Toxicon:X 8 (2020) 100058

Contents lists available at ScienceDirect

Toxicon: X

journal homepage: www.journals.elsevier.com/toxicon-x

Quantification of zearalenone and α-zearalenol in swine liver and reproductive tissues using GC-MS

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ARTICLE INFO

Keywords:
Mycotoxin
Zearalenone
α-ZEL
GC-MS
Reproductive tissue
Liver
Fusarium
Co-products
Swine
Prolapse

ABSTRACT

The mycotoxin zearalenone (ZEN) is a common contaminant of swine feed which has been related to a wide range of reproductive anomalies in swine, such as pelvic organ prolapse, anestrous, and pseudopregnancy. New information is needed to understand how ZEN and related metabolites accumulate in swine reproductive tissues. We conducted a feeding study to track ZEN and the metabolite α-zearalenol (α-ZEL) in swine liver and reproductive tissues. Thirty pubertal gilts were randomly assigned one of three treatments, with ten pigs in each treatment group: (1) base feed with solvent for 21 days, (2) ZEN-spiked feed for seven days followed by base feed with solvent for 14 days, and (3) ZEN-spiked feed for 21 days. At the end of the trial, liver, anterior vagina, posterior vagina, cervix, uterus, ovaries, and broad ligament were collected from pigs. ZEN was found in the anterior vagina, posterior vagina, cervix, and ovaries, with significantly higher concentrations in the cervix relative to other reproductive tissues. ZEN and α-ZEL were found in liver tissue from pigs in each treatment group. Our results show that ZEN accumulates more in the cervix than other reproductive tissues. The presence of ZEN in reproductive tissues may be indicative of ZEN-related reproductive symptoms. Future work could examine how ZEN concentrations vary in reproductive tissues as a factor of the pigs age, weight, sex, or parity, to establish parameters that make pig more sensitive to ZEN.

1. Introduction

Swine feed is composed of a variety of cereal crops which may be infected by mycotoxin-producing *Fusarium spp.* (Goswami and Kistler, 2004; Gruber-Dorninger et al., 2019). Mycotoxins in cereals persist through feed preparation (Bryden, 2012), and depending on storage conditions (Miller, 1995), continue to be produced until being fed to livestock. In addition, distiller's dried grains with solubles (DDGS), a co-product of ethanol, are becoming an increasingly common ingredient in swine feed due to the high nutrient density relative to corn (Jung et al., 2013; Spiehs et al., 2007). DDGS have been found to contain an increased concentration of mycotoxins relative to the original crop (Agyekum et al., 2014; Gruber-Dorninger et al., 2019; Khatibi et al., 2014). Mycotoxins contributed though DDGS alone are known to have a costly impact on the swine industry, contributing to upwards of $147 million in losses annually (Wu and Munkvold, 2008). With recent reports of increasingly common reproductive anomalies in swine herds (Stock et al., 2017), there are concerns regarding mycotoxin contamination in DDGS.

The mycotoxin zearalenone (ZEN) is one of a variety of mycotoxins produced by fungi in the genus *Fusarium* (Caldwell et al., 1970), which may adversely affect swine health. A recent survey showed ZEN was present in 56% of finished feed samples and 75% of DDGS worldwide (Gruber-Dorninger et al., 2019). ZEN behaves as a pseudo-estrogen, leading to a wide range of reproductive issues in swine of all ages and sex (Kanora and Maes, 2010; Yang et al., 2020; Zinedine et al., 2007). After consumption, ZEN may be metabolized into α-zearalenol (ZEL), β-ZEL, α-zearalanol, β-zearalanol, or zearalenone (Zöllner et al., 2002). ZEN and α-ZEL have especially high binding affinities for estrogen receptors in swine (Fitzpatrick et al., 1989), leading to changes in the regulation of sex hormones (Zheng et al., 2019). ZEN and metabolites are conjugated to glucuronic acid during entero-hepatic metabolism

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https://doi.org/10.1016/j.toxcx.2020.100058
Received 4 May 2020; Received in revised form 3 September 2020; Accepted 7 September 2020
Available online 9 September 2020

(Minervini and Dell'Aquila, 2008), and lose their estrogenic potency (Frizzell et al., 2015), allowing for excretion after 4–5 days (Olsen et al., 2009).

Methods for quantifying ZEN and metabolite concentrations in biological samples are important in determining mycotoxin exposure in animals for clinical or research purposes. Kwaśniewska et al. has previously reviewed methods used for determination of ZEN and metabolites in tissue samples (Kwaśniewska et al., 2015). Many methods require samples to undergo similar homogenization, extraction, and clean-up steps prior to instrumental analysis. Liquid (LC) and gas chromatography (GC) are common instrumental methods capable of separating ZEN and metabolites from other compounds, and when combined with mass spectrometry (MS), can be used to identify ZEN and metabolites by ion mass. While LC methods are common in recent literature (Kwaśniewska et al., 2015; Singh and Mehta, 2020), gas chromatography may be capable of providing similar sensitivity (Kinani et al., 2008). However, research involving the application of GC-MS to the analysis and ZEN and metabolites in biological samples is limited (Blokland et al., 2006). More research is needed to explore the use of GC-MS in the analysis of ZEN and metabolites in biological samples.

ZEN-related reproductive anomalies range from relatively mild symptoms such as vulva swelling or pseudopregnancy, to more severe symptoms such as prolapse, infertility, and embryonic death (Kanora and Maes, 2010; Yang et al., 2020). Some symptoms disappear when contaminated feed is replaced (Blaney, B.J., Bloomfield, B.C, Moore, 1984), while others may persist and lead to euthanasia of the animal. Sows that are euthanized for reproductive related anomalies contribute to approximately $5200 in losses annually per 1000 sows, with unknown additional expenditures associated with piglet loss, veterinary care, and labor (Stock et al., 2017). In order to address rising concerns with ZEN-related reproductive anomalies, there is a need for more information regarding the transport of ZEN and α-ZEL throughout swine reproductive tracts.

Based on previous literature, we hypothesized that (1) non-conjugated ZEN and α-ZEL would be present in reproductive tissues of pigs fed high concentrations of ZEN, (2) conjugated and non-conjugated ZEN and α-ZEL will be present in the liver of pigs metabolizing ZEN, and (3) ZEN and α-ZEL concentrations will vary among feeding study treatment groups and tissue type. To test these hypotheses, we performed a swine feeding trial wherein three groups of pubertal gilts were fed controlled amounts of ZEN over a 21-day period. The first group was fed non-spiked swine feed for 21 days, the second group was given feed spiked with 6 mg ZEN for 7 days, followed by 14 days on non-spiked feed, and the third group was given feed spiked with 6 mg ZEN for 21 days (Fig. 1). The specific objectives of this study were to (1) develop a method for GC-MS quantification of ZEN and α-ZEL in swine reproductive tissues, and (2) compare ZEN and α-ZEL concentrations between tissues and treatment groups.

2. Materials and methods

2.1. Swine feeding study

A swine feeding study, in which known amounts of ZEN were added to swine feed, was conducted to determine ZEN and α-ZEL concentrations in swine reproductive tissues. Methods and procedures for this feeding study were approved by the Virginia Tech Institutional Animal Care and Use Committee. Thirty cross-bred pubertal gilts, approximately six months old, with an average weight (± standard deviation) of 106.9 ± 17.4 kg were housed individually and randomly assigned to one of three treatments, with 10 pigs in each treatment group: (Treatment 1) base feed with evaporated solvent for 21 days, (Treatment 2) feed spiked with 6 mg ZEN for 7 days followed by base feed with evaporated solvent for 14 days, or (Treatment 3) feed spiked with 6 mg ZEN for 21 days (Fig. 1).

To prepare for administering treated feed, 20 g concentrated portions of ZEN-spiked and solvent-only feed were prepared prior to the study. Each morning, the concentrated portion (20 g) of feed was added to a 207 g of non-treated feed, and mixed thoroughly to form a 227 g (0.5 lb) 'treatment ration'. After the treatment ration was consumed, pigs were given an additional 2.0 kg (4.5 lb) non-treated feed. Eating behavior of the pigs was monitored to ensure that pigs consumed the treatment ration, with negligible amounts of feed leftover in the trough.

Concentrated portions of feed spiked with either 6 mg ZEN or evaporated solvent were prepared by modifying a commercially available grower diet (Big Spring Mill, Elliston, VA). ZEN-spiked feed was prepared by adding 600 μL of 10 mg mL^{-1} ZEN working solution to 20 g grower diet. The 10 mg mL^{-1} ZEN solution was prepared by dissolving crystalline ZEN (≥98% purity, J&K Scientific, Beijing, China) in acetonitrile (Thermo-Fisher, Waltham, MA, USA). Solvent-only feed was prepared by adding 600 μL acetonitrile to 20 g of commercial grower diet. For both forms of concentrated feed, solvent was evaporated under a fume hood overnight, then feed was stored at room temperature in a dark, dry area until feeding. Treated feed was stored for less than 60 days. Drinking water was provided to each pig for *ad libitum* consumption.

Overall, pigs consuming ZEN-spiked feed were given 6 mg ZEN alongside 2.3 kg (5 lb) feed, for a concentration of 2.67 μg g^{-1} ZEN. The 6 mg dose of ZEN was selected based on literature that reported estrogenic symptoms in pubertal gilts consuming 3–10 μg g^{-1} ZEN (Zimmerman et al., 2019). Six mg ZEN was intended to be added to 2 kg feed for a final concentration of 3 μg g^{-1} ZEN, however, the amount of feed was ultimately increased to 2.3 kg (5 lb), while maintaining the dose of 6 mg ZEN per day.

2.2. Sample collection and storage

At the end of the 21-day trial pigs were slaughtered at a local abbatoir. Reproductive tracts were collected after slaughter, and dissected into the anterior vagina, posterior vagina, cervix, broad

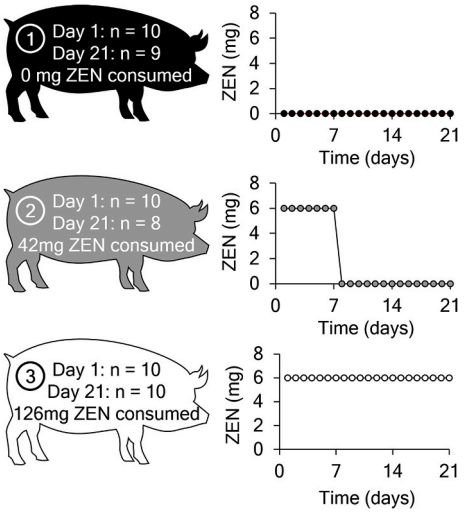

Fig. 1. Feeding trial timeline for each treatment group. Thirty pigs were randomly divided into one of three treatment groups, with 10 pigs in each group. Pig silhouettes show the number of pigs (n) participating in each treatment group at the beginning (day 1) and end (day 21) of the trial, and the total amount of supplemented ZEN consumed by the end of the trial. Plots to the left of each pig show a timeline of how ZEN was administered to pigs in each treatment group. Pigs participating in treatment 1 were fed a commercial diet with no additional ZEN for 21 days. Pigs participating in treatment 2 were fed a commercial diet spiked with 6 mg of ZEN for 7 days, followed by commercial diet with no added ZEN for 14 days. Pigs participating in treatment 3 were fed a commercial diet spiked with 6 mg ZEN for 21 days.

ligament, uterus, and ovaries (Fig. 2). Excess fatty or ligamentous tissue was trimmed away. Follicular fluid was drained from the ovaries prior to freezing so that result reflected concentrations within tissue only. Reproductive tissue samples were then packaged in freezer bags (Ziploc, SC Johnson, Racine, WI, USA), and stored at −20 °C until analysis.

A small portion of liver (approximately 4.0 × 4.0 cm) was also collected from each pig at slaughter. Liver tissue was placed in a 15 mL conical tube (Genesee Scientific, San Diego, CA, USA), quick-frozen in liquid nitrogen, and stored at −80 °C.

2.3. Extraction

While still frozen, anterior vagina, posterior vagina, cervix, ovaries, broad ligaments, and liver specimens were finely minced. Minced tissue from both ovaries was combined. Due to its large size, a 2–3 cm cross-section of the folded, frozen, uterus was finely minced, as opposed to analyzing the whole organ. Three sub-samples were collected from each tissue specimen. Tissue specimen were deemed suitable for analysis only if three sub-samples could be collected; smaller samples which could not be sub-sampled in triplicate were excluded from analyses.

Liver tissue was analyzed for total ZEN and α-ZEL concentrations, including ZEN and α-ZEL glucuronides (Fig. 3). The enzymatic hydrolysis method used to de-conjugate glucuronides was adapted from De Boer et al. (De Boer et al., 2005). Each sub-sample of tissue (1 g) was homogenized in 0.64 mL pH 5.0 acetate buffer (1 M, BioWorld, Fisher Scientific, USA). Homogenization was carried out using a Polytron PT 10–35 with PTA-10T generator (Kinematica AG, Luzern, Switzerland). After homogenization, 10 μL of β-glucuronidase (*Helix pomatia*, H-2, Millipore Sigma, USA) was added to the mixture, incubated at 37 °C for 2 h, then allowed to cool to room temperature. Methods for extraction of ZEN and α-ZEL from enzyme-hydrolyzed liver tissue were adapted from standard mycotoxin analysis methods previously described by Khatibi et al. (Khatibi et al., 2014). Once cooled, 3.36 mL of acetonitrile was added to the homogenized solution, forming an 84% (v/v) extraction solution of acetonitrile and acetate buffer, which was then shaken (200 rpm) for 1 h at room temperature. The shaken mixture was passed over two C18 columns (1.5 g mixture of aluminum oxide and C18 in a 1:3 ratio). Aluminum oxide (active, neutral, 0.063–0.200 mm particle size range) was acquired from Sigma Aldrich (St. Louis, MO, USA), and C18 was acquired from VWR (Radnor, PA, USA). After passing through the columns, 2 mL of combined effluent was evaporated to dryness at 55 °C under air flow.

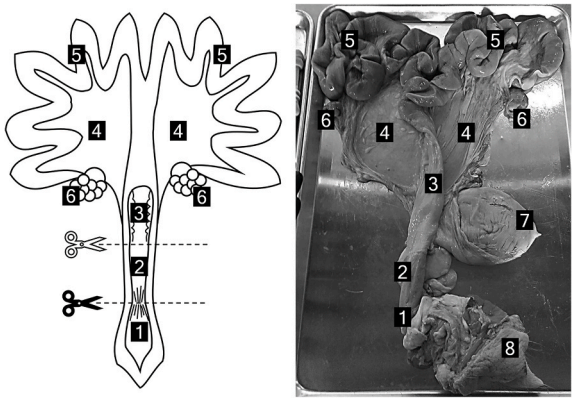

Fig. 2. The swine reproductive tract depicted as a line-drawing (left) and a greyscale image (right). In the left image, black scissors indicate where the anterior and posterior vagina were separated, while the white scissors indicate where the anterior vagina and cervix were separated. In the right image, reproductive tissue is shown from a pig that was not involved in the feeding trial, which shows organ positions in reference to the bladder and vulva. The posterior vagina is partially shielded by vulva tissue in the right image. Corresponding numbers on both images indicate tissue types. Legend: (1) posterior vagina, (2) anterior vagina, (3) cervix, (4) broad ligament, (5) uterus, (6) ovaries, (7) bladder, (8) vulva.

Reproductive tissue was analyzed for non-conjugated ZEN and α-ZEL concentrations (Fig. 3). The amount of tissue collected per sub-sample varied depending on tissue size: 1 g sub-samples were collected of ovaries and broad ligament, 2 g sub-samples were collected of of anterior and posterior vaginas, and 5 g sub-samples were collected of cervix and uterus. Sub-samples were then homogenized in an extraction solution of 84% (*v/v*) acetonitrile in DI water. The volume of extraction solution added to each sub-sample was equal to four times the mass of tissue: 4 mL to ovaries and broad ligament, 8 mL to anterior and posterior vagina, and 20 mL to cervix and uterus. Enzymatic hydrolysis was not included in reproductive tissue analyses because of the low binding affinity of ZEN-glucosides to estrogen receptors (Frizzell et al., 2015). Homogenized tissue was shaken at room temperature for 1 h at 200 rpm. The shaken mixture was passed over one C18 column for every 4 mL of extraction solution. Conditioning the C18 column is not necessary for this method (Khatibi et al., 2014). After passing through the column, 2 mL of effluent from broad ligament, anterior vagina, posterior vagina, cervix, and uterus sub-samples, and 1 mL of effluent from ovary sub-samples, were evaporated to dryness at 55 °C under air flow.

2.4. Derivatization

Derivatization methods were adapted from Kinani et al. (Kinani et al., 2008). Evaporated broad ligament, anterior vagina, posterior vagina, cervix, and uterus effluent was derivatized with 100 μL *N,O*-Bis (trimethylsilyl)trifluoroacetamide (BSTFA, Sigma Aldrich, St. Louis, MO, USA) at 90 °C for 10min, then resuspended in 500 μL of 500 ng mL^{-1} Mirex (Sigma Aldrich, St. Louis, MO, USA) in isooctane. The reaction was quenched with 1000 μL DI water and vortexed until the isooctane layer was clear. Evaporated ovary and liver effluent was derivatized with 50 μL BSTFA at 90 °C for 10min, and re-suspended in 250 μL of 500 ng mL^{-1} Mirex working solution in isooctane. The reaction was quenched with 500 μL water and vortexed until the isooctane layer of solvent was clear. For all tissue types, a 125 μL aliquot of the isooctane layer was transferred to a vial for GC-MS analysis. For quality assurance, effluent from recovery experiments was evaporated and derivatized alongside experimental samples.

All analyses were carried out with tissue-specific matrix-matched calibration standards ranging from 5 to 500 ng g^{-1}. Crystalline ZEN (≥98% purity) and α-ZEL (≥98% purity) used for calibration and method validation were acquired from Cayman Chemical (Ann Arbor, MI, USA).

2.5. GC-MS parameters

Analyses were carried out on an Agilent 6890/5975 GC-MS system. An autosampler (Agilent 7683B) was used for splitless 1 μL injections of sample through a non-tapered liner (Agilent, Santa Clara, CA, USA), onto an HP-5MS column (0.25 mm inner-diameter by 0.25 μm film diameter thickness by 30 mm length, Agilent, Santa Clara, CA, USA), using helium (Ultra High Purity, Airgas, Radnor, PA) as a carrier gas.

Inlet temperature was set at 250 °C. For analysis of anterior vagina, posterior vagina, cervix, broad ligament, and uterus samples, initial oven temperature of 140 °C was held for 0.5 min then ramped at a rate of 20 °C/min to 300 °C and held for 3.0 min (Fig. 4). For ovary and liver samples, a modified oven method was used; the initial oven temperature of 140 °C was held for 0.5 min, ramped at a rate of 10 °C/min to 300 °C, and held for 0.5 min. The oven method was modified for ovary and liver samples because preliminary experiments using the original oven method showed co-eluting peaks in chromatograms which could potentially interfere with ZEN and α-ZEL quantification. The co-eluting peaks were successfully separated from ZEN and α-ZEL using the

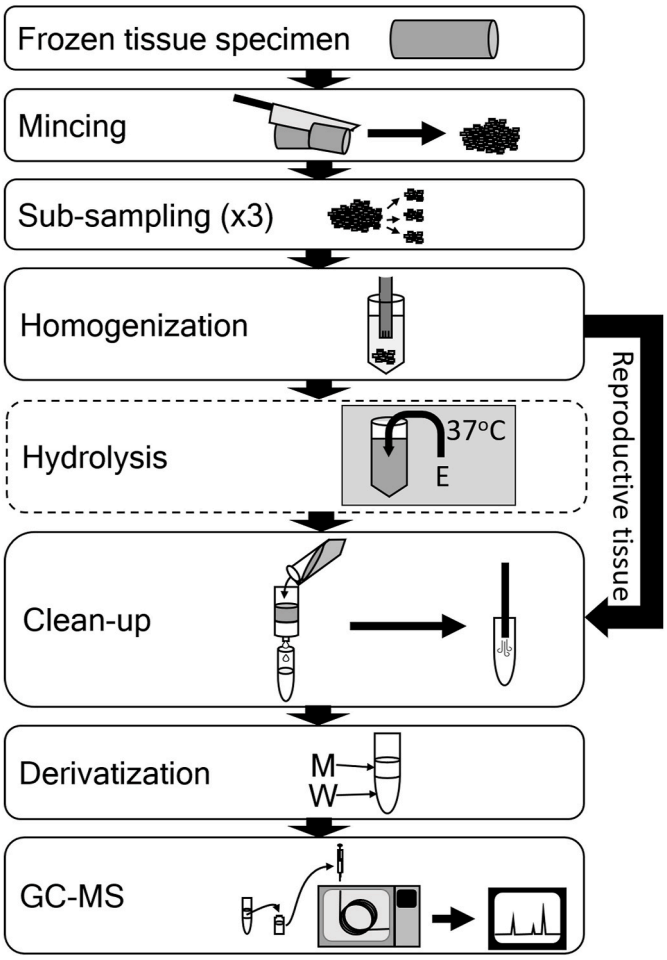

Fig. 3. A flow chart showing key events involved in processing tissue for GC-MS analysis. Tissues were finely minced while still frozen, and sub-sampled in triplicate. Liver tissue sub-samples were homogenized in acetate buffer and incubated for 2 h at 37 °C with β-glucuronidase enzyme. Following incubation, acetonitrile was added to each sub-sample to create an 84% (v/v) solution, then shaken for 1 h at room temperature. Reproductive tissue sub-samples were homogenized in a solution of 84% (v/v) acetonitrile and water, skipping the enzyme hydrolysis step. Samples were then shaken for 1 h at room temperature. After shaking, all tissue sub-samples were passed over at least one C18 clean-up column, and a known volume of effluent was evaporated to dryness. The evaporated sub-samples were re-suspended in a solution of 500 ng mL^{-1} mirex in isooctane, and the reaction was quenched with water. The isooctane layer of each sub-sample was then transferred to the GC-MS for analysis.

modified oven method. After method completion, the oven was held at 335 °C for 2.5 min.

The mass spectrometer was run in selected ion monitoring (SIM) mode. Mirex was used as an internal standard, at 500 ng g^{-1}. Table 1 describes mass-charge (m/z) ratios of quantifier and qualifier ions, as well as proportions used for identifying analytes of interest. Representative quantifier and qualifier ion chromatograms are shown in Fig. 4.

2.6. Statistical analyses

All statistical analyses were carried out using Minitab 19 (v19.2, State College, PA, USA). A Kruskal-Wallis test was used to compare average ZEN and α-ZEL concentrations between treatment groups within each tissue type. A separate Kruskal-Wallis test was used to compare average, overall, ZEN and α-ZEL concentrations between tissue types. Due to a lack of suitable non-parametric post-hoc tests, a one-way analysis of variance (ANOVA) combined with Fisher's LSD was also performed to compare overall ZEN and α-ZEL concentrations between tissue types.

2.7. Method validation

A series of preliminary experiments were conducted to develop and refine methods for detecting ZEN and α-ZEL in swine tissues using GC-MS. Reproductive tracts and liver were collected from pubertal gilts of similar age and weight, which were not involved in the feeding study. Anterior and posterior vaginal tissue samples were combined for validation experiments. All tissue used for validation was analyzed prior to the recovery experiment to confirm that neither ZEN nor α-ZEL could be determined on resulting chromatograms. Recovery of ZEN and α-ZEL was performed in triplicate at the limit of quantification (LOQ), 50 ng g^{-1} and 100 ng g^{-1}. Frozen tissues from each tissue type were minced as previously described, then spiked with the appropriate volume of 1 mg g^{-1} ZEN and α-ZEL working solution. Working solutions of 1 μg g^{-1} ZEN and α-ZEL were made by diluting 100 μg g^{-1} standards solution (Romer Labs, Newark, DE, USA) in appropriate volumes of acetonitrile. Extraction and derivatization procedures for each tissue type were then carried out as previously described.

Tissue specific matrix-matched calibration standards (5–500 mg g^{-1} ZEN and α-ZEL) were made by evaporating 2 mL of blank tissue extract from the validation experiments with the appropriate volume of 1 mg g^{-1} ZEN and α-ZEL working solution. LOQ was determined as the lowest calibration standard with a signal to noise ratio greater than three. Repeatability of quantification at the LOQ was determined by repeatedly injecting a calibration standard equivalent to the LOQ and determining relative standard deviation (RSD) by dividing the standard deviation of the determined concentrations, by the expected concentration.

3. Results

3.1. Swine feeding study

Thirty pigs participated in the feeding study, but only 27 pigs completed it. Three pigs were removed from the trial for unrelated health reasons that interfered with treatment consumption. Of the 27 pigs that completed the trial, nine were from treatment 1, eight were from treatment 2, and ten were from treatment 3 (Fig. 1). Though 27 full reproductive tracts were collected, individual tissue specimens were not included in this study if they were too small to be sub-sampled in triplicate, or if they could not be separated from the surrounding fatty tissue. In preliminary experiments, excess fatty tissue was found to affect analytical performance. A total of 25 anterior vagina, 13 posterior vagina, 19 broad ligament, 19 pairs of ovaries, 27 uteri, and 27 cervix specimens were analyzed. The number of sub-samples tested per group are reported in Fig. 5.

3.2. ZEN and α-ZEL in tissues

ZEN peaks were observed in GC-MS chromatograms for samples of the anterior vagina, posterior vagina, cervix, ovaries, and liver. No ZEN peaks were observed in the uterus or broad ligament. α-ZEL peaks were observed in the liver, but were not observed in any reproductive tissues. Chromatograms were qualitatively evaluated for significant analyte peaks (Fig. 5). Quantitative analyses were conducted only with peaks which corresponded to concentrations greater than the LOQ.

In the anterior vagina, an average of 25.4 ± 6.0 ng g^{-1} ZEN was present across treatment groups (Fig. 6), representing four of the 21 sub-samples analyzed. Of the four quantifiable sub-samples, three sub-samples were from the treatment 1 group and one sub-sample was from the treatment 2 group. An additional nine sub-samples were found to have significant chromatographic ZEN responses which quantified below the LOQ (Fig. 5). Of the nine sub-samples which quantified below the LOQ, one sub-sample was from the treatment 1 group, three sub-

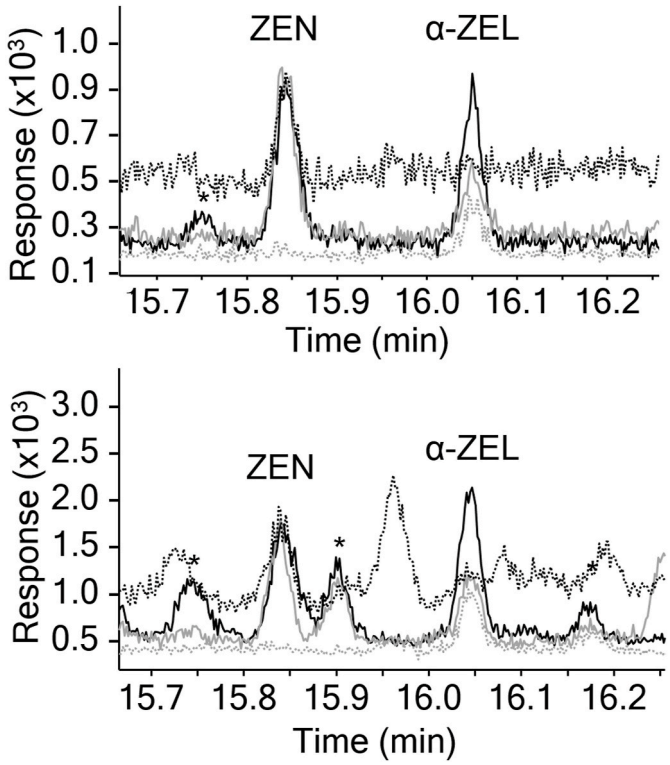

Fig. 4. Quantifier and qualifier ion chromatograms resulting from a calibration standard (upper) and representative liver sample (lower). ZEN is quantified by the 333.15 m/z (black, solid line) chromatogram, and qualified with the 305.2 m/z (grey, solid line) and 429.3 m/z (black, dashed line) chromatograms; α-ZEL is quantified with the 305.2 m/z (grey, solid line) chromatogram and qualified with the 333.15 m/z (black, solid line) and 446.1 m/z (grey, dashed line) chromatograms. GC-MS methods were optimized to separate ZEN and α-ZEL from co-eluting chromatographic peaks (*) with similar quantifier ions. The upper chromatogram show a 25 ng g^{-1} ZEN and α-ZEL calibration standard with liver tissue background; the lower chromatogram shows a representative liver sample found to contain 28.2 ng g^{-1} ZEN and 38.5 ng g^{-1} α-ZEL.

Table 1
Mass spectrum ions used for identification and quantification of Mirex, ZEN, and α-ZEL.

Compound name	Quantifier ion (m/z)	Qualifier ion (m/z)	Qualifier ion (m/z)
Mirex	271.80 (100%)	275.8 (32.8%)	n.a.
ZEN	333.15 (100%)	305.2 (84.5%)	429.3 (66.6%)
α-ZEL	305.2 (100%)	333.15 (47.3%)	446.3 (35%)

n.a.: not applicable. (%) indicates the percentage of the major ion response.

samples were from the treatment 2 group, and 5 sub-samples were from the treatment 3 group. No α-ZEL was identified in any anterior vagina sub-samples.

In the posterior vagina, an average of 21.5 ± 3.0 ng g^{-1} ZEN was present across treatment groups (Fig. 6), representing four of the 18 sub-samples analyzed. All four quantifiable sub-samples were from the treatment 3 group. An additional four sub-samples were found to have significant chromatographic ZEN responses which quantified below the LOQ (Fig. 5). Of the four sub-samples which quantified below the LOQ, two sub-samples were from the treatment 1 group, and two sub-samples were from the treatment 2 group. No α-ZEL was identified in any posterior vagina sub-samples.

In the cervix, an average of 81.3 ± 11.9 ng g^{-1} ZEN was present across treatment groups (Fig. 6), representing 24 of the 81 sub-samples analyzed. Of the 24 quantifiable sub-samples, eight sub-samples were from the treatment 1 group, eleven sub-samples were from the treatment 2 group, and five sub-samples were from the treatment 3 group. An additional two sub-samples were found to have significant chromatographic ZEN responses which quantified below the LOQ (Fig. 5). Of the two sub-samples which quantified below the LOQ, one sub-sample was from the treatment 1 group, and one sub-sample was from the treatment 2 group. No α-ZEL was identified in any cervix sub-samples.

In the ovaries, an average of 22.0 ± 4.3 ng g^{-1} ZEN was present across treatment groups (Fig. 6), representing four of the 57 sub-samples analyzed. Of the four quantifiable sub-samples, one sub-sample was from the treatment 1 group, one sub-sample was from the treatment 2 group, and two sub-samples were from the treatment 3 group. An additional eleven sub-samples were found to have significant chromatographic ZEN responses which quantified below the LOQ (Fig. 5). Of the eleven sub-samples which quantified below the LOQ, three sub-samples were from the treatment 1 group, seven sub-samples were from the treatment 2 group, and one sub-sample was from the treatment 3 group. No α-ZEL was identified in any ovary sub-samples.

In the liver, an average of 34.9 ± 6.5 ng g^{-1} ZEN and 37.9 ± 6.3 ng g^{-1} α-ZEL was present across treatment groups (Fig. 6). Three of the 66 liver sub-samples analyzed contained quantifiable concentrations of ZEN. Of the three sub-samples containing ZEN, one was from the treatment 1 group, and two were from the treatment 3 group. An additional nine sub-samples were found to have significant chromatographic responses for ZEN which quantified below the LOQ (Fig. 5). Of the nine sub-samples which qualified ZEN below the LOQ, three were from the treatment 1 group and six were from the treatment 3 group. Eight of the 66 liver sub-samples analyzed contained quantifiable concentrations of α-ZEL. Of the eight sub-samples containing α-ZEL, one was from the treatment 2 group, and seven were from the treatment 3 group. An additional seven sub-samples were found to have significant chromatographic responses for α-ZEL which quantified below the LOQ (Fig. 5). Of the seven sub-samples which quantified α-ZEL below the LOQ, three were from the treatment 1 group, and four were from the treatment 3 group. A total of 17 liver sub-samples were found to contain ZEN or α-ZEL, of which ten sub-samples had both ZEN and α-ZEL present. In treatment group 1 there were five sub-samples with both ZEN and α-ZEL present, two sub-samples with only ZEN present, and one sub-sample with only α-ZEL present. In treatment group 2, there were no sub-samples with ZEN present, and only one sub-sample with α-ZEL present. In treatment group 3, there were seven sub-samples with both ZEN and α-ZEL present, no sub-samples with only ZEN present, and two

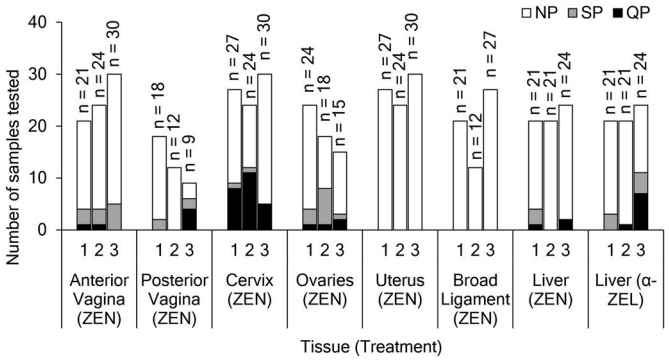

Fig. 5. A stacked bar plot showing the qualitative results of GC-MS analysis for either ZEN or α-ZEL, of each tissue type and between treatment groups. The total number of sub-samples tested (n) is shown above each bar. Each bar is then divided into different colored segments representing the number of sub-samples in that group with an analyte response below the LOQ with no significant peak (NP), a significant chromatographic analyte response below LOQ (SP), and a quantifiable analyte response (QP). Treatment groups are indicated below each bar, within each tissue type: (1) base feed with solvent for 21 days, (2) ZEN-spiked feed for seven days followed by base feed with solvent for 14 days, and (3) ZEN-spiked feed for 21 days.

sub-samples with only α-ZEL present.

A Kruskal-Wallis test was used to compare median concentrations of ZEN and α-ZEL among tissue types and treatment groups. When comparing median analyte concentrations between treatment groups, no significant differences were found ($p = 0.15$). When comparing median overall concentrations between tissue types, at least one group was found to have a significantly different concentration of ZEN or α-ZEL ($p = 0.04$). Without a suitable post-hoc test to determine which group is significantly different, data was also analyzed using a one-way ANOVA with a Fisher's LSD. The one way ANOVA comparing average overall ZEN and α-ZEL concentration between tissue types provided similar results to the Kruskal-Wallis test ($p = 0.02$), and the Fisher's LSD determined that the average concentration of ZEN in the cervix was significantly greater than ZEN concentrations in the anterior vagina, posterior vagina, and ovaries, as well as the α-ZEL concentration determined in the liver. The average ZEN concentration in the liver was found to be similar to analyte concentrations across all analyzed tissue types.

3.3. Method validation

Results of method validation are summarized in Table 2. ZEN and α-ZEL peaks were successfully recovered at acceptable rates (75–120%). Repeatable recovery at the LOQ (10 ng g^{-1} ZEN and α-ZEL in reproductive tissue, 25 ng g^{-1} ZEN and α-ZEL in liver) was demonstrated in each tissue type (RSD < 20%).

4. Discussion

Mycotoxin contamination in swine feed is known to contribute to a variety of adverse health effects in herds. ZEN, specifically, contributes to a range of reproductive anomalies that disrupt breeding programs (Kanora and Maes, 2010; Zinedine et al., 2007), and in severe cases such as prolapse, lead may lead to culling (Iida et al., 2019; Stock et al., 2017). While the specific financial loses attributed to ZEN are not known, increasing incidences of POP in sows is raising concerns about the potential role of ZEN in reproductive tissues. This study presents new information regarding the accumulation of ZEN and its metabolite α-ZEL in reproductive tissues.

ZEN was found in swine reproductive tissues including the anterior vagina, posterior vagina, cervix, and ovaries. While there are no studies

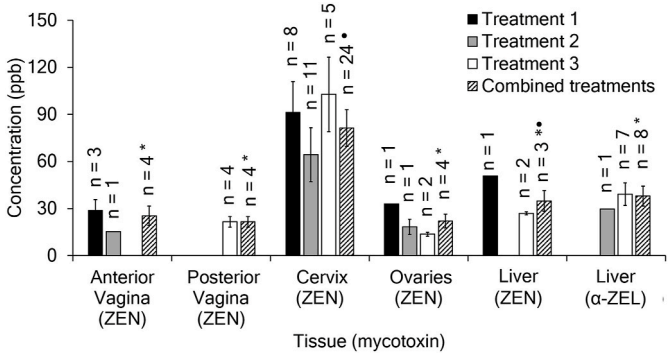

Fig. 6. A bar plot showing the average ZEN and α-ZEL concentrations in different tissues. ZEN was quantified for the anterior vagina, posterior vagina, cervix, ovaries, and liver, and α-ZEL was quantified for the liver. Neither ZEN nor α-ZEL was present for quantification of the uterus or broad ligament (not shown). The total number of quantifiable sub-samples (n) for each tissue type and within each treatment group is listed above each bar. Different colors and patterns represent the three treatment groups: (1) base feed with solvent for 21 days, (2) ZEN-spiked feed for seven days followed by base feed with solvent for 14 days, and (3) ZEN-spiked feed for 21 days. An additional bar (striped) shows average ZEN and α-ZEL concentration across all three treatment groups. Similar analyte concentrations between tissues are denoted by * and ● (p=0.02).

which directly quantify ZEN or α-ZEL in affected reproductive tissues, anomalies related to ZEN consumption have been reported in the vagina and ovaries (Kanora and Maes, 2010). Anomalies related to the cervix have not been previously reported. ZEN and α-ZEL have, however, been reported in reproductive tissues of pigs which did not display reproductive symptoms (Gajecka et al., 2012). Changes that occur as a result of consuming low-levels of ZEN do not necessarily produce symptoms, but may still affect tissues in a manner that does not affect animal performance (Gajęcka et al., 2016). In this study, pigs did not develop any reproductive anomalies, so we cannot determine if the identified ZEN concentrations in reproductive tissue are related to tissue health or performance.

ZEN was found in a greater proportion of cervix sub-samples than the other analyzed reproductive tissues, and was found to have a significantly greater concentration overall. The greater proportion and concentration of ZEN in cervix tissue suggests that the cervix accumulates more ZEN than other reproductive tissues. To our knowledge, the cervix has not been reported to experience changes related to ZEN-exposure, though it may have some involvement in ZEN-linked conditions such as prolapse and changes to fertility. ZEN competes with estrogen (Fitzpatrick et al., 1989), and estrogen has been linked to changes in cervical tissue weight and morphology (Elridge-White et al., 1989; Necaise, 2012). The accumulation of ZEN in cervical tissue may have implications for tissue performance and overall reproductive health.

Though α-ZEL was observed in liver samples, α-ZEL was not found in any reproductive tissue types studied. Previous studies have shown that, in pigs, ZEN and α-ZEL are excreted in similar concentrations (Gajęcka et al., 2016; Olsen et al., 2009; Zöllner et al., 2002). It has been established that α-ZEL has a similar, if not higher, binding affinity to estrogen receptors than ZEN (Fitzpatrick et al., 1989; Katzenellenbogen et al., 1979; Shier et al., 2001), so it is unexpected that ZEN was found in reproductive tissues while α-ZEL was not. Previously reported ZEN and α-ZEL concentrations in the uterus and ovaries are lower than the LOQ established for this study, so it is possible that our methods were not sensitive enough to identify α-ZEL in these samples. Consequently, alternative methods such as LC-MS are warranted to examine potential trace concentrations of α-ZEL that might occur below our LOQ with our GC-MS method.

ZEN concentrations within each of the tissue types did not vary significantly between treatment groups. The presence of ZEN in the tissues of pigs from treatment 1, suggests that consuming low-level naturally occurring ZEN in feed saturates tissue to a similar extent as greater ZEN concentrations in feed. The concentration of naturally occurring ZEN is presumed to be significantly lower than the 2.67 μg g^{-1} ZEN that was spiked into feed in the treatment 2 and 3 groups (Binder et al., 2007; Pleadin et al., 2015; Zachariasova et al., 2014). Previous studies, however, have shown differences in the concentration of ZEN in tissues, between different ZEN treatments (Gajęcka et al., 2016; Zöllner et al., 2002). Parameters for this trial were based on literature indicating that pubertal gilts were likely to experience severe symptoms when consuming comparable concentrations of ZEN in their diets (Kanora and Maes, 2010). Our study may not have been able to distinguish these differences due to variations in feeding trial parameters such as age, weight, and treatment timeline.

Both ZEN and α-ZEL were found in liver sub-samples from pigs in each treatment group. The liver serves to metabolize and eliminate circulating ZEN and metabolites from pigs (Binder et al., 2017), therefore we presume the reported ZEN and α-ZEL concentrations includes both conjugated and non-conjugated ZEN and metabolites. ZEN-consumption has been associated with decreased liver performance and morphological changes, especially in combination with the common co-occurring mycotoxin, deoxynivalenol (Pistol et al., 2014; Skiepko et al., 2020). The concentrations of ZEN and α-ZEL reported in the liver are greater than previously reported values ranging <0.5–5.1 ng g^{-1} ZEN and <1.0–12 ng g^{-1} α-ZEL (Dänicke et al., 2005a; Dänicke et al., 2005b; Gajęcka et al., 2016; Schneweis et al., 2005; Zöllner et al., 2002).

Table 2
Method validation results.

Analyte	Tissue	LOQ (ng/g)	RSD of LOQ (%)	Average recovery at LOQ ± S.D. (%)	Average recovery at 50 ng/g ± S.D. (%)	Average recovery at 100 ng/g ± S.D. (%)
ZEN	Vagina	10.0	5.2	118.6 ± 9.6	85.7 ± 2.1	87.3 ± 9.4
	Cervix	10.0	6.3	92.1 ± 5.8	89.0 ± 6.0	87.9 ± 2.9
	Broad Ligament	10.0	9.9	82.6 ± 10.8	74.1 ± 3.1	79.6 ± 2.0
	Uterus	10.0	10.9	112.7 ± 6.5	95.9 ± 2.9	92.5 ± 2.0
	Ovaries	10.0	8.6	92.5 ± 9.7	97.6 ± 8.4	83.6 ± 0.8
	Liver	25.0	12.8	116.7 ± 9.2	103.5 ± 19.7	75.0 ± 15.5
α-ZEL	Vagina	10.0	9.7	76.8 ± 5.9	106.6 ± 2.7	98.0 ± 3.4
	Cervix	10.0	10.5	85.2 ± 5.9	97.3 ± 7.4	101.0 ± 4.6
	Broad Ligament	10.0	5.1	92.8 ± 3.3	75.4 ± 1.1	77.1 ± 2.0
	Uterus	10.0	17.2	102.1 ± 6.9	92.1 ± 1.7	82.6 ± 1.9
	Ovaries	10.0	13.7	100.6 ± 15.4	95.3 ± 5.2	94.5 ± 1.3
	Liver	25.0	9.7	94.6 ± 2.6	89.7 ± 11.3	85.1 ± 4.4

S.D.: standard deviation.

This variation may be attributed to factors such as animal age, size, and timeline. Average ZEN and α-ZEL concentrations were found to be statistically similar in our study, which is similar to trends reported in previous studies (Gajęcka et al., 2016; Zöllner et al., 2002). While overall ZEN and α-ZEL concentrations were similar across groups, it should be noted that treatment 3 pigs had the greatest proportion of sub-samples containing α-ZEL. Average overall concentrations of ZEN and α-ZEL were similar and were close to the method LOQ (25 ng g^{-1}), which may have contributed to the apparent discrepancy in quantifiable sub-sample proportions. Alternatively, the discrepancy may be a result of increased metabolism from ZEN to α-ZEL in the liver (Binder et al., 2017). Overall, the presence of ZEN and α-ZEL in the liver show that ZEN was metabolized and circulated in pigs from all three treatment groups, which may help to explain the similar ZEN concentrations observed between treatment groups in individual reproductive tissues.

We developed, validated, and implemented a new GC-MS method for analysis of ZEN and α-ZEL in swine liver and reproductive tissues. Previous studies have primarily relied on LC-MS for quantification of ZEN and related metabolites in tissues. However, GC-MS has been used for analysis of ZEN in grains (Khatibi et al., 2014; Tanaka et al., 2000) and sediment (Kinani et al., 2008). We found that the GC-MS LOQ for ZEN and α-ZEL (10 ng g^{-1}) is higher than what has been reported for LC-MS analysis of similar tissues (0.5 ng g^{-1} (Zöllner et al., 2002), 1 ng g^{-1} (Gajęcka et al., 2016)). This is an important drawback to consider when using GC-MS to analyze ZEN in tissues, as concentrations of ZEN in tissues are typically much lower than those reported in grains. However, to our knowledge, there has not been a direct comparison of GC-MS and LC-MS methods for ZEN and metabolite analyses.

Future studies are necessary to continue investigating the role of ZEN-contaminated feed in swine reproductive anomalies. Similar feeding trials may be repeated with larger sample sizes or extended treatment timelines. Boars, piglets, and sows are also susceptible to the effects of ZEN (Kanora and Maes, 2010), and may experience more confounding factors related to reproduction, especially sows who have carried multiple litters. These studies would provide broader representation of the types of pigs being affected by ZEN in industry, which could help to determine if pigs are more vulnerable to ZEN at certain life stages.

CRediT authorship contribution statement

Erica Pack: Conceptualization, Investigation, Formal analysis, Data curation, Writing - review & editing, Writing - original draft. **Jacob Stewart:** Conceptualization, Investigation, Formal analysis, Data curation, Writing - review & editing, Writing - original draft. **Michelle Rhoads:** Conceptualization, Investigation, Formal analysis, Data curation, Writing - review & editing, Writing - original draft. **James Knight:** Conceptualization, Investigation, Formal analysis, Data curation, Writing - review & editing, Writing - original draft. **Raffaella De Vita:** Conceptualization, Investigation, Formal analysis, Data curation, Writing - review & editing, Writing - original draft. **Sherrie Clark-Deener:** Conceptualization, Investigation, Formal analysis, Data curation, Writing - review & editing, Writing - original draft. **David G. Schmale:** Project administration, Funding acquisition, Conceptualization, Investigation, Formal analysis, Data curation, Writing - review & editing, Writing - original draft.

Declaration of competing interest

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank the Virginia Tech Swine Center for providing housing for the pigs involved in this study, the Virginia Tech Meat Center for facilitating euthanization of the pigs, Dr. Kiho Lee for providing reproductive tissue for method validation, Niki McMaster for assisting in development of GC-MS methods, and all student volunteers who aided in caring for pigs throughout the feeding study. Funding for research was provided by Pratt Award #444347, Mycotoxin-Contaminated Ethanol Co-Products As Swine Feed: Clinical Signs, Diagnosis and Return to Reproductive Health, and the Virginia Tech Graduate Research and Development Program.

Abbreviations

GC-MS	Gas Chromatography Mass Spectrometry
ZEN	Zearalenone
ZEL	Zearalenol
BSTFA	*N,O*-Bis(trimethylsilyl)trifluoroacetamide
DDGS	Distillers' Dried Grains with Solubles
ANOVA	Analysis of Variance
LOQ	Limit of Quantification

Ethical statement

Methods and procedures for this animal feeding study were approved by the Virginia Tech Institutional Animal Care and Use Committee (IACUC) protocol number #18–118.

Virginia Tech is committed to providing humane care for and ensuring the well-being of animals used in research and teaching at this university by our faculty, staff, and students. This commitment is guided by the ethical principles described in the "U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training" and in applicable federal regulations, guidelines, and policies, including, but not limited to, the federal Animal Welfare Act, the "Public Health Service Policy on Humane Care and Use of Laboratory Animals," and the Institute for Laboratory Animal Research, a unit of the U.S. National Research Council's "Guide for the Care and Use of Laboratory Animals."

For operational purposes and as required by federal law, this commitment is vested in the IACUC which operates under an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare

within the U.S. Department of Health and Human Services.

Virginia Tech is also a registered research facility with the United States Department of Agriculture Animal and Plant Health Inspection Service's Animal Care unit and its facilities and program of animal care are inspected annually by a federal inspector.

All teaching uses as well as any research involving vertebrate animal species (excluding human subjects), regardless of funding source, are under the purview of the Virginia Tech IACUC. Animals cannot be obtained or used without prior protocol review and approval by the IACUC. As per federal law, the IACUC has the authority to approve, disapprove, or require modifications to be made to protocols to ensure regulatory compliance, and also has the authority to suspend or terminate ongoing protocols if it is determined that inappropriate or unapproved activities involving animals are taking place.

The Virginia Tech IAUC reports to the Vice President for Research and Innovation, who, as the designated "Institutional Official," is responsible for ensuring to federal regulatory officials, on behalf of the university, that issues of non-compliance are satisfactorily rectified.

- Virginia Tech's Animal Welfare Assurance # is A-3208-01 (expr. 7-31-2021)
- USDA-APHIS-AC Registration Certificate # 52-R-0012 (expr. 10-01-2021)

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