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The optimisation of a rapid method for the determination of lasalocid in poultry feed using supercritical fluid extraction and high performance liquid chromatography

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Abstract

A simple and rapid method, using supercritical fluid extraction with off-line high-performance liquid chromatography for the isolation and determination of lasalocid in poultry feed is described. Lasalocid is widely used as a coccidiocidal drug in poultry and for increased feed efficiency as well as for weight gain in ruminant animals, namely cattle and sheep. Results show good repeatability with a minimum quantification level of 0.5 μg g^{-1} and mean 'spiked' recovery of 100% using poultry feed ($n=32$) spiked with lasalocid in the range of 0.5–125 μg g^{-1}. Carbon dioxide used as a supercritical fluid is proposed as an alternative isolation method to the current solvent extractions, which generally require lengthy clean up procedures prior to the assay.

Keywords: Lasalocid; Poultry feed; Supercritical fluid extraction; HPLC

1. Introduction

Lasalocid was one of the first ionophores isolated by Berger, Rachlin, Scott, Sternbach, and Goldberg (1951). It has a broad-spectrum anti-coccidial activity which was reported by Brossi (1969). The high degree of activity against the *Eimeria* species makes it a very effective coccidiostat used for poultry. It is also used for growth stimulating properties in ruminant animals including cattle, pigs and sheep, by improving their feed utilisation and weight gain (Bartley, Herod, Bechtle, Sapienza, & Brent, 1979; Mitrovic & Schildknecht, 1974). At a dose of 75–125 mg kg^{-1} in feed, lasalocid is very effective against the upper intestinal species of coccidia and does not show weight depression in chicks when compared with other ionophores, and often allows better weight gain (Reid, Johnson, & Dick, 1975). Lasalocid is also effective against the major *Eimeria* species in turkey coccidiosis at a dose of 125 mg kg^{-1} and at a dose of 112 mg kg^{-1} in cattle coccidiosis (Fitzgerald & Mansfield, 1979). Lasalocid is most active against the earliest endogenous stages of coccidia, with little activity against schizogony or gametogony in chicken. Lasalocid is coccidiocidal and is active against coccidia in vitro and in vivo (Long, 1982).

Lasalocid has a fluorescent chromophore which is readily determined by HPLC using a fluorescence detector having an excitation λ_{max} between 308 and 315 nm, and fluorescence emission λ_{max} between 400 and 430 nm. The fluorescence is highly sensitive to the pH of the solvent. When the pH is changed from 8.3 to 3.2 the fluorescence intensity decreases by two orders of magnitude. Several HPLC methods based on fluorescence detection have been reported (Horii, Miyahara, & Momma, 1990; Kaykaty & Weiss, 1983; Tarbin & Shearer, 1992; Weiss et al., 1983).

Other methods (Elliott, Kennedy, & McCaughey, 1998), which are currently available for lasalocid assay include: TLC-bioautography using the organism *Bacillus subtilis* as the inoculum (MacDonald et al., 1979; Vanderkop, & MacNeil, 1990). ELISA (Kennedy, Blanchflower, & O'Dornan, 1995), GC–MS (Weiss, Kaykaty, & Miwa, 1983), LC–MS–MS and LC–MS (Blanchflower & Kennedy, 1995; Horii, Miyahara, & Maruyama, 1991). The incubation period and the clean-up procedures of these methods add considerably to the time required for the complete assay of lasalocid.

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The aim of this study was to develop and optimize an alternative method that was rapid, simple and easily applicable to poultry feed, and extending the method to eggs, chicken tissue, chicken liver and beef tissue. Such a method would be very useful for the rapid screening of lasalocid in feeds and foodstuffs.

Lasalocid should not be present in feeds intended for laying and breeding stock or during the withdrawal period prior to the slaughter of poultry, to prevent lasalocid residues in animal products used for human consumption. The monitoring and surveillance of drugs in the food production at different stages required by the European Legislations using new, rapid and reliable method would be of immense benefit in the control and use of drug residues in the food chain, particularly where current methods are tedious, expensive and require the use and disposal of large volumes of solvents. This method provides a measure to control lasalocid from entering the food chain mainly through poultry feed.

2. Experimental

2.1. Reagents and materials

Poultry feed free from coccidiostats was obtained from the Charnwood Milling Co. (Framlingham, Suffolk, UK. The feed was homogenised and stored at room temperature. Fresh eggs were obtained from the local supermarket, they were pooled together by mixing (beating) and stored at 4 °C or at refrigerator temperature. Fresh chicken, chicken liver and beef were obtained from a local butcher; the tissues were homogenised and stored at −20 °C or at freezer temperature. The pooled liquid egg and the tissues were checked for the presence of lasalocid by the supercritical fluid extraction–high-performance liquid chromatography (SFE–HPLC) method described in the study.

2.2. Reagents

Solvents: acetonitrile, methanol, HPLC grade, Ethanol absolute (Rathburn, Walkerburn, UK); ethyl acetate, HPLC grade, (Fisons, Loughborough, UK); sodium sulphate anhydrous, Analytical grade, (Aldrich, Gillingham, Dorset, UK; silica gel for column chromatography, grade 60, 230–400 mesh, 60 Å, (Aldrich, Gillingham, Dorset, UK); sodium hydroxide, 1 M.

Modifier: (ethanol/sodium hydroxide/ethyl acetate); ethanol 90% v/v was prepared by mixing 9 parts absolute ethanol with 1 part of deionised water; (i) Ethanol/sodium hydroxide solution: this was prepared by mixing ethanol 90% v/v with 1 M sodium hydroxide in the ratio 9:1; (ii) Ethyl acetate: modifier solution made up with mixing the above solutions (i) and (ii) in the ratio of 2:1. This modifier was used throughout the study; Lasalocid sodium, analytical standard, (Sigma, UK).

2.2.1. Lasalocid standard solution

A 25 mg portion of the reference standard was weighed into a 25-ml volumetric flask and dissolved in methanol and made to volume, mixed well and stored in the refrigerator and protected from light.

2.2.2. Spiking of samples

Poultry feed: 1 g samples were spiked with 25, 50, 75, 100 and 125 μl of the above standard solution (1 mg ml^{-1}) representing 25–125 mg kg^{-1} of lasalocid dosed onto the control feed samples. Eggs and tissues: 1 g samples were spiked with 50, 100 and 200 μl of a 10-fold diluted solution of the above standard solution (1 mg ml^{-1}) with methanol representing 5–20 ppm lasalocid dosed onto the control egg and tissue samples. Eggs and tissues, 1 g samples were spiked with 50, 100 and 200 μl of a hundred-fold diluted solution of the above standard solution (1 mg ml^{-1}) with methanol, representing 0.5, 1.0 and 2.0 mg kg^{-1} of lasalocid onto the control egg and tissue samples.

After the spiking the samples were left to stand for at least 1 h to equilibrate prior to the extraction by the SFE.

2.3. Apparatus

2.3.1. HPLC

Mobile phase of acetonitrile, flow rate of 1 ml min^{-1}; Fluorescence detection, excitation λ_{max} at 310 nm and fluorescence emission λ_{max} at 430 nm; column, APEX Silica 5 μ, (150×4.6 mm i.d), Jones Chromatography, (Hengoed, mid Glamorgan, UK), injection volume of 20 μl; detector, Hewlett Packard 1046 A; integrator, Chromjet, Spectra Physics; chart speed of 10 cm min^{-1}.

2.3.2. SFE

The supercritical fluid extractor used was an ISCO model 260 D, marketed by Jones Chromatography, UK. Several of the SFE parameters were optimised prior to the extraction of lasalocid. These included (1) choice of modifier (methanol, methanol/water, ethanol, ethanol/water, acetonitrile, acetonitrile/water, hexane, ethyl acetate, ethanol/ethyl acetate, acetone, diethyl ether, methanol/diethylamine, tetrahydrofuran, methanol/tetrahydrofuran, ethyl acetate/tetrahydrofuran, *n*-propanol, *n*-butylacetate, diethanolamine/acetonitrile/tetrahydrofuran, ethanol/ethylacetate/sodium hydroxide), (2) temperature (40–90 °C), (3) pressure (3000–6500 psi) and (4) extraction time 5–80 min in static and dynamic and combination of both modes (5) selection of packing materials for column, (alumina, florisil, Sephadex L20, cellulose, sodium sulphate, glass beads, silica). The final optimised SFE conditions used throughout the study for the extraction of lasalocid were (1) extraction

temperature of 50 °C, (2) extraction pressure (constant) of 4000 psi and (3) restrictor temperature of 80 °C and (4) carbon dioxide flow rate of 1.5–2 ml min^{-1}.

3. Methods

3.1. Extraction by supercritical carbon dioxide

3.1.1. Preparation of silica column

One gram of silica was placed into a 10-ml extraction cartridge and 2 ml of ethyl acetate was added to the silica. The mixture was tapped gently to give a compact slurry, this column preparation was used for all the extractions in the study.

3.1.2. Extraction of poultry feed using the supercritical fluid (carbon dioxide)

A 1 g portion of the feed sample was placed on the prepared silica column in the extraction cartridge and 5 ml of modifier solution (ethanol/ethyl acetate/sodium hydroxide) was added to the cartridge. The feed was extracted for 20 min, the first 5 min in the static mode and the following 15 min in the dynamic mode. This extraction was repeated once more (5 min in the static mode and 15 min in the dynamic mode) with the addition of a further 5 ml of modifier to the cartridge. The combined extract was collected in a 20 ml volumetric flask containing ~2.5 ml of methanol. After both extractions, the extract was made to the mark with methanol and filtered through 0.45-μm syringe filter. This was analysed by the HPLC.

3.1.3. Extraction of egg by the supercritical fluid method

Two grams of anhydrous sodium sulphate were placed on a prepared silica column in the extraction cartridge, 1 g of liquid egg was applied directly to the sodium sulphate layer and both were mixed with a small spatula to form a paste. The sodium sulphate was used as a drying agent to prevent restrictor blockage due to extracted water forming ice during decompression, and also to allow for sample dispersion within the SFE cartridge (Burford, Hawthorne, & Miller, 1993; Parks & Maxwell, 1994). The spatula was washed with 5 ml of the modifier solution into the cartridge. Each extraction was performed as for the feeds, i.e. 5 min static SFE followed by 15 min dynamic SFE carried out twice. The combined extract following both extractions (static and dynamic) was collected in a 20 ml volumetric flask containing ~ 2.5 ml of methanol and made up to the mark with methanol. The solution was filtered through a 0.45 μm filter prior to quantification by HPLC.

For samples dosed at 0.5 and 1 mg kg^{-1} lasalocid, the combined extract was collected in a tube (100×5 mm i.d) to avoid splashing of extract when a 5 ml volumetric flask was used to collect the extract. After both extractions, the final volume was made to mark in a 5 ml volumetric flask by evaporating the extract by placing in a water bath at 55 °C under a stream of nitrogen. This was filtered prior to quantification by HPLC.

3.1.4. Extraction of chicken tissue, chicken liver and beef tissue

Two grams of anhydrous sodium sulphate were placed on a prepared silica column in the extraction cartridge, 1 g of homogenised tissue was applied directly on top of the sodium sulphate layer. A further 1 g of anhydrous sodium sulphate was applied on top of the tissue to sandwich the sample. The tissue was mixed with the sodium sulphate using a small spatula to give a paste and the same extraction procedure followed as for the egg sample.

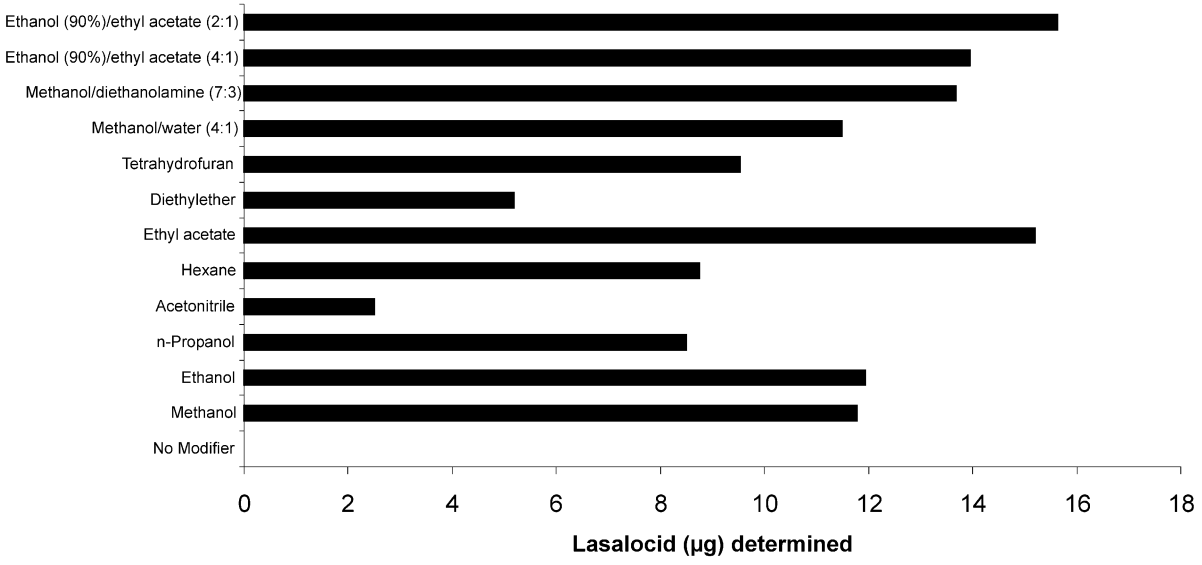

Fig. 1. Extraction of lasalocid using different modifiers.

3.2. Optimisation

The efficiency of the extraction of lasalocid by SFE using carbon dioxide as the supercritical fluid is dependent mainly on the following parameters. i.e. modifier, pressure and the temperature used during the extraction process. The effect on the recovery of lasalocid from spikes using different parameters was initially investigated in order to obtain the optimum conditions for the extraction of lasalocid by the SFE.

3.3. The effect of the use of modifiers for the extraction of Lasalocid

25 μg of lasalocid standard was immobilized on 2 g of silica in an extraction cartridge and was extracted by supercritical CO_2 at 60 °C using a pressure of 5000 psi. The extraction was carried out without any modifiers and also with 5 ml of different solvents or solvent mixtures used as modifiers. The extraction was done in the following mode, i.e 10 min in the static mode followed by 20 min in the dynamic mode using a flow of 1.5–2 ml min^{-1}.

4. Results

4.1. Effect of different pressures on lasalocid extraction

A pressure of 4000 psi was found to be the optimum for the extraction of lasalocid at 50 °C. This was demonstrated by using different modifiers, i.e methanol, ethanol, ethanol (90%), ethanol/ethyl acetate (2:1) and extracting lasalocid at 3000, 4000, 5000 and 6000 psi (Figs. 1 and 2).

4.2. Effect of temperature on the extraction of lasalocid

4.2.1. Selection of temperature

The highest recoveries were obtained at 50 °C as shown by this study. The optimum temperature of 50 °C was demonstrated in all the cases that were investigated in the range 40–70 °C. The trend showed that starting from 40 °C, the recovery peaked up at 50 °C, then dropped with a rise in temperature irrespective of the nature of the modifier either a single solvent or a mixture of solvents used for the purpose of modifying the supercritical fluid (Fig. 3).

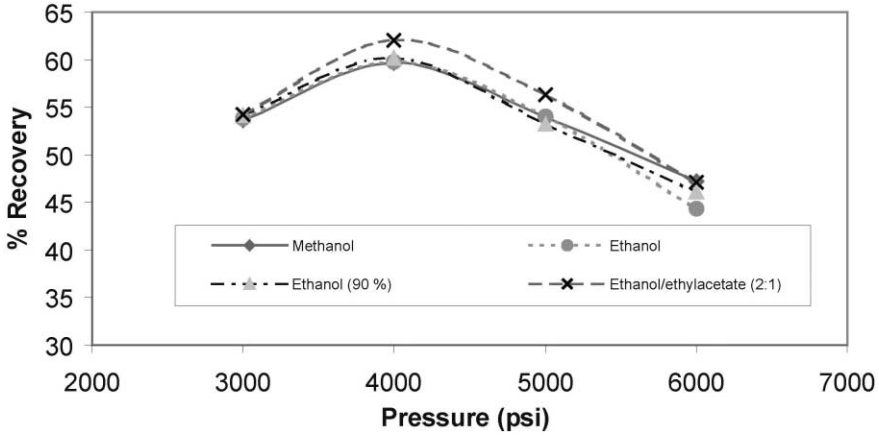

Fig. 2. Extraction of lasalocid at different pressures.

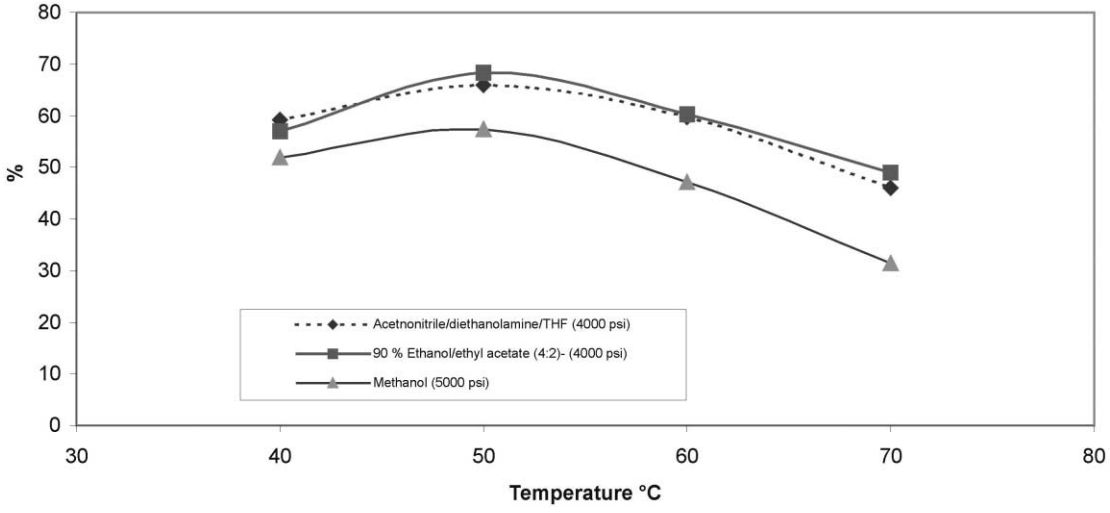

Fig. 3. Extraction of lasalocid at different temperatures.

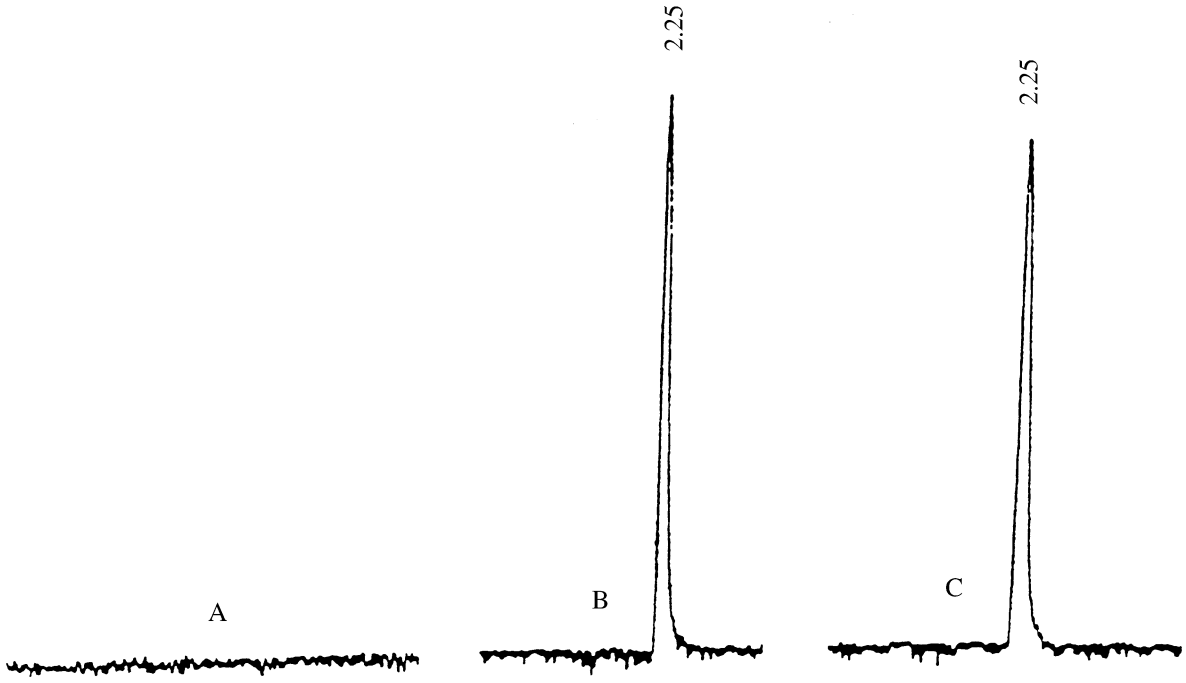

Fig. 4. HPLC chromatograms following SFE extraction of: (A) feed sample (control); (B) external lasalocid standatd 125 ppm; (C) feed sample spiked with 125 ppm lasalocid.

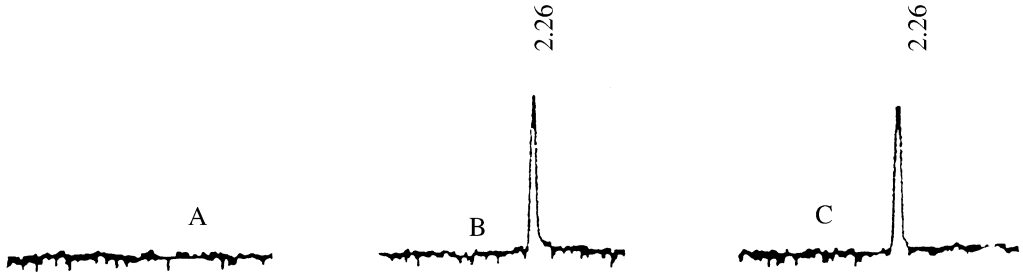

Fig. 5. HPLC chromatograms (attenuation 8) following SFE extraction of: (A) chicken muscle sample (control); (B) external lasalocid standard 10 ppm; (C) chicken muscle sample spiked with 10 ppm lasalocid.

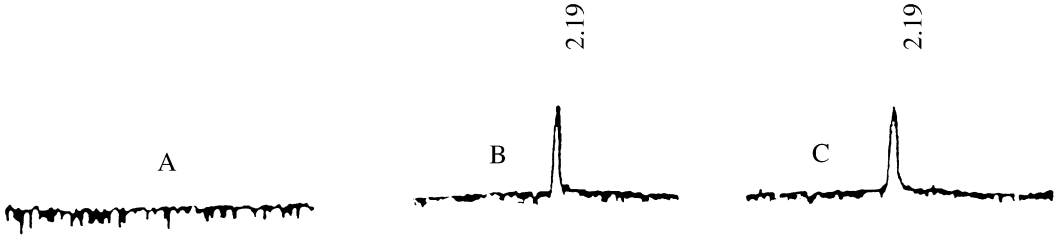

Fig. 6. HPLC chromatograms (attenuation 4) following SFE extraction of: (A) chicken liver sample (control); (B) external lasalocid standard 5 ppm; (C) chicken liver sample spiked with 5 ppm lasalocid.

The optimum SFE conditions obtained were:

modifier: ethanol/sodium hydroxide/ethyl acetate pressure: 4000 psi temperature: 50 °C extraction time: 5 min static followed by 15 min dynamic. This is carried out twice with a further addition of 5 ml of modifier.

These parameters were used to extract spiked poultry feed, animal tissues and eggs.

5. Results and discussion

Table 1 shows the actual determinations and the percentage recoveries of lasalocid from feeds, eggs, chicken tissue, chicken liver and beef tissue. Each of the samples showed no significant matrix interferences. The results (Figs. 4–6) show that lasalocid was successfully extracted

Table 1

Added lasalocid (ppm)	Lasalocid determined (ppm)					% Lasalocid recovered				
	Poultry feed	Egg	Chicken tissue	Chicken liver	Beef	Poultry feed	Egg	Chicken tissue	Chicken liver	Beef
0.5	0.48	0.51	0.53	0.45	0.46	96.0	102.0	106.0	90.0	92.0
	0.49	0.51	0.50	0.45	0.49	98.0	102.0	100.0	90.0	98.0
	0.51	0.44	0.45	0.48	0.52	102.0	88.0	90.0	96.0	104.0
	0.47	0.50	0.48	0.45	0.51	94.0	100.0	96.0	90.0	102.0
1.0	1.02	0.97	1.02	1.00	1.08	102.0	97.0	102.0	100.0	108.0
	1.10	1.01	1.01	0.99	1.08	110.0	101.0	101.0	99.0	108.0
	0.93	0.91	1.00	0.99	1.06	93.0	91.0	100.0	99.0	106.0
	1.06	0.94	0.98	1.02	1.03	106.0	94.0	98.0	102.0	103.0
2.0		2.20	2.08	1.90	2.05		110.0	104.0	95.0	98.0
		2.05	2.10	1.90	2.20		103.0	105.0	95.0	98.0
		1.96	1.97	1.89	1.95		98.0	99.0	95.0	98.0
		2.05	1.95	1.95	2.15		103.0	98.0	98.0	108.0
5.0		4.9	4.9	4.7	4.7		98.0	98.0	94.0	94.0
		5.3	5.3	4.7	4.9		106.0	106.0	94.0	98.0
		5.0	5.2	5.2	4.9		100.0	104.0	104.0	98.0
		5.1	4.8	4.9	4.7		102.0	96.0	98.0	94.0
10	10.10	9.9	10.4	10.2	9.4	101.0	99.0	104.0	102.0	94.0
	10.40	9.6	10.0	9.4	9.3	104.0	96.0	100.0	94.0	93.0
	10.50	9.6	9.9	9.3	9.8	105.0	96.0	99.0	93.0	98.0
	10.10	9.4	10.0	9.4	10.1	101.0	94.0	100.0	94.0	101.0
20		19.6					98.0			
		19.4					97.0			
		19.0					95.0			
		18.9					95.0			
25	26.0					104.0				
	24.2					97.0				
	24.9					100.0				
	25.0					100.0				
50	45.0					90.0				
	50.0					100.0				
	48.5					97.0				
	51.8					104.0				
75	73.8					98.0				
	76.7					102.0				
	76.5					102.0				
	78.0					104.0				
100	97.5					98.0				
	101.5					102.0				
	101.0					101.0				
	104.0					104.0				
125	123.5					99.0				
	126.5					101.0				
	124.8					100.0				
	123.9					99.0				

Table 2
Summary of results

Matrix	No. of samples (*n*)	Added lasalocid range (ppm)	Recovery range %	Mean recovery %	S.D.	RSD %
Poultry feed	32	0.5–125	93–110	100	3.98	3.97
Eggs	24	0.5–20	88–110	99	4.78	4.85
Chicken tissue	20	0.5–10	90–106	100	3.93	3.92
Chicken liver	20	0.5–10	90–104	96	4.06	4.23
Beef	20	0.5–1.0	92–108	101	5.63	5.61

using the SFE method. The mean recoveries of lasalocid by the SFE method for poultry feed, eggs, chicken tissue, chicken liver and beef tissue with a range of added lasalocid are summarised in Table 2 and were 100, 99, 100, 96 and 101%, respectively. The consistency of the mean recoveries of lasalocid from a wide range of added lasalocid from these matrices indicated the versatillity of the method. The main benefits of the SFE method include a significantly reduced overall analysis time, which is less than 90 min (extraction time: 40 min, HPLC determination 2–3 min, sample preparation for level less than 1 ppm, 30 min). The SFE extract did not require any further clean-up procedure prior to the HPLC analysis (current extraction method require solvent–solvent portioning followed by a clean-up regime). The method requires a low volume of solvents (20 ml) for the complete extraction and determination of lasalocid, which is economical, and the relatively low toxicity of the solvents representing little hazards from exposure to solvents.

6. Conclusion

This study has shown that the SFE method can be used as an alternative to current extraction methods used for the isolation of lasalocid from poultry feed and animal matrices. The main advantage of the SFE method over the conventional extraction procedures is that the lasalocid is selectively isolated, i.e the method functions both as an extraction and clean-up process. Therefore, this represents a simple and rapid method which can be adapted for lasalocid determination in poultry feed which is the main source for the occurrence of lasalocid residues in animal products. The approved method for the determination of lasalocid in poultry feeds (Analytical Methods Committee, 1995) has the scope for the determination of lasalocid in the range of 30–150 mg kg^{-1} which uses 100 ml of acidified methanol for the extraction of 5 g of feed. The determination involves several steps such as swirling, extraction by using sonication, cooling, time to allow for suspension to settle, filtration prior to HPLC analysis. Hence the SFE–HPLC method compares favorably in that a lower sample weight is used and the quantification level is much lower.

The prescribed limit of 0.1 mg kg^{-1} of lasalocid residues by the UK Legislation in animal tissues and eggs was not achievable using the SFE–HPLC method. However, there is scope to improve the method by using larger extraction cells such that larger sample weight can be extracted by the SFE and the consistent recoveries on animal products dosed at the level of 0.5 mg kg^{-1} with lasalocid has been demonstrated in this study.

Another method for the detection of lasalocid in animal matrices and eggs has been developed and reported (Matabudul, Conway, & Lumley, 2000) which can detect lasalocid at levels of 10 ng g^{-1} in animal tissues and eggs by HPLC with fluorescence detection and can be used for regulatory purposes.

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