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A rapid method for the determination of lasalocid in animal tissues and eggs by high performance liquid chromatography with fluorescence detection and confirmation by LC-MS-MS

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A simple and rapid method has been developed for the extraction of lasalocid from chicken muscle, eggs and liver and kidney from chicken, pig, sheep and calf. This method allows the screening of a large number of samples, *i.e.* 30–40 within a working day, and has an overall analysis time of 90 min. Lasalocid standard solution can be detected at 1 ng ml^{-1} by both HPLC-fluorescence (HPLC-F) and LC-MS-MS; the limit of quantification in fortified samples by the described method is 1 ng g^{-1}. Results show good repeatability and mean 'spiked' recoveries by HPLC-F in the range of 10 to 200 ng g^{-1} (ppb) of 103, 87, 107, 97, 97, 103, 93, 109 and 100% in chicken muscle, chicken liver, egg, pig liver, pig kidney, sheep liver, sheep kidney, calf liver and calf kidney, respectively. For concentrations between 1 and 6 ng g^{-1} of spiked lasalocid in eggs and chicken liver by LC-MS-MS, the average recoveries were 76 and 59%, respectively.

Introduction

Coccidiosis poses severe health problems to several classes of livestock, and has a significant impact economically on the farmer if no control is in place for its prevention. This disease is caused by protozoa of the genus *Eimeria*. The symptoms include anorexia, loss of weight and haemorrhagic and mucoid diarrhoea,[1] and animals may become emaciated, dehydrated, weak and listless.[2] Mortality from coccidiosis is usually associated with severe diarrhoea which causes loss of electrolytes and dehydration.

Lasalocid is widely used on farms to prevent coccidiosis and to improve feed conversion in poultry as well as in ruminant animals. At a dose of 75–125 mg kg^{-1} (ppm) in the feed,[3] it prevents coccidiosis caused by *Eimeria necatrix*, *E. tenella*, *E. acervulina*, *E. brunetti*, *E. maxima* and *E. mivati* in broiler chickens; when fed at a dose of 90–125 mg kg^{-1} in the feed, it prevents coccidiosis in pheasants and turkeys caused by *E. duodenalis*, *E. colchici*, *E. phasiani* and *E. gallopavonis*, *E. adenoides*, *E. dispersa*, respectively. The withdrawal period is 5 days prior to slaughter for poultry and 7 days for pheasant. Lasalocid is not permitted in feed for breeding and laying stock. For cattle and sheep, lasalocid is used for feed conversion efficiency as well as an anticoccidial agent at a daily dose of 1 mg kg^{-1} body weight. Lasalocid is very effective against the pathogenic species *E. bovis* and *E. zuernii* in cattle aged less than 2 years. The addition of 40 mg kg^{-1} lasalocid in the feed of Holstein bull calves showed an increased body weight gain of 8% when compared to controls fed on unmedicated diets.[4] In sheep, the most common infective species are *E. ahsata*, *E. ovinoidalis* and *E. ovina* which are equally inhibited by lasalocid.[5]

Due to its widespread use on the farm, there is a risk that lasalocid residues may be present in animal products intended for human consumption. Suitable methods must therefore be available for monitoring its presence in animal tissues resulting from improper use or cross contamination. In the UK the Advisory Group on Veterinary Residues (AGVR) has prescribed a differential level (DAL) of 100 ng g^{-1} (ppb) in animal tissues and eggs. The US Food and Drug Administration (FDA)[6] has set the following limits: 300 ng g^{-1} in chicken skin with adhering fat, 700 ng g^{-1} in cattle liver and rabbit liver.

Several methods have been reported for the determination of lasalocid in different matrices such as feed, eggs, tissue and liver. These methods generally use solvent extraction followed by a clean-up stage, and determination is achieved by different types of chromatography, namely thin layer chromatography (TLC—bioautography),[7,8] using *Bacillus subtilis* as the inoculum, GC-MS,[9] LC-MS, LC-MS-MS[10,11] and HPLC,[12–16] methods based mainly on fluorescence detection.

The aim of this study was to develop a rapid method which could be used to examine a large number of samples from diverse biological matrices, *i.e.* muscle tissue, liver and kidney from different animal species and eggs, with specific detection and an appropriate limit of quantification. HPLC with fluorescence detection (HPLC-F) may be specific but it requires a run time of about 20 min, whereas LC-MS has been demonstrated to give better specificity than HPLC-UV/fluorescence systems and run times may be reduced. For confirmation purposes, however, several diagnostic ions are required, and their generation by cone voltage fragmentation or source collision-induced dissociation (CID) has to be carefully considered, particularly if the analyte is present at trace levels. Source CID is prone to interference from the matrix or other co-eluting analytes. The non-specificity and non-selectivity of this technique (because it fragments all species of ion in the ion beam) can limit the use of LC-MS unless the analyte of interest has been well separated from interferences either by a thorough clean-up or by a complex HPLC separation system. If the analyte is present at high levels, source CID can provide useful data.

LC-MS-MS provides a solution by filtering out unwanted ions and it generates product or daughter ions which are unambiguously derived from the analyte. Thus LC-MS-MS

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allows for greater specificity, selectivity and sensitivity and provides more confidence in the analytical results obtained. The requirement for complex chromatographic separation is also substantially reduced and simple isocratic systems can provide rapid analysis of samples. Using LC-MS-MS, the chromatographic run time can be reduced to less than 10 min. The development of off-axis or orthogonal source designs (such as the Z-Spray™) also allows the analysis of 'dirty' samples, *i.e.* minimal sample clean-up is required.

Experimental

Samples

Animal tissues and eggs were obtained from local retail outlets and were examined for the presence of lasalocid by the described methods. The tissues and eggs were homogenized and stored at −30 and 4 °C, respectively.

Solvents

The following solvents were used: methanol, acetonitrile, tetrahydrofuran (HPLC grade, Aldrich, Riedel-de-Haën, Seelze, Germany); trifluoroacetic acid (Sigma, St. Louis, Montana, USA); sodium sulfate anhydrous (analytical grade, Aldrich, Gillingham, Dorset, UK); ammonium acetate (AR grade, BDH, Poole, Dorset, UK); acetic acid glacial (BDH); lasalocid sodium salt (≈97%) (analytical standard, Sigma).

Ammonium acetate (0.01 M) was adjusted to pH 4.5 by the following method: 0.771 g of ammonium acetate was dissolved in 990 ml of deionized water in a 1 l volumetric flask; the pH was adjusted to 4.5 with glacial acetic acid and the final volume was made up to the mark; this was filtered through a 0.45 μm membrane filter.

Lasalocid standard solutions

Stock solution (1 ml = 1 mg lasalocid) 0.0258 g of lasalocid sodium salt (97% purity) (equivalent to 25 mg lasalocid) was dissolved in methanol and made up to the mark in a 25 ml volumetric flask. This solution is stable for up to 3 months and should be kept refrigerated.

Working solution(a) (1 ml = 10 μg or 1 μl = 10 ng lasalocid): 250 μl of the stock solution was pipetted into a 25 ml volumetric flask and diluted to the mark with acetonitrile. (b) (1 ml = 1000 ng or 1 μl = 1 ng lasalocid): 25 μl of the stock solution was pipetted into a 25 ml volumetric flask and diluted to the mark with acetonitrile. The working solutions should be prepared weekly and kept refrigerated.

Spiking of samples

Samples spiked at 0–400 ng g^{-1}: 5 g portions of blank material (*i.e.* material which had been shown to contain no detectable lasalocid by the developed method) were spiked with 0, 5, 12.5, 25, 50, 100 and 200 μl of the working standard lasalocid solution (a). This represents levels of 0, 10, 25, 50, 100, 200 and 400 ng g^{-1} in spiked samples.

Samples spiked at 0–6 ng g^{-1}: 5 g portions of blank material (as above) were spiked with 0, 5, 10, 20 and 30 μl of the working standard lasalocid solution (b). This represents levels of 0, 1, 2, 4 and 6 ng g^{-1} in spiked samples.

The spiked samples were left to stand for 1 h prior to the extractions.

Lasalocid standard solutions in acetonitrile equivalent to the above spike levels were run with the spiked samples for both HPLC-F and LC-MS-MS.

Apparatus

The following apparatus was used: polypropylene tubes, Falcon tubes (15 and 50 ml) (Becton Dickinson Labware, France); long flat-end spatula (205 × 10 mm, stainless steel); centrifuge up to 4000*g* (Jouan CR422, St. Herblain, France); Turbo-Vap evaporator (40 °C) with nitrogen supply (Zymark, USA); mechanical shaker (JK IKA Labortechnik, Staufen, Germany).

For HPLC, the following apparatus and conditions were used: mobile phase of acetonitrile–ammonium acetate buffer pH 4.5 (90 + 10); flow rate, 1 ml min^{-1}; excitation wavelength, 310 nm; emission wavelength, 430 nm; injection volume, 50 μl; column, Luna, phenylhexyl (250 × 4.6 mm id) 5 μm fitted with a phenylhexyl guard column (Phenomenex, Cheshire, UK); fluorescence detector (Merck Hitachi model F-1050, sensitivity at 100); pump (Waters 600) with autosampler (Hewlett Packard, series 1050); integrator (Hewlett Packard with Chemstation software).

Determination of lasalocid by HPLC with fluorescence detection

Method for lasalocid at the level of 10–200 ng g^{-1} A 5 g portion of liver/kidney/egg was weighed in a 50 ml Falcon tube, and 15 g of anhydrous sodium sulfate was added to the tube. With the flat end of a long spatula, the tissue/egg was mixed until a powdery or granular mixture was obtained. This was best achieved by mixing the sodium sulfate with the sample for about 1 min and leaving to stand for about 5 min. When all the water had been absorbed by the sodium sulfate, the solid mixture was dispersed with the spatula. The granular mixture was not allowed to set at the bottom of the tube; if this occurred, the tube was inverted and, with a tapping action, the solid mixture was dislodged to allow maximum contact with the solvent. An aliquot of 10 ml acetonitrile was added to the mixture and this was vortexed for 30 s. The tube was placed on a horizontal shaker for 30 min at 300 motions min^{-1}. The mixture was centrifuged at 4000*g* for 15 min. An aliquot of the supernatant was filtered through a 0.45 μm syringe filter and was analysed by HPLC-F and LC-MS-MS.

Method for lasalocid at the level of 1–6 ng g^{-1} in eggs and chicken liver An aliquot of 7.5 ml of the supernatant of the above extract was transferred to a 15 ml graduated tube and was evaporated to 1 ml in a Turbo-Vap at 40 °C. The concentrated extract was filtered through a 0.45 μm syringe filter and the determination was carried out by HPLC-F or LC-MS-MS.

Quantification of the spiked matrices was performed against the external lasalocid standard solutions used as a calibration curve, and the linearity of the corresponding calibration curves by HPLC-F and LC-MS-MS gave a correlation coefficient (R^2) ≥ 0.99.

Determination of lasalocid by LC-MS-MS

Equipment for LC-MS-MS The HPLC equipment consisted of a Waters Alliance 2690 system (Waters Ltd, Watford, Hertfordshire, UK), connected to a Micromass Quattro LC tandem mass spectrometer with a Z-Spray™ API source operating in positive ion electrospray (ESP) mode (Micromass UK Ltd, Altrincham, Cheshire, UK).

Chromatographic conditions The mobile phase was based on that used in the method described by Blanchflower and Kennedy,[17] with the following composition: acetonitrile, methanol, tetrahydrofuran, water and trifluoroacetic acid (67 : 10 : 10 : 13 : 0.1 v/v); column, phenylhexyl, Luna C18(2), 3

μm (150 mm × 2.1 mm id) (Phenomenex, Cheshire, UK); flow rate, 200 μl min^{-1}; injection volume, 15 μl.

Optimization of MS-MS conditions A solution of lasalocid sodium (10 μg ml^{-1} in the mobile phase) was infused at a rate of 10 μl min^{-1} into the detector and the capillary/cone voltages were adjusted to yield the $[M + Na]^+$ ion (*m/z* 613) (see Fig. 1). Further MS-MS experiments were performed to generate the major product (daughter) ion fragments of *m/z* 377 and 577; both of these ions were used for confirmatory and quantification purposes. The narrow bore column allows eluant from the column to be transferred directly into the electrospray interface without the need for flow splitting.

The following parameters were used: capillary voltage, 3.5 kV; cone voltage, 50 V; source temperature, 140 °C; collision energy, 36 eV; collision gas pressure, 2.3 e^{-3} mbar.

Sample extracts and standard solutions were injected into the system, and a single peak for lasalocid was observed at about 6.4 min. The product (daughter) ion fragments were monitored by multiple reaction monitoring (MRM) mode.

Results and discussion

Table 1 shows the actual determinations by HPLC-F of lasalocid from different animal tissues and eggs. The mean recoveries of lasalocid by this method for chicken muscle, chicken liver, eggs, pig liver, pig kidney, sheep liver, sheep kidney, cattle liver and cattle kidney within the range 0–200 ng g^{-1} of added lasalocid are summarized in Table 2 and are as follows: 103, 87, 107, 97, 97, 103, 93, 109 and 100%, respectively. The consistency of the mean results from different matrices indicates the versatility and robustness of the method.

Table 3 shows the determinations of added lasalocid in the range 0–6 ng g^{-1} by the described LC-MS-MS procedure; the mean recoveries for spiked chicken liver and eggs were 59 and 76%, respectively. The lower recoveries can be attributed to the concentration stage where the extract has to be dried to a low volume using a Turbo-Vap at 40 °C under a stream of nitrogen.

Table 4 shows the results obtained at 0–400 ng g^{-1} of spiked lasalocid in eggs by both HPLC-F and LC-MS-MS with

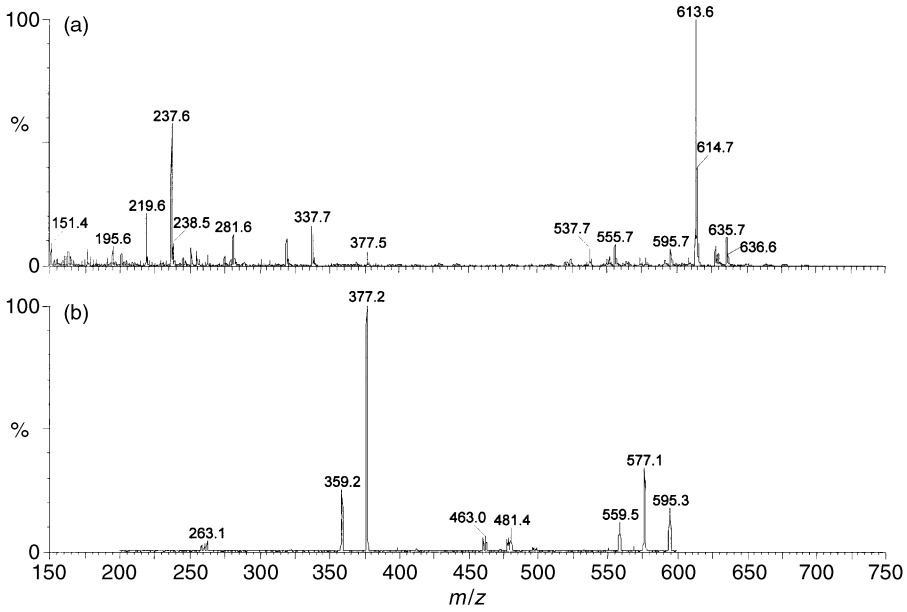

Fig. 1 (a) Lasalocid $[M + Na]^+$ pseudo-molecular ion spectrum. (b) Daughter ion fragments from parent $[M + Na]^+$.

Table 1 Determination of lasalocid (ng g^{-1}) in spiked samples by HPLC-F

Spike level/ ng g^{-1}	Chicken muscle	Chicken liver	Egg	Pig liver	Pig kidney	Sheep liver	Sheep kidney	Calf liver	Calf kidney
0 (×4)	0	0	0	0	0	0	0	0	0
10		11.1	11.9						
		10.8	11.9						
		11.3	9.6						
		10.6	9.5						
25	25.4	20.0	27.2	21.0	25.6	21.4	23.4	27.9	20.6
	27.2	20.2	27.2	21.0	25.6	19.2	24.4	25.9	26.0
	27.4	19.0	29.4	20.6	25.6	26.2	23.6	27.0	26.4
	27.6	21.4	29.0	20.8	24.4	27.0	23.6	24.1	25.8
50	49.8	41.2	52.6	55.3	48.8	54	41.4	55.5	50.0
	52.0	41.0	53.6	54.8	48.0	52.4	40.8	55.6	49.0
	52.0	41.4	57.0	55.7	47.6	53.4	41.0	54.3	52.2
	52.0	42.4	54.0	54.8	47.4	54.2	41.4	56.5	50.4
100	97.4	80.8	101.2	100.8	97.2	106.6	100.6	110.3	99.6
	101.4	81.2	101.8	99.1	96.6	104.2	101.0	110.5	98.6
	101.8	83.8	110.0	99.5	96.0	107.2	101.0	111.3	104.2
	101.6	83.0	104.2	100.8	94.6	104.4	99.0	111.2	100.0
200	194.8	156.2	201.4	188.4	192.8	208.8	210.6	222.9	202.0
	202.4	156.2	204.4	186.0	191.4	208.2	192.4	217.5	203.4
	202.4	161.0	223.2	186.5	189.2	210.6	188.4	219.5	206.0
	201.2	156.0	205.8	185.9	188.8	207.8	175.2	217.3	198.0

Table 2 Summary of results[a]

Matrix	No. of samples (*n*)	Added lasalocid range/ng g^{-1}	Recovery range (%)	Mean recovery (%)	*s* (%)	RSD (%)
Chicken muscle	20	0–200	97.4–109.6	102.7	4.00	3.89
Chicken liver	24	0–200	76.0–110.8	86.8	11.95	13.78
Eggs	24	0–200	95.3–118.7	107.5	7.09	6.60
Pig liver	20	0–200	82.4–111.3	96.8	10.14	10.48
Pig kidney	20	0–200	94.4–102.4	97.1	2.81	2.90
Sheep liver	20	0–200	76.8–108.0	102.7	8.68	8.46
Sheep kidney	20	0–200	82.0–101.0	92.8	7.09	7.64
Calf liver	20	0–200	96.4–111.2	109.1	3.99	3.66
Calf kidney	20	0–200	82.4–105.6	100.3	5.31	5.29

[a] *s* = standard deviation; RSD = relative standard deviation.

Table 3 Determination of lasalocid by LC-MS-MS

Lasalocid spike/ng g^{-1}	Lasalocid determined in chicken liver/ng g^{-1}	Lasalocid determined in eggs/ng g^{-1}
0 (×4)	0	0
1	0.6	1.1
	0.8	0.8
	0.8	0.8
2	0.7	0.8
	0.8	1.3
	1.1	1.3
	1.6	2.1
	1.2	1.5
4	1.7	2.7
	1.7	2.6
	2.9	3.5
	2.6	3.2
6	2.7	2.8
	2.7	3.2
	4.1	4.9
	3.1	4.8
Mean recovery (%)	59.4	76.4

recoveries of 94 and 87%, respectively. Figs. 2–4 show the chromatograms obtained by both HPLC-F and LC-MS-MS. No matrix interferences were observed in the spiked samples and there were no other peaks at the respective retention times in any of the non-spiked controls assayed by both detection methods.

Blanchflower and Kennedy[11] reported mean recoveries in the range 76–88% in eggs fortified between 5 and 50 ng g^{-1} using LC-electrospray-MS for quantification. Su *et al.*,[18] using an HPLC-F method, obtained recoveries in the range 90–98% in bovine and chicken tissues and the reported limit of detection was 5 ng g^{-1}. Tarbin and Shearer,[15] also using an HPLC-F method, obtained recoveries between 66 and 78% in chicken tissues and 76% in eggs.

The described method compares well with the above methods in terms of recovery, and is also relatively simple in that a single extraction step is required for the quantification of levels above 10 ng g^{-1}, with a concentration step required at levels of less than 10 ng g^{-1}. This method was developed for the rapid screening of lasalocid in animal tissues and eggs to avoid the need for any clean-up process or lengthy extraction. The main advantages of this method are the number of samples that can be analysed (up to 40 samples can be assayed by one analyst in a day) and the small amount of acetonitrile used for the extraction of a sample (10 ml, with no additional use of any other

Table 4 Determination of lasalocid (0–400 ng g^{-1}) by HPLC-F and LC-MS-MS

Spike level/ng g^{-1}	HPLC-F/ng g^{-1}	Mean/ng g^{-1}	*s*/ng g^{-1}	RSD (%)	LC-MS-MS/ng g^{-1}	Mean/ng g^{-1}	*s*/ng g^{-1}	RSD(%)
0 (×5)	0				0			
50	44.6	44.5	0.36	0.81	41.6	42.6	2.36	5.54
	44.2				40.9			
	44.4				40.9			
	45.1				40.9			
	44.3				46.6			
100	96.5	96.1	0.48	0.50	89.2	86.2	2.23	2.59
	95.3				85.3			
	96.3				87.2			
	96.4				83.1			
	96.2				86.1			
200	190.1	190.1	1.46	0.77	170.4	178.4	6.29	3.52
	187.8				177.2			
	190.0				187.8			
	191.7				177.1			
	191.0				179.8			
400	388.8	389.0	1.87	0.48	354.2	352.0	4.74	1.35
	390.6				355.6			
	391.1				354.8			
	387.9				351.6			
	386.6				344.0			
Mean recovery (%)	94.4				87.2			
s (%)	3.29				3.27			
RSD (%)	3.48				3.75			

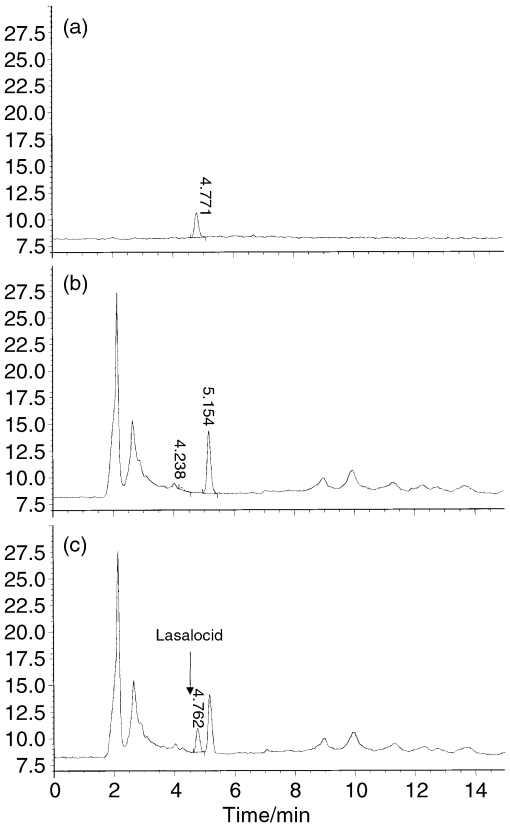

Fig. 2 HPLC-F: (a) lasalocid standard (5 ng ml^{-1}); (b) egg blank; (c) egg spiked with lasalocid at 10 ng g^{-1}.

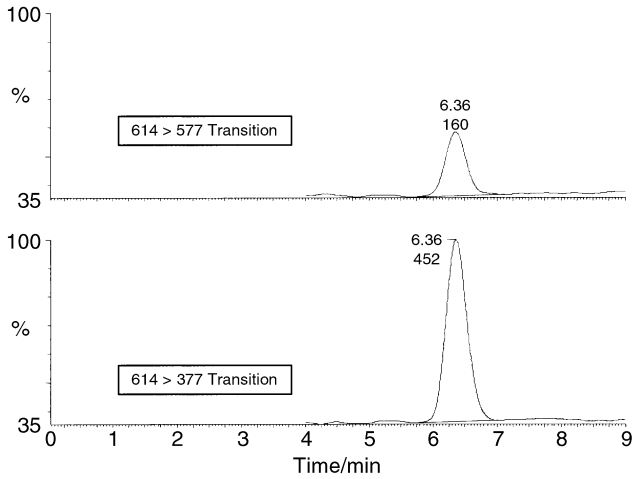

Fig. 3 Egg with spiked lasalocid at 2 ng g^{-1}.

chemicals or chromatographic columns generally required for the subsequent clean-up stage). Sample preparation takes about 2–3 h, with the extraction, centrifugation and evaporation taking a total of 90 min; the evaporation process takes 45 min. The run time for HPLC-F is about 18 min and 8 min for LC-MS-MS. This represents a simple, rapid, efficient and versatile method which can be adapted to the routine analysis of lasalocid in animal tissues and eggs.

Conclusions

We have described a method for the determination of lasalocid in different animal matrices and eggs by HPLC-F and LC-MS-MS. The method has been used successfully by this laboratory for the screening of lasalocid in livers and eggs submitted for

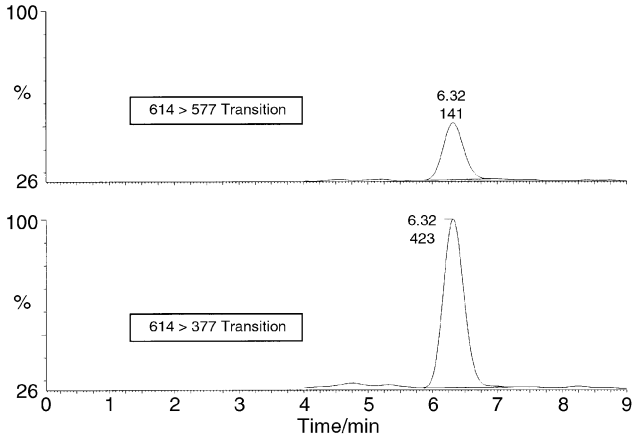

Fig. 4 Chicken liver with incurred lasalocid residue at ~1 ng g^{-1}.

examination over the last 12 months. The limit of quantification is 1 ng g^{-1} and the method has wide applicability, *i.e.*, animal tissues and eggs containing 1–400 ng g^{-1} of lasalocid with the use of the appropriate calibration curve. The quantification of all putative positive samples must be corrected for recovery based on the response of the fortified samples against the external lasalocid standards. The HPLC-F procedure was used for the primary screening of samples, and putative positive samples for the presence of lasalocid were confirmed by both LC-MS-MS and HPLC-F. The results obtained by both methods showed no significant differences, indicating the robustness, sensitivity and specificity of the described method. Both methods can be used either independently or in combination for the detection of lasalocid at levels well below the prescribed DAL of 100 ng g^{-1} in different biological matrices.

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