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A method for the determination of lasalocid, an antibiotic and coccidiostat, in grease and fat is described. The manufacture of lasalocid produces a grease-like residue as a waste byproduct. Recently this byproduct has been shown to have been illegally introduced into the animal feed chain. Therefore, a quantitative and confirmatory procedure to analyse for lasalocid in this matrix is needed. A portion of grease/oil sample was extracted into hexane-washed acetonitrile, and a portion of the extract was then applied to a carboxylic acid solid-phase extraction (SPE) column for concentration and clean-up. The SPE column was washed with additional hexane-washed acetonitrile and ethyl acetate/methanol, after which lasalocid was eluted with 10% ammoniated methanol. The eluate was evaporated to dryness, redissolved in (1:1) acetonitrile–water and filtered through a PTFE syringe filter. Confirmation and quantitation of lasalocid in the final extract employed a triple quadrupole LC-MS/MS. The method was applied to grease and oil samples containing from 0.02 to 34 000 mg kg^{-1} of lasalocid.

Keywords: lasalocid grease; animal feed; contamination; LC-MS analysis

Introduction

Lasalocid is one of several ionophore antibiotics used worldwide for the prevention and treatment of coccidiosis and as a growth promoter in poultry (chicken and turkey), cattle, rabbits and sheep. It is not approved for swine, and is extremely toxic to horses. It is the active ingredient in the feed additives Bovatec and Avatec (USFDA NADA 096-298). Published tolerances exist for lasalocid in approved tissues (USFDA CFR 556.347).

In August 2014, about 50 000 turkeys died at a farm in Michigan from an overdose of lasalocid in their feed. Potentially contaminated turkey and swine feed was analysed as part of the investigation by the Michigan Department of Agriculture and Rural Development (MDARD) in conjunction with the USFDA's Center for Veterinary Medicine (CVM). The analysed turkey feed contained much higher levels of lasalocid than stated on the label. Also, the supposedly unmedicated swine feed contained over 1500 g t^{-1} (tonne) of lasalocid, and over 20 000 hogs had already consumed the product. The ultimate source of the feed contamination seemed to come from grease that was used in the manufacture of the feed. Grease and oil can often be used as an ingredient in animal feed. For example, recycled grease from restaurants is sometimes used for this purpose, but this grease is not allowed to contain any antibiotics. The fat used in feed supplies calories, makes the feed more palatable, and improves nutrient and vitamin absorption (Vetlineindia 2015; www.Vetlineindia.com/feed-oil-fat.html).

When lasalocid is biosynthesised, it produces a grease-like waste stream (see the results and discussion). This oily waste stream is only meant to be used as an energy source in biofuels. It is not approved to enter the food/feed stream in any way. However, since it is not a good manufacturing practice (GMP)-regulated product, practices used for its labelling and subsequent disposition are less stringent. It does appear that a lasalocid oily waste stream somehow entered the food arena in the above case (USFDA Recall Notice 2014). More than 100 farms in eight states may have been affected. Therefore, it was necessary to develop an analytical method to analyse for lasalocid in this very challenging matrix. The analytical method would need to be able to determine lasalocid at very high levels for grease/oil samples that represented the undiluted byproducts of lasalocid biosynthesis. In addition, the method would need to be able to determine sub-mg kg^{-1} values of lasalocid in order to trace contaminated grease samples that may have been subsequently diluted with non-contaminated grease during processing and ultimate distribution. There are several recent LC-MS analytical methods for lasalocid and other ionophores such as monensin in the literature, but these apply to feed (Turnipseed et al. 2001; Storey et al. 2008; Boscher et al. 2010; Huang et al. 2011; Vincent et al. 2011; AOAC 2013; Rokka et al. 2013) or tissue (AOAC 2012) or to milk and eggs (Spisso et al. 2010; Thompson et al. 2011). Because this recent form of lasalocid contamination seems to be a new one, there are no methods published specifically for lasalocid analysis in grease.

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Materials and methods

Chemicals and reagents

Deionised water was 18 MΩ·cm, prepared from a MilliQ water system from Millipore (Bedford, MA, USA). Methanol, acetonitrile and water (for the LC mobile phase) were LC-MS Optima; glacial acetic acid and ethyl acetate were obtained from Fisher Scientific (Houston, TX, USA). Hexane and ammonium hydroxide ACS grade 28–30.0% NH_3 were from EMD Millipore (Gibbstown, NJ, USA). Sodium acetate trihydrate and sodium bicarbonate were from Sigma Aldrich (St. Louis, MO, USA). Lasalocid A sodium salt was from Sigma Aldrich (a Fluka product), provided as a glass ampoule containing 86.9 μg ml^{-1} lasalocid in acetonitrile (reported as the free acid and corrected for purity). Volumetric glassware (5, 10 and 25 ml) was class A or equivalent. Disposable polypropylene 15- and 50-ml centrifuge tubes were from VWR International (Radnor, PA, USA). Nitrogen gas was grade 9.5 or better. The SPE columns were wide-pore CBX disposable extraction columns, catalogue # 7217-06 from J.T. Baker through VWR International. No substitution should be made for this SPE. The Teflon syringe filters were Pall acrodisc 13 mm with 0.2 μm PTFE membranes from Gelman through VWR. The syringes were disposable plastic, latex free, 1 ml from Becton-Dickinson (Rutherford, NJ, USA). Eppendorf variable pipetters (5–1000 μl volume) were from Brinkmann Instruments (Westbury, NY, USA).

Equipment

The ultrasonic cleaner with a 3.7 L water bath was from Cole-Parmer (Vernon Hills, IL, USA). The water bath with nitrogen evaporator was from Organomation (Berlin, MA, USA). The centrifuge with refrigeration capability was from the International Equipment Co. (Needham Heights, MA, USA). The mechanical multi-tube shaker/vortex mixer was from Fisher Scientific (Pittsburgh, PA, USA). The liquid chromatograph-mass spectrometer system was a Thermo Fisher (Chelmsford, MA, USA) TSQ quantum triple-quadrupole mass spectrometer coupled to a Surveyor liquid chromatography pump and autosampler. Thermo Fisher Excalibur software was used for all data acquisition. The MS source and collision energy parameters were optimised using a syringe pump to introduce at 10 μl min^{-1} a lasalocid standard solution (at a 10 μg ml^{-1} concentration in the diluent) into a stream of mobile phase at 250 μl ml^{-1} via a T-union. Positive mode ESI voltage was 3000 V; the capillary temperature was 300°C. The sheath gas was 50; the auxiliary gas was 5 (both nitrogen arbitrary units). The Q1 and Q3 peak widths were 0.7 amu full width at half maximum (FWHM); the Q2 collision gas pressure was 1.5 torr argon. No skimmer offset was used. The scan time was 0.4 s. The precursor selected in Q1 was the $[M + Na]^+$ adduct of lasalocid with m/z = 613.3. Collision energies were optimised for three product ion transitions (613.3 → 377.3, 613.3 → 577.2, and 613.3 → 595.4 m/z). The tube lens offset value was 91. The LC column was a YMC-Pack ODS-AQ 100 × 2.0 mm with a 3 μ particle size from YMC America (Allentown, PA, USA). The mobile phase was 10% (1 mM sodium acetate + 2% acetic acid in water) + 90% acetonitrile (premixed). The column flow rate was isocratic at 0.250 ml min^{-1}. The column oven temperature was 35°C. The injection volume was 10 μl (partial loop injection). The MS divert valve was switched from 'waste' to 'detector' just before the lasalocid peak eluted (around 2 min). A 5-min runtime was used.

Preparation of reagents

A total of 10% ammoniated methanol was prepared by adding 10 ml ammonium hydroxide to 90 ml methanol in a 100 ml mixing cylinder and mixed. The ethyl acetate–methanol (8:1) solution was prepared by adding 10 ml methanol plus 80 ml ethyl acetate into a 100-ml mixing cylinder and mixed. The hexane-washed acetonitrile was prepared in a 250 ml separating funnel by adding 125 ml acetonitrile plus 40 ml hexane and vigorously shaken for 10 s. The phases were allowed to separate. The lower acetonitrile phase was used for extraction of grease/oil samples. The HPLC buffer (1 mM sodium acetate + 2% acetic acid in water) was prepared by dissolving 0.136 g sodium acetate trihydrate plus 20 ml acetic acid plus 980 ml LC-MS water into a 1000 ml mixing cylinder and mixed. The final HPLC mobile phase was prepared by adding 100 ml of the sodium acetate/acetic acid buffer plus 900 ml acetonitrile into a 1000 ml mixing cylinder and mixed. Neutralised methanol was prepared by adding sodium bicarbonate (1 g) to 100 methanol (100 ml) and mixed.

Preparation of standard and spiking standard solutions

A 0.500 ml aliquot of the 86.9 μg ml^{-1} reference standard lasalocid received in the glass ampoule was diluted to 5 ml with neutralised methanol to form the stock and spiking standard solution at a concentration of 8.69 μg ml^{-1}. This stock solution was stable for 6 months when refrigerated. Six separate solvent standards were then prepared by diluting the stock standard with (1 + 1) acetonitrile/water to give standards ranging from 0.2 to 0.004 μg ml^{-1} concentrations. These standards were stable for 1 month when refrigerated. Using the extraction procedure (see below), this gave a solvent standard range equivalent to 1 to 0.020 mg kg^{-1} respectively. Sample (1 g) was extracted with 5 ml, and 1 ml of that extract was taken through the SPE column with the final volume also being 1 ml). Thus, for a 1 mg kg^{-1} level standard:

$$\left(\left(\left(\frac{1\mu g}{g}\right)\times 1g\right)\div 5mL\right)\times\left(\frac{1mL}{1mL}\right)=0.2\mu g/mL$$

The dilution factor to convert μg ml^{-1} to mg kg^{-1} equivalent is 5 ml g^{-1}. For low-level spikes (1 mg kg^{-1} range) the stock standard solution was used to prepare wet spikes using a blank matrix control (see below). For example, a 0.39 mg kg^{-1} spike was prepared by adding 0.045 μl of the 8.69 μg ml^{-1} stock standard to 1 g of control fat.

Sample preparation and extraction

A 1 ± 0.03 g portion of product was weighed into 15-ml polypropylene tubes. The tubes were centrifuged at 4000 rpm for 1 min to draw down the product to the base of the tubes. Then 5.0 ml hexane-washed acetonitrile were added to each tube. The tube was then placed in a heated 50 ± 5°C water bath for 6 min. The tubes were mixed vigorously using a vortex mixer for approximately 10 min. The tubes were then centrifuged at 6000 rpm for 10 min at 5°C. CBX SPE columns were conditioned by allowing methanol (3 ml) followed by hexane-washed acetonitrile (3 ml) to drip through the column using an SPE manifold. The conditioned SPE columns were not allowed to dry. Atop each SPE column, hexane-washed acetonitrile (1 ml) was applied followed by extract (1 ml) and a slight vacuum was applied. The SPE columns were then washed with the following solutions: hexane-washed acetonitrile (1 ml) followed by ethyl acetate/methanol (2 ml) (8:1). A slight vacuum was maintained throughout the washing. The lasalocid was eluted with 10% ammoniated methanol (4.5 ml) into a 12 × 75 mm disposable glass test tube. The eluate was then evaporated to dryness in a 50 ± 5°C water bath with a nitrogen purge. The remaining residue was dissolved 1.0 ml of acetonitrile–water (1:1). Each glass test tube was then vortexed for 10 s followed by sonication for 10 s and vortexed again for another 10 s. A portion of this solution was filtered through a 0.2 μ PTFE syringe filter into a reduced volume HPLC injection vial. Rendered duck fat or olive oil was used for blank matrix controls and spiked matrix controls.

For grease samples that contain higher than 1000 mg kg^{-1} of lasalocid

Some grease samples may contain very high levels of lasalocid (one sample tested in our laboratory contained 34 000 mg kg^{-1} of lasalocid – see the results and discussion). For samples containing > 1000 mg kg^{-1} lasalocid, it was found that SPE column clean-up was no longer necessary and that the large dilution factor would give a clean extract. For example, for a sample containing 34 000 mg kg^{-1} lasalocid: a 1 ± 0.03 g portion of product was weighed into a 50 ml polypropylene tube. The tube was centrifuged at 4000 rpm for 1 min to draw down the product to the base of the tube as before. Then 25.0 ml of hexane-washed acetonitrile were added to the tube. The tube was placed in a heated 50 ± 5°C water bath for 6 min. The tube was vigorously mixed using a vortex mixer for approximately 10 min and centrifuged at 6000 rpm for 10 min at 5°C. Then a 0.100 ml portion of this extract was diluted to 100.0 ml and 0.100 ml of this dilution were further diluted to 5.00 ml (all dilutions used 1 + 1 acetonitrile–water). A portion of this solution was filtered through a 0.2 μ PTFE syringe filter into a reduced volume HPLC injection vial. With this dilution, the final sample concentration was in the range of the solvent standard curve as prepared above:

$$\begin{aligned}(((34,000\mu g/g)\times 1g)\div 25mL)\times(0.100mL\div 100.0mL)\\ \times(0.100mL\div 5.00mL)=0.027\mu g/mL\end{aligned}$$

The dilution factor would be 1.25×10^6 ml g^{-1} in this example. For samples containing such high levels of lasalocid it was not possible to prepare accompanying lasalocid spikes at equivalent levels (the purchased stock standard was not concentrated enough). In this case, controls were extracted and diluted per the sample, and the final dilution was wet-spiked with lasalocid standard at approximately the midpoint of the solvent standard curve. Such matrix standards showed no appreciable matrix suppression in the LC-MS response. For samples that contained between 1 and 1000 mg kg^{-1} of lasalocid, SPE column clean-up was used as before except that the extract was additionally diluted to give a final injection vial concentration approximately at the midpoint of the solvent standard curve. For lasalocid quantitation, the area of the MS product ion 377.3 *m/z* was used. Linear regression was done using a six-point external solvent standard curve, using μg ml^{-1} as the units for the standard concentration. Any positive unknown sample lasalocid concentration was then calculated (in μg ml^{-1}) using this curve and then converted to mg kg^{-1} using the dilution factor associated with that particular sample's extraction. For MS confirmation of identity, two product ion ratios (area counts for qualifier ions *m/z* 577 and 595 compared with the quantitation ion *m/z* 377) were used (see the results and discussion).

Results and discussion

Biosynthesis of lasalocid

The commercial manufacture of Lasalocid produces an oily waste byproduct sometimes referred to as 'Lascadoil' or 'lasalocid oil'. Lasalocid is synthesised by a *Streptomyces* genus of bacteria. This predatory soil bacterium produces two-thirds of all antibiotics made. In order to produce commercially a specific antibiotic using

this bacteria, the cluster of genes responsible for the antibiotic of choice must be identified on the bacteria's chromosome or plasmid. Techniques to promote the expression of these genes, while repressing other sets of genes that would produce unwanted antibiotics, are then performed. Antibiotic production on an industrial level is dependent upon the fermentation conditions: specific nutrients, pH and temperature all must be optimised. A specific mutant strain of *Streptomyces, S. lasaliensis*, will produce only lasalocid if the right precursors and short-chain fatty acid units are used in the starting fermentation mix (Podojil et al. 1989; Migita et al. 2008). After the exponential growth phase of the lasalocid-producing bacteria, a high proportion of longer-chain fatty acids (with higher melting points) are produced as part of the cell's membrane and membrane transport system. The oily byproduct is termed Lascadoil. The lasalocid pharmaceutical component is meant to have been extracted out of this mix, but some amounts can still remain. This oil can then be processed by the biofuel industry.

Applicability of the method

Because of the nature of the lasalocid contamination into the food stream and the need to trace its ultimate fate, the analytical method developed would need to analyse for lasalocid in different types of oil/fat/grease samples. The samples received for lasalocid testing in our laboratory varied widely. Some samples were nearly clear, only slightly viscous liquids. Others were of medium to highly viscous liquids with light brown to dark brown colours. Still other samples were dark brown soft-solid products. Evidently the original Lascadoil could have a complex path. There might be undiluted Lascadoil containing very high amounts of lasalocid in *Streptomyces* fatty acid cell residue. There might be samples of Lascadoil further diluted with recycled restaurant and/or vegetable oil as well as Lascadoil samples possibly diluted with turkey fat – all of which might contain much lower levels of (diluted) lasalocid. Further, there might be Lascadoil diluted with other complex oils (such as glycerin, tallow and soybean oil) from biodiesel and other processing. Because the sample matrices to be analysed were so variable and complex, the use of matrix-matched standards for quantitation was impossible – solvent standards were therefore used. Validation and recovery studies for all these matrices over the six-order-magnitude range of lasalocid ultimately found was also not practical. The primary use of the described method was to determine the complex fate of the Lascadoil product which may have been diluted many different times with different substances. With multiple sequential dilutions of Lascadoil possible, it was essential that the method be able to detect lasalocid contamination at lower levels (from 0.2 to 1 mg kg^{-1}). For this reason the validation and recovery studies (see below) focused on sub-mg kg^{-1} level of lasalocid. It was decided to use rendered duck fat and olive oil as the matrix control blanks (and spikes) in the described method to match the intended samples as much as possible. In addition, some of the grease samples themselves were also spiked with lasalocid in order to ascertain some idea of the recovery of this analyte in samples that would contain the highest level of interfering compounds. As lasalocid has no tolerance in food, any sample that contained > 1 mg kg^{-1} lasalocid would be considered grossly contaminated. Recovery studies and validation at these higher levels was therefore not necessary. Spiking samples at parts per thousand levels would also not be practical. To monitor the precision of the instrument response, a midpoint solvent standard (from the initial solvent standard curve) was re-injected every 10 injections. The response of this standard (using the main quantitation ion) varied by less than 10%.

Results

Several samples of various grease/oil/fat samples related to the dead turkeys were analysed in our laboratory for lasalocid using the described method. Lasalocid was quantitated and confirmed to be present by LC-MS/MS in the majority of samples analysed at levels ranging from 0.02 to 34 000 mg kg^{-1}. The minimum quantitation level (MQL) was established at 0.02 mg kg^{-1} lasalocid. A typical sample chromatogram of a grease sample containing 0.4 mg kg^{-1} of lasalocid is shown in Figure 1. None of the matrix control samples (duck fat or vegetable oil) had any interference in their associated LC-MS/MS chromatograms. Before any samples were analysed, a series of lasalocid matrix control spikes were analysed. The spikes were set at < 1 mg kg^{-1} levels as these levels would be the most difficult to recover in the difficult matrices tested. This data are presented in Table 1. The recoveries of spiked lasalocid for duck fat and olive oil were > 80%. However, when the grease samples were spiked, the recovery was in the 55–65% range for sub-mg kg^{-1}-level lasalocid spikes. As this very dirty grease sample matrix was so challenging, this level of recovery seems acceptable. The specific brand of SPE columns used was critical: other brands tried in our laboratory did not seem to clean-up the grease matrix as effectively. Other carboxylic acid SPE columns were tried, but did not retain lasalocid from the applied acetonitrile sample extract. The Baker CBX columns were the only weak cation-exchange phase SPE columns that retained lasalocid dissolved in acetonitrile. In samples containing higher levels of lasalocid, its yellow colour could be seen on the SPE column before elution. This retention allowed further rinsing of the SPE column to eliminate interfering fats and oils. Strong cation-exchange SPE columns (SCX) were not used because they retained all compounds too efficiently – it was difficult to separate differentially the lasalocid from charged

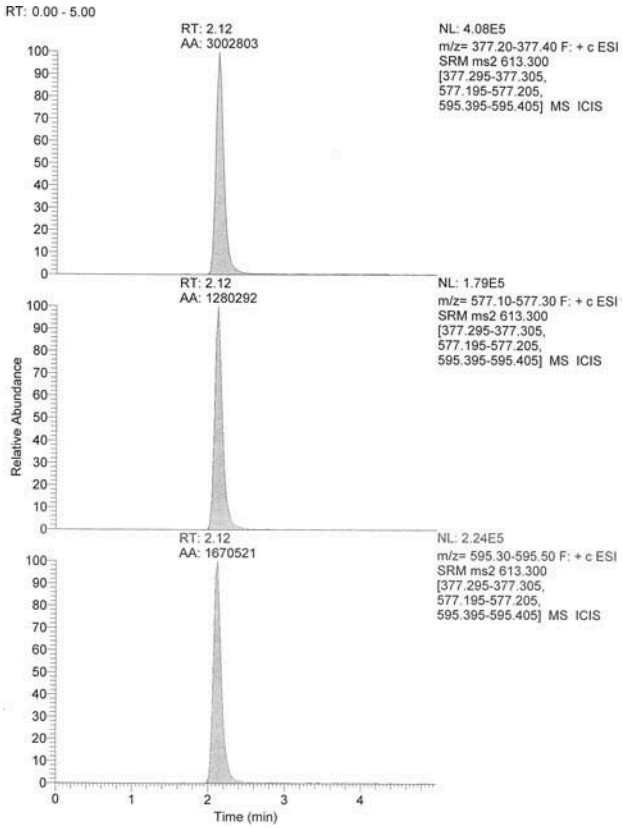

Figure 1. Chromatogram of a grease sample containing 0.4 mg kg^{-1} of lasalocid.

Table 1. Recoveries of sub-mg kg^{-1} level lasalocid spikes in various fat matrices.

Fat matrix	Spike level (mg kg^{-1})	Replicates analysed	Average recovery (% RSD)
Duck fat	0.2	5	99 (10)
Duck fat	0.4	9	95 (11)
Duck fat	0.8	5	98 (3)
Olive oil	0.4	5	81 (5)
Grease sample	0.2	4	57 (3)
Grease sample	0.4	4	67 (3)

interfering compounds when using this phase SPE column.

Good linearity was obtained throughout the validation for lasalocid with corresponding r^2 values ≥ 0.995 for the solvent standard curve injected. Lasalocid can be chromatographed using a common LC-MS non-buffered mobile phase such as acetonitrile and 0.1% formic acid; however, when this is done the analyte peak has substantial tailing on a traditional reverse-phase analytical column. When a buffered mobile phase of pH 4.5 is used instead, a better peak shape is the result. Heptafuorobutyric acid has also been used to improve lasalocid peak shape (Huang et al. 2011). Also it was our experience that while the sodiated adduct of lasalocid will always be the dominant precursor ion (even if just water or formic acid is used in the LC mobile phase), better reproducibility of the lasalocid standard response is achieved if a small amount of sodium salt is added to the mobile phase. Any sample chromatogram that had an MS^2 lasalocid quantitation ion response above the MQL within a 5% matching retention time of standard lasalocid was further examined for its qualifier ion ratios. The MQL was established such that all three product ions would have a signal-to-noise ratio of > 10 to 1 compared with the blank matrix control. Standard lasalocid ion ratios for 577/377 and 595/377 *m/z* averaged 43% and 56% respectively. For a presumptive positive lasalocid sample injection, its two product ion ratios must fall within an absolute ±20% of the average ratios of the standard curve average injected in order to meet confirmation of identity guidelines. The criteria used were from the USFDA's *Guidelines for Mass Spectrometry for Confirmation of the Identity of Animal Drug Residues* (USFDA 2003).

Conclusions

The described method is acceptable to quantitate and confirm for the presence of lasalocid in most types of food-related oil and/or grease samples where lasalocid contamination might be a concern. The procedure applies to a wide range of lasalocid levels for a very challenging matrix, and uses a rapid, simple clean-up.

Disclosure statement

No potential conflict of interest was reported by the authors.

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