Determination of principal ergot alkaloids in swine feeding

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Abstract

BACKGROUND. Ergot alkaloids are secondary metabolites produced by fungi in the genus *Claviceps*. They contaminate a large variety of cereals, such as rye, triticale, wheat and barley. The ingestion of contaminated cereals might cause adverse health effects in humans and animals. In fact, pigs, cattle, sheep, and poultry are involved in sporadic outbreaks and, although there are several studies about occurrence of ergot alkaloids in grain cereals, there are scarce studies focused on compound feed.

RESULTS. Twelve ergot alkaloids have been quantified in 228 feed samples intended for swine. The analytes were extracted using QuEChERS with Z-Sep+ as sorbent in the clean-up step, which reduced the matrix effect, allowing limits of quantification between 2.1-21.7 µg/kg. The analytes were subsequently quantified by UHPLC-MS/MS. A total of 29 samples (12.7%) revealed contamination by at least one ergot alkaloid, and among contaminated samples, 65% were contaminated by more than one. Only 6 of 12 target ergot alkaloids showed concentrations above the limit of quantification. The concentrations for individual ergot alkaloids ranged between 5.9 µg/kg for ergosinine to 145.3 µg/kg for ergometrine (the predominant ergot alkaloid), while the total ergot alkaloid content ranged from 5.9 to 158.7 µg/kg.

CONCLUSIONS. The occurrence of ergot alkaloids in feed samples in Spain seems to be lower than in other regions of Europe. All the samples fulfilled current recommendations of the feed industry about practical limits for ergot alkaloids in pig feeds. This suggests that the feeds are safe for pig consumption, regarding the presence of ergot alkaloids.

Keywords: feed; pig; ergot alkaloids; liquid chromatography; mass spectrometry; QuEChERS

1. INTRODUCTION

Ergot alkaloids (EAs) are secondary metabolites produced by a wide range of mycotoxigenic fungi mainly in the genus *Claviceps*. These fungi parasitize the seed heads of living plant (cereals, such as rye, triticale, wheat and barley) at the time of flowering, forming a dark mass of mycelia (ergot body or sclerotia) containing the alkaloids. EAs are indole derivatives that present diverse structures but share a common tetracyclic ergoline ring system methylated on nitrogen N6 and different substitutions on C8. Most important EAs are derivatives of lysergic acid, and their toxicity is linked to their structural similarity with dopamine, noradrenaline, adrenaline and serotonin, enabling binding to the biogenic amine receptor and the interruption of neurotransmission.[1,2,3] Although based on median lethal dose (LD_{50}) EAs exhibit moderate oral acute toxicity, the ingestion of contaminated cereals might cause adverse health effects in humans and animals, as the well-known ergotism. These effects cover a broad spectra of symptoms (including convulsions, gangrenous, vomiting, fever or alterations in endocrine function, among others) depending on the species.[4] In the last decades, pigs, cattle, sheep, and poultry have been involved in sporadic outbreaks, with a negative economic impact in producers and livestock industry.[5,6,7]

There are more than fifty known EAs but, based on the most common EAs produced by *Claviceps purpurea* and their higher prevalence in cereals, only some of them are considered relevant for monitoring purposes. Those EAs are the major compounds: ergometrine (Em), ergosine (Es), ergotamine (Et), ergocornine (Eco), ergokryptine (Ekr) and ergocristine (Ecr). They have a stereocenter on position C8, and the aforementioned *(R)*-epimers can undergo a reversible epimerization into the corresponding *(S)*-inine epimers: ergometrinine (Emn), ergosinine (Esn), ergotaminine (Etn), ergocorninine (Econ), ergokryptinine (Ekrn) and ergocristinine (Ecrn).[8] The proportion of individual alkaloids is extremely variable within ergot bodies and their relative toxicity, which depends on their configuration, is not yet well understood. Currently, only the *(R)*-epimers are considered biologically active and toxics, while the *(S)*-epimers have almost no pharmacological effects; however, due to their rapid epimerization, and in order to avoid underestimation of the total content of EAs, the determination of both *(R)*- and *(S)*-epimers is mandatory.[9]

In order to protect animal health, European Union (EU) has established a maximum content of 1000 mg/kg of rye ergot sclerotia (*Claviceps purpurea*) in feed materials and compound feed containing unground cereals,[10] while different countries around the world have fixed different tolerance limits.[11,12] However, some recent studies reveal that feed contamination at levels of sclerotia close to this regulatory limit could have adverse effects on animal, suggesting that this limit should be revised.[13]

EAs as such are still not regulated, and there are discrepancy among authors on the correlation between sclerotia content and concentration of total EAs in the samples. Thus, while several studies describe a linear correlation between the content of sclerotia and the EA concentrations in cereal samples,[14,15,16] according to other study this correlation is difficult to establish at low concentrations of EAs (below 350 µg/kg grain).[17] On the other hand, other studies concluded that a reliable prediction of the EA content based on sclerotia is difficult, and effects on animal health correlated better with EAs than with sclerotia content.[18,19] In this sense, the establishment of maximum limits for EAs in food and feed is under study and, in the meantime and based on available data, the EFSA has estimated a group acute reference dose of 1 µg/kg body weight (BW) and a group tolerable daily intake of 0.6 µg/kg BW per day for avoiding vasoconstrictive effects of EAs in livestock.[8] Nevertheless, a recent study revealed that even low concentrations of EAs in the diet (100-200 µg/kg) have shown to adversely affect animal growth performance.[20]

Focusing on pigs, the ergotism is generally associated with poor performance, loss of appetite, agalactia, impact on reproduction, neonatal mortality, impaired liver function and gangrenous, among others.[6,11,21] These broad symptoms may make difficult to identify a possible intoxication due to the ingestions of EAs. Taking into account that, as stated before, no specific regulation about EA content in feeds is currently available, feed industry recommends practical limits for EAs in pig feeds to reduce negative effects on health and performance from 200 to 500 µg/kg for lower and higher level, respectively.[22] However, a recent study suggested that a long term consumption of even lower concentrations of EAs could induce deleterious effects in the liver and intestine in pigs.[13]

Concerning recent occurrence studies about EAs, although the occurrence of main mycotoxins in feed has been extensively studied around the world,[23,24,25,26] only few of these works included EAs.[19,26,27] Moreover, most of the papers including determination of EAs are focused on cereal grains,[14,28,29,30,31,32,33,34] being scarce those including processed feeds.[19,26,27] According to the recent available results, the data reported by EFSA in 2017, where a total of 654 feed samples collected in five different countries (Czech Republic, Croatia, Slovenia, the Netherlands and the United Kingdom) between 2011 and 2016, revealed that in more than 50% of these samples, not a single EAs was found above the limit of quantification (LOQ). It must be highlighted that most of the samples included in that study were grains of cereals or forages, being very limited the occurrence data on compound feeds.[16] Even a higher incidence of EAs was found in feeds from Kenya (including 16 dairy and 27 poultry feeds, and 24 feed ingredients), where up to 73% of the samples were contaminated by EAs (LOD =0.4 µg/kg), with concentrations between 0.4-285.7 µg/kg (mean concentration = 46.8 µg/kg).[27] Lower incidence were found in other studies, as a recent survey including 517 ground cereal samples from Slovenia, which revealed contamination in 17% of the samples, but with higher concentrations, ranging from 14 to 4217 µg/kg (mean concentration = 448 µg/kg).[33] On the opposite, other studies reported lower incidence of

contamination by EAs, as in 343 samples of feedingstuffs collected between 2008-2012 in Czech Republic and United Kingdom, where mean concentrations of units or tenths of µg/kg were found,[19] or in 32 poultry, swine, cattle, horse and lamb feed samples from Spain, where contamination by EAs was below LOQ (20 µg/kg) in all the samples.[26] These results highlight the differences in the production of EAs depending on climatic and geographical conditions. Also, the influence of composition is an important factor, as some grains (as rye) are more prompt to *Claviceps* contamination, while others (as maize) shows a very low incidence.

Regarding methods of analysis, alternative analytical techniques have been proposed for determination of EAs in feeds, such as infrared spectroscopy,[35,36] or liquid chromatography (LC) coupled to fluorescence detection,[37,38] although LC coupled to tandem mass spectrometry (LC-MS/MS)[26,19,26] is usually chosen for their unequivocal confirmation. Concerning sample treatment, extractions with different solvents or QuEChERS are generally proposed for extraction of EAs from cereal grains. Conditions of both, treatment and analysis of the samples, must be established in order to minimise epimerization of major EAs. The usual methods for determination of EAs (including sample treatments and analytical techniques) have been reviewed by Crew.[39] An important aspect that must be taking into account when optimising the method is the composition of the matrix to avoid a high matrix effect. In this sense, compound feed is a complex matrix based mainly on cereals (as rye, maize, wheat or barley), but also including other components, such as animal or vegetable oils, meals, molasses, minerals and vitamins, among others, in different proportion, depending on the animal stage (fattening pigs, sows, piglets, gilts, etc.).

Taking into account the above exposed, the scarce data about the presence of EAs in compound feed, and the lack of sample treatment specifically optimised for such a complex matrix, the aim of this work was the development and validation of an analytical method based on LC-MS/MS for the detection and quantification of the main twelve EAs (*R* and *S* epimers) in compound feed. Moreover, the method was applied in the analysis of 228 samples intended for fattening pigs, sows, piglets and gilts. To the best of our knowledge, this is the first time that such an extensive study has been performed in compound feed, and the data will be helpful to evaluate the incidence of EAs in this matrix, their co-occurrence, and the exposure of Spanish pigs to these contaminants.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

All reagents were of analytical reagent grade and solvents were LC-MS grade. Acetonitrile (MeCN) and methanol (MeOH) were obtained from VWR BDH Prolabo (West Chester,

Pennsylvania, USA), ammonium carbonate from Fluka (Buch, Switzerland) and formic acid was supplied by MERCK (Darmstadt, Germany).

Ultrapure water used throughout the work was obtained from a Milli-Q water purification system (18.2 MΩ cm^{-1}, Milli-Q Plus system, Millipore Bedford, MA, USA).

Z-Sep+ sorbent for clean-up was supplied from Supelco (Bellefonte, PA, USA); C-18 and EMR (Agilent Technologies, Santa Clara, California, USA) and Oasis HLB prime cartridges (Waters, Barcelona, Spain) were also tested. Before injection into the LC system, samples were filtered using syringe filters (25 mm, 0.2 µm nylon membrane) supplied by VWR.

2.2. Standards

Fine film dried standards of ergosine (Es), ergocornine (Eco), ergokryptine (Ekr), ergocristine (Ecr) and the corresponding epimers, ergosinine (Esn), ergocorninine (Econ), ergokryptinine (Ekrn) and ergocristinine (Ecrn), were purchased from Techno Spec (Barcelona, Spain); ergometrine (Em), ergotamine (Et) and their corresponding epimers, ergometrinine (Emn) and ergotaminine (Etn) were supplied by Romer Labs (Getzersdorf, Austria). As indicated by the manufacturer, these standards were reconstituted in 5 mL of MeCN, to obtain concentrations of 500 µg/mL for the main EAs and of 125 µg/mL for the epimers. Due to the rapid epimerisation of EAs in solution, defined volumes of individual or mixed standard solutions were pipetted into amber glass tubes, evaporated to dryness under a stream of nitrogen and stored at -20ºC. Under these conditions, the stability of standards have been reported to be at least one year.[40] The residues were reconstituted in the required amount of solvent immediately before use.

2.3. Instrumentation and equipment

EAs determination was carried out using an Agilent 1290 Infinity LC (Agilent Technologies) assembled with on-line degasser, autosampler, a binary pump and a column oven locating a Zorbax Eclipse Plus RRHD C18 column (50 mm×2.1 mm, 1.8 µm) linked to a triple quadrupole (QqQ) mass spectrometer API 3200 (AB Sciex, Toronto, ON, Canada) with electrospray ionization (ESI). Data processing were performed using the Analyst Software version 1.5 with schedule multiple reaction monitoring (MRM) TM Algorithm (AB Sciex).

For sample treatment, a high speed solids crusher with a grinding degree 50-300 mesh and a rotation speed of 25000 r/min (Model 250A from Hukoer, China), a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA), an evaporator system (System EVA-EC, from VLM GmbH, Bielefeld, Germany) and a universal 320R centrifuge (HettichZentrifugen, Tuttlingen, Germany) were used.

2.4. Samples

A total of 228 samples of swine feed were included in this study. Samples (approx. 500 g) were collected at different farms and manufacturing industries around Spain from February to August 2017 and sent directly to the laboratory by the veterinarians responsible for those farms or feed mills. Every sample was classified according to the following criteria:

- Animals to which it was destined: 71 samples were compound feed destined to fattening pigs, 42 to sows, 111 to piglets, 2 to gilts, and 2 samples of maize (grain given to the fattening pigs in two farms).
- Presentation: 2 samples of maize (grain) and 226 compound feed samples: 183 were presented as flour and 43 as pellet.
- Sampling point: 77 samples were collected from a silo, 29 from a feeder, 25 from a sack and 6 were stored in bulk without container in warehouse. No information was provided for the rest of the samples.

Samples were milled and homogenized using a laboratory grinder. Finally, all samples were stored, protected from light at room temperature until analysis.

2.5. Sample preparation

For EAs extraction a previously reported method for analysis of cereals was adapted.[32] Briefly, 1.0 g of homogenized sample were placed into a 50-mL screw cap test tube with conical bottom, and the analytes were extracted with 10 mL of MeCN and ammonium carbonate (85:15, v/v), shaking by vortex for 3 min. Every sample was centrifuged (12830 ×g, 5 min at 4°C) and 5 mL of the upper layer was placed into a 10-mL screw cap test tube with conical bottom with 0.15 g of Z-Sep+ sorbent for clean-up. Then, the 10-mL tube was shaken for 3 minutes and centrifuged (12830 ×g, 5 min at 4°C). Finally, 2 mL of the upper layer was evaporated to dryness under a gentle stream of N_2 and the residue was dissolved with 1 mL of MeOH:water (50:50, v/v). The solution was filtered through a 0.22 µm nylon membrane filter before injection into the UHPLC-MS/MS system.

2.6. Determination of ergot alkaloids by UHPLC-MS/MS

The chromatographic separation of EAs was carried out using a C18 Zorbax Eclipse Plus RRHD column (50 x 2.1 mm, 1.8 µm). The composition and gradient of the mobile phase were optimized in order to obtain a good resolution between peaks at the least possible time of analysis. This mobile phase consisted of eluent A (aqueous solution with 0.3% formic acid, v/v), and eluent B (MeOH with 0.3% formic acid, v/v) at flow rate of 0.4 mL/ min. The eluent gradient profile was as follows: 0-6 min: 30-50% B; 6-9 min: 50% B; 9-10 min: 50-30% B; 10-12 min: 30% B. The column temperature was set at 35 °C and the injection volume was 5 µL. The autosampler

temperature was maintained at 4ºC in order to minimize epimerization and the sample sequence was limited to 24 hours [41].

The MS method used multiple reaction monitoring (MRM) conditions and positive electrospray ionization (ESI+) mode. The MS parameters were as follows: temperature of the source: 500 °C; collision gas (nitrogen): 5 psi, voltage of the ion spray: 5 kV, curtain gas (nitrogen): 30 psi, GAS 1 and GAS 2 (both of them nitrogen): 50 psi.

The experiments were carried out using different declustering potential (DP), entrance potential (EP), collision cell exit potential (CXP) and collision energy (CE) for each analyte (see Table 1). The monitored ions of the target analytes were the protonated molecules $[M+H]^+$ for all of them, except for the epimers Esn, Etn, Econ and Ecrn where the signal at *m/z* corresponding to $[M-H_2O+H]^+$ was higher than that of the protonated molecules, in concordance with Diana Di Mavungu et al. (2012).[41] Under optimum conditions, EAs were separated and detected in less than 7 min. Figure 1 shows a chromatogram of a spiked feed sample obtained under these conditions.

3. RESULTS AND DISCUSSION

3.1. Optimization of sample treatment

In this work, different methods previously described for the extraction of mycotoxins were compared and investigated for the determination on EAs in feed. Specifically, the following methods were tested:

Method 1: Described for the extraction of EAs from rye, based on an extraction with ethyl acetate/MeOH/0.2 M ammonium bicarbonate pH 8.5 (30 min), phase separation, evaporation, reconstitution in MeOH/MeCN/water, addition of hexane, centrifugation and analysis of the aqueous phase.[29]

Method 2: Proposed by Guo et al. (2016) for the extraction of EAs from cereal-based products, based on a QuEChERS, followed by a dispersive solid phase extraction (dSPE) using C18 as sorbent for cleaning-up.[32]

Method 3: A QuEChERS-based extraction with MeCN with 5% formic acid, followed by the addition of salts (4 g $MgSO_4$, 1 g NaCl) without further clean-up, described for the simultaneous determination of EAs and other mycotoxins in wheat and maize.[30]

The results (summarized in Figure 2) were compared in term of extraction efficiency (calculated as: 100×signal of spiked sample/signal of spiked cleaned-up extract), and matrix effect (calculated

as: 100×(signal of spiked cleaned-up extract– signal of spiked matrix-free injection solvent)/signal of spiked matrix-free injection solvent).

Although method 1 provided the lowest matrix effects for most of the EAs, it was quite tedious and the throughput was worst compared to methods 2 and 3, with no improvements on extraction efficiency or matrix effect. Method 2 and 3 where both based on QuEChERS, using different conditions for extraction: MeCN/ammonium carbonate (method 2) followed by dSPE, and MeCN/formic acid (method 3) without dSPE. Those differences could cause the variations in the extraction efficiency reported by both methods, as basic media could increase the extraction of EAs reported by method 2. Moreover, as no clean-up was performed in method 3, matrix effect was the higher for most of EAs. It must be considered that method 3 was proposed for the extraction of 23 mycotoxins (including EAs), and the recoveries reported for EAs were around 60%, in accordance with results showed in Figure 2. Considering all these aspects, and as the method 2 showed the best extraction efficiency for most of EAs with an acceptable matrix effect, it was selected henceforth.

To further improve the extraction efficiency and decrease matrix effect of method 2, different sorbents used in the clean-up step based on dSPE were tested: C-18 (chosen sorbent in method 2 and recommended for removal of nonpolar interferences), Z-Sep+ (zirconium-based sorbent), and a polymer known as EMR (Enhanced Matrix Removal), both recommended for cleaning-up challenging matrices, especially those with high contents of fat, as the case of feeds with fat contents up to 10%. The results are shown in Figure 3. As can be seen, the use of C-18 sorbent involved a higher matrix effect in all cases, while lower matrix effects were obtained using Z-Sep+ sorbent. In addition, Z-Sep+ showed also the best extraction efficiency, so this sorbent was selected as optimum.

3.2. Method validation

In order to validate the analytical method, enantiomeric stability during analysis, linearity, limits of detection (LODs) and quantification (LOQs), matrix effect, precision and trueness (in terms of extraction efficiency) were evaluated. The method validation data are summarized in Table 2.

The enantiomeric stability during analysis was study by injection of standard solutions of EAs before, in the middle and at the end of every sequence of analysis. This confirms that, during the analysis time the rate of epimers were constant. Moreover, long sequences of analyses were avoided.

The matrix effect was investigated at three concentration levels (10, 25 and 100 μg/kg). A value of 0% indicates no effect of the matrix on the signal, while a value below 0% means suppression

of the signal due to the presence of the matrix. The obtained values ranged from -5.5 to -25.4%, being the highest for Emn and Econ. Based on these results, and taking as a reference the commonly accepted criteria that matrix effects higher than 20% (absolute value) must be compensated, procedural calibration was selected to carry out the quantitative analysis.[42]

Therefore, to assess the method linearity, procedural calibrations were performed by spiking blank feed samples with five different concentrations levels (from 10 to 150 μg/kg). Each level was prepared in duplicate an injected twice. Good linearity was observed for all EAs in the studied ranges with determination coefficients (R^2) above 0.99. The LODs and LOQs were determined as 3×signal-to-noise ratio (S/N) and 10×S/N, respectively. LOQs ranged between 2.1 μg/kg (Emn) and 21.7 μg/kg (Etn) and although no regulatory limits have been established for EAs in feed, these results could indicate the suitability of the proposed analytical method for EAs quantification at low μg/kg levels.

The trueness (evaluated as recovery) and precision in term of repeatability (intra-day precision) and intermediate precision (inter-day precision) were evaluated at three concentration levels (10, 25 and 100 μg/kg, except for Em and Etn, where only two levels were evaluated). To assess the repeatability, three blank feed samples (experimental replicates) were spiked at each concentration level and the whole procedure was applied on the same day. Each sample was injected in triplicate (instrumental replicates). Intermediate precision was evaluated with a similar procedure, with samples analysed in four different days (four experimental replicated injected in triplicate). The recovery values ranged between 70 and 90%, while the precision of the method (expressed as relative standard deviation of peak areas) was always lower than 13%.

3.3. Occurrence of ergot alkaloids in feed samples

The proposed methodology was applied to the determination of EAs in 228 feed samples intended for feeding pigs. For identification of analytes, SANTE guidelines recommendations were followed.[43] In the occurrence study, samples with analyte concentrations lower than LOD were considered negatives. Samples with concentrations higher than LOD were considered as positives and included in the estimation of incidence (percentage of positive samples). Finally, only samples with concentrations higher than LOQ were considered for estimation of EA concentration values. A summary of the results is showed in Table 3.

A total of 29 samples (12.7%) revealed contamination by EAs above the LOD, while 25 samples (11%) showed concentrations above the LOQs. These results showed a much lower incidence of these compounds in Spain than in Central or North Europe (where about 50% samples analysed showed contamination by EAs)[16] or Kenya where, according to a recent work, 70% of analysed feeds samples where contaminated with EAs;[27] however, they are in agreement with the lower

incidence of EAs reported in a recent paper by Babič et al. (contamination by EAs in 17% of the analysed cereal grain intended for animal feeding in Slovenia),[33] a study by Zachariasova et al., (low occurrence of EAs in feed samples form Czech Republic and United Kingdom)[19], and another study that revealed no contamination of EAs in 32 Spanish feed samples.[26]

The total EA concentrations ranged from 5.9 to 158.7 µg/kg, in accordance with the values reported by the EFSA report for compound feed (up to 191 µg/kg),[16] by Kemboi et al. (0.4-285.7 µg /kg)[27], and by Zachariasova et al.,[19] which reported maximum concentrations of tens up to hundreds of µg/kg for EAs in complex compound feeds for pigs. However, they are much lower than those reported by Babič et al. in cereal grains (concentrations up to 4217 µg/kg).[33]

These discrepancies on incidence and concentration of total EAs among the different reported results could be explained by different factors which have impact on individual EA production, such as ingredients of compound feeds, and the large regional and climatic influences, as *Claviceps purpurea* development is favoured by high rainfall and humidity. Moreover, the differences between grain cereal and compound feeds could be due to the influence of processing, as compound feeds could show a lower concentration of EAs than cereal grains.[16,44]

Among the twelve EAs included in the study, only 6 of 12 target EAs (Em, Emn, Et, Es, Esn and Ecr) showed concentrations above LOQ, while Eco was detected in one sample, with a concentration lower than the LOQ. Em was the most frequent EA (detected in 18 samples), and also showed the highest concentration (ranging between 18.6 and 145 µg/kg), followed by Esn (detected and quantified in 11 samples), Et (detected and quantified in 8 samples) and Ecr (detected and quantified in 5 samples). These results are slightly different from the EFSA report (where the EAs most commonly detected were Et, Es and Ecr[16]), from Zachariasova et al., (only Ecr, En, Ekr and Eco were detected in complex compound feed for pigs from Czech Republic and United Kingdom),[19] or from Kemboi et al. (where Ecrn, Ecr and Em were prevalent in feeds from Kenya),[27] but are similar to the results obtained by Babič et al., where the most frequent EAs were Em, Es and Ecrn.[33] Moreover, in accordance to EFSA report,[16] the main EAs were found at higher concentrations than their epimers, except for Esn. Moreover, the main EAs and their corresponding epimers were detected together in scarce samples. Specifically, Em and Emn were detected in only one sample and Es and Esn in 3 samples. These conclusions suggest that the effects of storage should be taken into account, as prolonged storage at higher temperatures can increase the epimerization.[11] Other factors, such as heating and processing could be significant, as stated recent study that reveals that heating can change the proportion of epimers.[44]

Moreover, the 65.5% of positive samples (19 from 29) revealed the co-occurrence of several EAs (see Figure 4). It should be noted that the 48% of samples were contaminated with 2 EAs, being the most common the appearance of Em with Esn. A summary of co-occurrence in positive

samples is shown in Table 4. These co-occurrence should be a matter of concern, as additive or synergistic effect can increase the toxicity of mycotoxins.[45]

Considering the available information about the samples, of 29 contaminated samples, 11 were for piglet (10% of total piglet feed samples), 9 for fattening pigs (6.4% of total fattening pigs feed samples) and 9 for sows (21.4% of total sows feed samples). The feed presented as pellet showed a higher percentage of contaminated samples (8 samples, 18.6% of total pellet samples) than the feeds presented as flour (21 samples, 11.5% of total flour samples). Regarding the sampling point, 6 contaminated samples were collected from a silo, 4 from a sack and only 1 sample were stored in bulk without container in warehouse. For the rest of the positive samples, this information was not available.

As a summary, all the analysed samples showed concentrations lower than the recommended limits of the feed industry for EAs in pig feeds to reduce their negative effects (from 200 to 500 µg/kg for lower and higher limit, respectively), and could be considered as safe.[22] However, some samples showed concentrations that should not be considered irrelevant, as some authors have reported negative effects (as a reduction of the growth efficiency of livestock) with even low concentrations (<100 µg/kg) of total EAs in the diet, for a long term consumption.[11] Although EAs acute intoxication in pigs is rarely reported in farms,[6] it is obvious that EAs are a source of concern for livestock and should be regularly monitored.

4. CONCLUSIONS

A method based on UHPLC-MS/MS and QuEChERS extraction has been optimised and validated for the determination of the main EAs in 228 compound feed samples, in order to obtain good recoveries and decrease matrix effect in such a complex matrix. Compared to other reported methods using common sorbents (as C18 or PSA), the use of Z-Sep+ in the dSPE clean-up step reduced the signal suppression (lower than 20% in most cases), with good recoveries and precision for all the analytes. The analysis revealed that only 12.7% of the samples were contaminated with EAs. The occurrence of these compounds in feed samples in Spain seems to be lower than in other regions of Europe, where around 50% of the samples showed contamination by EAs (according to the EFSA report), but are in accordance with other recent reported studies. All the samples fulfilled current recommendations of the feed industry about practical limits for EAs in pig feeds. This suggests that the tested feeds are safe for pig consumption, regarding the presence of EAs. However, some samples showed concentrations higher than 150 µg/kg, which, according to some studies, could have negative effects in long term consumption. EU has established maximum content only for rye ergot esclerotia; however, taking into account that feed

is a processed commodity and that esclerotia content is not easy to estimate in this matrix, setting maximum limits for EAs content in addition to esclerotia could be useful for controlling these contaminants. Besides, considering the relative incidence of EAs, the individual toxicity of each EA should be considered to stablish tolerable levels of ergot contamination in cereal grains for food and feed.

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