sciendo

J Vet Res 67, 603-610, 2023
DOI:10.2478/jvetres-2023-0070

Development, in-house validation and application of a method using high-performance liquid chromatography with fluorescence detection (HPLC-FLD) for the quantification of 12 ergot alkaloids in compound feeds

Ewelina Kowalczyk✉, Krzysztof Kwiatek

Department of Hygiene of Animal Feedingstuffs, National Veterinary Research Institute, 24-100 Puławy, Poland
ewelina.kowalczyk@piwet.pulawy.pl

Received: July 17, 2023 Accepted: December 5, 2023

Abstract

Introduction: Ergot alkaloids (EAs) are toxic substances naturally produced by *Claviceps* fungi. These fungi infest a wide range of cereals and grasses. When domestic animals are exposed to EAs through contaminated feeds, it is detrimental to them and leads to significant economic losses. For that reason, it is important to monitor feed for the presence of EAs, especially with methods enabling their determination in processed materials. **Material and Methods:** Ergot alkaloids were extracted with acetonitrile, and dispersive solid phase extraction (d-SPE) was used for clean-up of the extracts. After evaporation, the extracts were reconstituted in ammonium carbonate and acetonitrile and subjected to instrumental analysis using high-performance liquid chromatography with fluorescence detection. The developed method was validated in terms of linearity, selectivity, repeatability, reproducibility, robustness, matrix effect, limits of quantification and detection and uncertainty. The EA content of 40 compound feeds was determined. **Results:** All the assessed validation parameters fulfilled the requirements of Regulation (EU) 2021/808. At least one of the monitored alkaloids was determined in 40% of the samples. The EAs with the highest incidence rate were ergocryptine, ergometrinine and ergocornine. The total concentrations of EAs ranged from under the limit of quantification to 62.3 μg kg^{-1}. **Conclusion:** The results demonstrated that the developed method was suitable for simultaneously determining twelve EAs in compound feed and could be used for routine analysis.

Keywords: ergot alkaloids, compound feed, HPLC-FLD, d-SPE.

Introduction

Ensuring feed safety is increasingly vital in guaranteeing the safety of the entire food chain. Consequently, monitoring feeds for both natural and anthropogenic contaminants has become a critical aspect of the safety assurance system. One group of naturally occurring contaminants that may result in detrimental health impacts on animals and economic losses are ergot alkaloids (EAs).

Ergot alkaloids hold significant importance in the history of both toxins and pharmaceuticals as some of the most noteworthy natural products. They are produced by ergot sclerotia, which are dormant structures of ergot fungi that are seed-like or spur-shaped objects and are produced specifically by *Claviceps* species (20). *Claviceps purpurea* is the most widespread of these species in Europe, and it can infect more than 400 plant species. Among cereals, especially rye, wheat, triticale, barley, millet and oats are considered to be susceptible (10).

When cereals or grass with sclerotia are harvested, it can result in contamination (5). Human ergotism has been almost completely eliminated thanks to more efficient modern grain cleaning techniques; however, it remains a significant disease in the veterinary field primarily affecting sheep, cattle, pigs and chickens (4).

The immediate effect of EA ingestion is the appearance of symptoms of neurotoxicity such as agitation, constricted or dilated pupils, muscle weakness, trembling and stiffness. Ergot alkaloids affect various neurotransmitter receptors, notably those for adrenaline, dopamine and serotonin. This interaction between EAs and neurotransmitter receptors can have

© 2023 E. Kowalczyk, K. Kwiatek. This is an open access article distributed under the Creative Commons Attribution-NonCommercial-NoDerivs license (http://creativecommons.org/licenses/by-nc-nd/3.0/)

both short-term and long-term consequences (11). Ergopeptines such as ergovaline or ergotamine can cause adrenergic blockage, resulting in potent and long-lasting vasoconstriction. This can lead to reduced blood flow, intense burning pain, oedema, cyanosis, dry gangrene, and even the loss of hooves in cattle or limbs in humans. Prolactin secretion may also be reduced in livestock through EA activity on dopamine receptors in the pituitary. This reduction in serum prolactin levels can lead to various reproductive issues, especially in horses, including agalactia, poor conception, late-term foal losses, dystocia and thickened placentas (20). Maruo *et al.* (16) indicated that continued ingestion of EAs, even at lower concentrations, could also have harmful effects on the liver and intestines of pigs over an extended period of time.

In animal nutrition, compound feeds (whether complementary or complete) represent a large amount of the total feed consumed by farmed animals, but the available data on the occurrence of EAs in these commodities were scarce and did not allow a reliable exposure estimate to be made (11). Even though the awareness of the possibility of contamination with ergot alkaloids has increased, there are still cases of poisoning among animals caused by these toxins (23). Particular attention should be given to screening compound feeds and processed cereals, as visually inspecting them for the presence of ergot sclerotia is impossible. Regardless of the opportunity for gross inspection existing or not, the issue extends beyond merely visually detecting the presence of ergot sclerotia; the dust and small particles that remain after the presence of sclerotia can also be a source of contamination (7, 11). Therefore, to determine contamination with EAs, processed cereals and grains require more complex testing methods, which mainly involve chromatographic techniques.

Most often, liquid chromatography combined with fluorescence or mass detectors is used for determination of EAs. In the case of these contaminants, both techniques have been proved to have sufficient sensitivity and specificity (12, 18). Although there are over 50 known EAs, only a select few are deemed pertinent for monitoring because of their higher prevalence in cereals and their production by *Claviceps purpurea*. According to Recommendation 2012/154/EU, among the EAs selected for monitoring are ergocristine, ergocristinine, ergotamine, ergotaminine, ergocryptine, ergocryptinine, ergometrine, ergometrinine, ergosine, ergosinine, ergocornine and ergocorninine (1, 7). Monitoring food for the same group of EAs is also obligatory according to Regulation 2023/915 establishing the maximum levels of particular contaminants in food commodities (9).

The aim of the study was to develop a simple method for the determination of these 12 ergot alkaloids in compound feeds based on liquid chromatography and fluorescence detection. The developed and validated method was applied to the analysis of 40 compound feed samples.

Material and Methods

Chemicals and reagents. Ammonium carbonate, ammonia and trichloroacetic acid were purchased from Avantor Performance Chemicals (formerly POCH, Gliwice, Poland). Acetonitrile and methanol were from J.T. Baker (Deventer, the Netherlands). Water was purified with the Milli-Q water purification system (MilliporeSigma, Burlington, MA, USA). Primary-secondary amine (PSA) sorbent was provided by Supelco (Bellefonte, PA, USA). Activated carbon, octadecyl (C18) sorbent and formic acid were purchased from Sigma Aldrich (St. Louis, MO, USA). The relevant 12 EAs, namely ergocristine (CAS: 511-08-0), ergocristinine (CAS: 511-07-9), ergotamine (CAS: 113-15-5), ergotaminine (CAS: 639-81-6), ergocryptine (CAS: 511-09-1), ergocryptinine (CAS: 511-10-4), ergometrine (CAS: 60-79-7), ergometrinine (CAS: 479-00-5), ergosine (561-94-4), ergosinine (CAS: 596-88-3), ergocornine (CAS: 564-36-3) and ergocorninine (CAS: 564-37-4), were purchased from Alfarma (Řež, Czech Republic).

Standard solutions. Stock standard solutions of each alkaloid were prepared in acetonitrile at a concentration of 1,000 μg mL^{-1}. All solutions were stored ≤−18°C. Mixed working standard solutions containing all alkaloids at concentrations of 10 and 1 μg mL^{-1} were subsequently prepared from the stock standard solutions.

Method optimisation. The development of the method involved optimising the extraction, purification and instrumental conditions. For extraction optimisation, solvents based on acetonitrile, such as acetonitrile alone, acetonitrile with 1% ammonia, with 0.1% formic acid, with 0.1% trichloroacetic acid, and in combination with ammonium carbonate (in a ratio of 85:15 v/v) were tested. Methanol and methanol with 0.1% formic acid were among the other solutions evaluated for extraction.

For purification purposes, different amounts of dispersive solid-phase extraction (d-SPE) sorbents such as PSA (0.2–0.4 g), C18 (0.2–0.3 g) and activated carbon (0.01–0.04 g) were assessed. Instrumental optimisation focused on designing gradient conditions that would ensure a good separation of all analysed EAs.

Feed samples. The 40 compound feed samples examined in the study were collected during veterinary inspections in Poland in 2022. Feed samples were represented mainly by compound feeds for swine and poultry. The materials were ground, homogenised and passed through a 1-mm sieve before analysis.

Sample preparation. Feed samples of 5 g were weighed into 50 mL polypropylene Falcon tubes and 25 mL of acetonitrile was added. Subsequently the samples were mixed on a horizontal shaker for 30 min. Next, all samples were centrifuged for 10 min at 4,000 × *g*. Supernatants were transferred to new Falcon tubes containing 0.4 g of PSA and 0.019 g of activated carbon and were shaken for a further 10 min. Afterwards, the samples were centrifuged again for 10 min at 4,000 × *g*. A total of 4 mL of purified extract was passed through nylon filters, transferred to smaller vials and evaporated

to dryness in a nitrogen stream. Residues were reconstituted in 200 μL of acetonitrile and 200 μL of 0.001 M ammonium carbonate, and the dissolved samples were then passed through double PVDF filters before being transferred to chromatographic vials.

Instrumental parameters. For the analysis of the samples, HP 1100 Series separation modules from Agilent Technologies (Santa Clara, CA, USA) were used. The modules included were a degasser system, binary pump, automatic injector and column thermostat. A fluorescence detector was used for visualisation. The excitation and emission wavelengths were 330 nm and 420 nm, respectively. The separation of the compounds was carried out on a Gemini NX-C18 column of 100 mm × 4.6 mm with 2.6 μm particle size (Phenomenex, Torrance, CA, USA) coupled with a C18 guard column (Phenomenex). The column was thermostatted at 20°C. The mobile phase containing 0.001 M ammonium carbonate in water (A) and acetonitrile (B) was used in the following gradient mode: 0 min 30% B, 0–5 min 55% B, 5–13 min 55% B, 13–19 min 65% B, 19–22 min 70% B, 22–22.5 min 30% B, and maintenance of 30% B until 26 min had elapsed. The flow rate was 0.7 mL min^{-1} and the injection volume was 20 μL.

Identification and quantification. Identification of the 12 ergot alkaloids was made by retention time comparisons with those of the respective reference standards. For the quantification, calibration curves were used which had been prepared by spiking blank feed samples with standards solution before the extraction procedure. Curves for all monitored alkaloids were in the 10–1,000 μg kg^{-1} range. The calibration curves were constructed by plotting the peak area versus the alkaloid concentrations and were prepared freshly before every analysis.

Method validation. The developed method was validated in house according to the requirements in Regulation 2021/808 (8). Various validation parameters such as linearity, selectivity and matrix effect, recovery, repeatability, reproducibility, limits of detection (LOD) and quantification (LOQ), robustness and uncertainty were evaluated to assess the utility of the method. Feed that did not contain any of the monitored alkaloids was used as the blank matrix to prepare the fortified samples. For the evaluation of the linearity of the method, blank samples were spiked before extraction at 10, 20, 50, 100, 250, 500 and 1,000 μg kg^{-1} concentrations. The linearity was proved if the coefficient of determination R^2 was higher than 0.99. In order to evaluate the matrix effect, blank feed samples were spiked after a d-SPE clean-up procedure at a concentration of 50 μg kg^{-1}; also a standards solution of the same concentration was prepared. For the calculation of matrix effect (%), an equation was applied producing the ratio of the analyte peak area in the extract of the blank sample spiked with the standards solution to the analyte peak area of the standards solution, multiplied by 100.

Selectivity was evaluated by analysis of the set of blank feed samples to check the possible presence of interferences in the retention time of the monitored alkaloids. For the purpose of recovery and repeatability evaluation, a set of six blank feed samples was spiked at 10, 250 and 1,000 μg kg^{-1} concentrations. Recovery was calculated by dividing the determined levels with the spiked level and multiplying by 100. For the repeatability assessment, the standard deviation (SD) and coefficient of variation (CV) were calculated for each analysed level.

Reproducibility was assessed by spiking two other sets of blank feed samples at the same concentrations as for repeatability and analysing them on different days with the same instrument, and overall SD and CV values were calculated for each level.

The limit of detection and limit of quantification were determined on the basis of signal to noise ratio (S/N = 3 for LOD and S/N = 10 for LOQ).

To determine the robustness of the method, the Youden procedure was applied. Blank feed samples were fortified at the concentration of 50 μg kg^{-1} and subjected to analysis in order to evaluate the effect of the seven chosen variables which were altered. The effect of amount of PSA (0.4 g or 0.5 g), amount of activated carbon (0.019 or 0.025 g), type of syringe filter (PVDF or nylon), duration of mechanical shaking (15 min or 10 min), flow rate (0.8 mL min^{-1} or 0.7 mL min^{-1}) and the concentration of the ammonium carbonate in the mobile phase (0.001 M or 0.015 M) were evaluated. Student's *t*-test was used to determine the impact of changes in individual parameters on the results.

The uncertainty of the method was estimated by identification and quantification of the uncertainty components of the overall analytical process as indicated in EURACHEM/CITAC (6). The expanded uncertainty was expressed as a percentage value (P-value = 0.05; k = 2).

Results

Method optimisation. Acetonitrile was chosen as the most efficient extraction solvent. Different proportions of d-SPE sorbents were analysed for purification. It was found that 0.2 g PSA and 0.01 g activated carbon were not sufficient to remove the matrix impurities (Fig. 1). Only increased amounts of primary secondary amine and activated carbon, 0.4 g and 0.019 g respectively, produced a satisfactory cleaning effect. The mobile phase, which allowed a good separation of all analysed alkaloids, consisted of 0.001 M ammonium carbonate in water and acetonitrile. The injection volume for sufficient sensitivity was 20 μL. Adequate intensity and selectivity of the signal was achieved by choosing excitation and emission wavelengths of 330 nm and 420 nm, respectively. A chromatogram yielded by the optimised method showing a clear peak separation is given in Fig. 2B.

Method validation. The procedure proved to be linear in the investigated range (10–1,000 μg kg^{-1}) as the obtained R^2 for all individual alkaloids was higher

than 0.99 (Table 1). The analysis of blank feed samples revealed that the method was selective, as no interferences were detected in the retention times of the analysed compounds (Fig. 2A). The recovery for the ergot alkaloids was in the range of 85.2 to 117.8% (Table 2). The coefficient of variation representing the repeatability of the method was in the 1.2–9.2% range. Also, satisfactory CV values for reproducibility were obtained, and were in the 2.2–12.4% range. The matrix effect varied from 42.4% to 132.8%. The uncertainty of the determination of the particular compounds depending on the level ranged from 4.5% to 36.3%.

The signal-to-noise approach was used to establish the limit of detection, which was within a range of 0.8 to 2.6 μg kg^{-1}, depending on the compound. Ergocorninine had the lowest detection limit, while ergometrinine and ergotaminine had the highest. Although the limit of quantification was determined using the signal-to-noise approach (Table 1), the lowest calibration level was ultimately used as the method's quantification limit.

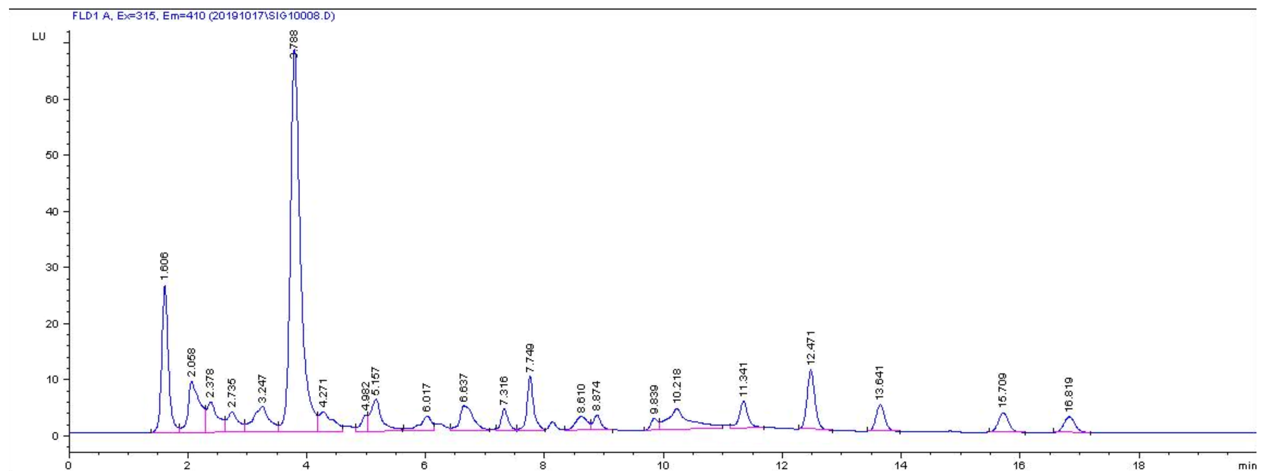

Fig. 1. Example chromatogram obtained in the course of method optimisation. A 0.2 g mass of primary-secondary amine and 0.01 g of activated carbon were used for clean-up. LU – luminescence unit

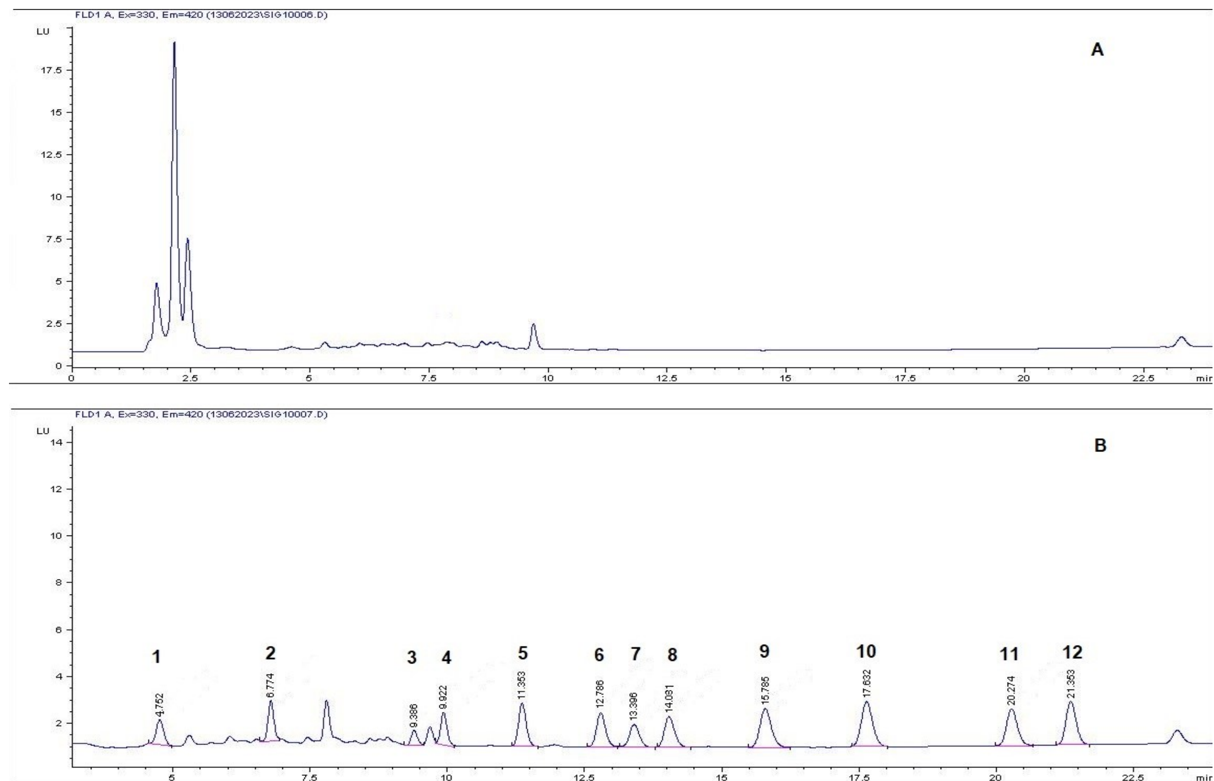

Fig. 2. Chromatograms of A – blank feed sample; B – blank feed spiked at the concentration of 10 μg kg^{-1}
1 – ergometrine; 2 – ergometrinine; 3 – ergosine; 4 – ergotamine; 5 – ergocornine; 6 – ergocryptine; 7 – ergocristine; 8 – ergosinine; 9 – ergotaminine; 10 – ergocorninine; 11 – ergocryptinine; 12 – ergocristinine

Table 1. Validation results for determination coefficient (R^2), matrix effect, limit of detection (LOD) and limit of quantification (LOQ) and uncertainty of analysed ergot alkaloids

	Linearity		Matrix effect (%)	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	Uncertainty (%) (at concentration:)		
	R^2					10 μg kg^{-1}	250 μg kg^{-1}	1,000 μg kg^{-1}
ergocristine	0.9992	y = 3.313x−14.6219	65.2	2.2	3.8	17.1	15.4	16.8
ergocristinine	0.9996	y = 3.8130x−27.8254	97.1	1.0	2.0	24.9	4.5	7.3
ergotamine	0.9998	y = 25522x−22.9181	132.8	2.2	4.6	30.6	16.2	11.6
ergotaminine	0.9998	y = 8.3582x−45.1085	68.7	2.6	4.3	36.3	11.4	7.0
ergocryptine	0.9984	y = 1.5186x−8.3205	85.4	2.1	5.0	20.9	10.3	12.9
ergocryptinine	0.9997	y = 4.2522x−29.9617	94.1	1.6	3.5	26.3	4.9	10.0
ergometrine	0.9903	y = 3.3136x−14.6219	42.3	2.5	4.7	32.9	22.8	23.9
ergometrinine	0.9902	y = 3.6036x−12.6925	58.3	2.6	4.8	28.3	31.5	26.4
ergosine	0.9999	y = 2.1023x−9.7773	81.5	1.7	4.1	20.8	9.7	10.2
ergosinine	0.9995	y = 2.6077–16.4461	97.0	2.4	4.2	31.3	15.7	10.9
ergocornine	0.9996	y = 3.3136x–14.6219	67.7	2.2	4.6	24.6	12.3	14.1
ergocorninine	0.9996	y = 4.2199x−30.8679	99.9	0.8	1.8	24.4	5.7	11.0

Table 2. Validation results for recovery, repeatability and within-laboratory reproducibility

	Recovery (%) (at concentration:)			Repeatability (%) (at concentration:)			Reproducibility (%) (at concentration:)		
	10 μg kg^{-1}	250 μg kg^{-1}	1,000 μg kg^{-1}	10 μg kg^{-1}	250 μg kg^{-1}	1,000 μg kg^{-1}	10 μg kg^{-1}	250 μg kg^{-1}	1,000 μg kg^{-1}
ergocristine	102.8	94.5	95.8	3.7	7.9	9.2	8.1	5.4	7.3
ergocristinine	110.8	99.5	102.5	4.3	2.5	2.9	6.1	2.2	2.7
ergotamine	114.0	92.6	99.7	6.3	2.3	4.8	6.2	3.3	5.8
ergotaminine	117.8	96.2	102.4	1.2	3.9	1.4	3.4	4.2	2.5
ergocryptine	104.0	98.0	97.5	2.6	5.5	8.9	9.7	4.8	6.0
ergocryptinine	112.0	100.7	104.0	3.6	2.5	2.3	5.4	2.3	3.1
ergometrine	115.8	89.9	110.8	4.1	6.0	4.0	4.6	5.3	5.1
ergometrinine	111.9	85.2	112.6	3.3	4.3	4.7	7.7	5.5	4.0
ergosine	107.1	96.8	102.8	3.5	3.7	5.3	7.6	3.6	4.2
ergosinine	113.8	93.2	100.9	3.7	4.8	6.4	7.1	3.8	5.4
ergocornine	101.5	94.9	95.6	4.2	1.8	7.2	12.4	3.7	5.9
ergocorninine	110.2	99.6	104.5	3.9	3.2	1.9	6.7	2.8	3.1

Table 3. Concentrations (μg kg^{-1}) of ergot alkaloids determined in 16 positive compound feeds

Sample No.	Err	Errine	Ert	Ertine	Erp	Erpine	Erm	Ermine	Ers	Ersine	Erc	Ercine	∑ EAs
1	< LOQ			< LOQ	< LOQ			23.2					23.2
2					< LOQ			22.2				< LOQ	22.2
3	< LOQ			< LOQ	< LOQ		15.5					< LOQ	15.5
4							16.4	15.7					32.1
5		< LOQ									24.4		24.4
6						< LOQ						< LOQ	-
7		< LOQ									< LOQ		-
8		< LOQ									< LOQ		-
9					< LOQ	< LOQ						< LOQ	-
10					31.0			27.4					58.4
11					< LOQ			18.3	10.4				28.7
12								38.3		24.0			62.3
13			< LOQ	< LOQ	< LOQ	< LOQ							-
14					< LOQ				< LOQ	< LOQ	< LOQ		-
15	< LOQ	< LOQ			< LOQ			11.9					11.9
16					< LOQ	16.7							16.7

Err – ergocristine; Errine – ergocristinine; Ert – ergotamine; Ertine – ergotaminine; Erp – ergocryptine; Erpine – ergocryptinine; Erm – ergometrine; Ermine – ergometrinine; Ers – ergosine; Ersine – ergosinine; Erc – ergocornine; Ercine – ergocorninine; ∑ EAs – sum of all ergot alkaloids

As far as the robustness is concerned, all examined changes in the parameter values did not affect the results with any statistical significance, which confirms the robustness of the method to small variations in the evaluated parameters.

Levels of EAs in feed samples. To claim that a sample was positive, the determined concentration had to be higher than the LOD of the method. However, for quantification, only the values which were above the LOQ were taken into consideration. A 40% proportion of all analysed samples was determined as positive and contained at least one of the monitored alkaloids. Quantification was carried out for 62.5% of the positive results, which constituted 25% of all samples. The determined concentrations of the total sum of the alkaloids varied from < LOQ μg kg^{-1} to 62.3 μg kg^{-1}. The mean contamination was at the level of 7.4 μg kg^{-1} and the median for all samples was zero. The mean and median for the contaminated samples for which concentrations were above the LOQ were 29.5 μg kg^{-1} and 23.8 μg kg^{-1}, respectively. The most frequently detected EAs were ergocryptine (in 25% of samples) and ergometrinine (in 17.5% of samples). Ergocristinine, ergocryptinine, ergocornine and ergocorninine were detected in 10% of the samples, and ergotamine was the least abundant alkaloid (in 2.5% of the samples). The concentrations of individual compounds and the total content of EAs in each positive compound feed are shown in Table 3. Among all EAs the highest concentrations were determined for ergometrinine (38.3 μg/kg^{-1}) and ergocryptine (31 μg kg^{-1}). All positive samples contained from two to five alkaloids, and at least three alkaloids were detected in half of the 16 positive feed samples. However, five detected alkaloids (ergotamine, ergocristine, ergotaminine, ergocorninine and ergocristinine) were below the LOQ values.

Discussion

The most commonly used approach in the extraction of ergot alkaloids involves solvents such as acetonitrile in combination with ammonium carbonate (15, 19). The ratio of the two solvents is usually 85 : 15 (v/v) or 84 : 16 (v/v) (1, 3, 14, 15). This combination was also tested during the method development stage. However, an increase in the sensitivity of the method was required, and for that purpose, the extract needed to be evaporated under a nitrogen stream. Although the approach yielded good recoveries of all alkaloids, concentrating the extract proved to be time-consuming because of the evaporation constraints of solvent mixtures containing water.

Among other solvents or combinations of solvents that were tested for the efficient extraction of ergot alkaloids were acetonitrile, acetonitrile modified with ammonia or formic or trichloroacetic acid, methanol, and methanol in combination with formic acid. As the acidic and basic modifiers did not improve recoveries of any of the alkaloids, we decided to select acetonitrile as the final extraction solvent. Schummer *et al.* (21) also used acetonitrile as a single extraction solvent, and overnight deep freezing was used as a clean-up; however, this approach did not give good results for clean-up of compound feeds.

In the case of these feeds, the purification step becomes crucial in eliminating potential interferences because of the composition complexity. We decided to test dispersive solid-phase extraction sorbents as the clean-up, as they have been used as part of the QuEChERS procedures which were also applied in EA determination (1, 15). For this purpose, different combinations of PSA, C18, and activated carbon were assessed for the most efficient clean-up effect. While activated carbon is a good adsorbent that can eliminate many impurities from the matrix, using too large an amount may reduce the recoveries of certain alkaloids, particularly ergometrine and ergometrinine; therefore, the amount applied should be balanced between reasonable recoveries and satisfactory clean-up effect. For that reason, the final sorbent combination used in the protocol consisted of 0.4 g of PSA and 0.019 g of activated carbon.

Application of the Gemini NX-C18, 100 mm × 4.6 mm, 2.6-μm-particle-size column with the combination of the mobile phase consisting of ammonium carbonate and acetonitrile provided good separation of all analysed compounds.

The developed method was validated in house. All assessed parameters fulfilled the requirements of EU Regulation 2021/808 (8). The values of the coefficients of variation obtained for both repeatability and reproducibility of the method were below 20%. In addition, recoveries obtained for all compounds at all levels were within the recommended range of 70–120%. For compensation of the matrix effect, calibration curves were prepared based on blank feed matrix. The method proved to be linear for all compounds, as the coefficient of determination was higher than 0.99. Low limits of detection and quantification were obtained for the method ranging from 0.8 to 2.6 μg kg^{-1} and from 1.8 to 5.0 μg kg^{-1}, respectively. However, as the demand for establishing trueness and precision at LOQ levels is increasing, we made the decision to apply the lowest validation level of 10 μg kg^{-1} as the quantification limit for all analysed ergot alkaloids.

The proposed protocol was applied for the determination of the ergot alkaloids in 40 compound feed samples. The estimated percentage of positive samples (40%) determined in our study is higher than outcomes reported by Arroyo-Manzanares *et al.* (1), who determined ergot alkaloids in 12.7% of all analysed samples. A lower incidence rate of ergot alkaloids was also determined by Babič *et al.* (2), who reported 17% of positive samples. However, other authors showed higher percentages of positive samples in tested feed. For example, Poapolathep *et al.* (19) indicated over 40% of swine feeds being positive for EAs and over 50% of

dairy feeds. Seventy percent of composite feed and grass silage samples analysed by Di Mavungu *et al.* (5) were contaminated with EAs. In a Dutch study, the percentage of compound feeds positive for EAs reached 83% (17).

In our study, the most commonly occurring ergot alkaloids were ergocryptine, ergocryptinine, ergometrinine, ergocornine, ergocorninine and ergocristinine. However, in other studies the alkaloid profile was different. For example, according to the EFSA report from 2017, the most frequently detected EAs in feed samples from European countries were ergotamine, ergosine and ergocristine (11). Among the most frequently occurring alkaloids in swine feeds analysed by Arroyo-Manzanares *et al.* (1) were ergometrine, ergometrinine, ergotamine, ergosine, ergosinine and ergocristine. Poapolathep *et al.* (19) tested swine and dairy feed and found ergocryptine, ergocryptinine, ergosine, ergosinine, ergotamine, ergotaminine, ergocristine and ergocristinine. All positive samples contained multiple alkaloids. In many cases more than three alkaloids were detected. Many of the detected alkaloids were found to be below the LOQ of the method, and in many cases only one was quantitatively determined. Of all the monitored alkaloids, ergometrinine was the most usual one found at quantifiable levels. Among other alkaloids that were quantitatively determined were ergometrine, ergosine, ergosinine, ergocornine, ergocryptine and ergocryptinine.

In the literature there are numerous reports of research focusing on cereals for human consumption. However, there are a relatively low number of articles focusing on ergot alkaloids in feed, especially compound feeds. Because cereals will only be part of compound feed ingredients, it might be expected that the levels of EAs in compound feeds will be lower than the levels in feed materials comprised purely of unprocessed cereals. In our study, the overall measurable concentrations ranged from 15.5 to 62.3 μg kg^{-1}. The concentrations reported by other authors were in variable ranges, and in the case of Arroyo-Manzanares *et al.* (1) were from 5.9 to 158.7 μg kg^{-1}. An EFSA report indicated concentrations reaching 191 μg kg^{-1} (11), and Kemboi *et al.* (13) reported ergot alkaloids in the range 0.4–154.5 μg kg^{-1} and 0.6–285.7 μg kg^{-1}, depending on the month of sampling. Poapolathep *et al.* (19) determined EAs in swine feeds in a 0.25–100.55 μg kg^{-1} range and in dairy feed in a 0.26–210.53 μg kg^{-1} range. Mulder *et al.* (17) reported contamination of compound feed with an average concentration of 77.0 μg kg^{-1} and a maximum of 583.0 μg kg^{-1}. Authors who conducted testing of unprocessed cereals usually noted higher maximum concentrations. Di Mavungu *et al.* (5) gave the highest concentration of EAs in a composite feed sample as 1,145.0 μg kg^{-1}, and Babič *et al.* (2) determined EAs in a range from 4.0 to 4,217.0 μg kg^{-1}. Mulder *et al.* (17) reported contamination of unprocessed feed reaching 1,231.0 μg kg^{-1}.

Generally, in the case of ergot alkaloids, there might be an additional difficulty in comparing obtained results, and reported results are often not in line with each other. The explanation for that might be the dependence of the variability of the ergot alkaloids in sclerotia on many different factors, especially environmental ones. Weather conditions are one of the important elements affecting both the amount and the composition of EAs in sclerotia (3). Additionally, the subjection of compound feeds to many processing steps, including high temperature treatments, is another possible differentiator of one sample of feed from another. It has already been observed that heating contaminated cereal products, for example by baking them, favours epimerisation and even degradation of EAs (22). In addition, there is huge variability in the type and proportions of cereals used in compound feed production. Therefore, the final composition of ergot alkaloid contamination may even not be the same in a compound feed and in the unprocessed cereals used in its production.

A method suitable for the determination of the 12 most relevant ergot alkaloids in compound feeds was developed and subsequently validated in house. The validation parameters assessed in the study met the requirements outlined in EU Regulation 2021/808, which confirmed the suitability of the method for the determination of EAs in compound feeds. The method was employed to analyse 40 compound feed samples. Among these samples, 16 (40% of the total) were found to contain at least one of the monitored alkaloids. However, only 10 samples could be quantified for ergot alkaloid concentration, as their levels exceeded the method's limit of quantification. The sum of determined concentrations ranged from below the LOQ to 62.3 μg kg^{-1}. The most frequently detected EAs were ergocryptine (25% samples), ergometrinine (17.5% samples), ergocristinine, ergocryptinine, ergocornine and ergocorninine (10% samples). Even though the EA concentrations detected can be considered relatively low, the high percentage of positive results indicates a need for additional information on the typical contamination level, focusing on feed materials used for the production of compound feed.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Financial Disclosure Statement: The study was supported by the National Veterinary Research Institute.

Animal Rights Statement: None required.

References

1. Arroyo-Manzanares N., Rodríguez-Estévez V., García-Campaña A.M., Castellón-Rendón E., Gámiz-Gracia L.: Determination of principal ergot alkaloids in swine feeding. J Sci Food Agric 2021, 101, 5214–5224, doi: 10.1002/jsfa.11169.

2. Babič J., Tavčar-Kalcher G., Celar F.A., Kos K., Červek M., Jakovac-Strajn B.: Ergot and Ergot Alkaloids in Cereal Grains Intended for Animal Feeding Collected in Slovenia: Occurrence, Pattern and Correlations. Toxins 2020, 12, 1–18, doi: 10.3390/toxins12110730.
3. Bryła M., Ksieniewicz-Woźniak E., Podolska G., Waśkiewicz A., Szymczyk K., Jędrzejczak R.: Occurrence of ergot and its alkaloids in winter rye harvested in Poland. World Mycotoxin J 2018, 11, 635–646, doi: 10.3920/WMJ2018.2322.
4. Craig A.M., Klotz J.L., Duringer J.M.: Cases of ergotism in livestock and associated ergot alkaloid concentrations in feed. Front Chem 2015, 18, 3, 1–6, doi: 10.3389/fchem.2015.00008.
5. Di Mavungu J.D., Malysheva S.V., Sanders M., Larionova D., Robbens J., Dubruel P., Van Peteghem C., De Saeger S.: Development and validation of a new LC–MS/MS method for the simultaneous determination of six major ergot alkaloids and their corresponding epimers. Application to some food and feed commodities. Food Chem 2012,135, 292–303, doi: 10.1016/j.foodchem.2012.04.098.
6. Eurachem/Co-operation on International Traceability in Analytical Chemistry: *Guide: CG 4 Quantifying Uncertainty in Analytical Measurement*, Third Edition, edited by S.L.R. Ellison, A. Williams, Eurachem/CITAC, LGC, Teddington, 2012.
7. European Commission: Commission Recommendation 2012/154/EU of 15 March 2012 on the monitoring of the presence of ergot alkaloids in feed and food. OJEU L 2012, 77, 55, 16/03/2012, 20–21, https://eur-lex.europa.eu/legal-content/EN/TXT/ PDF/?uri=CELEX:32012H0154.
8. European Commission: Commission Implementing Regulation (EU) 2021/808 of 22 March 2021 on the performance of analytical methods for residues of pharmacologically active substances used in food-producing animals and on the interpretation of results as well as on the methods to be used for sampling and repealing Decisions 2002/657/EC and 98/179/EC. OJEU L 2021, 180, 64, 21/05/2021, 84–109, https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32021R0808.
9. European Commission: Commission Regulation (EU) 2023/915 of 25 April 2023 on maximum levels for certain contaminants in food and repealing Regulation (EC) No 1881/2006. OJEU L 2023, 119, 66, 05/05/2023, 103–157, https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32023R0915.
10. European Food Safety Authority, Panel on Contaminants in the Food Chain (CONTAM), Alexander J., Benford D., Boobis A., Ceccatelli S., Cottrill B., Cravedi J.-P., Di Domenico A., Doerge D., Dogliotti E., Edler L., Farmer P., Filipič M., Fink-Gremmels J., Fürst P., Guérin T., Knutsen H.K., Machala M., Mutti A., Rose M., Schlatter J., van Leeuwen R.: Scientific Opinion on Ergot alkaloids in food and feed. EFSA J 2012, 10, 2798, 1–158, doi: 10.2903/j.efsa.2012.2798.
11. European Food Safety Authority, Arcella D., Gomez Ruiz J.A., Innocenti M.L., Roldan R.: Scientific report on human and animal dietary exposure to ergot alkaloids. EFSA J 2017, 15, 4902, 1–53, doi: 10.2903/j.efsa.2017.4902.
12. Holderied I., Rychlik M., Elsinghorst P.W.: Optimized Analysis of Ergot Alkaloids in Rye Products by Liquid Chromatography-Fluorescence Detection Applying Lysergic Acid Diethylamide as an Internal Standard. Toxins 2019, 11, 1–9, doi: 10.3390/toxins11040184.
13. Kemboi D.C., Ochieng P.E., Antonissen G., Croubels S., Scippo M.L., Okoth S., Kangethe E.K., Faas J., Doupovec B., Lindahl J.F., Gathumbi J.K.: Multi-Mycotoxin Occurrence in Dairy Cattle and Poultry Feeds and Feed Ingredients from Machakos Town, Kenya. Toxins 2020, 12, 1–16, doi: 10.3390/toxins12120762.
14. Kokkonen M., Jestoi M.: Determination of ergot alkaloids from grains with UPLC-MS/MS. J Sep Sci 2010, 33, 2322–2327, doi: 10.1002/jssc.201000114.
15. Krska R., Stubbings G., Macarthur R., Crews C.: Simultaneous determination of six major ergot alkaloids and their epimers in cereals and foodstuffs by LC-MS-MS. Anal Bioanal Chem 2008, 391, 563–576, doi: 10.1007/s00216-008-2036-6.
16. Maruo V.M., Bracarense A.P., Metayer J.P., Vilarino M., Oswald I.P., Pinton P.: Ergot Alkaloids at Doses Close to EU Regulatory Limits Induce Alterations of the Liver and Intestine. Toxins (Basel) 2018, 10, 183, doi: 10.3390/toxins10050183.
17. Mulder P.P.J., van Raamsdonk L.W.D., van Egmond H.J., Voogt J., van Brakel M.W., van der Horst G.M., de Jong J.: Dutch survey ergot alkaloids and sclerotia in animal feeds. 2012, RIKILT Report 2012.005. https://edepot.wur.nl/234699.
18. Oellig C.: Lysergic acid amide as chemical marker for the total ergot alkaloids in rye flour – Determination by high-performance thin-layer chromatography–fluorescence detection. J Chromatogr A 2017, 1507, 124–131, doi: 10.1016/j.chroma.2017.05.043.
19. Poapolathep S., Klangkaew N., Zhang Z., Giorgi M., Logrieco A.F., Poapolathep A.: Simultaneous Determination of Ergot Alkaloids in Swine and Dairy Feeds Using Ultra High-Performance Liquid Chromatography-Tandem Mass Spectrometry. Toxins 2021, 13, 724, 1–11, doi: 10.3390/toxins13100724.
20. Schardl C.L.: Introduction to the Toxins Special Issue on Ergot Alkaloids. Toxins (Basel) 2015, 7, 4232–4237, doi: 10.3390/toxins7104232.
21. Schummer C., Brune L., Moris G.: Development of a UHPLC-FLD method for the analysis of ergot alkaloids and application to different types of cereals from Luxembourg. Mycotoxin Res 2018, 34, 279–287, doi: 10.1007/s12550-018-0322-5.
22. Schummer C., Zandonella I., van Nieuwenhuyse A., Moris G.: Epimerization of ergot alkaloids in feed. Heliyon 2020, 6, 1–10, doi: 10.1016/j.heliyon.2020.e04336.
23. Waret-Szkuta A., Larraillet L., Oswald I.P., Legrand X., Guerre P., Martineau G.: Unusual acute neonatal mortality and sow agalactia linked with ergot alkaloid contamination of feed. Porc Health Manag 2019, 5, 1–5, doi: 10.1186/s40813-019-0131-z.