determination of ergot alkaloids in cereals, extraction has been performed with non-polar organic solvents under alkaline conditions or with polar solvents under acidic conditions, as described in 1.5.3. In this work, for the first time, a mixture of acetonitrile + aqueous solvent at a ratio of 84+16 (v/v) (a polar alkaline solvent) was used. This ratio has already successfully been applied in mycotoxin analysis and particularly for trichothecene mycotoxins (Krska *et al.*, 1998).

Based on the chromatographic conditions used by Lehner et al. (2005) ammonium carbonate (200 mg/L, 3.03 mmol; pH = 8.9 ± 0.3., Fluka ref. 74415) was used as aqueous phase of the extraction solvent. 5 g of ground sample were placed in a 60 mL amber sample jar with a PTFE screw cap and extracted for 30 min with 25 mL of the extraction mixture on a horizontal shaker. During preliminary experiments, different cereals (barley, oats, wheat, rye) and several processed food products were spiked and extracted with this extraction mixture. Reasonable recoveries of 80-110 % were obtained for all matrices investigated during the course of this initial experiments. The extraction efficiency of acetonitrile + ammonium carbonate buffer 84+16 (v/v) was compared with acidic MeOH/0.25%H_3PO_4 (40+60) (Ware *et al.*, 2000) and neutral ACN/NH_4Ac (1+2) (Mohamed *et al.*, 2006) at a sample-solvent ratio of 5g + 25 mL and an extraction time of 30 min with three replicate measurements each. Naturally contaminated barley (high level, up to 25 mg/kg ergot alkaloids) and a low level contaminated rye product (rye crispbread, up to 16 µg/kg) were used as commodities in this study. The extracts obtained were only diluted (rye 1:1, barley 1:50) and filtered but no clean-up with PSA (see 3.6.2.) was performed prior to the end determination by LC-MS/MS. Extraction with ACN/NH_4Ac (1+2) required subsequent centrifugation at 15,000 rpm at 4°C for 30 min to enable separation of the sample from the solvent.

Figures 48 and 49 demonstrate that the highest concentrations of the 12 ergot alkaloids tested were found after extraction with acetonitrile + ammonium carbonate buffer 84+16 (v/v) for both matrices, and particularly for the rye sample. To prove that the higher concentrations were the result of higher extraction efficiency rather than the result of matrix effects in mass spectrometry, standard additions at a contamination level of 10 mg/kg ergot alkaloid for the highly contaminated barley were carried out. Reasonable recoveries for all 12 ergot alkaloids of 91-121 % were obtained for these spiked samples and confirmed the high efficiency of the selected extraction mixture.

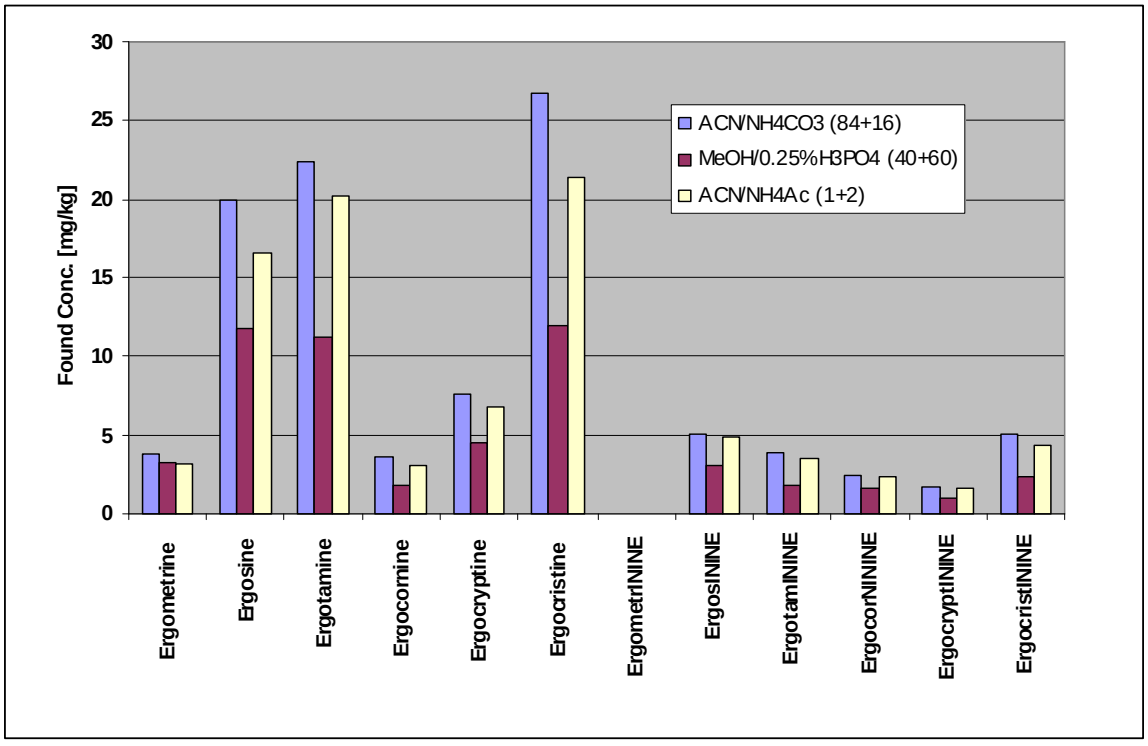


Figure 48 Extraction of ergots from barley (high level) with alkaline, acidic, or neutral solvent mixtures.

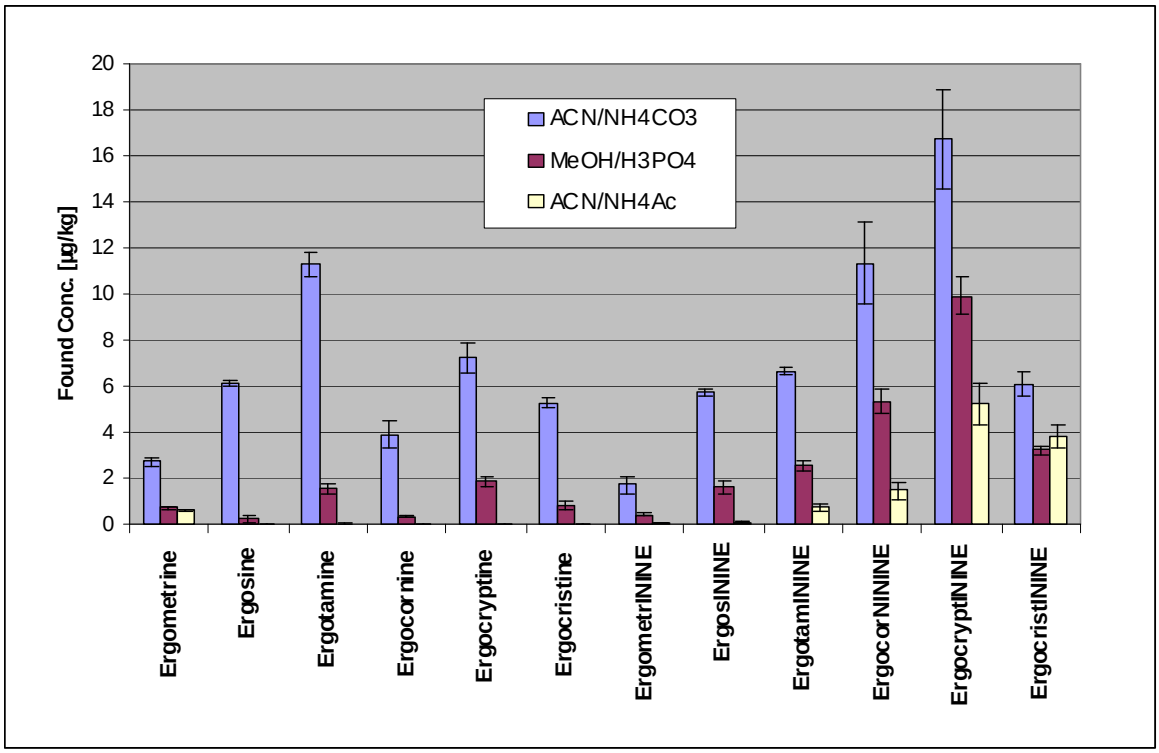


Figure 49 Extraction of ergots from rye (low level) with alkaline, acidic, or neutral solvents.

Comparison of three different sample-solvent ratios (5 g + 15 mL, 5 g + 25 mL and 5 g + 50 mL) and three different extraction times (30, 60 and 90 min) was carried out using the most efficient extraction mixture, acetonitrile + ammonium carbonate buffer 84+16 (v/v). When investigating different sample-solvent ratios, different dilutions of 1:25 for 5 g barley + 25 mL solvent mixture, 1:50 for 5 g + 50 mL and 1:82.5 for 5 g + 15 mL ensured comparable conditions for all three ratios with uniform end dilutions of 1:50. Different sample-solvent ratios may not only influence the efficiency of the extraction but also affect the final MS detection. More concentrated extracts (in case of a higher sample-solvent-ratio) can result in enhanced signal suppression. In order to eliminate this comparable conditions were adjusted through the use of uniform end dilutions for all three sample-solvent-ratios. Only by employing this approach could possible differences in the concentrations measured be clearly identified as resulting from the different sample-solvent ratios used. Again, the extracts obtained were further diluted (rye 1:1, barley 1:50) and filtered but no clean-up was performed prior to end determination by LC-MS/MS.

Figure 50 to 53 clearly show that neither the sample-solvent-ratio nor the extraction times significantly affected the extraction of the 12 ergot alkaloids from the rye and barley samples.

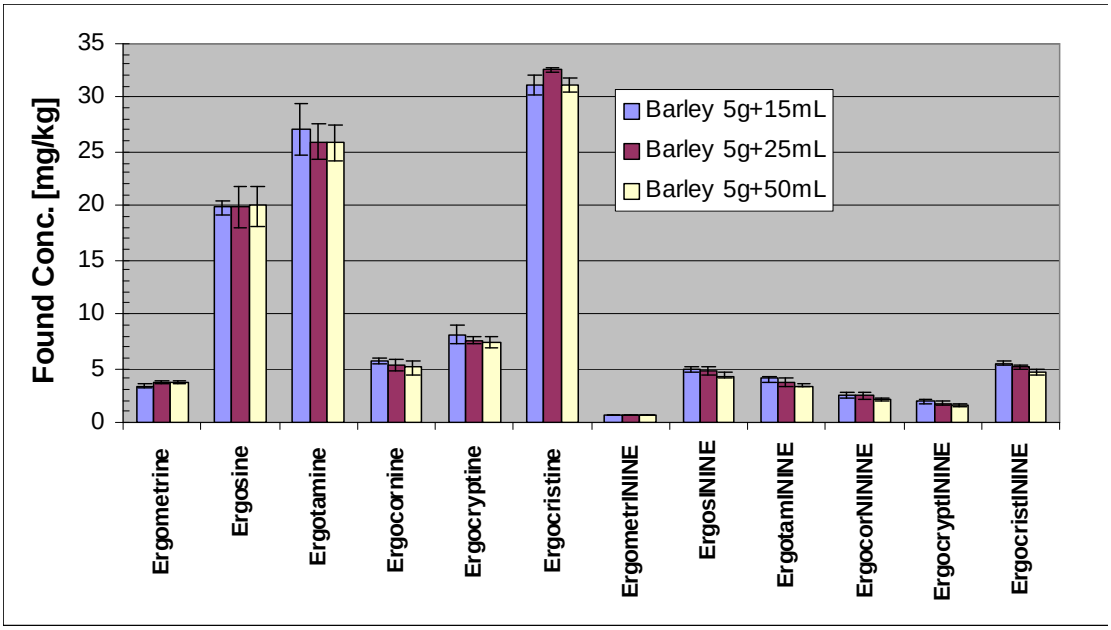


Figure 50 Extraction of naturally contaminated barley with three different sample-solvent mixtures.

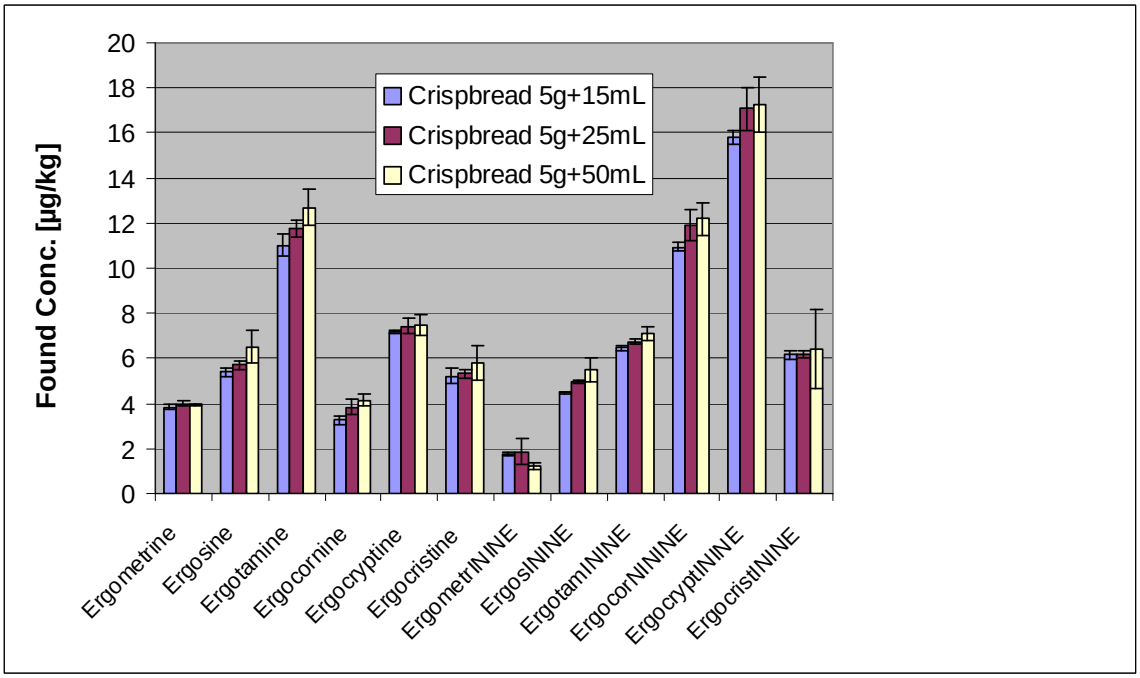


Figure 51 Extraction of naturally contaminated rye (low level) with three different sample-solvent mixtures (5 g + 15 mL, 5 g + 25 mL, 5 g + 25 mL) acetonitrile + ammonium carbonate buffer 84+16 (v/v).

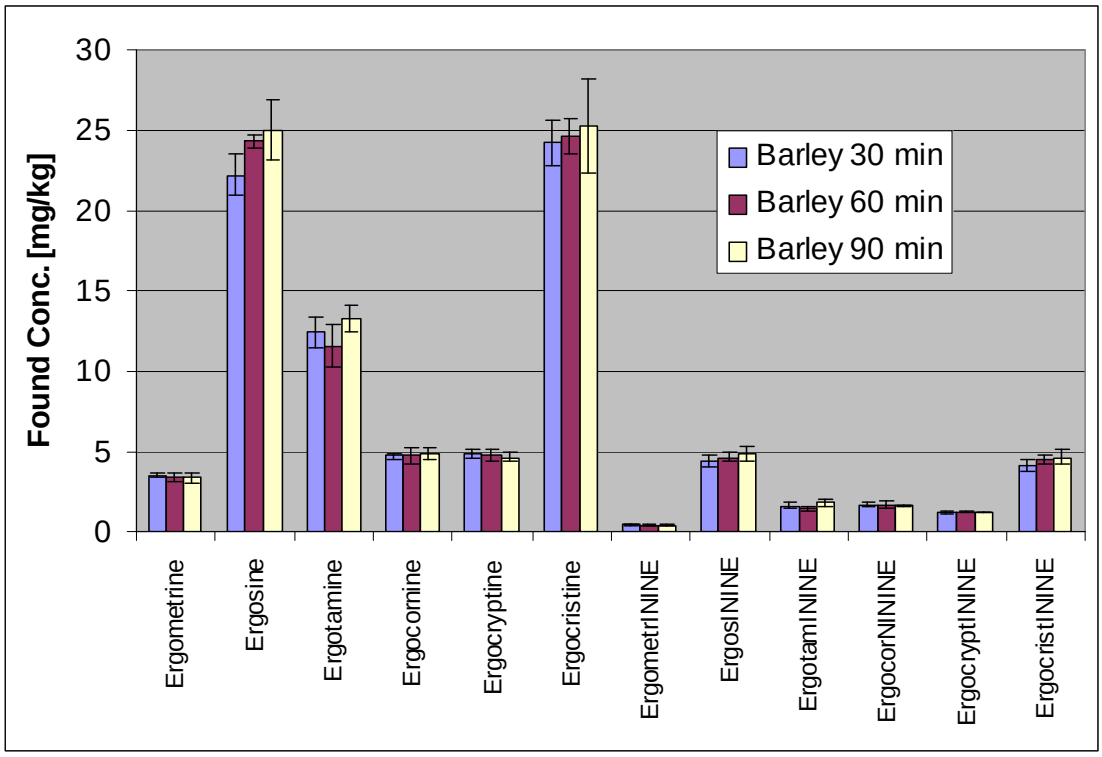


Figure 52 Extraction of naturally contaminated barley (high level) with three different extraction times (30, 60 and 90 min).

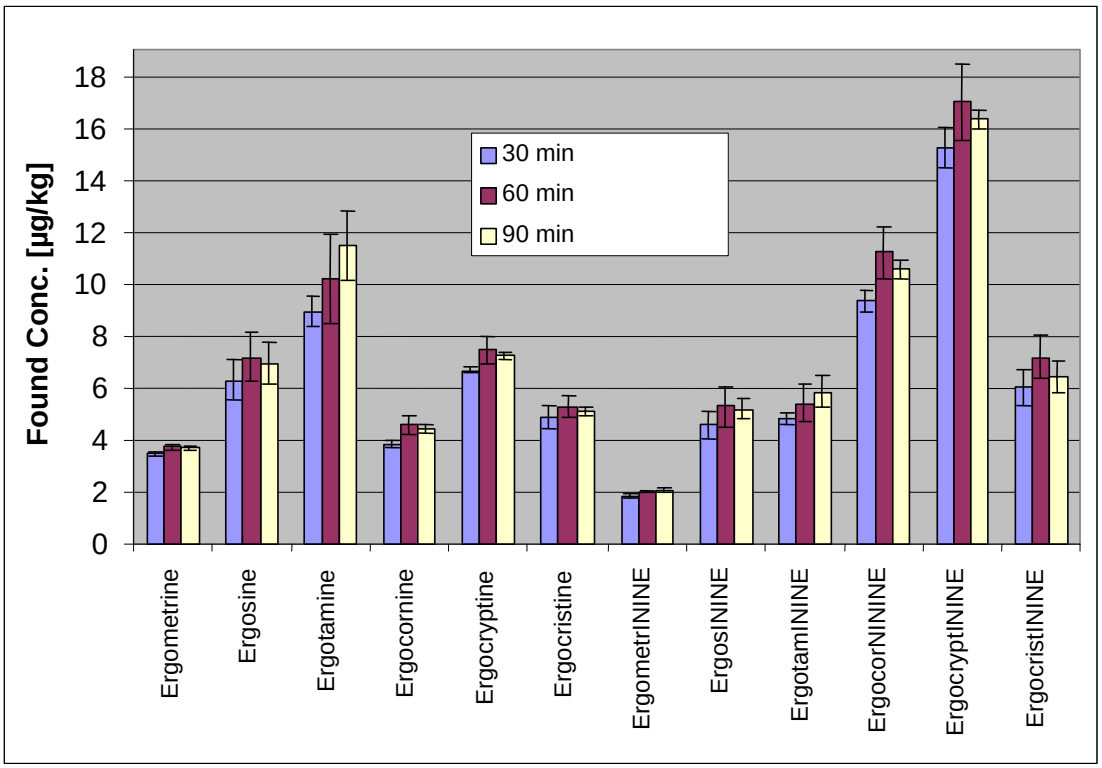


Figure 53 Extraction of naturally contaminated rye (low level) with three different extraction times (30, 60 and 90 min) with acetonitrile + ammonium carbonate buffer 84+16 (v/v).

Based on the results obtained from the optimisation of the extraction procedure the following extraction conditions were chosen for all further analyses: 5 g sample + 25 mL acetonitrile + ammonium carbonate buffer (200 mg/L) 84+16 (v/v), 30 min extraction time.

3.6.2. Optimisation of the clean-up

Sample preparation and especially clean-up procedures have always been the major bottleneck in any analytical procedure for the determination of chemical contaminants in food products. Solid-phase extraction (SPE) has become the most important technique for the simultaneous enrichment and clean-up of the analytes of interest. Common adsorbents including C8, C18, and ion-exchange have been applied for the analyses of various matrices for contaminants and residues. Recently, the availability of dual quality polymeric SPE adsorbents such as hydrophilic–lipophilic balance (HLB) made simultaneous enrichment and clean-up of analytes in biological, environmental, and food matrices possible (Koesukwiwat *et al*., 2007).

In this work, besides HLB and ion-exchange SPE (MCX) columns (van de Streene *et al*., 2006) also a more rapid procedure called dispersive solid-phase extraction was used, in which a primary secondary amine (PSA) sorbent is employed. Dispersive SPE is based on the SPE methodology, but the sorbent is directly added to the extract, vortexed and subsequently filtered or centrifuged. PSA is a weak anion exchanger sorbent with the ability to remove fatty acids, sugars and some other matrix co-extractives that form hydrogen bonds. This procedure omits many time consuming steps such as conditioning or washing, commonly employed in traditional SPE methods. Dispersive SPE using PSA is known from a multiresidue method developed by Anastassiades *et al*. (2003) for pesticide analysis. This method was given the acronymic name QuEChERS that reflected its major advantages procedure (=**qu**ick, **e**asy, **ch**eap, **e**ffective, **r**ugged and **s**afe). In this work, the potential of dispersive SPE using PSA was for the first time studied for the determination of the selected ergot alkaloids.

Initial SPE experiments were mainly carried out with 40 ng/mL calibrants of the 12 ergot alkaloids (corresponding to a concentration of 200 µg/kg ergot alkaloids in cereals) and blank wheat extracts spiked at a level of 200 µg/kg. Hydrophilic-Lipophilic Balance Sorbent reversed-phase sorbent (Oasis®, HLB, 200 mg), mixed-mode cation-exchange and reversed-phase cartridges for bases (Oasis® MCX, 150 mg) and multifunctional Mycosep® columns for Ochratoxin A (#229, Romer Labs) were evaluated. Calibrants containing the six EFSA-ergot alkaloids (ergometrine, ergosine, ergotamine, ergocornine, ergocryptine, ergocristine) were prepared in acetonitrile + ammonium carbonate buffer (200 mg/L) 1+2.

HLB (200 mg) cartridges were pre-conditioned with successively 3 mL methanol and 3 mL ammonium carbonate buffer (200 mg/L). After having loaded 3 mL of the calibrant, the cartridges were washed with successively 3 mL water and 3 mL acetonitrile + water (1+10). Finally, ergot alkaloids were eluted with 6 mL methanol + acetonitrile (1+2). The recovery of each analyte was calculated from the ratios obtained from peak area of these experiments to the one of calibrants at the same concentration injected directly onto the LC-MS/MS. Almost no epimerisation and convenient average recoveries of almost 100% were obtained with the HLB column for all ergot alkaloids except for the most polar ergometrine. The latter was not sufficiently retained on the column and completely eluted already during the washing step with water.

MCX SPE cartridges were pre-conditioned with successively 3 mL methanol and 3 mL ammonium carbonate buffer (200 mg/L). After having loaded 4 mL of the calibrant, the cartridges were washed with successively 4 mL water and 3 mL methanol. Finally, ergot alkaloids were eluted with 6 mL 5% NH_3 in methanol or in acetonitrile, respectively. All ergot alkaloids tested were completely recovered from the column with no matrix interferences during the final MS detection. In order to avoid epimerisation, the applied calibrants were not acidified and thus not positively charged at N-6 which does not favour the retention through the ion-exchange mechanism. Nevertheless, all analytes were retained on the column. This might be due to the slight amount of reversed-phase contribution from the MCX column phase and/or due to some charge which may have been introduced into the molecules even under neutral/slightly alkaline conditions. Final elution under the described alkaline conditions (5% NH_3), however, strongly promoted epimerisation to the *–inines* of up to 27% ergosinine as can be seen from Figure 54. In contrast to that, freshly prepared or properly stored ergot alkaloids in acetonitrile did not show any epimerisation at all (see Figure 55).

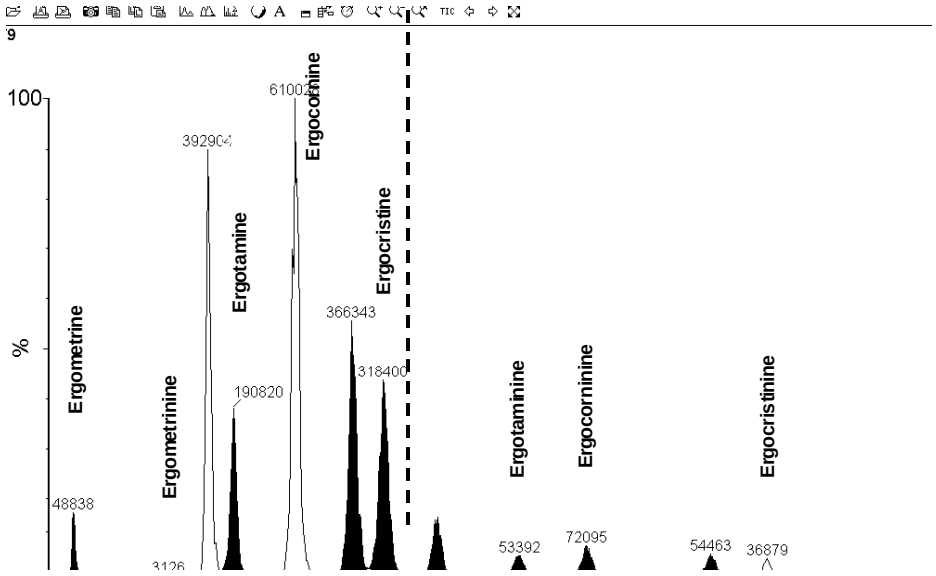
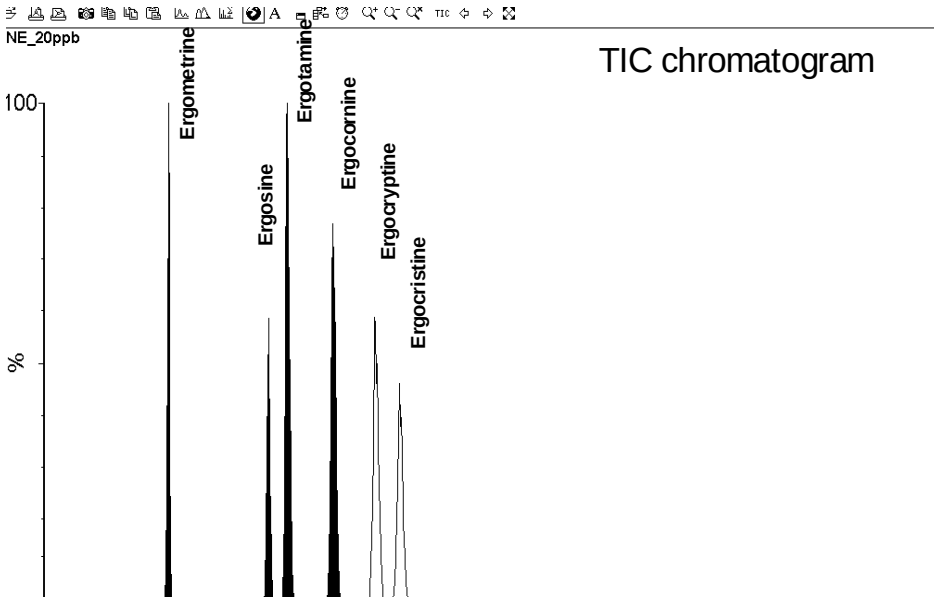


Figure 54 LC-MS/MS TIC chromatogram of ergometrine, ergosine, ergotamine, ergocryptine and ergocristine after elution from Oasis® MCX column.



Note epimerisation of up to 27% for ergosinine in ergosine

Figure 55 LC-MS/MS TIC chromatogram of ergometrine, ergosine, ergotamine, ergocryptine and ergocristine in pure acetonitrile. Note no epimerisation

A one step clean-up using Mycosep® multifunctional columns (#229, Romer Labs) which has been designed for the determination of ochratoxin A in cereals (Buttinger *et al*., 2004) was employed with extracts of ergot alkaloids in acetonitrile + ammonium carbonate buffer (200 mg/L) 84+16 (v/v). However, recoveries of this highly OTA specific column was not more than 50% for the ergot alkaloids tested.

Finally, dispersive SPE using primary secondary amine (PSA) was studied as a rapid one step clean-up for the selected ergot alkaloids. After 30 min extraction of 5 g sample with 25 mL acetonitrile + ammonium carbonate buffer (200 mg/L) 84+16 (v/v) the extract was filtered through Whatman filter Nr. 54 and subsequently subjected to dispersive solid phase extraction. For that purpose 1 mL of the filtered extract were 45 sec vortexed in a 4 mL screw capped amber glass vial containing 50 mg PSA material (Varian Bondesil PSA, 40 µm) and finally filtered through a PTFE syringe filter (Klarity syringe filter, 0.22 µm) prior to LC-MS/MS detection. Although preliminary experiments employing PSA clean-up for the six major ergot alkaloids indicated minor epimerisation of maximum 12 % for ergotamine and ergosine, the validation study (see 3.8.) which also involved the epimers (-inines) of these alkaloids did not reveal any significant epimerisation. Moreover, convenient recoveries at higher precision were obtained with the new PSA clean-up (see scheme in Figure 56). Interestingly, using PSA as normal SPE column material (HF BondElut LRC PSA, 500 mg) gave recoveries significantly higher than 100% during preliminary studies and were rejected for this reason and also for its higher cost compared to the dispersive PSA material.

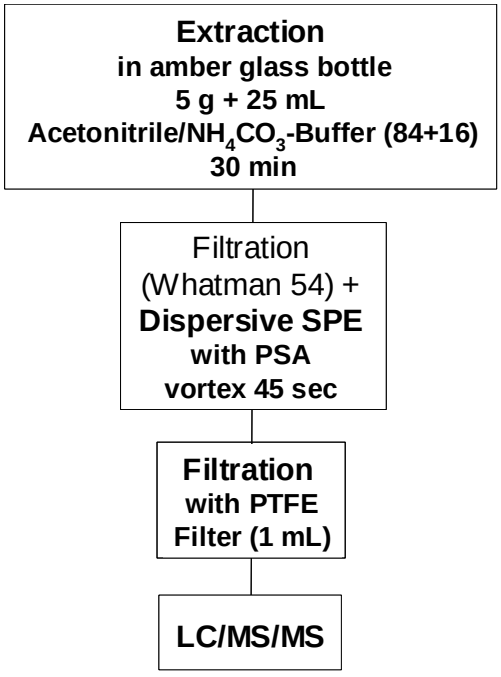


Figure 56 Analytical procedure for the determination of the selected ergot alkaloids with LC-MS/MS after dispersive SPE using PSA.

3.7. Investigation of matrix effects

Specific Objective: To investigate the effect of co-extracted compounds on the detected analytical response for each analyte.

3.7.1. Investigation of signal suppression/enhancement

In order to evaluate the influence of the matrix on the mass spectrometric detection, cleaned (PSA-clean-up, see 3.6.2.) and non-cleaned-up extracts of blank wheat and malted milk biscuits were each spiked at 9 different concentration levels in the µg/L range with a relative concentration of 1:2:4:10:25:50:100:250:500 (each in duplicate) which correspond to concentrations between 5 and 250 µg ergot alkaloid per kg matrix. As zero level the extract of the blank matrix was employed. The resulting linear calibration functions were compared to that of a calibrant containing no matrix. The signal suppression/enhancement (SSE) was calculated according to the following equation:

$$SSE\ (\%) = 100 * slope_{spiked\ extract} / slope_{liquid\ standard}$$

This procedure was used to reveal and to compare the effects/losses arising from ion suppression/enhancement in cleaned and non cleaned-up sample extracts. Figures 57 and 58 show the obtained results for SSE (%) for the spiked extracts of wheat and malt milk biscuit Ideally, a ratio of 1 is obtained which corresponds to an SSE of 100% and indicates no matrix effects over the tested concentration range.

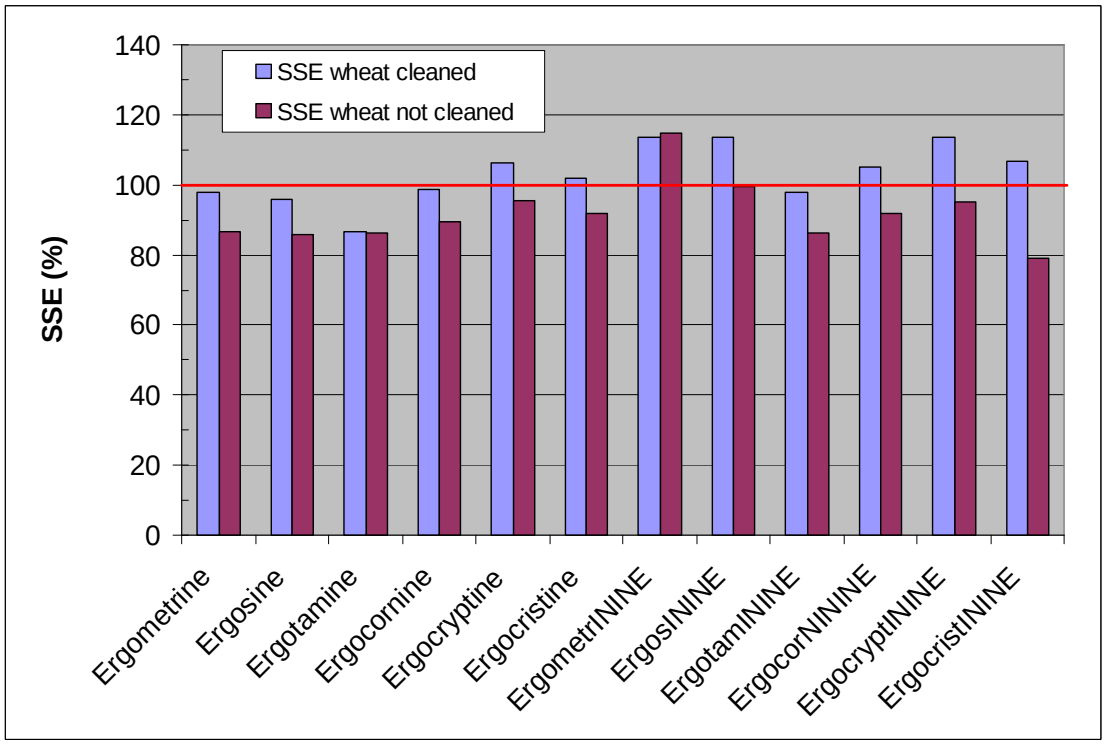


Figure 57 Signal suppression effect (SSE) in wheat extract

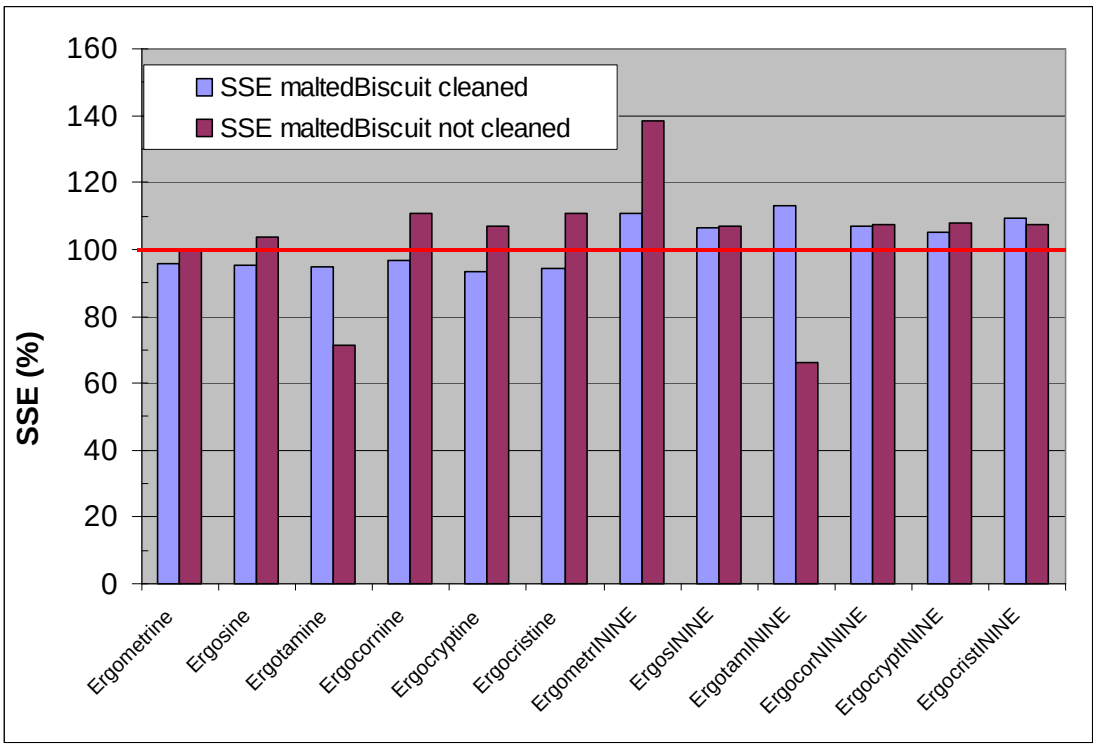


Figure 58 Signal suppression effect (SSE) in malted milk biscuit extract

Neither figure (57 and 58) shows severe matrix effects for any of the 12 ergot alkaloids. However, a tendency towards underestimation of the ergot alkaloid concentration can be observed for both matrices. In case of non-cleaned-up wheat underestimations of 3-21% were

observed (SSE = 80-86%) for seven analytes including ergometrine, ergosine and ergocornine. For PSA cleaned-up samples SSEs were mostly in the range of 100% with slight enhancement effects for ergometrinine, ergosinine and ergocryptinine (SSEs ~110%).

For malted milk biscuits a different picture was observed (see Figure 58): Whereas no significant differences between cleaned and non-cleaned-up extracts were observed for most ergot alkaloids, signal reductions for non-cleaned-up samples of 24% and 47% compared to cleaned-up samples were obtained for ergotamine and ergotaminine, respectively. In contrast to these underestimations, an SSE of 139% was obtained for ergometrinine.

Summarising, it can be concluded that although only minor matrix effects were generally observed, the positive effect of the PSA clean-up was clearly visible through calculations of the SSE for cleaned and non-cleaned-up extracts of wheat and malted milk biscuits.

3.7.2. Quality of chromatographic separation

As already demonstrated in Figures 4 and 54, a good and rapid chromatographic separation with baseline separated peaks was achieved under the described HPLC conditions (see 3.2.). Moreover, no matrix interferences were observed in any of the 10 different food products for which the new LC-MS/MS method was validated (see 3.8.). As an example the ESI(+)LC-MS/MS SRM chromatogram of blank wheat spiked at a level of 5 µg/kg is shown in Figure 59.

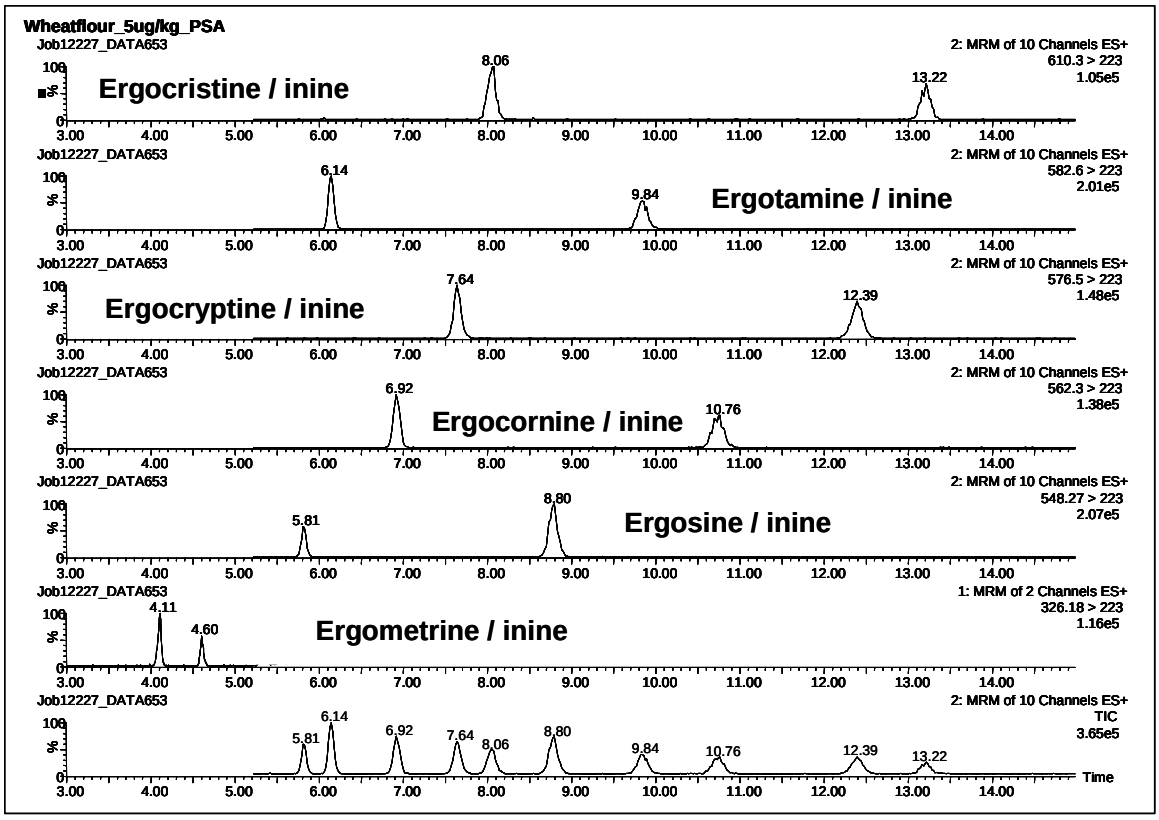


Figure 59LC-ESI-MS/MS SRM chromatogram of blank wheat spiked at 5 µg/kg

During SRM of the transition *m/z* 576=>223 for α–ergocristine (Rt=7.64 min) an additional peak occurred at Rt=7.92 min in case of low level naturally contaminated rye samples. We explain this peak with the presence of the ergocryptine β-isomer (Müller *et al*., 2006), for which, however, no standard is available. Figure 60 shows an LC-ESI-MS/MS SRM chromatogram of this particular transition obtained from naturally contaminated rye flour, rye crisp bread and multi grain crackers and from an ergocryptine/inine calibrant. A similar chromatogram is shown in Figure 61 for all five EFSA ergopeptides and their epimers in naturally contaminated rye crisp bread. Despite the close elution of both isomers, Figures 60 and 61 clearly demonstrate the good separation even of these similar analytes.

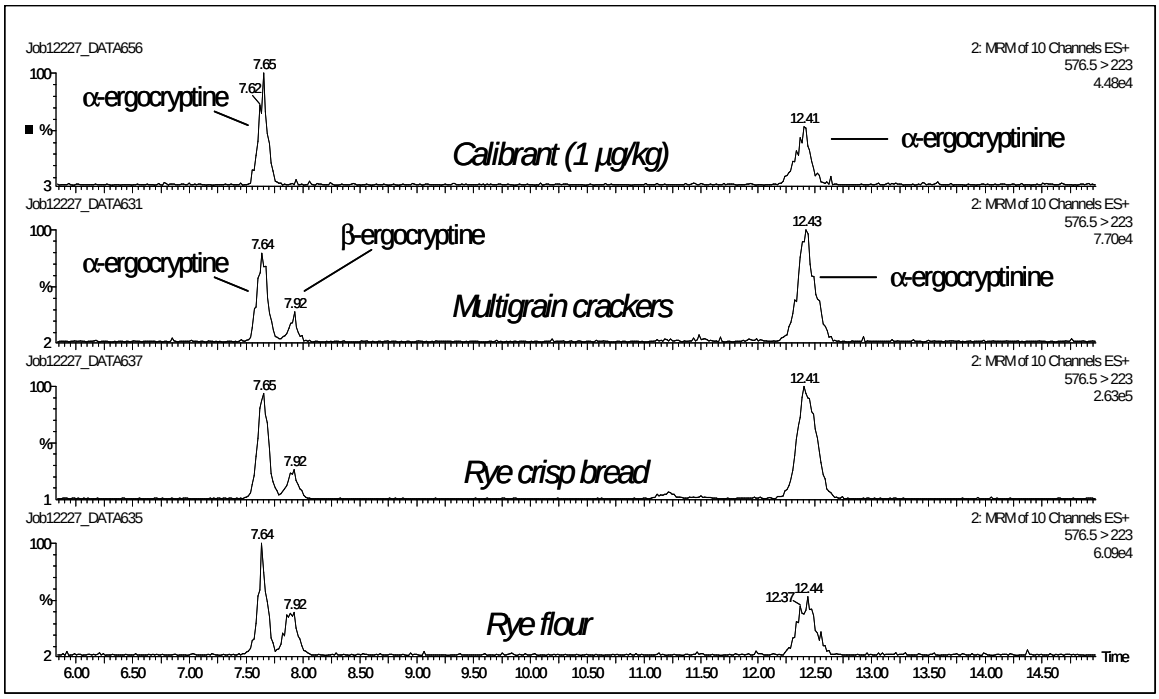


Figure 60 Ergocryptine β-isomer in calibrant and naturally contaminated samples.

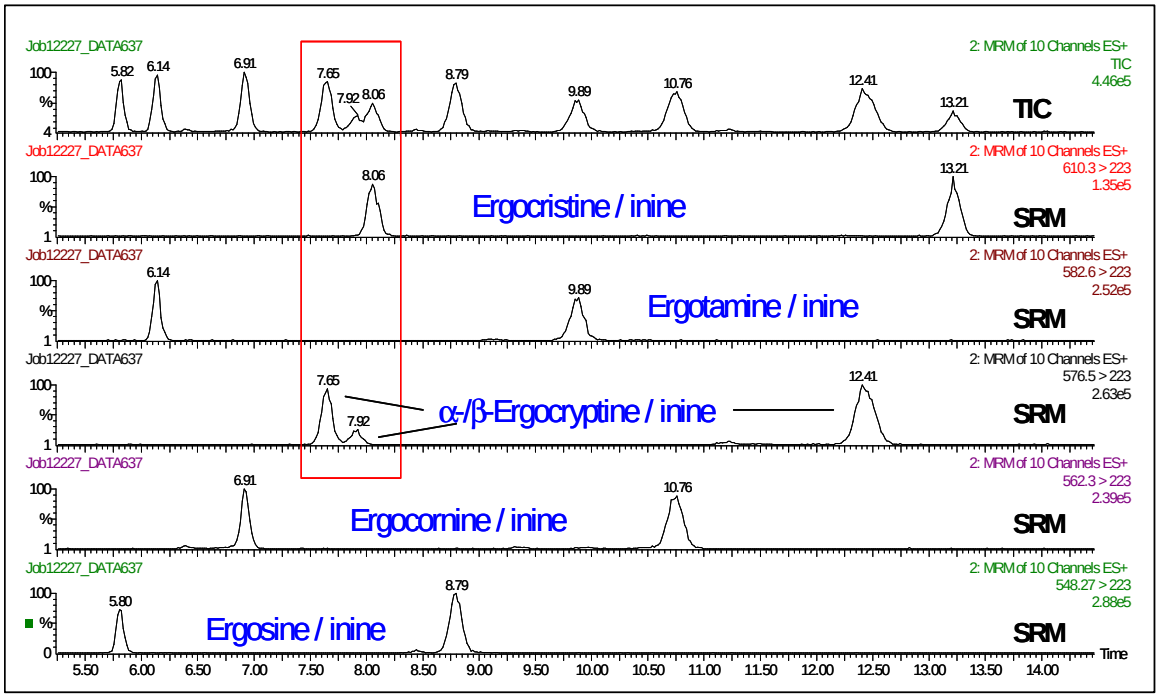


Figure 61 LC-ESI-MS/MS SRM chromatogram of (α-/β-)ergocryptine/inine in naturally contaminated rye samples

In Figure 61, an overlap of β-ergocryptine and ergocristine can be recognised in the TIC (upper chromatogram of Figure 61). However, when the chromatograms of the SRM transitions are displayed separately, α- and β-ergocryptine and ergocristine are well resolved.

3.8. Validation of HPLC-MS/MS method for the analysis of ergot alkaloids

Specific Objective: To validate the HPLC-MS/MS method developed, recording precision, recovery, selectivity, robustness, LOD, LOQ and measurement uncertainty.

3.8.1. Calibrants and chemicals

Crystalline ergot alkaloids were purchased from Prof. Miroslav Flieger, Laboratory of Physiology and Genetics of Fungi of the Institute of Microbiology, Academy of Sciences of the Czech Republic (flieger@kav.cas.cz). Acetonitrile and water (fluorescence grade) were supplied by Fisher Scientific (UK). Ammonium carbonate (p.a. for HPLC) was obtained from Fluka (UK).

Individual stock solutions of six ergot alkaloids, ergocristine, ergotamine, ergocornine, ergosine, α-ergocryptine, ergometrine (as ergometrine hydrogenmaleate) and their corresponding six epimers ergocristinine, ergotaminine, ergocorninine, ergosinine, ergocryptinine and ergometrinine in acetonitrile were prepared at levels of 50-200 µg/mL. From these individual stock solutions mixed calibrants of all 12 ergot alkaloids were freshly prepared through dilution with acetonitrile at levels of 0.1, 0.2, 1, 2, 4, 10, 20 and 40 ng/mL These concentrations correspond to concentrations of the individual ergot alkaloid in the sample between 0.5 and 200 µg/kg. All solutions were stored in dark brown glass vials in a freezer at -24°C in darkness to prevent any isomerisation problems.

3.8.2. Spiking

Ground samples (5 g) were spiked by adding 25, 250 and 500 µL, respectively, of a 1 µg/mL mixed calibrant containing all 12 ergot alkaloids (3.8.1.) under dim conditions. To avoid epimerisation the spiked samples were not stored over night but extracted approximately 20 min after the spiking.

3.8.3. Extraction and clean-up

The calibrants described in 3.8.1. were analysed in duplicate for external calibrations during validation and analysis of naturally contaminated samples. As described in 3.6.2. dispersive SPE using primary secondary amine (PSA) was used as a rapid one step clean-up for the selected ergot alkaloids. After 30 min extraction of 5 g ground sample with 25 mL acetonitrile + ammonium carbonate buffer (200 mg/L) 84+16 (v/v) the extract was filtered through Whatman filter Nr. 54 and subsequently subjected to dispersive solid phase extraction. For

that purpose 1 mL of the filtered extract were 45 sec vortexed in a 4 mL screw capped amber glass vial containing 50 mg PSA material (Varian Bondesil PSA, 40 µm) and finally filtered through a PTFE syringe filter (Klarity syringe filter, 0.22 µm) prior to injection of 10 µl into the LC-MS/MS system (for details see SOP in Annex 3).

3.8.4. Validation design

Validation was carried out for 10 different matrices on 6 different days. The selected matrices (samples) were obtained from supermarkets and local farmers around York and included 1 baby food, 4 types of cereals and 5 processed foods (see Table 12). Due to the enormous effort which would have been required for a full validation for 10 different matrices on 6 different days, a special analytical scheme was developed: In total 4 different concentration levels were covered by this validation plan: (5 µg/kg, 50 and 100 µg of each of the 12 ergot alkaloids per kg sample plus very low level = blank samples). On each of the 6 independent validation days 5 of the matrices were analysed at levels 5 and 100 µg/kg and 5 of the matrices at the levels "blank" and 50 µg/kg, respectively. Thus, during the whole validation period of 6 days, 120 measurements (20 on each day) were carried out at 4 different concentration levels. In Table 12 the scheme for the measurements on day 1 is presented as an example. Fuller details are provided in Annex 4.

Ergot alkaloids were considered as positively identified in the samples when the following criteria were met: i) the chromatographic retention time of the analyte corresponded to that of the calibrants within a ±2% tolerance; (ii) the presence of a signal was identified at each of the two diagnostic transition reactions (quantifier and qualifier ion, see 3.3.3.) and (iii) the peak area ratio from these two channels was within the tolerance of ±20% which was set out in 2002/657/EC of the mean ratio from the calibrants. To be acceptable for quantification the residuals at each concentration for each calibrant channel must not exceed 30 % and the coefficient of determination (r2) must be ≥ 0.99.

Calibration curves for each analyte were constructed by plotting the analyte concentration versus the signal intensity (area) of the analyte using the Micromass MassLynx version 4.0 software.

Table 12. Validation scheme and the classification of the 10 different matrices

Matrix	Concentration	Classification
Oat and bran flakes	50	Processed food (oats)
Pearl barley	50	Cereal (barley)
Rye flour	100	Cereal (rye)
Rusk biscuits	100	Baby food (wheat)
Malted milk biscuits	100	Processed food (wheat, malt)
Oat meal	Blank	Cereal (oats)
Oat meal	50	Cereal (oats)
Rye crispbread A	Blank	Processed food (rye)
Wheat flour	Blank	Cereal (wheat)
Multigrain crackers	5	Processed food (wheat, barley)
Rye flour	5	Cereal (rye)
Rusk biscuits	5	Baby food (wheat)
Pearl barley	Blank	Cereal (barley)
Rye crispbread B	5	Processed food (rye)
Rye crispbread B	100	Processed food (rye)
Rye crispbread A	50	Processed food (rye)
Multigrain crackers	100	Processed food (wheat, barley)
Oat and bran flakes	Blank	Processed food (oats)
Malted milk biscuits	5	Processed food (wheat, malt)
Wheat flour	50	Cereal (wheat)

Whereas the matrices “oatbran flakes”, “pearl barley”, “oat meal” and “wheat flour” contained ergot alkaloids at levels lower than the LOD of the method, the samples “multigrain crackers”, “rye flour” “rye crispbread A”, “rye crispbread B” and “baby rusk biscuits” contained levels of up to 5 µg/kg. In case of “malted milk biscuit” ergosine was present at a concentration of 21 µg/kg with a sum of ergot alkaloids of 95 µg/kg as shown in Figure 62.

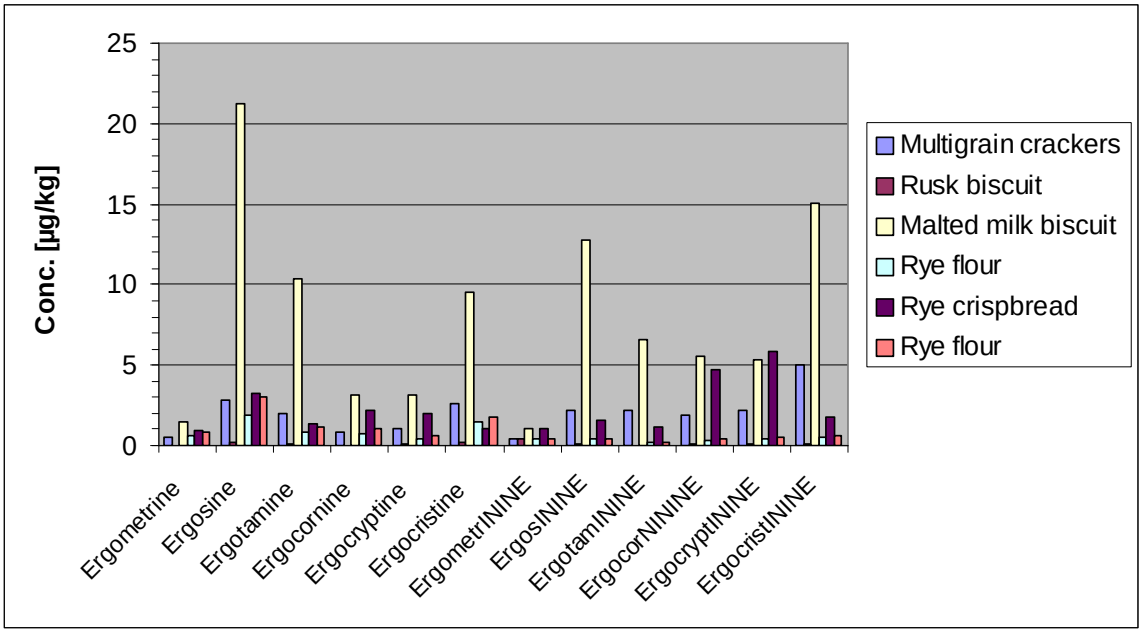


Figure 62 Levels of ergot alkaloids in the "blank" samples (matrices) used during validation

3.8.5. Validation results

Performance of the method was assessed by estimating the relation between analyte concentration and standard uncertainty. Hence the relation between analyte concentration and expanded uncertainty and estimates of limit of detection and limit of quantification were derived for each analyte.

3.8.5.1. Calibration results

Calibration curves for all analytes were linear over the working range of 0.1 – 40 ng /mL which corresponds to a concentration of 0.5 - 200 µg ergot alkaloid per kg matrix. Squared correlation coefficients (r^2) for the eight point calibration curves were 0.9973 (Ergometrine), 0.9985 (Ergosine), 0.9979 (Ergotamine), 0.9982 (Ergocornine), 0.9973 (Ergocryptine), 0.9982 (Ergocristine) and 0.9984 (Ergometrinine), 0.9956 (Ergosinine), 0.9905 (Ergotaminine), 0.9888 (Ergocorninine), 0.9894 (Ergocryptinine), 0.9905 (Ergocristinine).

3.8.5.2. Recoveries

Figure 63 depicts the mean recoveries obtained for each of the 12 ergot alkaloids in the 10 different matrices at the spiking levels 5, 50 and 100 µg/kg. 85 out of 90 recovery measurements were between 70 and 105%.

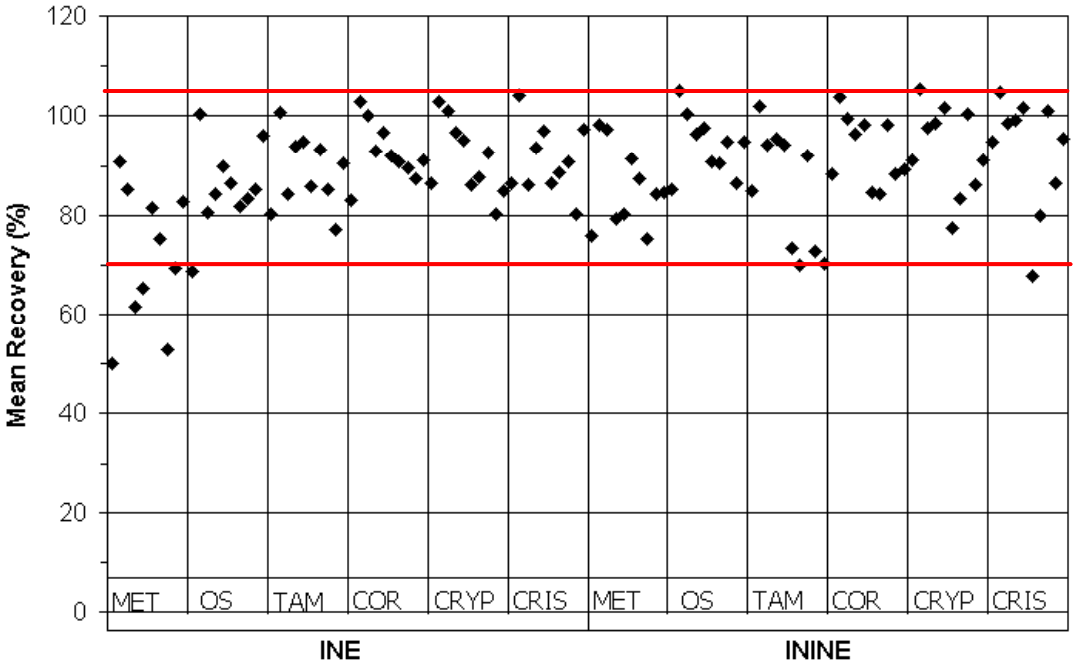


Figure 63 Mean recoveries obtained for each of the12 ergot alkaloids in the 10 different matrices.

The red lines indicate recovery range between 70 and 105%

The spiking levels were 5, 50 and 100 µg/kg. In each "ergot alkaloid column" of the figure each point represents the recovery achieved in one of the 10 matrices over 6 different days (n=6). (MET = ergometrine/inine, OS=ergosine/inine, TAM=ergotamine/inine, COR=ergocornine/inine, CRYPT=ergocryptine/inine, CRIS=ergocristine/inine). No trend in recovery for different spiking levels was observed during preliminary calculations. Therefore for clarity the mean recovery shown was obtained from a pool of data for the three concentration levels used with each of the 10 different matrices.

Only five recovery results - four for ergometrine and one for ergocristinine - were below 70% with only two values for ergometrine in rye crisp bread lower than 60% as shown in Table 13.

For a few matrices the recoveries for ergometrine and ergometrinine were comparatively low and the expanded uncertainty high. Despite this the LOQs for ergometrinine were surprisingly good. Further research would be needed to provide an no explanation for this.

Table 13. Five recovery results for which recoveries of less than 70% were obtained

Sample	Analyte	Recovery %
Rye Crispbread A	Ergometrine	50
Rye Crispbread A	Ergometrine	53
Multigrain crackers	Ergometrine	62
Oat and bran flakes	Ergometrine	65
Oatmeal	Ergocristinine	68

3.8.5.2. Limit of Quantification (LOQ)

The LOQ is the smallest amount of analyte in a test sample that can be quantitatively determined with suitable precision and accuracy under previously established method conditions (AOAC 2006). Given a fitness for purpose criterion that the relative standard uncertainty associated with results should be less than RSU_{MAX}, (relative standard uncertainty encompasses precision and accuracy), and the relation between concentration and standard uncertainty shown in Equation 1, a limit of quantification (LOQ) is given by:

$$LOQ = \sqrt{\frac{u_0^2}{RSU_{MAX}^2 - RSU^2}}$$

Figure 64 shows estimates of LOQ where the maximum acceptable standard uncertainty is equal to 0.25.

The limit of detection is defined as the lowest concentration that will be detected with probability 1-β given a false positive rate α (ISO 1997), for α=β=0.025

Table 2 in Annex 4 shows all LOQs and LODs achieved for the individual ergot alkaloids. Convenient LOQs between 0.17 and 2.78 µg/kg were obtained dependent on the ergot alkaloid and the matrix with LODs almost equalling the LOQs. A summary of all LOQs is depicted in Figure 64, which demonstrates that the achieved LOQ is dependent on the analyte but almost independent from the matrix. This also proves the validity of the approach of pooling 10 different matrices for all 12 ergot alkaloids during the validation study.

In many cases LOQ is close to or equal to LOD. This means that results with sufficiently low uncertainty can be produced for all concentrations down to the point where they are censored

and reported as ‘not detected’. This is a feature of trace analyses where the upper limit for fit for purpose relative standard uncertainties is relatively large (>20%).

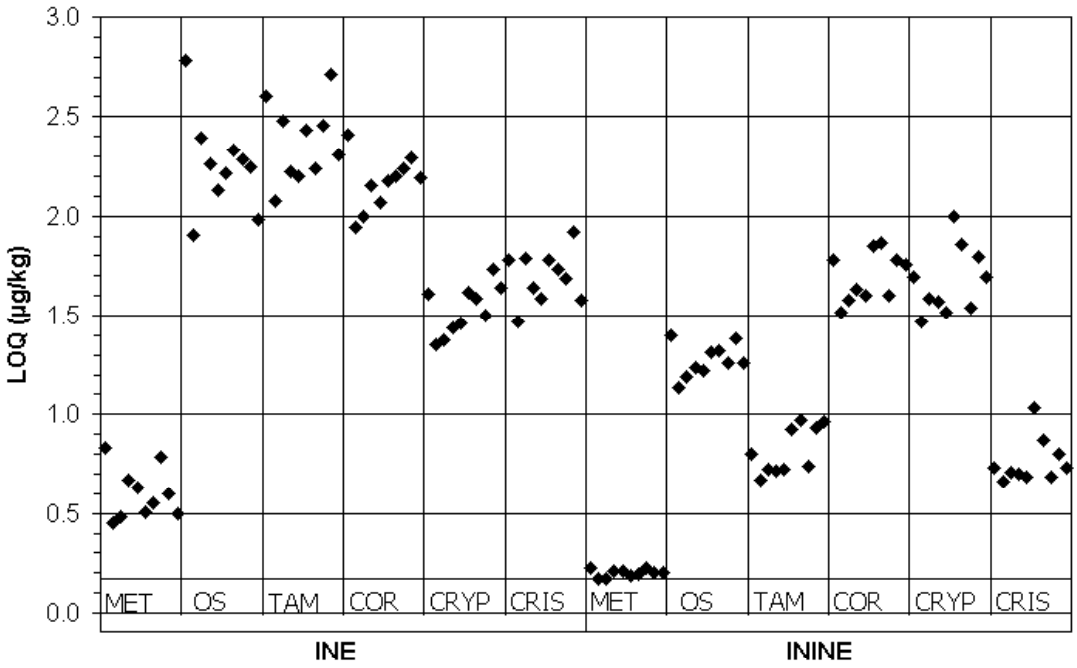


Figure 64 Limits of Quantification (LOQs) for each of the 12 ergot alkaloids in the 10 different matrices.

In each “ergot alkaloid column” of the figure each point represents the LOQ obtained in one of the 10 matrices. (MET = ergometrine/inine, OS=ergosine/inine, TAM=ergotamine/inine, COR=ergocornine/inine, CRYPT=ergocryptine/inine, CRIS=ergocristine/inine)

Estimates of s_0, RSD, and uncertainty are described in detail in Annex 4.

Table 14 shows the lowest and highest LOQs which were obtained for the *–ine* ergot alkaloids which range from 0.45 µg/kg (ergometrine in wheat rusk biscuits) to 2.78 µg/kg (ergosine in rye crisp bread).

Table 14 Lowest and highest limits of Quantification (LOQs) for the *–ine* alkaloids

Ergot	Sample	Matrix	LoQ ug/kg
Ergometrine	Rusk biscuit	Processed wheat	0.45
Ergometrine	Wheat flour	Cereal (wheat)	0.50
Ergotamine	Rye flour	Cereal (rye)	2.71
Ergosine	Rye crispbread	Processed rye	2.78

Table 15 shows the lowest and highest LOQs which were obtained for the *–inine* ergot alkaloids which range from 0.17 µg/kg (ergometrine in wheat rusk biscuits) to 2.00 µg/kg (ergocryptinine in oatmeal).

Table 15 Limits of Quantification (LOQs) for the *–inine* ergot alkaloids

Ergot	Sample	Matrix	LoQ ug/kg
Ergometrinine	Rusk biscuit	Processed wheat	0.17
Ergometrinine	Oatmeal	Cereal (oats)	0.18
Ergocorninine	Rye crispbread	Processed rye	1.78
Ergocryptinine	Oatmeal	Cereal (oats)	2.00

The LC/ESI(+)-MS/MS SRM chromatograms after PSA clean-up obtained from wheat spiked at a level of 1 µg/kg and rye spiked at a level of 5 µg/kg for each of the 12 ergot alkaloids are depicted in Figures 65 and 66. These Figures underline the performance of the developed method, i.e. excellent chromatographic separation in less than 14 minutes combined with high sensitivity.

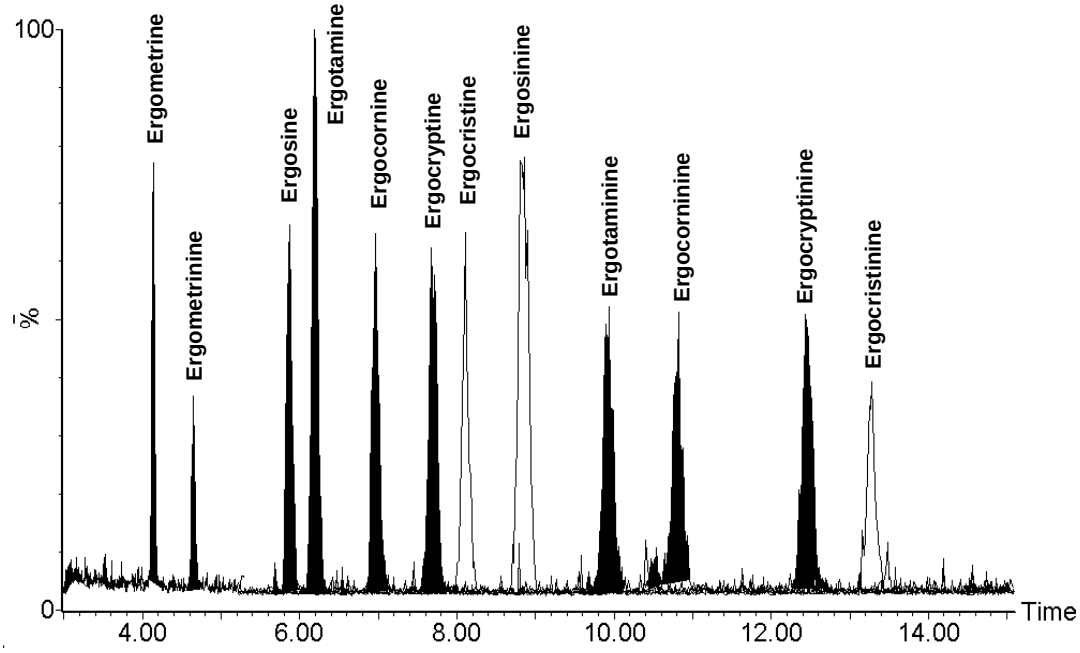


Figure 65 LC/ESI(+)-MS/MS overlaid SRM chromatograms of 12 quantifier transitions (MH+=>223) at a spiking level of 1 µg/kg in wheat for each of the 12 ergot alkaloids.

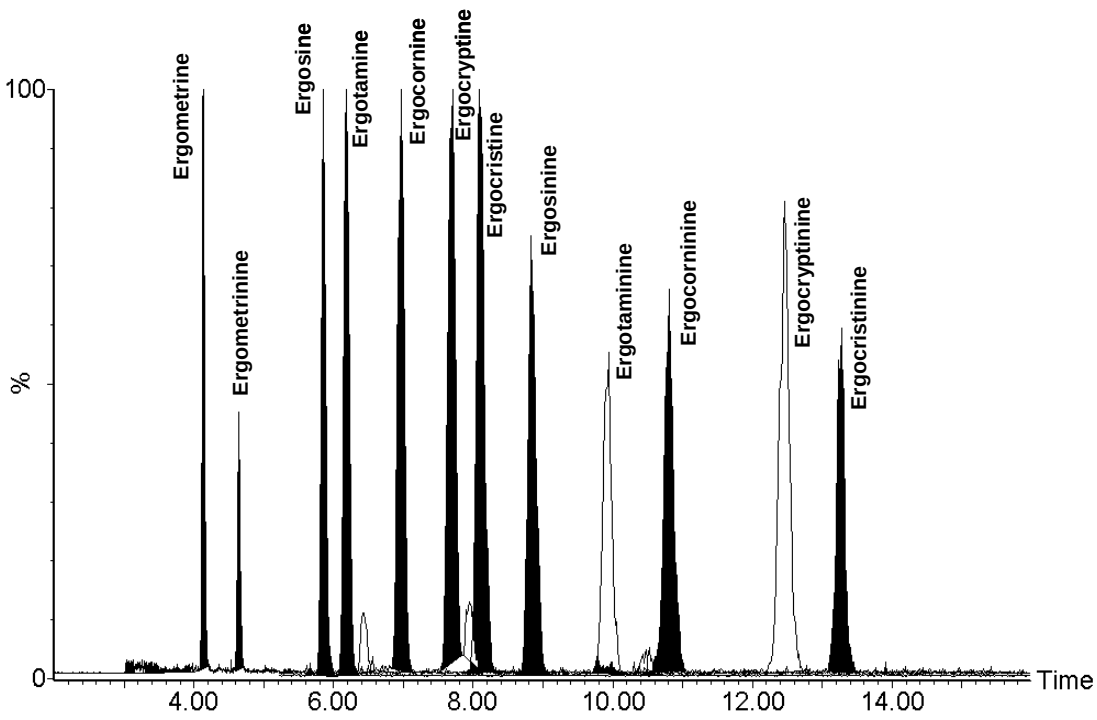


Figure 66 LC/ESI(+)-MS/MS overlaid SRM chromatograms of 12 quantifier transitions (MH+=>223) at a spiking level of 5 µg/kg in rye for each of the 12 ergot alkaloids

3.8.5.3 Instrumental limit of quantification

Based on the often applied definition of LOD and LOQ as the concentration for which signal-to-noise-ratios (S/N) of 3 and 10 respectively are achieved, the desired instrumental LOQ of 1µg/kg was achieved for all ergot alkaloids. This is demonstrated in Figure 67 for the two analytes for which the weakest signals were obtained, i.e. ergometrinine and ergocristinine (compare also with Figure 65) at a spiking level of 1 µg/kg in wheat. In both cases a S/N-ratio of greater than 10 was achieved. This estimate of instrumental LOQ does not reflect the performance of the whole method, which is illustrated in Figure 64.

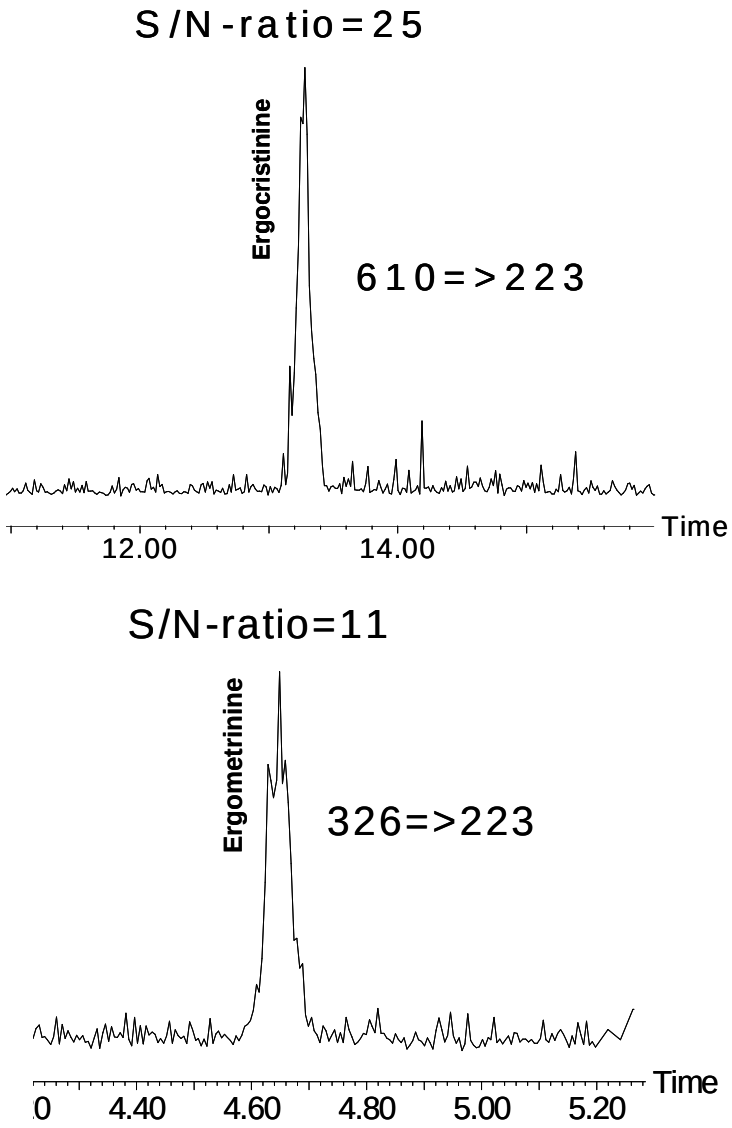


Figure 67 LC/ESI(+)-MS/MS SRM chromatogram showing S:N ratios for ergocristinine (top) and ergometrinine (bottom) spiked at1 µg/kg in wheat

3.8.5.4. Standard uncertainty and expanded uncertainty

A linear regression of within-product standard deviation associated with each alkaloid against concentration was used to gain estimates of variation at low concentrations (expressed as a standard deviation (s0) and variation at high concentrations (expressed as a relative standard deviation RSD). S0 was used to estimate standard uncertainty at low concentrations (u0). An estimate of the relative standard uncertainty associated with results of the measurement of high concentrations of alkaloids (RSU) was gained by combining RSD for each alkaloid with the uncertainty associated with the purity of standards (see 3.4.) and the uncertainty associated with the mean recovery ($\overline{R}$) of each alkaloid in each product.

Table 2 in Annex 4 shows estimates of s0, RSD, recovery, uncertainty associated with standard purity, uncertainty associated with mean recovery, and estimates of u0 and RSU.

An estimate of the standard uncertainty u(x) associated with a particular measurement result x is given by:

$$u(x) = \sqrt{u_0^2 + x^2 \times RSU^2}$$ Equation 1 (Eurachem 2000)

An estimate of the expanded uncertainty U(x) equivalent to a confidence interval of approximately 95% is given by

$$U(x) = 2 \times u(x)$$ (Eurachem 2000)

For example given a measurement result of 0.80 µg kg-1 ergometrine in product 1 (rye crispbread), the standard uncertainty is given by:

$$u(x) = \sqrt{0.190^2 + 0.80^2 \times 0.098^2} = 0.209$$ µg/kg

Hence the expanded uncertainty is equal to 0.418 µg/kg, and the concentration of ergometrine should be reported as 0.80±0.42 µg/kg

For the same analyte in the same matrix giving a measurement result of 8.0 µg/kg

$$u(x) = \sqrt{0.190^2 + 8^2 \times 0.098^2} = 0.807$$

Hence the expanded uncertainty is equal to 1.6 µg/kg, and the concentration of ergometrine should be reported as 8.00±1.6 µg/kg

A summary of the calculated measurement uncertainties is depicted in Figures 68 and 69 which shows that the observed measurement uncertainties are dependent on the analyte but almost independent of the matrix.

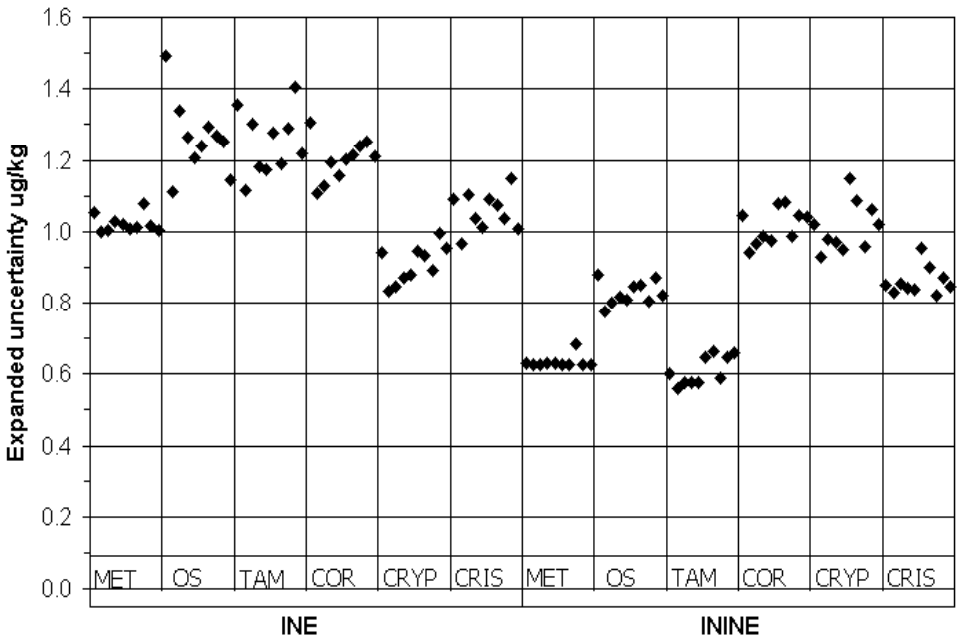


Figure 68 Expanded measurement uncertainties for each of the 12 ergot alkaloids in the 10 different matrices at 5 µg/kg.

In each "ergot alkaloid column" of the figure each point represents the expanded measurement uncertainty obtained in one of the 10 matrices. (MET = ergometrine/inine, OS = ergosine/inine, TAM = ergotamine/inine, COR = ergocornine/inine, CRYPT = ergocryptine /inine, CRIS=ergocristine/inine).

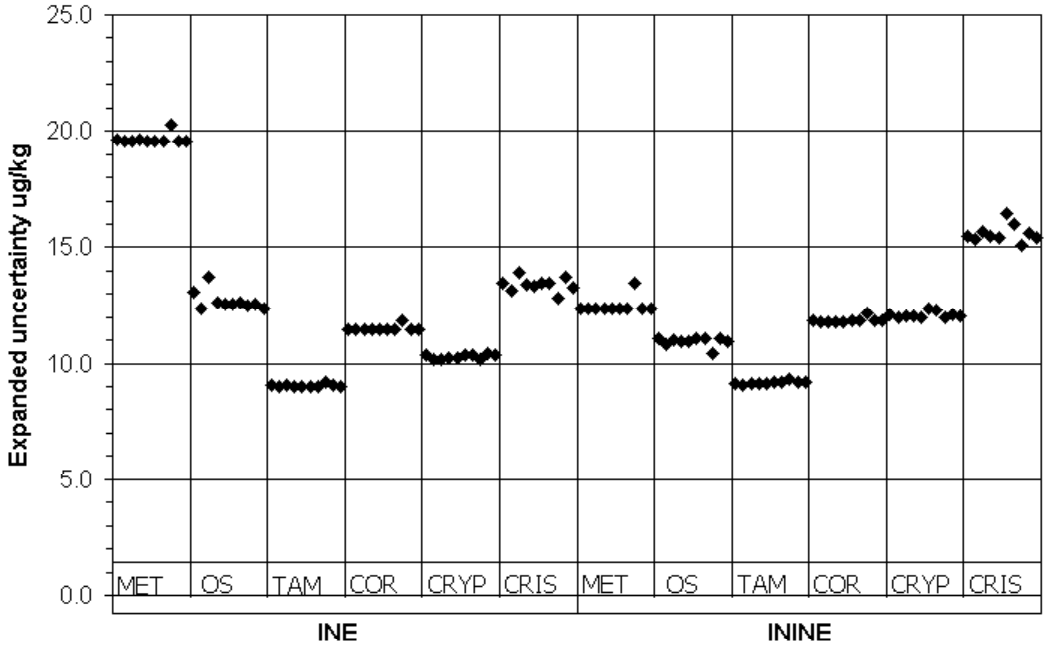


Figure 69 Expanded measurement uncertainties for each of the 12 ergot alkaloids in the 10 different matrices at 100 µg/kg.

In each "ergot alkaloid column" of the figure each point represents the expanded measurement uncertainty obtained in one of the 10 matrices. (MET = ergometrine/inine, OS = ergosine/inine, TAM = ergotamine/inine, COR = ergocornine/inine, CRYPT = ergocryptine /inine, CRIS = ergocristine/inine).

In Tables 16 and 17 the lowest and highest expanded uncertainties for the six –*ine* and six –*inine* – alkaloids, respectively, are summarised for the concentration levels 5 and 100 µg/kg. At a concentration level of 5 µg/kg the expanded measurement uncertainty can thus range from (5 ± 0.56)µg/kg (for ergotaminine in wheat rusk biscuits) to (5 ± 1.49)µg/kg (for ergosine in rye crispbread). Likewise, at a concentration level of 100 µg/kg very convenient measurement uncertainties from (100 ± 8.9)µg/kg (for ergotamine in wheat rusk biscuits) and (100 ± 20)µg/kg (for ergometrine in rye crispbread) were obtained.

Table 16 Lowest and highest expanded uncertainties at 5 µg/kg.

Ergot	Product	Matrix	U(5) ug/kg
Ergotaminine	Rusk biscuit	Processed wheat	0.56
Ergocryptine	Rusk biscuit	Processed wheat	0.83
Ergocryptinine	Oatmeal	Cereal (oats)	1.15
Ergosine	Rye crispbread	Processed rye	1.49

Table 17 Lowest and highest expanded uncertainties at 100 µg/kg.

Ergot	Product	Matrix	U(5) ug/kg
Ergotamine	Rusk biscuit	Processed wheat	8.9
Ergotaminine	Rusk biscuit	Processed wheat	9.0
Ergocristinine	Oatmeal	Cereal (oats)	16
Ergometrine	Rye crispbread	Processed rye	20

3.8.6. Comparability

3.8.6.1. Participants and samples

To check the comparability of the measurement results obtained with the newly developed method between laboratories and with an alternative procedure, a mini-intercomparison study between three laboratories from UK (CSL) = participant 1, Austria (IFA-Tulln) = participant 2 and Germany = participant 3 was organised in June 2007.

Three samples were analysed by the three participants. They were a high level barley containing an estimated level of 0.5 – 50 mg/kg ergot alkaloids, a mixture of this barley with wheat giving a low-level sample containing an estimated 5 – 500 µg/kg ergot alkaloids, and a low level sample of rye flour that contained less than 50 µg/kg ergot alkaloids.

3.8.6.2. Methods employed

Participant 1 (CSL) employed the new method described in this report.

Participant 2 (IFA-Tulln) used an almost identical method with the same extraction solvent but with no clean-up stage and a different manufacturer's LC-MS/MS instrument. Samples (10g) were extracted with 40 ml mobile phase solvent (acetonitrile/ammonium carbonate

buffer (200 mg/L) 84+16). The column was a Phenomenex Gemini 150 x 4,6 mm; 5µm particle size, and the mass spectrometer was an Applied Biosystems QTrap 4000.

Participant 3 used a different method based on Müller *et al.* (2006) which employed a completely different extraction solvent (Scott, *et al*., 1992) using a chlorinated solvent and final separation and detection by HPLC-FLD. The use of a different extraction solvent by participant 3 was an ideal way to reveal potential systematic errors.

The method employed by participant 3 was to extract 20 g sample with 100 ml dichloromethane/ethyl acetate/methanol/ammonia (conc.) 50:25:5:1 (v:v:v:v). 12 ml extract were cleaned up using a basic Al2O3 SPE column. The eluate was evaporated and redissolved in 5 ml acetonitrile/water (50:50). The HPLC system used a Phenomenex Gemini, 5 µm 250-4 column with a precolumn and a mobile phase of acetonitrile/water containing ammonium carbonate. The detector was FLD with an excitation wavelength of 245 nm and an emission wavelength of 418 nm.

3.8.6.3.Results

The results which have been obtained by the three participants for the three different samples are depicted in Figures 70 – 72.

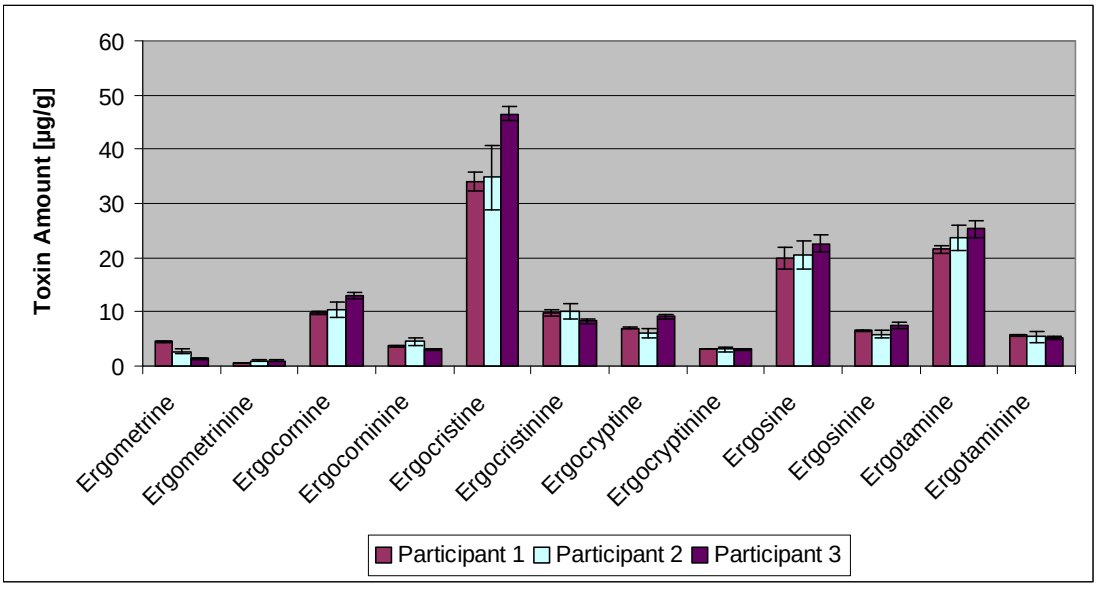


Figure 70 Results obtained for “barley high level”

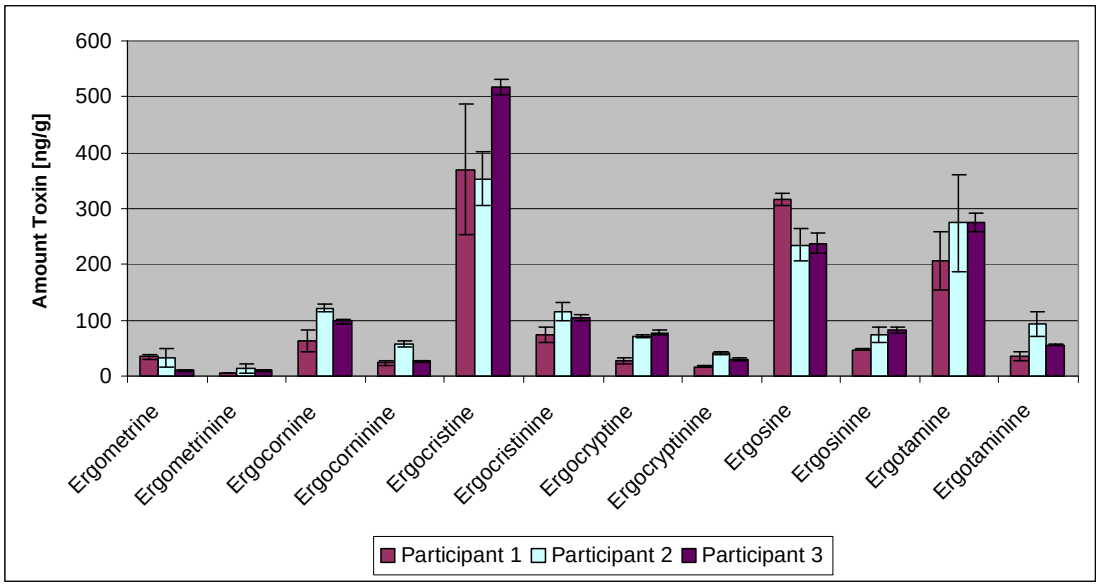


Figure 71 Results obtained for “barley wheat low”

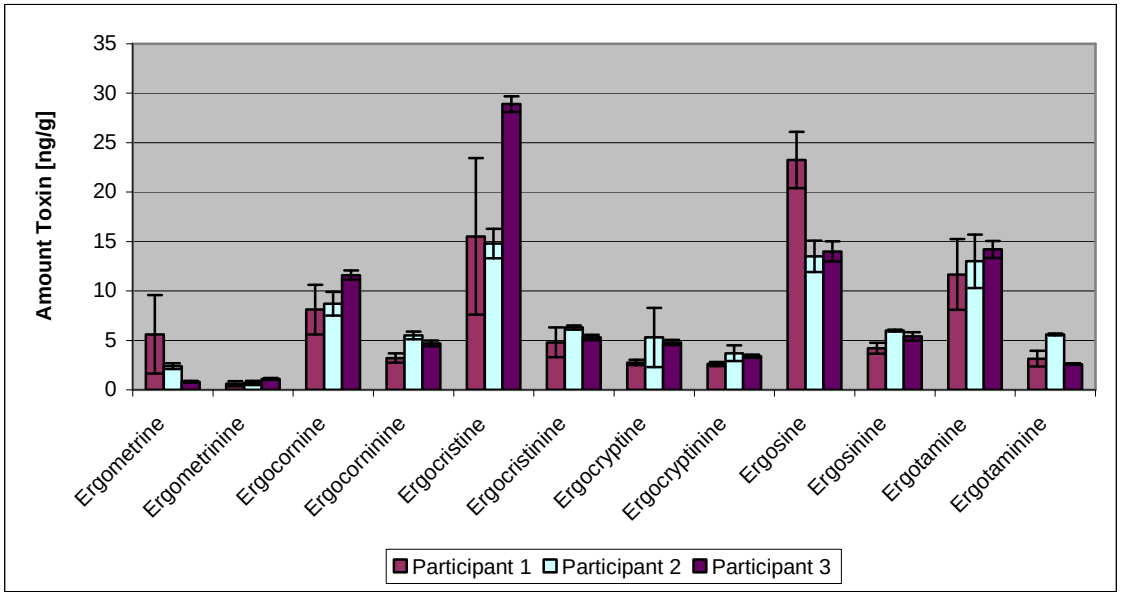


Figure 72 Results obtained for “rye flour”

Figures 70 –72 show a good comparability of measurement results with almost all deviations within the measurement uncertainties of the respective methods. The only exceptions are ergocristine in “rye flour” (participant 3: ca. 100% above mean value) and ergosine in “rye flour” (participant 1: ca. 90% above mean value). Minor deviations have also been obtained for ergocristine in “barley high level” (participant 3: ca. 25% above mean value) and ergosine “barley wheat low” (participant 1: ca. 25% above mean value). Overall and in view of the

lack of collaborative trials in the area of ergot alkaloids detection, the agreement of results is surprisingly good. Moreover, the results underline that the comparability of the newly developed method even with well established methods (participant 3) is satisfactory. However, the reasons for the deviations obtained for ergosine and particularly for ergocristine should be further investigated.

3.9. Conclusions

A method has been developed and validated for 10 different cereal and food samples which enabled the quantification of the 6 major ergot alkaloids defined by EFSA (ergometrine, ergotamine, ergosine, ergocristine, ergocryptine and ergocornine) and their corresponding epimers (-*inines*). A fast clean-up based on dispersive SPE using PSA material followed by a short chromatographic run (14 min, see figure 4) and SRM in ESI(+) mode resulted in convenient LOQs of 0.17 –2.78 µg/kg depending on the analyte and matrix. 85 of 90 recovery measurements over 6 measurement days, which were carried out for the 12 ergot alkaloids in 10 different matrices at the spiking levels 5, 50 and 100 µg/kg, were between 70 and 105%. Three values were still greater than 60%.

Moreover, highly satisfactory measurement uncertainties were obtained during the validation study which involved six measurement days with 120 independent measurements: At a concentration level of 5 µg/kg the expanded measurement uncertainty ranged from (5 ± 0.56)µg/kg to (5 ± 1.49)µg/kg, at a concentration level of 100 µg/kg from (100 ± 8.9)µg/kg and (100 ± 20)µg/kg. Both LOQs and measurement uncertainties of the method were dependent on the analyte but almost independent from the matrix which proves the validity of the approach of pooling 10 different matrices of all 12 ergot alkaloids during the validation study.

According to CEN Report CR 13505: 1999 Food Analysis – Biotoxins - Criteria of analytical methods of mycotoxins the obtained recoveries as well as the calculated measurement uncertainties are highly satisfactory: For example, the demanded performance characteristics for fumonisin B1 and B2 are: recoveries of 60 –120% with RSDr <30% for c<500 µg/kg and for deoxynivalenol 70 –110 % with RSDr <20% for c>100 µg/kg.

The comparability of the measurement results was finally investigated within a mini-intercomparison study which involved three laboratories. In general, the newly developed showed good comparability with the results obtained from the other two participants.

4. Standard Operating Procedure

see Annex 4

5. Dissemination of Research

Specific Objective: To prepare and submit a paper to a peer reviewed journal, describing the work carried out and the outcomes of the project.

The following papers will be submitted to peer reviewed journals:

- R. Krska, G. Stubbings, R. McArthur, C. Crews. A rapid LC/MS/MS method for the determination of 6 major ergot alkaloids and their epimers. *Anal. Bioanal. Chem.* (submitted in January 2008).
- M. Hafner, R. Krska, C. Crews, K. Fog-Nielsen. Stability of ergot alkaloid calibrants. *Journal of AOAC International (in preparation)*
- Krska, R., Berthiller, F., Schuhmacher, R., Nielsen, K. F. and Crews, C. (2007). Determination of ergot alkaloids: Purity assessment of standards and optimisation of extraction and clean-up conditions for food samples. In preparation for submission to: *J. AOAC Int*.
- Krska, R., Stubbings, G., Macarthur, R. and Crews, C. (2007). Rapid simultaneous determination of 6 major ergot alkaloids and their epimers in cereals and food stuffs by LC/MS/MS. In preparation for submission to: *Analytical and Bioanalytical Chemistry*.
- Krska, R and Crews, C. (2007) Significance, chemistry and determination of ergot alkaloids. *Food Additives and Contaminants* (in press).
- Crews, C., Krska, R and Berthiller, F. (2007) Determination of Pyrrolizidine alkaloids in ragwort (Senecio jacobaea) by liquid chromatography time-of-flight mass spectrometry (LC-TOFMS) supported by tandem mass spectrometry (LC-MS/MS). In preparation for submission to: J*ournal of the American Society for Mass Spectrometry*.
- Hafner, M., Sulyok, M., Schuhmacher, R., Crews, C. and Krska, R. (2007) Stability and epimerisation behaviour of ergot alkaloids in various solvents. Submitted to: *The World Mycotoxin Journal*.
- R. Krska, G. Stubbings, C. Crews Rapid simultaneous determination of 6 major ergot alkaloids and their epimers in cereals and food stuffs by LC/MS/MS. *Lecture at the Prague Food Symposium, Nov. 7-9, 2007.*

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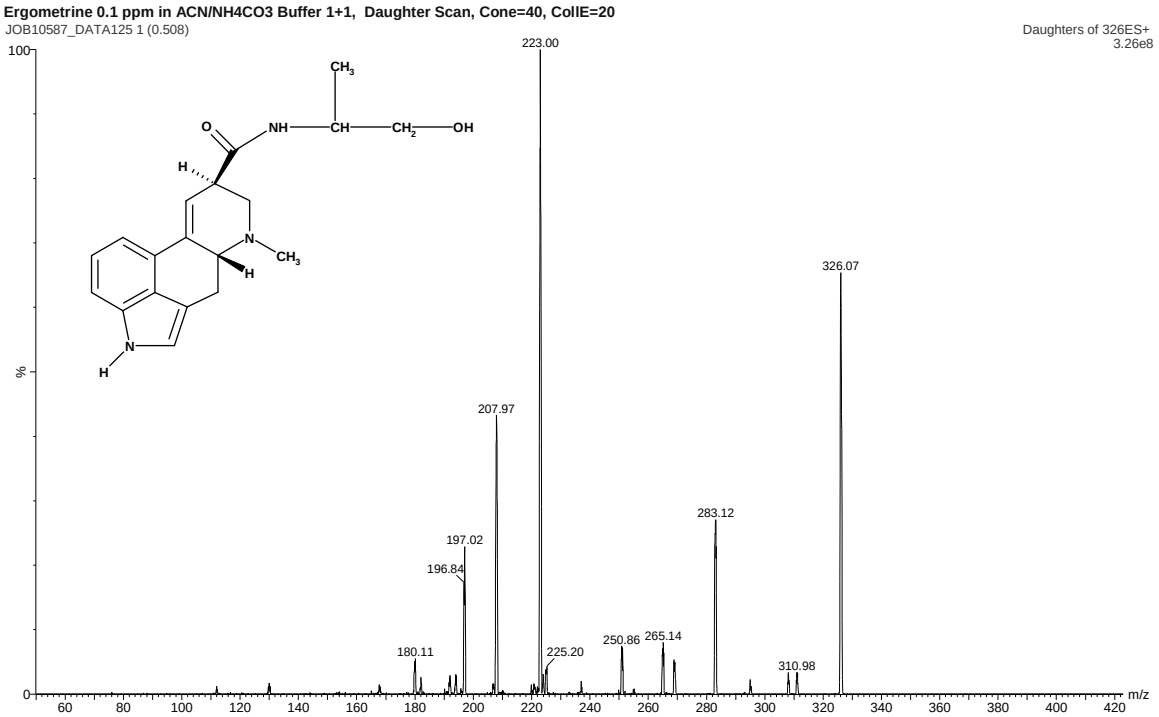
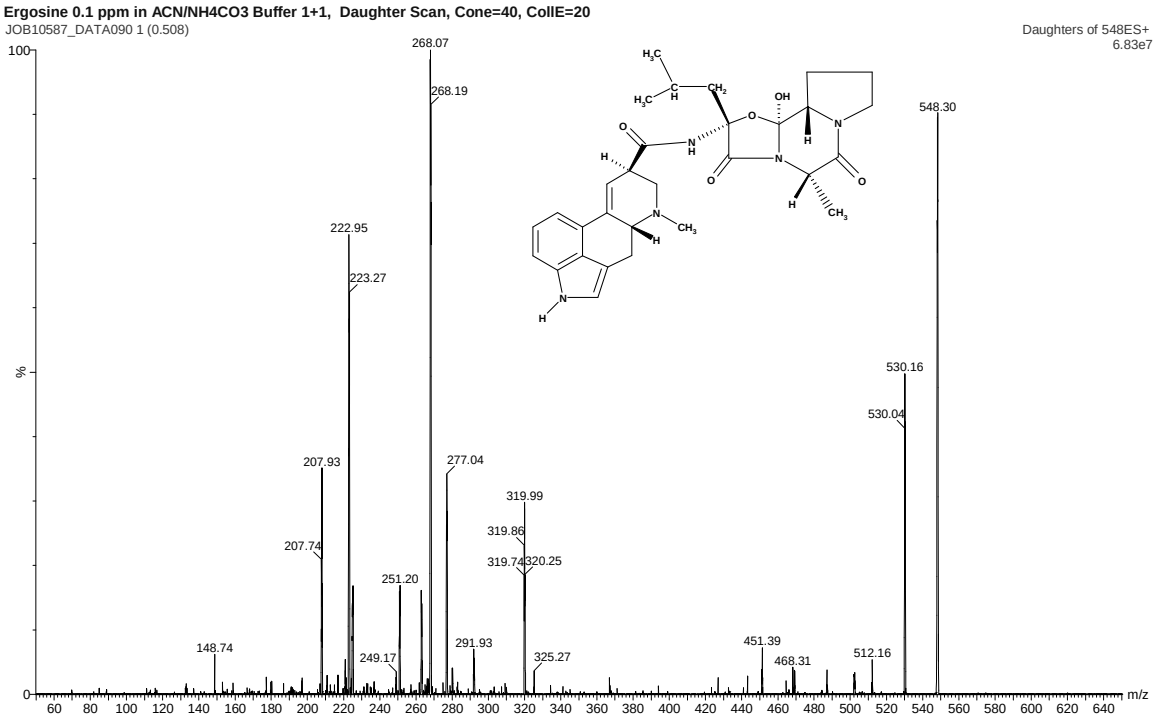
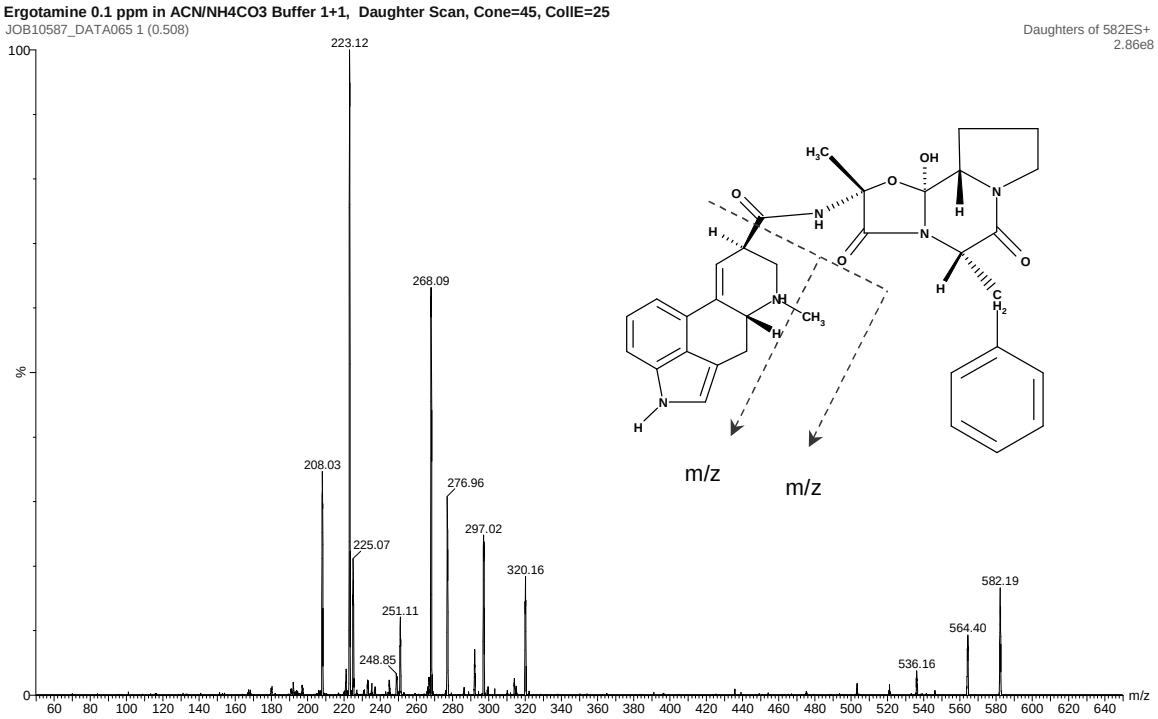
ANNEX 1: Product ion scans and mass spectra



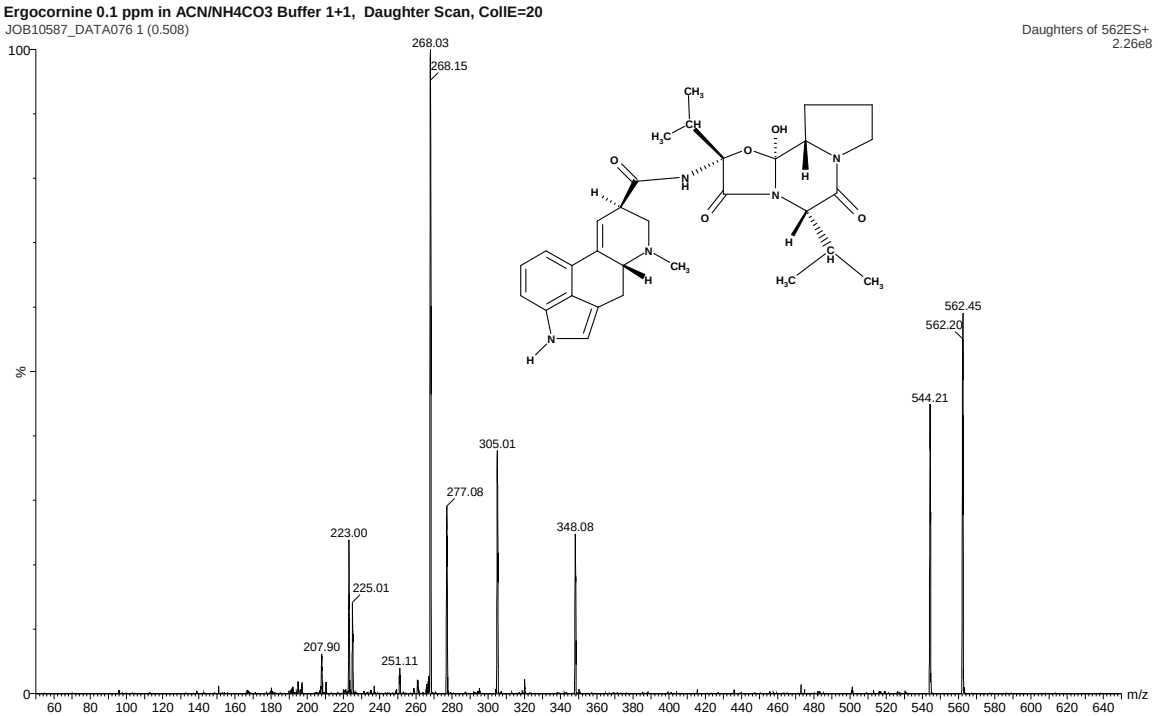
Annexe 1 Figure 1 Product ions of ergometrine



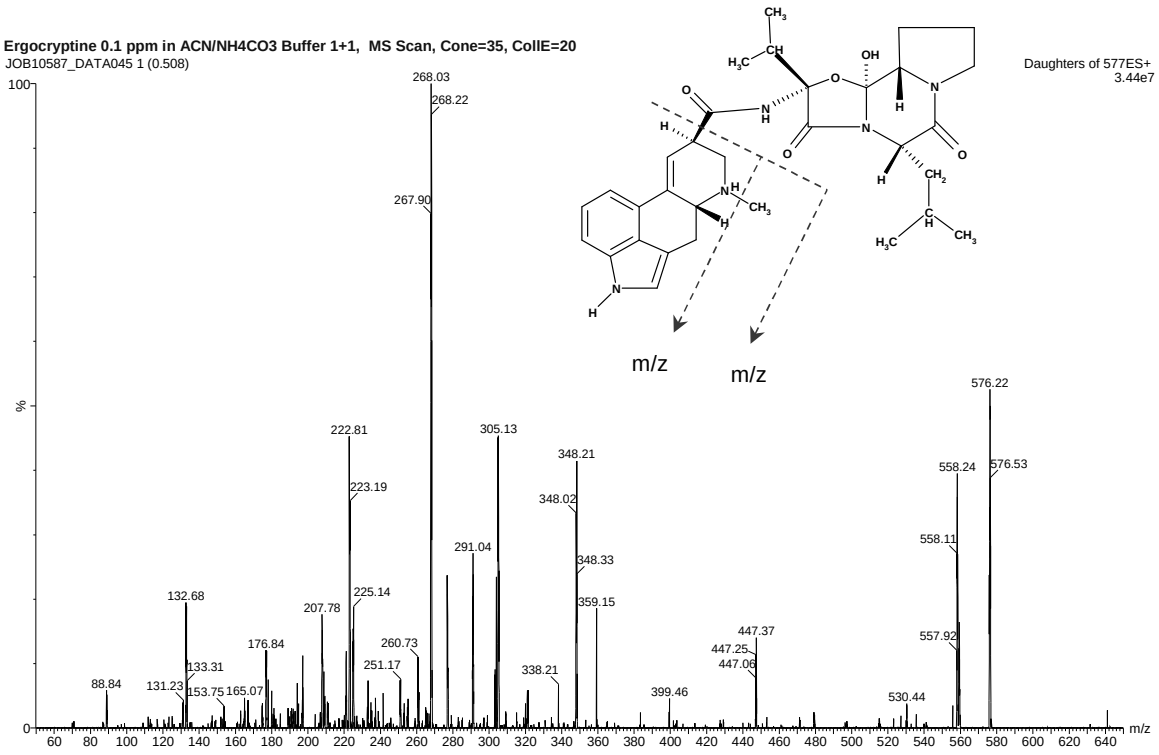
Annexe 1 Figure 2 Product ions of ergosine



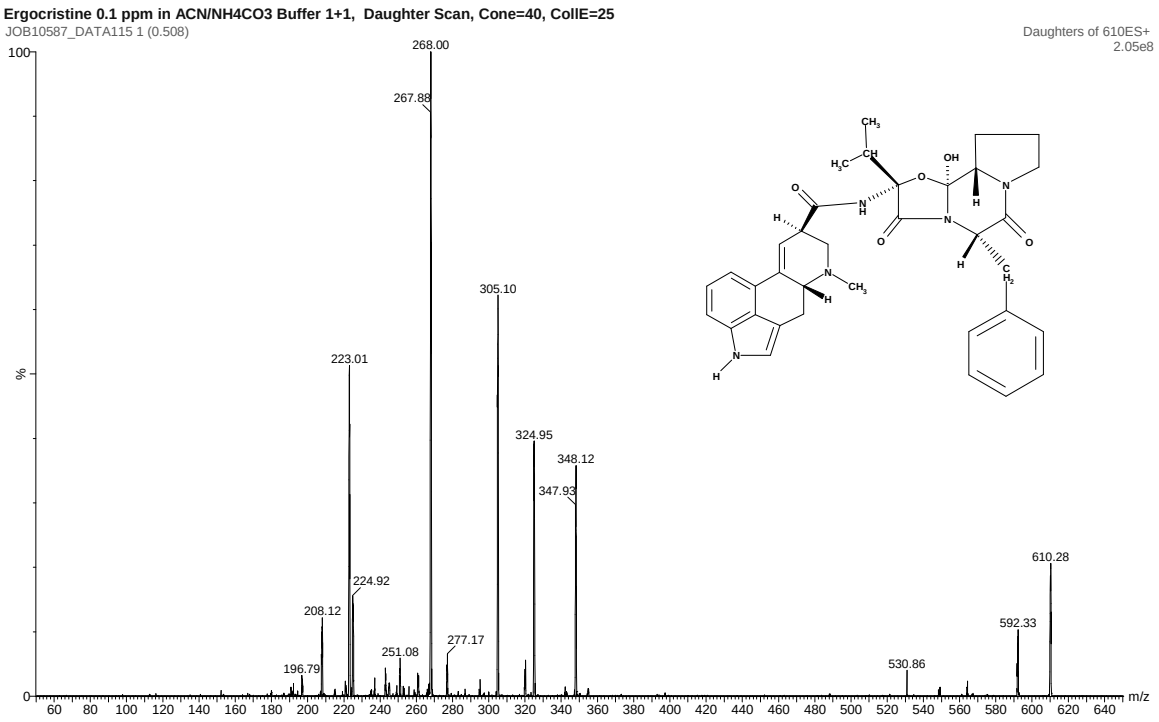
Annexe 1 Figure 3 Product ions of ergotamine



Annexe 1 Figure 4 Product ions of ergocornine

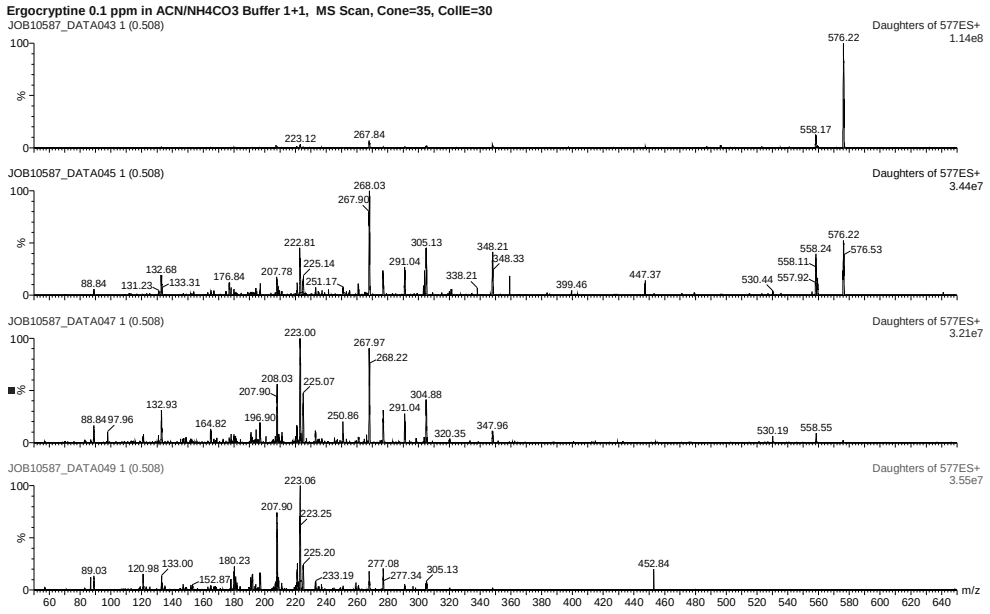


Annexe 1 Figure 5 Product ions of ergocryptine



Annexe 1 Figure 6 Product ions of ergocrystine

Infusion experiments with Ergocryptine:
Product ion scan at different collision energies



Annexe 1 Figure 7 Ergocryptine product ion changes with collision energy.

ANNEX 2: Detailed results and conditions for the stability studies

Annexe 2 Table 1. Recoveries for storage at -20°C compared to -80°C for each toxin in the seven solvents

Storage Temperature	ACN/ Buffer	$CHCl_3$	MeOH/ CH_2Cl_2	Stabilizing Solution	Extraction Mix	MeOH/ H_3PO_4	ACN
Ergometrin(in)e							
-20°C	100	112	104	106	97	91	103
+4°C	104	108	109	109	101	103	101
+20°C	100	99	114	114	100	105	100
Ergosin(in)e							
-20°C	94	110	99	100	99	95	104
+4°C	90	101	90	90	96	82	89
+20°C	87	99	89	89	91	81	67
Ergotamin(in)e							
-20°C	107	111	109	98	90	87	104
+4°C	93	96	99	99	87	93	87
+20°C	85	106	93	93	89	100	57
Ergocornin(in)e							
-20°C	103	104	107	91	101	93	103
+4°C	89	102	106	106	93	108	93
+20°C	89	101	84	84	93	93	79
Ergocryptin(in)e							
-20°C	90	106	114	100	96	100	106
+4°C	93	111	110	110	102	104	96
+20°C	90	112	115	115	107	103	82
Ergocristin(in)e							
-20°C	99	101	117	92	101	98	105
+4°C	101	106	106	106	98	105	94
+20°C	93	105	96	96	103	98	74

Recovery of the sum of –ine and –inine after storage for six weeks compared with storage at -80°C in the same solvent.

Annexe 2 Tables 2-7

Tables 2-7 list the percentages of the peak area of S-form (-inine) to the combined peak areas of the R- and S-forms. The columns below the header '0' week storage time for six replicate reference samples held at -80°C. The two columns below the 1, 3, and 6 week storage time headers have data for duplicate storage experiments.

Annexe 2 Table 2. Percentage of ergocorninine to total ergocorninine + ergocornine

	Storage Time [weeks]											
	0						1		3		6	
-80 ACN/Buffer 80/20	0.94	0.53	0.93	0.72	1.05	0.86						
-20℃ ACN/Buffer											0.94	0.97
+4℃ ACN/Buffer							2.51	2.26	**3.40**	3.63	7.10	7.17
+20℃ ACN/Buffer							5.64	5.40	**11.85**	11.88	23.55	23.13
+20℃ ACN/Buffer light							5.83	6.06				
-80 Chloroform	0.11	0.78	0.48	0.61	0.99	0.94						
-20℃ Chloroform											0.55	0.49
+4℃ Chloroform							0.57	0.73	**0.63**	0.34	0.90	1.29
+20℃ Chloroform							0.84	0.36	**0.72**	0.78	1.77	0.65
+20℃ Chloroform light							0.42	0.92				
-80 Methanol/CH2Cl2	6.56	0.92	0.83	0.70	0.94	1.02						
-20℃ Methanol/CH2Cl2											1.43	2.20
+4℃ Methanol/CH2Cl2							1.44	2.71	**5.33**	5.16	10.06	8.64
+20℃ Methanol/CH2Cl2							6.83	6.64	**18.36**	16.72	29.55	36.43
+20℃ Methanol/CH2Cl2 light							6.59	7.01				
-80 Stabilizing Solution	0.60	0.11	0.42	0.68	0.45	1.21						
-20℃ Stabilizing Solution											1.00	1.04
+4℃ Stabilizing Solution							0.83	1.31	**1.85**	0.62	1.54	1.11
+20℃ Stabilizing Solution							1.22	1.41	**3.78**	3.22	3.87	5.81
+20℃ Stabilizing Solution light							1.03	1.77				
-80 Extraction Mix	1.41	1.85	0.05	0.80	0.50	0.84						
-20℃ Extraction Mix											1.46	1.01
+4℃ Extraction Mix							1.36	1.08	**1.87**	1.75	3.52	3.21
+20℃ Extraction Mix							2.06	2.20	**3.48**	5.18	8.24	8.85
+20℃ Extraction Mix light							2.61	2.69				
-80 MeOH/H3PO4(aq)	0.91	0.38	1.32	0.78	0.54	0.56						
-20℃ MeOH/H3PO4(aq)											1.15	1.13
+4℃ MeOH/H3PO4(aq)							0.78	0.77	**0.85**	1.63	1.93	1.04
+20℃ MeOH/H3PO4(aq)							1.78	2.31	**3.29**	4.75	8.93	7.28
+20℃ MeOH/H3PO4(aq) light							2.96	2.50				
-80℃ Acetonitrile	0.82	0.80	0.80	0.85	0.94	0.82						
-20℃ Acetonitrile											0.98	0.82
+4℃ Acetonitrile							0.85	1.04	0.94	1.02	1.75	2.21
+20℃ (dark) Acetontrile							1.73	1.85	2.67	1.23	5.14	9.37
+20℃ (daylight) Acetonitrile							1.63	2.52				

Annexe 2 Table 3. Percentage of ergocristinine to total ergocristinine + ergocristine

	Storage Time [weeks]											
	0						1		3		6	
-80 ACN/Buffer 80/20	0.76	0.87	0.62	0.90	0.91	0.83						
-20℃ ACN/Buffer											0.89	1.17
+4℃ ACN/Buffer							3.11	4.05	**4.15**	3.69	7.24	5.73
+20℃ ACN/Buffer							6.32	5.88	**13.80**	12.03	24.84	23.37
+20℃ ACN/Buffer light							6.51	5.36				
-80 Chloroform	0.63	0.64	0.48	0.14	0.86	0.92						
-20℃ Chloroform											0.48	0.86
+4℃ Chloroform							0.76	0.00	**0.00**	0.65	1.24	1.01
+20℃ Chloroform							0.69	0.49	**0.87**	0.91	1.20	0.64
+20℃ Chloroform light							1.00	0.80				
-80 Methanol/CH2Cl2	8.78	1.09	1.55	0.31	1.87	1.95						
-20℃ Methanol/CH2Cl2											1.34	0.00
+4℃ Methanol/CH2Cl2							3.15	3.37	**6.30**	6.98	10.77	13.59
+20℃ Methanol/CH2Cl2							8.37	7.83	**22.70**	20.88	38.85	37.48
+20℃ Methanol/CH2Cl2 light							9.25	8.56				
-80 Stabilizing Solution	1.41	0.47	0.19	0.41	0.24	0.80						
-20℃ Stabilizing Solution											0.84	1.00
+4℃ Stabilizing Solution							0.44	0.68	**0.88**	0.69	1.49	1.50
+20℃ Stabilizing Solution							0.43	2.74	**3.16**	2.26	4.87	5.86
+20℃ Stabilizing Solution light							1.20	2.07				
-80 Extraction Mix	1.18	0.92	0.62	0.96	0.85	1.15						
-20℃ Extraction Mix											1.68	1.91
+4℃ Extraction Mix							1.46	1.96	**2.29**	2.50	4.45	4.97
+20℃ Extraction Mix							4.23	3.69	**6.89**	8.06	14.18	14.20
+20℃ Extraction Mix light							4.64	3.82				
-80 MeOH/H3PO4(aq)	0.74	0.43	0.76	0.67	0.81	0.26						
-20℃ MeOH/H3PO4(aq)											0.83	0.82
+4℃ MeOH/H3PO4(aq)							0.00	0.52	**0.00**	1.37	1.28	2.71
+20℃ MeOH/H3PO4(aq)							2.96	0.00	**5.59**	2.98	7.66	6.78
+20℃ MeOH/H3PO4(aq) light							3.04	4.99				
-80℃ Acetonitrile	0.80	0.80	0.87	0.91	0.85	0.83						
-20℃ Acetonitrile											1.17	0.87
+4℃ Acetonitrile							0.86	1.32	1.21	1.30	2.27	3.87
+20℃ (dark) Acetontrile							2.40	2.53	4.13	1.43	8.98	16.06
+20℃ (daylight) Acetonitrile							2.23	3.62				

Annexe 2 Table 4. Percentage of ergocryptinine to total ergocryptinine + ergocryptine

	Storage Time [weeks]											
	0						1		3		6	
-80 ACN/Buffer 80/20	0.67	0.91	1.08	1.26	0.88	0.83						
-20℃ ACN/Buffer											0.79	1.02
+4℃ ACN/Buffer							2.57	3.70	**3.45**	3.70	5.96	6.33
+20℃ ACN/Buffer							5.63	4.15	**10.60**	11.42	21.89	23.34
+20℃ ACN/Buffer light							4.59	5.59				
-80 Chloroform	0.47	0.77	0.86	0.57	0.48	0.86						
-20℃ Chloroform											0.43	0.47
+4℃ Chloroform							0.19	0.76	**0.30**	0.97	0.56	0.80
+20℃ Chloroform							0.54	0.59	**0.65**	1.03	0.76	0.60
+20℃ Chloroform light							0.46	0.61				
-80 Methanol/CH2Cl2	9.90	2.06	1.17	1.23	1.42	2.33						
-20℃ Methanol/CH2Cl2											1.74	1.78
+4℃ Methanol/CH2Cl2							3.69	3.96	**6.58**	6.88	13.61	10.58
+20℃ Methanol/CH2Cl2							7.53	7.55	**20.09**	21.87	34.35	34.92
+20℃ Methanol/CH2Cl2 light							9.90	8.84				
-80 Stabilizing Solution	1.07	0.70	1.05	0.91	0.89	1.42						
-20℃ Stabilizing Solution											1.26	1.54
+4℃ Stabilizing Solution							0.89	1.11	**1.60**	0.91	0.80	1.41
+20℃ Stabilizing Solution							1.73	2.01	**2.66**	2.54	4.28	6.23
+20℃ Stabilizing Solution light							1.46	1.32				
-80 Extraction Mix	0.93	1.02	0.97	1.38	0.76	0.91						
-20℃ Extraction Mix											0.87	1.03
+4℃ Extraction Mix							1.09	1.11	**1.92**	2.58	3.33	3.31
+20℃ Extraction Mix							2.72	2.38	**5.66**	5.60	10.09	10.48
+20℃ Extraction Mix light							2.70	3.48				
-80 MeOH/H3PO4(aq)	0.18	0.49	0.37	1.00	0.54	0.66						
-20℃ MeOH/H3PO4(aq)											1.05	1.14
+4℃ MeOH/H3PO4(aq)							0.99	1.13	**0.67**	1.64	0.90	1.52
+20℃ MeOH/H3PO4(aq)							1.81	1.65	**3.49**	3.49	7.05	7.22
+20℃ MeOH/H3PO4(aq) light							1.41	2.00				
-80℃ Acetonitrile	0.82	0.77	0.78	0.85	0.85	0.81						
-20℃ Acetonitrile											1.00	0.80
+4℃ Acetonitrile							0.85	1.10	1.09	1.14	1.97	2.55
+20℃ (dark) Acetontrile							1.81	1.86	2.98	1.26	5.99	9.76
+20℃ (daylight) Acetonitrile							1.89	2.55				

Annexe 2 Table 5. Percentage of ergometrinine to ergometrinine + ergometrine

	Storage Time [weeks]											
	0						1		3		6	
-80 ACN/Buffer 80/20	0.00	0.25	0.22	0.25	0.24	0.13						
-20℃ ACN/Buffer											0.22	0.15
+4℃ ACN/Buffer							0.42	0.44	**0.66**	0.64	1.40	1.13
+20℃ ACN/Buffer							0.52	1.15	**2.49**	1.81	5.05	4.74
+20℃ ACN/Buffer light							0.64	0.96				
-80 Chloroform	0.00	0.00	0.00	0.00	0.00	0.00						
-20℃ Chloroform											0.13	0.19
+4℃ Chloroform							0.05	0.09	**0.24**	0.00	0.00	0.15
+20℃ Chloroform							0.06	0.00	**0.09**	0.00	0.30	0.24
+20℃ Chloroform light							0.00	0.11				
-80 Methanol/CH2Cl2	0.00	0.00	0.00	0.00	0.00	0.00						
-20℃ Methanol/CH2Cl2											0.60	0.83
+4℃ Methanol/CH2Cl2							0.74	0.69	**0.00**	2.41	2.63	3.15
+20℃ Methanol/CH2Cl2							3.04	1.32	**5.39**	6.24	11.01	9.95
+20℃ Methanol/CH2Cl2 light							1.16	2.12				
-80 Stabilizing Solution	0.00	0.00	0.00	0.00	0.00	0.00						
-20℃ Stabilizing Solution											0.00	0.00
+4℃ Stabilizing Solution							0.52	0.17	**0.00**	0.00	0.00	0.00
+20℃ Stabilizing Solution							0.18	0.13	**0.00**	0.00	0.09	0.46
+20℃ Stabilizing Solution light							0.00	0.00				
-80 Extraction Mix	0.00	0.00	0.00	0.41	0.00	0.00						
-20℃ Extraction Mix											0.00	0.09
+4℃ Extraction Mix							0.43	0.49	**0.58**	0.41	0.71	1.00
+20℃ Extraction Mix							0.44	0.58	**1.33**	0.77	2.25	2.51
+20℃ Extraction Mix light							0.30	0.00				
-80 MeOH/H3PO4(aq)	0.00	0.00	0.00	0.00	0.00	0.00						
-20℃ MeOH/H3PO4(aq)											0.33	0.00
+4℃ MeOH/H3PO4(aq)							0.00	0.00	**0.00**	0.00	0.36	0.39
+20℃ MeOH/H3PO4(aq)							0.00	0.00	**0.00**	0.00	0.57	0.40
+20℃ MeOH/H3PO4(aq) light							0.53	0.36				
-80℃ Acetonitrile	0.07	0.12	0.13	0.16	0.16	0.18						
-20℃ Acetonitrile											0.11	0.16
+4℃ Acetonitrile							0.13	0.14	0.12	0.20	0.19	0.14
+20℃ (dark) Acetontrile							0.14	0.13	0.25	0.16	0.23	0.20
+20℃ (daylight) Acetonitrile							0.17	0.15				

Annexe 2 Table 6. Percentage of ergosinine to ergosinine + ergosine

	Storage Time [weeks]											
	0						1		3		6	
-80 ACN/Buffer 80/20	4.38	4.46	5.61	5.03	5.65	4.76						
-20℃ ACN/Buffer											6.08	5.84
+4℃ ACN/Buffer							6.31	6.49	**8.49**	9.70	12.49	12.52
+20℃ ACN/Buffer							8.90	11.31	**17.90**	18.27	25.40	26.43
+20℃ ACN/Buffer light							10.52	13.07				
-80 Chloroform	4.62	4.78	4.44	5.66	6.11	5.91						
-20℃ Chloroform											4.84	5.04
+4℃ Chloroform							5.56	4.58	**5.94**	5.61	6.76	5.61
+20℃ Chloroform							6.59	6.64	**6.04**	5.16	5.68	5.77
+20℃ Chloroform light							4.93	4.62				
-80 Methanol/CH2Cl2	15.42	4.98	5.80	5.79	4.88	6.85						
-20℃ Methanol/CH2Cl2											6.35	5.43
+4℃ Methanol/CH2Cl2							8.29	8.33	**12.71**	11.31	21.77	23.55
+20℃ Methanol/CH2Cl2							17.87	16.41	**29.21**	32.27	39.98	46.11
+20℃ Methanol/CH2Cl2 light							15.74	17.57				
-80 Stabilizing Solution	5.40	2.94	2.86	5.29	3.37	3.49						
-20℃ Stabilizing Solution											3.91	5.95
+4℃ Stabilizing Solution							4.90	3.90	**3.94**	7.38	5.95	4.10
+20℃ Stabilizing Solution							6.33	5.99	**12.76**	7.28	9.07	7.51
+20℃ Stabilizing Solution light							5.94	4.32				
-80 Extraction Mix	5.22	7.00	5.99	7.56	5.85	5.13						
-20℃ Extraction Mix											6.14	5.19
+4℃ Extraction Mix							6.46	7.38	**9.91**	10.29	9.60	11.87
+20℃ Extraction Mix							9.36	8.30	**14.90**	13.76	23.87	23.95
+20℃ Extraction Mix light							9.06	10.12				
-80 MeOH/H3PO4(aq)	3.60	3.24	4.27	5.75	3.78	3.92						
-20℃ MeOH/H3PO4(aq)											4.53	3.74
+4℃ MeOH/H3PO4(aq)							7.95	4.31	**5.43**	4.66	5.03	5.28
+20℃ MeOH/H3PO4(aq)							5.72	6.26	**5.17**	6.29	11.82	10.35
+20℃ MeOH/H3PO4(aq) light							4.97	4.00				
-80℃ Acetonitrile	9.73	9.72	9.54	9.67	9.23	9.86						
-20℃ Acetonitrile											9.51	9.59
+4℃ Acetonitrile							10.07	10.14	10.28	10.17	11.40	13.72
+20℃ (dark) Acetontrile							11.75	12.27	13.46	10.34	18.73	28.41
+20℃ (daylight) Acetonitrile							11.65	13.80				

Annexe 2 Table 7. Percentage of ergotaminine to ergotamine + ergotaminine

	Storage Time [weeks]											
	0						1		3		6	
-80 ACN/Buffer 80/20	1.27	1.65	1.84	1.98	1.62	1.96						
-20℃ ACN/Buffer											1.84	1.96
+4℃ ACN/Buffer							2.64	2.77	**4.83**	5.04	9.38	7.94
+20℃ ACN/Buffer							6.99	6.27	**14.66**	14.09	25.52	22.43
+20℃ ACN/Buffer light							7.05	7.93				
-80 Chloroform	1.27	0.78	1.48	1.30	1.06	1.67						
-20℃ Chloroform											1.20	1.57
+4℃ Chloroform							0.94	1.63	**1.02**	1.40	2.16	1.64
+20℃ Chloroform							1.30	1.45	**1.22**	1.70	1.90	1.67
+20℃ Chloroform light							1.39	1.90				
-80 Methanol/CH2Cl2	11.43	1.63	1.99	2.09	1.52	1.78						
-20℃ Methanol/CH2Cl2											2.96	2.49
+4℃ Methanol/CH2Cl2							3.94	4.11	**8.02**	8.85	13.47	15.47
+20℃ Methanol/CH2Cl2							7.89	11.93	**25.84**	25.52	34.46	40.27
+20℃ Methanol/CH2Cl2 light							11.43	13.21				
-80 Stabilizing Solution	1.00	1.10	0.74	1.23	1.06	1.38						
-20℃ Stabilizing Solution											1.60	1.41
+4℃ Stabilizing Solution							1.00	0.98	**1.40**	1.93	2.03	0.67
+20℃ Stabilizing Solution							2.24	3.16	**4.19**	3.24	6.15	6.87
+20℃ Stabilizing Solution light							1.97	2.33				
-80 Extraction Mix	1.36	1.82	1.13	1.51	1.25	1.78						
-20℃ Extraction Mix											2.37	1.97
+4℃ Extraction Mix							3.55	2.67	**4.17**	5.82	9.02	7.19
+20℃ Extraction Mix							6.14	5.33	**11.70**	13.94	21.89	22.93
+20℃ Extraction Mix light							6.06	6.59				
-80 MeOH/H3PO4(aq)	2.49	1.38	2.12	1.14	2.11	0.72						
-20℃ MeOH/H3PO4(aq)											1.77	2.17
+4℃ MeOH/H3PO4(aq)							1.52	2.19	**1.70**	1.74	2.64	3.99
+20℃ MeOH/H3PO4(aq)							2.01	2.53	**4.80**	6.21	9.00	7.89
+20℃ MeOH/H3PO4(aq) light							1.84	3.62				
-80℃ Acetonitrile	4.50	4.35	4.26	4.19	4.39	4.20						
-20℃ Acetonitrile											4.72	4.10
+4℃ Acetonitrile							4.49	5.03	4.92	4.76	6.42	9.59
+20℃ (dark) Acetontrile							6.55	7.17	9.63	5.48	17.39	31.66
+20℃ (daylight) Acetonitrile							6.70	8.83				

Annexe 2 Table 8 Percentage of ergometrinine to the sum of ergometrine and ergometrinine in five solvents for 12 injections made over an 18-hour period.

Injection No.	ACN	ACN/(NH4)2CO3 80+20	ACN/(NH4)2CO3 84+16	ACN/(NH4)HCO3 84+16	ACN/(NH4)Acetate 10+20
1	5.35	4.76	4.69	4.76	4.88
2	5.43	4.65	4.61	4.69	5.03
3	5.21	4.69	4.48	4.50	4.95
4	5.29	4.61	4.48	4.61	4.90
5	5.29	4.59	4.48	4.52	5.00
6	5.35	4.67	4.63	4.50	4.83
7	5.15	4.63	4.67	4.69	5.08
8	5.35	4.52	4.61	4.72	5.08
9	5.29	4.57	4.63	4.67	4.98
10	5.35	4.76	4.76	4.74	5.10
11	5.46	4.69	4.78	4.72	5.24
12	5.41	4.78	4.78	4.72	5.24

The time between injections was 84 minutes.

Annexe 2 Table 9 Percentage of ergosinine to the sum of ergosine and ergosinine in five solvents for 12 injections made over an 18-hour period.

Injection No.	ACN	ACN/(NH4)2CO3 80+20	ACN/(NH4)2CO3 84+16	ACN/(NH4)HCO3 84+16	ACN/(NH4)Acetate 10+20
1	4.57	4.27	4.18	4.17	4.55
2	4.76	4.41	4.33	4.31	4.76
3	5.03	4.48	4.46	4.48	4.81
4	4.98	4.42	4.48	4.59	4.85
5	5.08	4.42	4.63	4.52	4.78
6	5.24	4.44	4.63	4.59	5.05
7	5.24	4.55	4.48	4.65	4.98
8	5.49	4.61	4.55	4.69	5.00
9	5.52	4.57	4.65	4.61	5.08
10	5.59	4.65	4.59	4.63	5.32
11	5.56	4.72	4.67	4.72	5.15
12	5.99	5.03	4.61	4.69	5.29

The time between injections was 84 minutes.

Annex 3: Standard Operating Procedure

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HPLC-MS/MS method for the determination of ergot alkaloids

Issued by: C. Crews

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SAFETY

This method involves the use of several hazardous chemicals and procedures likely to produce a risk to the operator. The COSHH assessment should be consulted before applying this method.

General precautions

Protective clothing including laboratory coat (buttoned), safety spectacles and gloves should be worn at all times.

Samples should be regarded as a biological hazard. Direct contact with skin is best avoided and proper attention to hygiene must be maintained.

First Aid

Any injury must be reported, in the first instance, to a qualified First Aider and recorded. The First Aider will decide on further action. All accidents, incidents and near misses should be reported to the Health and Safety team.

INTRODUCTION

Ergot alkaloids are a group of toxins produced by several species of *Claviceps* fungi growing on cereals and forage grass. These toxins are a risk for consumers as they can enter the food chain. All ergot alkaloids share a common structure, the ergoline system and are divided into several classes, based on the presence of functional groups (Figures 1, Table 1).

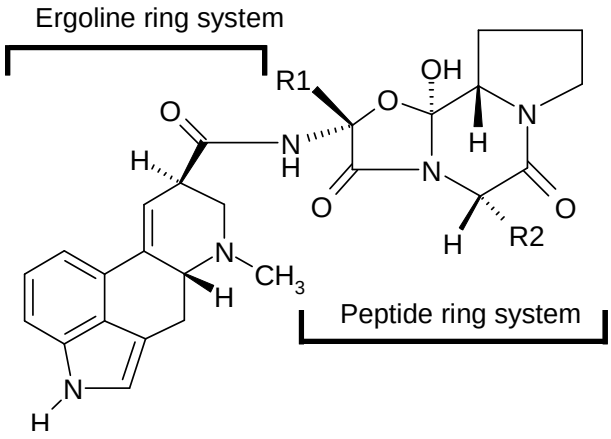


Figure 1: Structure of ergopeptines – see table 1 for substituents of common toxins. The chiral carbon atom C-8 is responsible for the epimerisation.

Table 1: Substituents of the major ergot alkaloid toxins (‘ines’)

Toxin	**Toxin Group**	**R 1**	**R 2**
Ergocornine	Ergopeptine	$CH(CH_3)_2$	$CH(CH_3)_2$
Ergometrine	Lysergic acid derivative		
Ergocristine	Ergopeptine	$CH_2C_6H_5$	$CH(CH_3)_2$
Ergotamine	Ergopeptine	$CH_2C_6H_5$	CH_3
Ergosine	Ergopeptine	$CH(CH_3)C_2H_5$	CH_3
Ergocryptine	Ergopeptine	$CH(CH_3)_2$	$CH_3CHCH_2CH_3$

N.B - The isomers of each of these compounds are nominally known as the ‘inines’.

1 SCOPE

The method is applicable to cereals and cereal-based food and feed products.

2 PRINCIPLE

Ergot alkaloids are extracted from cereals and cereal-based foods and feeds with buffer at pH 9 and cleaned up over a solid phase material prior to filtering and subsequent analysis by HPLC-MS/MS.

3 SAMPLING

Sub-samples of cereals and cereal-based foods and feeds are taken and stored at room temperature.

4 APPARATUS

General laboratory glassware is to be used except where stipulated. Unless otherwise stated volumetric glassware should be of grade 'A' quality. Laboratory equipment may be generic, e.g., pH meters, balances and vortex equipment.

4.1 Extraction bottles; 60 mL amber sampling jar and PTFE cap; Qmx, reference S00108 or equivalent.

4.2 Positive displacement pipettes; Gilson Microman M25, M50, M250 and M1000 or equivalent.

4.3 Sample shaker; AQS manufacturing Ltd., Cat. No. R100B, or equivalent.

4.4 Filter paper; Whatman 12.5 cm Hardened No. 54 or equivalent.

4.5 Screw cap 40 mL amber vials; Qmx, reference V0066 or equivalent.

4.6 Screw cap 4.0 mL amber vials; Qmx, reference V0068 or equivalent.

4.7 Plastic luer-lock syringe1 mL ; BD, Plastipak luer syringe, reference 300013 or equivalent.

4.8 PTFE plastic filters 13 mm x 0.22 µm; Qmx, reference Klarity F10030 or equivalent.

4.9 Screw cap 2.0 mL amber vials; Qmx, reference V0048 or equivalent.

4.10 LC-MS/MS e.g., Waters Quattro Ultima Pt triple quadrupole instrument, coupled to a Waters 2695 HPLC autosampler.

4.11 HPLC column; Phenomenex Gemini 5 µm C18 150 x 2.0 mm, 110 Å, Cat. No. 00F-4435-BO.

4.12 HPLC guard column; Phenomenex Gemini 5 µm C18, Cat No. AJ0-7596.

5 REAGENTS

5.1 Ergot alkaloid standards.

5.1.1 Ergometrine

5.1.2 Ergosine

5.1.3 Ergocornine

5.1.4 Ergocryptine

5.1.5 Ergotamine

5.1.6 Ergocristine

5.1.7 Ergometrinine

5.1.8 Ergosinine

5.1.9 Ergocorninine

5.1.10 Ergocryptinine

5.1.11 Ergotaminine

5.1.12 Ergocristinine

5.2 Acetonitrile; HPLC grade.

5.3 Water; HPLC/ Fluorescence grade.

5.4 Solid phase extraction material; Varian Bondesil PSA, 40 µm, 10 gm, part no. 12213023.

5.4 Ammonium carbonate (3.03 mmol/L; pH = 8.9 ± 0.3), e.g., Fluka ref. 74415, 250 g; Weigh 200 mg ± 2 mg into a weighing boat and transfer to a 1 L amber glass Duran bottle. Add 1 L ± 10 mL of water using a measuring cylinder. Shake the bottle vigorously to ensure all solid has dissolved. Check the pH of the solution.

5.5 Extraction solution. Acetonitrile:Ammonium carbonate (84:16); Measure separately using a measuring cylinder, 840 mL ± 10 mL of acetonitrile and 160 mL ± 10 mL of ammonium carbonate solution into a 1 L Duran bottle. Shake vigorously to mix.

6 STANDARDS

Stock Standards

6.1 Stock individual standard solutions at 100 µg/mL. Weigh 10 mg ± 0.2 mg of each of the solid standards (5.1.1 to 5.1.12) into glass weighing boats and transfer quantitatively into separate 100 mL volumetric flasks. Make up to volume with acetonitrile.

6.2 Standard 'ine' mixtures at 2 µg/mL. Take 2 mL of each of the stock standards of the 'ine' ergots (5.1.1 to 5.1.6) and transfer to a single 100 mL volumetric flask using a 2 mL glass pipette. Make up to volume with acetonitrile.

6.3 Standard 'inine' mixtures at 2 µg/mL. Take 2 mL of each of the stock standards of the 'inine' ergots (5.1.7 to 5.1.12) and transfer to a single 100 mL volumetric flask using a 2 mL glass pipette. Make up to volume with acetonitrile.

6.4 Standard complete mixture at 1 µg/mL. Take 10 mL of the 'ine' mixture (6.2) and transfer into to a 20 mL volumetric flask and make up to volume with the 'inine' mixture (6.3).

Calibration standards

Prepare the following calibration standards equivalent to 0.5 to 200 µg/kg alkaloid.

Pipette the following volumes of the 1 µg/mL mixture (6.4) into a 100 mL amber volumetric flask and make up to volume using acetonitrile. Stopper, invert and shake the flask; repeat several times to ensure mixing.

10 µL of the mix (6.4) into 100 mL acetonitrile to give 0.5 µg/kg equivalent.

20 µL of the mix (6.4) into 100 mL acetonitrile to give 1 µg/kg equivalent.

10 µL of the mix (6.4) into 10 mL acetonitrile to give 5 µg/kg equivalent.

20 µL of the mix (6.4) into 10 mL acetonitrile to give 10 µg/kg equivalent.

40 µL of the mix (6.4) into 10 mL acetonitrile to give 20 µg/kg equivalent.

100 µL of the mix (6.4) into 10 mL acetonitrile to give 50 µg/kg equivalent.

200 µL of the mix (6.4) into 10 mL acetonitrile to give 100 µg/kg equivalent.

400 µL of the mix (6.4) into 10 mL acetonitrile to give 200 µg/kg equivalent.

Analyse the 2 µg/mL stock solutions (6.2 and 6.3) by LC-MS/MS every 4 weeks to check that there has not been significant epimerisation. The areas of the peaks for the 'inine' epimers, should not exceed the area of the peaks for the 'ine' form and *vice versa* by more than 1 %. Epimerisation of these solutions may be gauged more easily if they are first diluted by a factor

of 100 in acetonitrile. To do this dilute the 2 µg/mL stock solutions (6.2 and 6.3) by taking 1 mL of each using a glass pipette and diluting each separately to 100 mL in a volumetric flask with acetonitrile.

7 PROCEDURE

Ergot alkaloids are sensitive to epimerisation by light and the procedures should be carried out in dim light with amber glass being used where possible. The samples should be analysed immediately after extraction and only if absolutely necessary stored overnight at 4 °C.

7.1 Extraction

Weigh 5 g ± 0.05 g sample into an amber glass sample bottle (4.1).

Prepare spiked samples. Add 250 µL of the 1 µg/mL standard mixture to a 'blank' sample using a positive displacement pipette (4.2). At least one blank and spike of the appropriate sample must be included with each batch. Spiked samples must be extracted immediately after spiking to limit epimerisation.

Add 25 mL of the extraction solution (5.5) to the bottle (4.1) using a 25 mL glass pipette.

Place sample bottles 'standing upright' in the shaker (4.3). Shake the samples for 30 minutes at moderate speed.

Whilst the samples are shaking prepare sufficient glass funnels containing folded 'filter paper (4.4) to filter the samples.

When the samples have finished shaking, shake each individually by hand for approximately 10 seconds prior to pouring through funnels and filter paper into 40 mL amber glass vials (4.5).

7.2 Clean-up

Remove 1 mL of sample, using a 1 mL displacement pipette (4.2), and transfer into a 4 mL amber glass vial (4.6) containing 50 mg ± 5 mg of the Varian Bondesil solid phase material (5.4).

Vortex each sample at high speed for 45 seconds.

Take up as much of the sample as possible using a plastic luer-lock syringe (4.7), fit a 13 mm PTFE 0.22 µm filter (4.8), and holding the syringe vertically allow any solid phase material to rest on the bottom of the syringe. Press the liquid through the filter into a 2 mL amber glass vial (4.9).

Using a Pasteur pipette transfer an aliquot of the sample into a 200 µL vial (4.10) and proceed to HPLC-MS/MS.

7.3 LC-MS/MS

Configure and operate the LC-MS/MS instrument in accordance with the manufacturer's instructions.

Each run sequence must contain a reagent blank, calibration solutions and samples. If possible a characterised reference material should be included.

7.4 HPLC system

Use a mobile phase: A = Acetonitrile (5.1); B = 3.03 mM/L ammonium carbonate solution (5.3). Flow rate 0.5 ml/min. with the gradient shown in Table 2. Injection volume = 10 µL.
Use an autosampler temperature of 15 °C ± 5 °C.

Table 2. HPLC Gradient.

Time (min)	A%	B%	Curve
0.0	5.0	95.0	6
1.0	17.0	83.0	1
2.0	47.0	53.0	1
10.0	54.0	46.0	1
15.0	80.0	20.0	1
16.0	5.0	95.0	1
21.0	5.0	95.0	1

7.5 MS/MS

Use a triple quadrupole mass spectrometer or equivalent in positive ion electrospray mode.

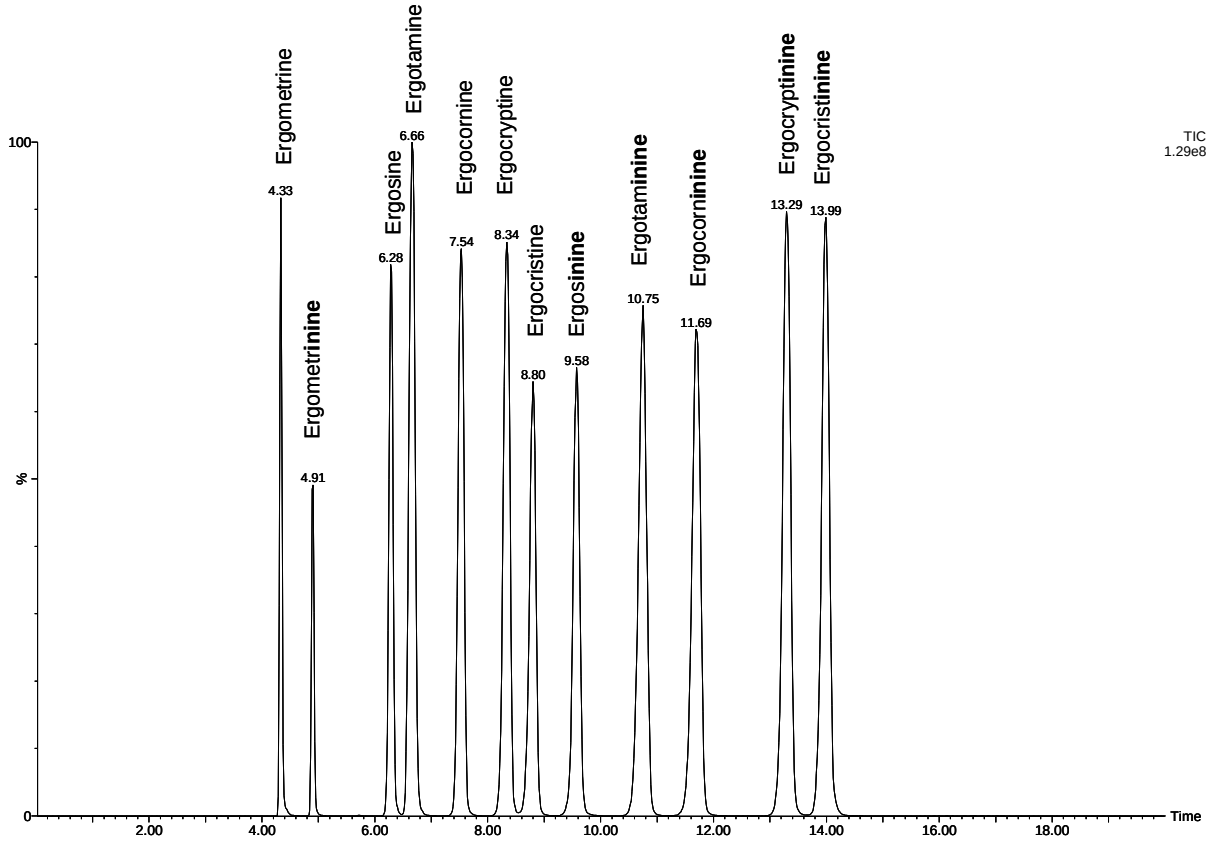
Set the acquisition mode to selected reaction monitoring (SRM), monitoring the transitions shown in Table 3.

Table 3. Collected transitions.

Ergot alkaloid	Precursor ion (m/z)	Product ion (m/z)
Ergometrine and ergometrinine	326.18	208
	326.18	223
Ergosine and Ergosinine	548.27	208
	548.27	223
Ergocornine and Ergocorninine	562.30	208
	562.30	223
Ergocryptine and Ergocryptinine	576.50	223
	576.50	268
Ergotamine and Ergotaminine	582.60	208
	582.60	223
Ergocristine and Ergocristinine	610.30	223
	610.30	268

A typical chromatogram is shown in Figure 2.

Figure 2. A typical LC-MS/MS chromatogram.



Selected transitions from the SRM used for quantitation and confirmation are given in Table 4.

Table 4. Ergot alkaloid retention times (Rt) and SRM transitions for quantification confirmation.

Compound	Rt (min)	SRM quantification ion.	SRM confirmation ion
Ergometrine	4.4	326 => 223	326 => 208
Ergometrinine	5.0	326 => 223	326 => 223
Ergosine	6.5	548 => 223	548 => 208
Ergosinine	9.9	548 => 223	548 => 208
Ergotamine	6.9	582 => 223	582 => 208
Ergotaminine	11.0	582 => 223	582 => 208
Ergocornine	7.8	562 => 223	562 => 208
Ergocorninine	12.0	562 => 223	562 => 208
Ergocryptine	8.6	576 => 223	576 => 268
Ergocryptinine	13.6	576 => 223	576 => 268
Ergocristine	9.1	610 => 223	610 => 268
Ergocristinine	14.2	610 => 223	610 => 268

7.4 Data Processing

Use the instrument data processing software to extract data from the appropriate SRM channels, integrate the peaks of interest and produce calibration curves with which to calculate the concentration of the ergot alkaloids in the samples.

Confirm the identity of the sample peaks by comparing the retention time, which should be within ± 2 % of the mean of the calibration standards, and the ratio of the signal for the confirmation channels to the signal for the quantification channel must be within ± 20 % of the mean ratio observed for the calibration standards.

ANNEX 4: Validation experimental design

Annexe 4 Table 1 validation experiment plan.

Day	Matrix	Concentration	Day	Matrix	Concentration
1	Oat and bran flakes	50 µg/kg	2	Pearl Barley	Blank
1	Pearl Barley	50 µg/kg	2	Wheat Flour	50 µg/kg
1	Rye Flour 2	100 µg/kg	2	Rye Bread	LOQ=1 µg/kg
1	Rusk Biscuits	100 µg/kg	2	Multigrain Crackers	LOQ=1 µg/kg
1	Malted Milk Biscuit	100 µg/kg	2	Rye Bread	100 µg/kg
1	Rye Flour 1	Blank	2	Rusk Biscuits	LOQ=1 µg/kg
1	Rye Flour 1	50 µg/kg	2	Rye Crispbread	50 µg/kg
1	Rye Crispbread	Blank	2	Pearl Barley	50 µg/kg
1	Wheat Flour	Blank	2	Oat and bran flakes	50 µg/kg
1	Multigrain Crackers	LOQ=1 µg/kg	2	Rye Crispbread	Blank
1	Rye Flour 2	LOQ=1 µg/kg	2	Rye Flour 1	Blank
1	Rusk Biscuits	LOQ=1 µg/kg	2	Malted Milk Biscuit	100 µg/kg
1	Pearl Barley	Blank	2	Oat and bran flakes	Blank
1	Rye Bread	LOQ=1 µg/kg	2	Rye Flour 2	100 µg/kg
1	Rye Bread	100 µg/kg	2	Rye Flour 2	LOQ=1 µg/kg
1	Rye Crispbread	50 µg/kg	2	Wheat Flour	Blank
1	Multigrain Crackers	100 µg/kg	2	Rye Flour 1	50 µg/kg
1	Oat and bran flakes	Blank	2	Malted Milk Biscuit	LOQ=1 µg/kg
1	Malted Milk Biscuit	LOQ=1 µg/kg	2	Rusk Biscuits	100 µg/kg
1	Wheat Flour	50 µg/kg	2	Multigrain Crackers	100 µg/kg
3	Rye Crispbread	Blank	4	Wheat Flour	Blank
3	Rye Crispbread	50 µg/kg	4	Rye Crispbread	50 µg/kg
3	Rye Flour 2	100 µg/kg	4	Rye Flour 2	100 µg/kg
3	Rye Bread	100 µg/kg	4	Multigrain Crackers	LOQ=1 µg/kg
3	Rusk Biscuits	100 µg/kg	4	Rye Bread	LOQ=1 µg/kg
3	Multigrain Crackers	100 µg/kg	4	Oat and bran flakes	50 µg/kg
3	Pearl Barley	50 µg/kg	4	Malted Milk Biscuit	LOQ=1 µg/kg
3	Pearl Barley	Blank	4	Malted Milk Biscuit	100 µg/kg
3	Malted Milk Biscuit	100 µg/kg	4	Oat and bran flakes	Blank
3	Oat and bran flakes	50 µg/kg	4	Rusk Biscuits	LOQ=1 µg/kg
3	Rusk Biscuits	LOQ=1 µg/kg	4	Pearl Barley	50 µg/kg
3	Rye Flour 2	LOQ=1 µg/kg	4	Wheat Flour	50 µg/kg
3	Malted Milk Biscuit	LOQ=1 µg/kg	4	Rye Flour 1	Blank
3	Wheat Flour	Blank	4	Rye Bread	100 µg/kg
3	Rye Flour 1	Blank	4	Pearl Barley	Blank
3	Rye Flour 1	50 µg/kg	4	Rye Flour 1	50 µg/kg
3	Rye Bread	LOQ=1 µg/kg	4	Rye Crispbread	Blank
3	Wheat Flour	50 µg/kg	4	Rusk Biscuits	100 µg/kg
3	Oat and bran flakes	Blank	4	Multigrain Crackers	100 µg/kg
3	Multigrain Crackers	LOQ=1 µg/kg	4	Rye Flour 2	LOQ=1 µg/kg

Annexe 4 Table 1 (continued) validation experiment plan.

Day	Matrix	Concentration	Day	Matrix	Concentration
5	Oat and bran flakes	Blank	6	Pearl Barley	50 µg/kg
5	Rye Flour 1	Blank	6	Wheat Flour	Blank
5	Malted Milk Biscuit	100 µg/kg	6	Multigrain Crackers	100 µg/kg
5	Multigrain Crackers	100 µg/kg	6	Rye Crispbread	50 µg/kg
5	Rusk Biscuits	100 µg/kg	6	Malted Milk Biscuit	100 µg/kg
5	Malted Milk Biscuit	LOQ=1 µg/kg	6	Rye Flour 1	Blank
5	Rusk Biscuits	LOQ=1 µg/kg	6	Multigrain Crackers	LOQ=1 µg/kg
5	Pearl Barley	Blank	6	Rye Bread	LOQ=1 µg/kg
5	Rye Crispbread	Blank	6	Malted Milk Biscuit	LOQ=1 µg/kg
5	Multigrain Crackers	LOQ=1 µg/kg	6	Rye Flour 1	50 µg/kg
5	Rye Bread	100 µg/kg	6	Oat and bran flakes	50 µg/kg
5	Oat and bran flakes	50 µg/kg	6	Rye Crispbread	Blank
5	Wheat Flour	50 µg/kg	6	Rusk Biscuits	LOQ=1 µg/kg
5	Rye Flour 2	LOQ=1 µg/kg	6	Oat and bran flakes	Blank
5	Rye Flour 2	100 µg/kg	6	Rye Flour 2	100 µg/kg
5	Wheat Flour	Blank	6	Rusk Biscuits	100 µg/kg
5	Rye Bread	LOQ=1 µg/kg	6	Wheat Flour	50 µg/kg
5	Pearl Barley	50 µg/kg	6	Pearl Barley	Blank
5	Rye Flour 1	50 µg/kg	6	Rye Flour 2	LOQ=1 µg/kg
5	Rye Crispbread	50 µg/kg	6	Rye Bread	100 µg/kg

Annexe 4 Table 2 Estimates of s_0, RSD, uncertainty associated with standard purity, mean recovery RSU, LOQ and LOD

Product	Analyte	S_0 (ug/kg)	RSD	Recovery (%)	RSU (purity)	RSU (recovery)	u_0 (ug/kg)	RSU	LOQ (ug/kg)	LOD (ug/kg)
1	Ergometrine	0.095	0.093	49.9	0.014	0.028	0.190	0.098	0.83	0.79
1	Ergosine	0.462	0.056	68.8	0.014	0.030	0.672	0.065	2.78	2.73
1	Ergotamine	0.514	0.040	80.3	0.014	0.015	0.640	0.045	2.60	2.58
1	Ergocornine	0.487	0.053	83.0	0.014	0.016	0.587	0.057	2.41	2.38
1	Ergocryptine	0.340	0.045	86.5	0.014	0.021	0.392	0.052	1.60	1.59
1	Ergocristine	0.370	0.060	86.3	0.014	0.027	0.428	0.068	1.78	1.75
1	Ergometrinine	0.041	0.054	76.0	0.014	0.027	0.054	0.063	0.22	0.09
1	Ergosinine	0.291	0.050	85.1	0.014	0.019	0.342	0.056	1.41	1.39
1	Ergotaminine	0.167	0.041	84.8	0.014	0.014	0.197	0.045	0.80	0.79
1	Ergocorninine	0.381	0.055	88.2	0.014	0.016	0.432	0.059	1.78	1.75
1	Ergocryptinine	0.375	0.055	91.1	0.014	0.021	0.412	0.061	1.70	1.67
1	Ergocristinine	0.165	0.072	94.9	0.014	0.025	0.173	0.078	0.73	0.71
2	Ergometrine	0.095	0.093	90.9	0.014	0.027	0.104	0.098	0.45	0.43
2	Ergosine	0.462	0.056	100.3	0.014	0.022	0.461	0.062	1.90	1.87
2	Ergotamine	0.514	0.040	100.8	0.014	0.014	0.510	0.044	2.07	2.05
2	Ergocornine	0.487	0.053	103.0	0.014	0.016	0.473	0.057	1.94	1.92
2	Ergocryptine	0.340	0.045	102.9	0.014	0.019	0.330	0.051	1.35	1.33
2	Ergocristine	0.370	0.060	104.3	0.014	0.023	0.354	0.066	1.47	1.44
2	Ergometrinine	0.041	0.054	98.3	0.014	0.027	0.042	0.062	0.17	0.07
2	Ergosinine	0.291	0.050	105.2	0.014	0.015	0.277	0.054	1.14	1.12
2	Ergotaminine	0.167	0.041	101.9	0.014	0.013	0.164	0.045	0.67	0.66
2	Ergocorninine	0.381	0.055	103.7	0.014	0.016	0.368	0.059	1.51	1.49
2	Ergocryptinine	0.375	0.055	105.4	0.014	0.019	0.356	0.060	1.47	1.45
2	Ergocristinine	0.165	0.072	104.9	0.014	0.023	0.157	0.077	0.66	0.64

Table 2 Estimates of s_0, RSD, uncertainty associated with standard purity, uncertainty associated with mean recovery RSU, LOQ and LOD

Product	Analyte	S_0 (ug/kg)	RSD	Recovery (%)	RSU (purity)	RSU (recovery)	u_0 (ug/kg)	RSU	LOQ (ug/kg)	LOD (ug/kg)
3	Ergometrine	0.095	0.093	85.2	0.014	0.027	0.111	0.098	0.48	0.46
3	Ergosine	0.462	0.056	80.4	0.014	0.037	0.575	0.068	2.39	2.34
3	Ergotamine	0.514	0.040	84.3	0.014	0.015	0.609	0.045	2.48	2.46
3	Ergocornine	0.487	0.053	100.0	0.014	0.016	0.487	0.057	2.00	1.97
3	Ergocryptine	0.340	0.045	100.9	0.014	0.019	0.336	0.051	1.38	1.36
3	Ergocristine	0.370	0.060	86.2	0.014	0.032	0.429	0.070	1.79	1.75
3	Ergometrinine	0.041	0.054	97.4	0.014	0.027	0.042	0.062	0.17	0.07
3	Ergosinine	0.291	0.050	100.4	0.014	0.018	0.290	0.055	1.19	1.17
3	Ergotaminine	0.167	0.041	94.1	0.014	0.014	0.177	0.045	0.72	0.72
3	Ergocorninine	0.381	0.055	99.5	0.014	0.016	0.383	0.059	1.58	1.55
3	Ergocryptinine	0.375	0.055	97.5	0.014	0.020	0.385	0.061	1.59	1.56
3	Ergocristinine	0.165	0.072	98.7	0.014	0.028	0.167	0.079	0.70	0.68
4	Ergometrine	0.095	0.093	61.5	0.014	0.028	0.154	0.098	0.67	0.64
4	Ergosine	0.462	0.056	84.4	0.014	0.025	0.548	0.063	2.26	2.23
4	Ergotamine	0.514	0.040	94.0	0.014	0.014	0.546	0.044	2.22	2.20
4	Ergocornine	0.487	0.053	93.0	0.014	0.016	0.524	0.057	2.15	2.12
4	Ergocryptine	0.340	0.045	96.6	0.014	0.020	0.351	0.051	1.44	1.42
4	Ergocristine	0.370	0.060	93.6	0.014	0.026	0.395	0.067	1.64	1.61
4	Ergometrinine	0.041	0.054	79.3	0.014	0.027	0.051	0.062	0.21	0.08
4	Ergosinine	0.291	0.050	96.3	0.014	0.017	0.303	0.055	1.24	1.22
4	Ergotaminine	0.167	0.041	95.5	0.014	0.014	0.175	0.045	0.71	0.70
4	Ergocorninine	0.381	0.055	96.3	0.014	0.016	0.396	0.059	1.63	1.61
4	Ergocryptinine	0.375	0.055	98.6	0.014	0.020	0.381	0.061	1.57	1.55
4	Ergocristinine	0.165	0.072	99.1	0.014	0.025	0.166	0.078	0.70	0.68

Table 2 Estimates of s_0, RSD, uncertainty associated with standard purity, uncertainty associated with mean recovery RSU, LOQ and LOD

Product	Analyte	S_0 (ug/kg)	RSD	Recovery (%)	RSU (purity)	RSU (recovery)	u_0 (ug/kg)	RSU	LOQ (ug/kg)	LOD (ug/kg)
5	Ergometrine	0.095	0.093	65.4	0.014	0.027	0.145	0.098	0.63	0.60
5	Ergosine	0.462	0.056	89.7	0.014	0.024	0.515	0.062	2.13	2.09
5	Ergotamine	0.514	0.040	94.9	0.014	0.014	0.541	0.044	2.20	2.18
5	Ergocornine	0.487	0.053	96.8	0.014	0.016	0.503	0.057	2.07	2.04
5	Ergocryptine	0.340	0.045	95.2	0.014	0.020	0.357	0.052	1.46	1.44
5	Ergocristine	0.370	0.060	97.0	0.014	0.025	0.381	0.067	1.58	1.55
5	Ergometrinine	0.041	0.054	80.2	0.014	0.027	0.051	0.063	0.21	0.08
5	Ergosinine	0.291	0.050	97.6	0.014	0.017	0.299	0.055	1.22	1.21
5	Ergotaminine	0.167	0.041	94.2	0.014	0.014	0.177	0.045	0.72	0.71
5	Ergocorninine	0.381	0.055	98.1	0.014	0.016	0.389	0.059	1.60	1.58
5	Ergocryptinine	0.375	0.055	101.8	0.014	0.019	0.369	0.060	1.52	1.50
5	Ergocristinine	0.165	0.072	101.7	0.014	0.024	0.162	0.078	0.68	0.66
6	Ergometrine	0.095	0.093	81.4	0.014	0.027	0.117	0.098	0.51	0.48
6	Ergosine	0.462	0.056	86.3	0.014	0.024	0.535	0.062	2.21	2.18
6	Ergotamine	0.514	0.040	85.9	0.014	0.014	0.598	0.044	2.43	2.41
6	Ergocornine	0.487	0.053	91.9	0.014	0.016	0.530	0.057	2.18	2.15
6	Ergocryptine	0.340	0.045	86.0	0.014	0.021	0.395	0.052	1.61	1.60
6	Ergocristine	0.370	0.060	86.4	0.014	0.027	0.428	0.068	1.78	1.74
6	Ergometrinine	0.041	0.054	91.3	0.014	0.027	0.045	0.062	0.18	0.07
6	Ergosinine	0.291	0.050	90.9	0.014	0.019	0.321	0.055	1.31	1.30
6	Ergotaminine	0.167	0.041	73.4	0.014	0.015	0.227	0.046	0.93	0.92
6	Ergocorninine	0.381	0.055	84.7	0.014	0.016	0.450	0.059	1.85	1.83
6	Ergocryptinine	0.375	0.055	77.4	0.014	0.024	0.485	0.062	2.00	1.97
6	Ergocristinine	0.165	0.072	67.8	0.014	0.037	0.243	0.082	1.03	1.00

Table 2 Estimates of s_0, RSD, uncertainty associated with standard purity, uncertainty associated with mean recovery RSU, LOQ and LOD

Product	Analyte	S_0 (ug/kg)	RSD	Recovery (%)	RSU (purity)	RSU (recovery)	u_0 (ug/kg)	RSU	LOQ (ug/kg)	LOD (ug/kg)
7	Ergometrine	0.095	0.093	75.3	0.014	0.027	0.126	0.098	0.55	0.52
7	Ergosine	0.462	0.056	81.9	0.014	0.025	0.564	0.063	2.33	2.29
7	Ergotamine	0.514	0.040	93.4	0.014	0.014	0.550	0.044	2.23	2.22
7	Ergocornine	0.487	0.053	90.9	0.014	0.016	0.536	0.057	2.20	2.17
7	Ergocryptine	0.340	0.045	87.8	0.014	0.021	0.387	0.052	1.58	1.56
7	Ergocristine	0.370	0.060	88.7	0.014	0.027	0.417	0.068	1.73	1.70
7	Ergometrinine	0.041	0.054	87.4	0.014	0.027	0.047	0.062	0.19	0.07
7	Ergosinine	0.291	0.050	90.5	0.014	0.019	0.322	0.055	1.32	1.30
7	Ergotaminine	0.167	0.041	69.8	0.014	0.015	0.239	0.046	0.97	0.96
7	Ergocorninine	0.381	0.055	84.2	0.014	0.016	0.453	0.059	1.86	1.84
7	Ergocryptinine	0.375	0.055	83.4	0.014	0.023	0.450	0.062	1.86	1.83
7	Ergocristinine	0.165	0.072	80.0	0.014	0.032	0.206	0.080	0.87	0.85
8	Ergometrine	0.095	0.093	52.8	0.014	0.038	0.180	0.101	0.79	0.75
8	Ergosine	0.462	0.056	83.4	0.014	0.023	0.554	0.062	2.29	2.25
8	Ergotamine	0.514	0.040	85.2	0.014	0.017	0.603	0.045	2.45	2.43
8	Ergocornine	0.487	0.053	89.5	0.014	0.022	0.544	0.059	2.24	2.21
8	Ergocryptine	0.340	0.045	92.7	0.014	0.019	0.366	0.051	1.50	1.48
8	Ergocristine	0.370	0.060	90.7	0.014	0.017	0.408	0.064	1.69	1.66
8	Ergometrinine	0.041	0.054	75.3	0.014	0.038	0.054	0.068	0.23	0.09
8	Ergosinine	0.291	0.050	94.8	0.014	0.005	0.307	0.052	1.26	1.24
8	Ergotaminine	0.167	0.041	92.1	0.014	0.017	0.181	0.046	0.74	0.73
8	Ergocorninine	0.381	0.055	98.2	0.014	0.022	0.388	0.061	1.60	1.58
8	Ergocryptinine	0.375	0.055	100.5	0.014	0.019	0.374	0.060	1.54	1.52
8	Ergocristinine	0.165	0.072	101.0	0.014	0.017	0.163	0.076	0.68	0.67

Table 2 Estimates of s_0, RSD, uncertainty associated with standard purity, uncertainty associated with mean recovery RSU, LOQ and LOD

Product	Analyte	S_0 (ug/kg)	RSD	Recovery (%)	RSU (purity)	RSU (recovery)	u_0 (ug/kg)	RSU	LOQ (ug/kg)	LOD (ug/kg)
9	Ergometrine	0.095	0.093	69.2	0.014	0.027	0.137	0.098	0.60	0.57
9	Ergosine	0.462	0.056	85.1	0.014	0.024	0.543	0.062	2.24	2.21
9	Ergotamine	0.514	0.040	77.1	0.014	0.015	0.666	0.045	2.71	2.69
9	Ergocornine	0.487	0.053	87.3	0.014	0.016	0.558	0.057	2.29	2.26
9	Ergocryptine	0.340	0.045	80.1	0.014	0.022	0.424	0.052	1.73	1.71
9	Ergocristine	0.370	0.060	80.1	0.014	0.030	0.462	0.069	1.92	1.88
9	Ergometrinine	0.041	0.054	84.1	0.014	0.027	0.049	0.062	0.20	0.08
9	Ergosinine	0.291	0.050	86.3	0.014	0.019	0.338	0.055	1.39	1.37
9	Ergotaminine	0.167	0.041	72.9	0.014	0.015	0.229	0.046	0.93	0.92
9	Ergocorninine	0.381	0.055	88.2	0.014	0.016	0.432	0.059	1.78	1.75
9	Ergocryptinine	0.375	0.055	86.0	0.014	0.021	0.437	0.061	1.80	1.77
9	Ergocristinine	0.165	0.072	86.4	0.014	0.027	0.191	0.079	0.80	0.78
10	Ergometrine	0.095	0.093	82.7	0.014	0.027	0.115	0.098	0.50	0.48
10	Ergosine	0.462	0.056	96.1	0.014	0.022	0.481	0.062	1.98	1.95
10	Ergotamine	0.514	0.040	90.6	0.014	0.014	0.567	0.044	2.30	2.28
10	Ergocornine	0.487	0.053	91.2	0.014	0.016	0.534	0.057	2.19	2.16
10	Ergocryptine	0.340	0.045	84.8	0.014	0.021	0.400	0.052	1.64	1.62
10	Ergocristine	0.370	0.060	97.4	0.014	0.024	0.380	0.066	1.57	1.55
10	Ergometrinine	0.041	0.054	84.6	0.014	0.027	0.048	0.062	0.20	0.08
10	Ergosinine	0.291	0.050	94.7	0.014	0.017	0.308	0.055	1.26	1.25
10	Ergotaminine	0.167	0.041	70.4	0.014	0.015	0.237	0.046	0.96	0.96
10	Ergocorninine	0.381	0.055	89.1	0.014	0.016	0.428	0.059	1.76	1.73
10	Ergocryptinine	0.375	0.055	91.2	0.014	0.020	0.412	0.061	1.70	1.67
10	Ergocristinine	0.165	0.072	95.3	0.014	0.024	0.173	0.077	0.73	0.71

Key	
Product	code
Rye Crispbread A	1
Rusk Biscuit	2
Malted Milk Biscuit	3
Multigrain crackers	4
Oat bran Flakes	5
Oatmeal	6
Pearl Barley	7
Rye Crispbread B	8
Rye Flour	9
Wheat Flour	10

S_0 (ug/kg)	
RSD	Relative standard deviation
Recovery (%)	Recovery (%)
RSU (purity)	Relative standard uncertainty associated with standard purity
RSU (recovery)	Relative standard uncertainty associated with recovery
u_0 (ug/kg)	
RSU	Relative standard uncertainty
LOQ (ug/kg)	Limit of quantification
LOD (ug/kg)	Limit of detection