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Analysis of ergot alkaloids – a review

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Abstract

Methods for detection and determination of ergot alkaloids in grains, grasses, feeds and grain foods are reviewed. They incorporate simple detection procedures - colorimetry, thin layer chromatography and enzyme-linked immunosorbent assay - or instrumental procedures such as liquid chromatography with fluorescence, mass spectrometric (MS) or MS/MS detection, capillary zone electrophoresis, and direct MS/MS.

Keywords: ergot alkaloids, grains, grasses, liquid chromatography, mass spectrometry

Introduction

Ergot alkaloids are produced by all species of the *Claviceps* genus, most notably by *C. purpurea* in sclerotia (ergots) growing on rye, wheat and other small grains (1, 2, 3, 4). Other important sources of these alkaloids are grasses infected with various endophytes or with *Claviceps* spp. (5). Ergot alkaloids are also produced by other fungi, for example *Penicillium* and *Aspergillus* (4). 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 (4). The main groups of natural ergot alkaloids are simple lysergic acid derivatives such as ergometrine (ergonovine), peptide alkaloids (ergopeptines, *e.g.* ergotamine, ergovaline), clavine alkaloids (which are hydroxyl- and dehydro- derivatives of 6,8-dimethylergoline, *e.g.* agroclavine) and lactam ergot alkaloids (*e.g.* ergocristam) (4). Structures of the ergot alkaloids more commonly occurring in ergoty grains are shown in Figure 1. The effects of ergot contaminated grains on humans in the Middle Ages are well known; human poisoning from ergot has also occurred in more recent times in France (1), India (6, 7) and Ethiopia (8). There are numerous reports of poisoning of farm animals by ergot contaminated feed (*e.g.* 9) and by endophyte-infected grasses (10, 11).

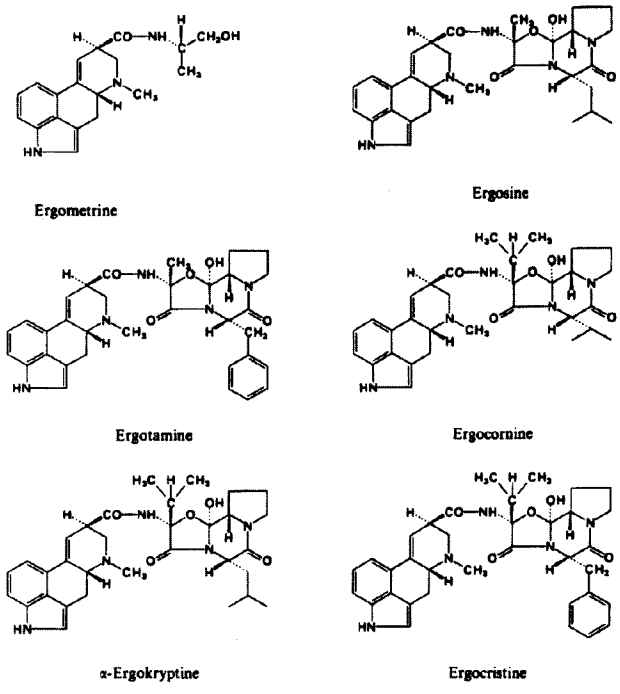

Figure 1. Structures of common ergot alkaloids

Before analysing an agricultural product using a chromatographic procedure, the analyst may wish to assess which alkaloids are likely to be present, with consideration given to the fungal source, pattern of alkaloid occurrence and the toxicities of the alkaloids (including distinguishing between toxic and less toxic epimers) (12). Such considerations do not apply if a colorimetric method of analysis estimating total alkaloids is used.

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

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of a grain sample (*e.g.* wheat, rye or barley), in Canada the minimum representative portion is 500 g and the optimum is 1000 g (13).

Extraction and Cleanup

Extracts of defatted ergots and ergot contaminated grain intended for colorimetric analysis or thin layer chromatography (TLC) (see sections below) have been prepared by extraction with chloroform-methanol-ammonium hydroxide, evaporation of the extract, and then partition of an ether solution of the residue with acid; after addition of ammonium hydroxide the alkaloids were re-extracted into chloroform (14, 15, 16, 17). A modified extraction procedure was used to analyse grains associated with human poisoning in India by TLC (6). However, all these methods have one or more disadvantageous step: defatting by using a Soxhlet apparatus, multiple extractions in the extraction and partition steps, or use of diethyl ether. If the van Urk colorimetric procedure (see section on Colorimetric Analysis) or TLC using van Urk's or other reagent for spraying the plate (see section on Thin Layer Chromatography) are used for semi-quantitative determination, more convenient extraction and liquid-liquid partition steps without these disadvantages could be taken from a liquid chromatographic (LC) method such as that of Ware *et al.* (18). This approach to analysing ergoty grains could be used in a developing country where LC equipment may not be available.

The extraction procedure used for determination of ergot alkaloids in agricultural products by enzyme-linked immunosorbent assay (ELISA) (see section on Enzyme-linked immunosorbent assay) is simple and no cleanup is needed, *e.g.* shaking with methanol-phosphate buffered saline solution containing Tween-20 (1:1, v/v) (19) or stirring with phosphate buffered saline solution (20, 21). Individual ergot alkaloids in grains and grain foods such as flour have generally been determined by LC-fluorescence detection (see section on Liquid chromatography). In these methods, extraction has been performed with a mixture of methylene chloride, ethyl acetate, methanol and 28% ammonium hydroxide (50:25:5:1, v/v) and the evaporated filtrate/centrifugate dissolved in ether or ether-methanol (35:5, v/v) before extraction into 0.5 N hydrochloric acid (22, 23, 24). The acid layer was washed with *n*-hexane, made alkaline with 28% ammonium hydroxide and re-extracted three times with methylene chloride. Ware *et al.* (18) and Fajardo *et al.* (25) simply extracted with 4% ammonium hydroxide-ethyl acetate (10 or 20:100, v/v) and cleaned up sample extracts by a similar acid-base partition or on a C_{18} Sep-Pak cartridge. An extraction solvent of methanol-0.25% conc. phosphoric acid (40:60, v/v) was used by Ware *et al.* (26). Other clean-up procedures include partition on an Extrelut column (27, 28), use of a strong cation exchange particle-loaded membrane extraction disk (26) and a silica gel HL column (29), which conveniently did not retain the inactive –inine isomers. All the foregoing methods gave satisfactory recoveries of individual ergot alkaloids determined in experiments with spiked matrices. Ergovaline is the predominant ergopeptine alkaloid in endophyte-infected tall fescue and other grasses. An LC–fluorescence method for ergovaline (using ergotamine as internal standard) was developed by Rottinghaus *et al.* (30): a freeze-dried sample was extracted with chloroform-0.001 M sodium hydroxide (9:1, v/v) and the filtered extract cleaned up on silica gel HL. Average recoveries of ergovaline were 85%. Extraction of ground tall fescue seeds with methanol yielded only 61-73% of the total extractable ergopeptine alkaloids determined by LC (31).

Analyses of agricultural commodities and foods for ergopeptine alkaloids must be carried out in subdued light to minimize formation of "lumiergopeptines", which are water addition products (32). It should also be noted that epimerization may occur in solution at room temperature leading to equilibrium mixtures (33). The degree of epimerization depends on the solvent, so stock standard solutions should be prepared in aprotic solvents such as chloroform and stored at <0 °C in amber vials. Working standard solutions should be prepared just before use. Lauber *et al.* (28) prepared stock standard solutions in methanol and stored them at -18°C. Standard solutions in methanol stored at -18°C were stable for at least one year (28). Ware *et al.* (26) advocated 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). Concentrations of the ergopeptine alkaloids ergotamine, ergocornine, α-ergocryptine and ergocristine in a mixed standard for use in LC were 10 µg/mL

and of ergonovine 1 µg/mL. When weighing standards, consideration should be given to possible solvent of crystallization (34, 35); the alkaloids should be dried overnight in vacuum at 70 °C and purity checked by UV measurement. Sources of standards were given by Lombaert (36).

Colorimetric Analysis

The usual colorimetric reaction for detecting ergot alkaloids, which has been used for extracts of grain ergots and *Triticale* grain artificially contaminated with 0.35% ergot (14, 15), is to add 1 mL 2% aqueous succinic acid to the evaporated extract, followed by 2 mL of modified van Urk's reagent - a solution of 200 mg *p*-dimethylaminobenzaldehyde and 0.15 or 0.2 mL of 10% aqueous ferric chloride in 100 mL 65% aqueous sulphuric acid. This positive reaction gives a blue colour. After 20 minutes at room temperature the absorbance at 580 nm is read versus a reagent blank. The modified van Urk reaction cannot distinguish between epimers of ergot alkaloids.

The van Urk reaction as modified by Michelon and Kelleher (37) replaced the ferric chloride with sodium nitrite, which resulted in greater sensitivity, more rapid reaction and greater reagent stability. Other colour reactions - with ninhydrin in aqueous sulphuric acid containing a trace of ferric chloride (38) and with diazotised 4-nitroaniline (39) - have only been applied to pharmaceutical preparations.

Thin Layer Chromatography (TLC)

The naturally occurring ergot alkaloids can be determined in extracts of ergots, grasses, grains and feeds by normal phase TLC. Those alkaloids possessing a C-9, C-10 double bond – including the major alkaloids found in grain ergots, but not the clavine alkaloids (40) - can readily be detected by their fluorescence under UV light. The excitation wavelength has been reported as 254 nm, 313 nm, 325 nm or 366 nm (40, 41, 42, 43, 44, 45) and the emission wavelength as 445 nm. As little as 15-100 ng of ergometrine (ergonovine) and 50-200 ng of peptide alkaloids could be determined using a chromatogram scanner (42). Detailed information on fluorodensitometric TLC determination of ergot alkaloids was given by Prošek *et al.* (43, 44), who preferred to use a deuterium lamp rather than a mercury lamp. Alkaloid spots located by UV could be eluted from the TLC plate and determined using van Urk's reagent (46) or vanillin/70% sulphuric acid (47).

Van Urk's reagent has been commonly used as a TLC spray reagent. This procedure was used to detect ergotamine, agroclavine, elymoclavine and other clavine alkaloids in ergots separated from bajra, rye and wheat in India (6, 7). Coloured spots formed with *p*-dimethylaminobenzaldehyde and hydrochloric acid vapours were measured by photodensitometry at 580 nm; 2 µg ergometrine could be determined (47). The pink spots have also been eluted from the TLC plate and measured at 550 nm in a spectrophotometer after addition of 1% sodium nitrite solution (7).

Other chemicals have been investigated as spray reagents for ergot alkaloids. The most sensitive of these appears to be an acidic aqueous solution of 0.5% glyoxylic acid containing 0.05% ferric chloride, which gave a deep blue spot with ergotamine and, after heating the sprayed plate at 110 °C, with lysergamide and certain ergot alkaloid derivatives; detection limits were 0.05-0.1 µg (49). No application of the glyoxylic acid spray to ergots or contaminated grains has been reported but would appear to be worthwhile. The π-acceptors 7,7,8,8-tetracyanoquinodimethane, 2,4,7-trinitrofluorenone, 2,4,5,7-tetranitrofluorenone, 2,4-dinitrofluorobenzene and 2,3-dichloro-5,6-dicyanobenzoquinone gave various colours with ergotamine (50); the latter reagent had the best detection limit – 8 µg/50 mm^2. Other possibly useful spray reagents for ergopeptine alkaloids include 0.05 g xanthydrol (9-hydroxyxanthene) in 15 mL acetic acid followed by a mixture of 3% hydrogen peroxide and 37% hydrochloric acid (1:1, v/v) to give a blue-violet spot after drying; 0.3% sodium 1,2-naphthoquinone-4-sulphonate in ethanol-water (1:1, v/v) followed by 10% hydrochloric acid and heating to form a red-purple spot; and 0.2% 2-nitroso-1-naphthol followed by 10% hydrochloric acid and heating, which gave a blue-black spot (51, 52). Detection limits with the latter two reagents were 1-3 µg.

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 (40), Röder *et al.* (53), Fowler *et al.* (54) and Lobo *et al.* (55) using

silica gel and alumina thin layers and several solvent systems illustrated the difficulty in achieving this objective for the 12 main alkaloids usually found in rye ergots, *i.e.* ergometrin(in)e, ergosin(in)e, ergotamin(in)e, ergocornin(in)e, α-ergokryptin(in)e and ergocristin(in)e, even with 2-dimensional TLC; α-ergokryptine and ergocristine were particularly inseparable. However, separation of all these ergot alkaloids, as well as some clavine alkaloids was achieved on two kinds of silica gel with solvent systems of chloroform-benzene-ethanol (40:10:3, v/v) and chloroform-methanol-acetic acid (90:5:1, v/v) (56). The same ergot alkaloids, as well as β-ergokryptine, ergostine and their isomers, were separated on silica gel impregnated with formamide using a mobile phase of diisopropyl ether-tetrahydrofuran-toluene-diethylamine (70:15:15:0.1, v/v), but D-lysergic and D-isolysergic acids did not migrate while their amides (ergine and erginine) had the same Rf values as ergometrine and ergometrinine, respectively (41). On silica gel layers, acetone-0.1 M ammonium carbonate-ethanol (32.5:67.5 :1, v/v) separated 10 of the 12 alkaloids usually found in ergots as well as four dihydro derivatives (47).

Enzyme-linked Immunosorbent Assay (ELISA)

Immunoassay is an attractive proposition as a method to screen for ergot alkaloids in agricultural crops and grain flour. The specificity for ergot alkaloids varies depending on the antibody used. A competitive inhibition ELISA was developed using polyclonal antibodies which recognized peptide ergot alkaloids having a phenylalanine moiety, such as ergotamine, ergostine and ergocristine (19); these could be detected in endophyte-infected tall fescue seeds, and 10 ng ergotamine/g could be detected in spiked rye, wheat and millet. Subsequently monoclonal antibodies were developed which recognized a much wider range of ergot alkaloids – any with an ergoline ring – so the competitive inhibition ELISA could detect ergovaline and other ergot alkaloids in endophyte-infected tall fescue, including clavine alkaloids (20, 57). For the analysis of ergovaline in tall fescue plants, Shelby and Kelley (20) reported a correlation (r^2=0.62) between ELISA and LC analyses, with no false positives by ELISA Similarly, Hill and Agee (57) reported a correlation (r^2=0.87); however, for another ELISA applied to tall fescue there was a lack of agreement with LC (58). ELISA was also useful for screening milled grains and could detect 10 ng/g of ergonovine (ergometrine) added to ergot free flour (21).

Dihydro ergot alkaloids are not usually encountered in ergot and endophyte-infected grasses but in sorghum ergot (*Claviceps africana*). The main toxic alkaloid is dihydroergosine, which could be detected by ELISA at 10 ng/g and quantitated at concentrations of 100 ng/g in sorghum grain or mixed feed (59). Using a monoclonal antibody, there was little cross reactivity with ergotamine and dihydroergotamine; analyses of 44 samples of sorghum grain for dihydroergosine showed excellent agreement with LC determinations over a 10-250,000 ng/g range.

Liquid Chromatography (LC)

Initially ergopeptine alkaloids and clavine alkaloids were determined using normal phase LC with UV detection at one or more of several wavelengths from 225 nm to 320 nm (60, 61, 62, 63, 64). A basic alkylamine stationary phase was used with a neutral mobile phase; a silica gel stationary phase was used with a neutral or acidic mobile phase; and a hydroxymethyl modified porous polystyrene employed a basic mobile phase containing triethylamine. Good separation of the alkaloids under study was obtained. However, reversed phase LC with fluorescence detection is the usual mode for determining ergot alkaloids in grain ergots, grain foods and endophyte-infected tall fescue. Excitation wavelengths were in the range 235-250 nm (22, 24, 25, 30, 65) or 310-360 nm (11, 16, 26, 31, 65, 66, 67, 69). Good sensitivity and detection limits of 0.5 ng of ergosine, ergotamine, ergocornine, α-ergokryptine and ergocristine and 0.1 ng of ergometrine (22) and 0.15 ng for ergovaline (31) have been reported. The response factor for ergometrine was about twice that of the other five grain ergot alkaloids (16). Reported detection limits for individual ergot alkaloids in grains and grain foods were of the order of 0.04-0.46 ng/g (27, 68) or 1-2 ng/g (24, 26). Limits of quantitation in rye flour were 0.12-1.37 ng/g (lower for the –inine isomers) (68); the limit of quantitation reported by Lombaert (36) was about 1 ng/g for each alkaloid in grains and grain products and 4 ng/g for infant

cereals (69). Ultraviolet (UV) detection at 225-254 nm (15, 22, 67, 70, 71) or 310-311 nm (16, 72) has also been used; response was better at the lower wavelength (16). Electrochemical detection was a sensitive additional means of detecting ergot alkaloids by reversed phase LC (ergocriptine, ergonovine and ergotamine were tested) (65).

Isocratic mobile phases used for reversed phase LC of ergot alkaloids include 0.02 M 1-heptanesulfonic acid:acetonitrile (60:40, v/v) containing 1% acetic acid (C_{18} column) (25, 36), 43% (v/v) acetonitrile in aqueous ammonium carbonate (0.2 g/L) with a C_8 column (22), acetonitrile: ammonium carbamate buffer (0.2 g/L) (1:1, v/v) with a phenyl-hexyl column (68) and 0.05 M dibasic ammonium phosphate-acetonitrile (55:45, v/v) with a polystyrene-divinylbenzene polymer resin (18). A neutral mobile phase of acetonitrile-phosphate buffer pH 7 (50:50, v/v) was used on a C_{18} column by Magg and Ballschmiter (72). Various reversed phase solvent or flow rate gradient systems have also been used (11, 23, 24, 27, 28, 31, 72). In general, good separations of ergopeptine alkaloids were achieved. LC quantitation of ergonovine may be difficult in certain foods because of a large interfering peak (24, 25) and recoveries may be lower than for the other ergot alkaloids (24).

A simple means of confirmation of the identity of ergot alkaloids is to heat the remaining extract in 2% acetic acid and observe by LC with fluorescence detection the appearance of the ergopeptinine isomers (29).

LC-positive ion electrospray ionization (ESI) MS/MS was used to determine ergonovine, ergotamine, ergocornine, α-ergokryptine and ergocristine in rye flour (73) and rye bread (74) and also ergotamine in blood, urine and hair (75). Novel ergoline alkaloids were found by this technique in grass and forage feed samples associated with animal poisoning (76). Ergot alkaloids were included in a LC-ESI MS/MS multimycotoxin method for analysis of wheat and maize (77). Shelby *et al.* (67) used LC-positive ion ESI MS to identify ergot alkaloids – including ergine, ergovaline, ergosine and ergonine – in endophyte-infected tall fescue; ergonovine and ergotamine were not found. LC-fast atom bombardment MS was used to confirm ergonovine in endophyte-infected drunken horse grass (*Achnatherium inebrians*), in which high levels of ergonovine and lysergic acid amide (but not ergovaline) were detected by LC (11). Clavine alkaloids have also been identified by LC-MS (78).

Other Procedures for Detection of Ergot Alkaloids

A mixture of ergometrine, ergotamine and their epimers was separated by capillary zone electrophoresis in 0.1 M phosphate buffer (pH 2.5) with detection at 206 nm (79). Addition of γ-cyclodextrin (30 mM) to the background electrolyte increased both the migration time and the resolution of the alkaloids. Separation of five natural ergopeptine alkaloids (ergometrine not included) and their epimers could not be achieved without both 20 mM β-cyclodextrin and 8 mM γ-cyclodextrin in the 50 mM phosphate buffer (pH 2.5); detection was by UV at 214 nm or laser induced fluorescence with an excitation wavelength of 325 nm (80). Capillary zone electrophoresis was applied to the analysis of rye ergot sclerotia but not yet to grain foods.

Supercritical fluid chromatography with UV detection at 280 nm or electron impact MS has been applied to the identification of a number of clavine alkaloids from *Claviceps purpurea* (81).

Gas chromatography (GC) is not very useful for determination of ergopeptine alkaloids (82) 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. GC-MS has not been applied to the determination of ergot alkaloids in foodstuffs and has only been used in pharmaceutical and forensic areas and, in one instance, for confirmation of identity of the alkaloids in grain foods (83). Clavine alkaloids do not have amide linkages so have been chromatographed by GC (84).

MS/MS (without any LC separation) can be used to identify ergot alkaloids directly from extracts of ergot, ergot-contaminated pelleted or ground feed, rye flour and endophyte-infected tall fescue and perennial rye grass (29, 85, 86, 87, 88, 89), but there is no distinction between the epimeric alkaloids. In contrast to MS/MS, MS alone, including chemical ionization MS (90), has not been applied to analysis of extracts of ergot or grasses (10).

Near-infrared spectroscopy has been used to determine total ergot alkaloid content of tall fescue with calibration by immunoassay (91); stockpiled tall fescue infected with a toxic endophyte had to be omitted from the population of samples.

Conclusion

A wide variety of methods is available for detection and determination of ergot alkaloids in grains, grasses, feeds and grain foods. None of these methods has been validated by interlaboratory study. They incorporate simple detection procedures - colorimetry, TLC and ELISA - or instrumental procedures such as LC with fluorescence and MS detection, capillary zone electrophoresis and direct MS/MS. The former type of detection procedure could be used in developing countries, preferably using extraction and cleanup procedures developed for LC methods.

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