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Determination of monensin, salinomycin and narasin in muscle, liver and eggs from domestic fowl using liquid chromatography–electrospray mass spectrometry

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Abstract

A method is presented for the simultaneous determination of the three ionophores, monensin, salinomycin and narasin in muscle, liver and eggs from domestic fowl. Samples are homogenised in methanol and any ionophores present are extracted into toluene–hexane. The extracts are concentrated and injected into a bench-top electrospray LC–MS system. Chromatography is carried out using an end-capped reversed-phase column, with a mobile phase consisting a mixture of acetonitrile, methanol, tetrahydrofuran, trifluoroacetic acid and water. Using single-ion monitoring, the ionophores can be detected down to the 1 ng/g level.

Keywords: Monensin; Salinomycin; Narasin; Antibiotics

1. Introduction

Monensin, salinomycin and narasin belong to a group of polyether antibiotics produced naturally by certain strains of *Streptomyces*. They are relatively large molecules with molecular masses of 671, 751 and 765 for monensin, salinomycin and narasin, respectively. The usual structural features include a carboxylic acid at one terminus and a hydroxyl group at the other. They readily form cyclic complexes with cations, particularly sodium, as shown in Fig. 1, and have the ability to cross biological membranes [1].

The main therapeutic application of the polyether ionophore antibiotics in veterinary medicine is for the prevention and treatment of coccidiosis in poultry. They are added to feeds as the sodium salts at fortification levels of 60–120 mg/kg. Monensin and narasin are supplied under the trade names Elancoban and Monteban, respectively, by Elanco Products and salinomycin is supplied as Sacox by Hoechst Animal Health. Monensin is recommended for use in broiler chickens and turkeys for fattening, provided a withdrawal period of three days is observed before slaughter. Narasin and salinomycin are recommended for use only in broiler chickens, with a withdrawal period of five days. While the use of these antibiotics can be beneficial, care needs to be taken to ensure that they are used at the correct concentrations and that they are not present in feed intended for other species, to which some of them may prove fatal.

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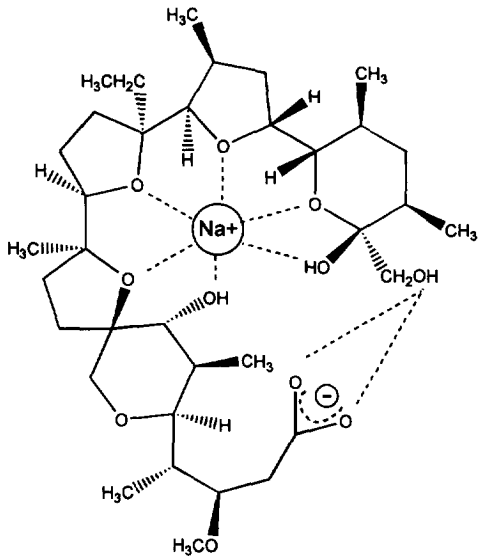

Fig. 1. Molecular representation of the cyclic structure of the monensin–sodium complex.

Several analytical methods have been published for the determination of one or more of these compounds in feed or tissues. Screening methods include those using thin-layer chromatography–bioautography [2] and enzyme-linked immunosorbent assays (ELISA) [3]. Most confirmatory assays which have been published use high-performance liquid chromatography. Since the ionophores do not possess any significant UV absorbance, derivatisation is generally required. Two derivatising agents, vanillin [4–6] and 9-anthryldiazomethane (ADAM) [7,8] have been used to form chromophores with absorbance at 520 nm and fluorescent derivatives, respectively. For confirmatory methods, however, it is preferable to use techniques which have the highest possible specificity, and this generally means some type of mass spectrometry. One method has been published [9] for the confirmation of monensin in chicken tissue using gas chromatography–mass spectrometry (GC–MS) but this was only applicable to samples containing more than 5 μg/g and levels of this magnitude are rarely, if ever, found in poultry receiving therapeutic doses of monensin. In addition, the ionophores are not good candidates for GC–MS because of their high molecular mass and low volatility.

We have already published a method [10] for the determination of the polyether antibiotic, lasalocid, in eggs using liquid chromatography–electrospray mass spectrometry (LC–EMS) and have also found the technique to be suitable for the determination of monensin, salinomycin and narasin in poultry tissue and eggs. In this paper, we describe a relatively simple and sensitive assay for the determination of these three ionophores in tissue and eggs, with a detection limit of about 1 ng/g. Samples are extracted with methanol and any ionophores present are partitioned into hexane–toluene. These extracts are then concentrated and injected directly into the LC–MS system and three ions are monitored for the sodium complexes of the three compounds.

2. Experimental

2.1. Chemicals

Acetonitrile, methanol and tetrahydrofuran were HPLC grade, and other reagents were analytical-reagent grade. Monensin, salinomycin and narasin were obtained as their sodium salts from Sigma (Poole, UK), Calbiochem-Novabiochem (Nottingham, UK) and as a gift from Lilly Research laboratories (Indianapolis, USA), respectively. Stock standards (1 mg/ml) of each in methanol were prepared, which are stable for up to three months stored at 4°C. Working standards (1 μg/ml and 100 ng/ml) of each were prepared in acetonitrile–water (75:25, v/v), stable for at least one month stored at 4°C.

2.2. HPLC system

The HPLC system consisted of a Merck-Hitachi Model L6000 pump and AS2000 autosampler (Merck, Poole, UK) and an Intersil 150×4.6 mm ODS-2 (5 μm particle size, endcapped) reversed-phase column (GL Sciences, Tokyo, Japan).

The mobile phase was a mixture of acetonitrile, methanol, tetrahydrofuran, water and trifluoroacetic acid (67:10:10:13:0.1, v/v). This was filtered and degassed using an HPLC solvent filter unit fitted with a 0.5-μm HVLP filter (Millipore-Waters, Watford, UK). The mobile phase was pumped through the HPLC system at a flow-rate of 1 ml/min. The

effluent from the HPLC column was directed into a low-volume T-piece, one outlet of which went to waste, with the other outlet being connected to the inlet of the electrospray probe. A short length of narrow bore PEEK tubing (150 mm×0.127 mm) was used at the waste outlet of the T-piece to give a split ratio of about 4:1 so that approximately 200 μl/min of the column effluent entered the LC–EMS system.

2.3. LC–EMS system

The bench-top quadrupole dedicated LC–MS system was a VG Platform (VG Biotech, Altrincham, UK) fitted with a Megaflow electrospray probe. The instrument was operated in the positive-ion mode for optimum sensitivity with the ionophores. Full scan data were collected in order to obtain spectra from standards and single-ion data (dwell time 0.5 s for each ion) were collected when analysing samples. The source of the instrument was maintained at 125°C and the flow-rates of the drying and nebulising gases were optimised at 500 and 15 l/h, respectively. The cone voltage was set at 25 V. The position of the electrospray probe and the MS parameters (low and high mass resolution and ion energy) were optimised to give maximum sensitivity and symmetrical peak shape (approximately 1 mass unit wide) using any of the solvent ions produced by the mobile phase. Once optimised, these rarely required changing.

2.4. Extraction procedure

Frozen tissue samples were pulverised using a domestic food blender and were stored at −20°C until analysis. Whole-egg samples were homogenised using a Silverson homogeniser (Silverson Machines, Chesham, UK) and either analysed fresh or also stored at −20°C until analysis. Portions (5 g) were weighed into 115×28 mm Quickfit centrifuge tubes. Tissue samples for recovery studies were also set up at this stage by spiking with the required volume of a mixed standard (1 μg/ml), allowing them to stand for 10 min and then treating them as for normal samples. Water (2 ml) and methanol (13 ml) were added to each sample followed by homogenisation for 30 s using a Silverson homogeniser. Any tubes containing tissue were then placed in an ultrasonic bath for 10 min and mixed. All tubes were then centrifuged for 10 min at 2000 *g*. Aliquots of the supernatants (2 ml) were transferred to 100×17 mm Quickfit tubes and sodium hydroxide solution (4 ml, 0.1 *M*) was added to each. The solutions were then extracted with a mixture (1:1, v/v) of hexane–toluene (2 ml and 1 ml) by inversion for 30 s and centrifuging for 10 min at 1500 *g*. The extracts were combined in 65×14 mm Quickfit tubes and evaporated to dryness under nitrogen at 60°C in a fume hood, using a needle manifold assembly. The residues were dissolved in acetonitrile–water (200 μl, 75:25, v/v) and transferred to autosampler vials for analysis by LC–EMS.

2.5. LC–EMS analyses

The HPLC and LC–EMS systems were operated for 15 min to allow for equilibration prior to sample analyses. The LC–EMS was set to collect multiple single-ion data for the ions at *m/z* 693, 773 and 787 for monensin, salinomycin and narasin, respectively. The autosampler was programmed to inject 20-μl aliquots of sample extracts and a mixed standard containing 100 ng/ml of each of the three ionophores. The standard was re-injected after each batch of four sample extracts. Peak-area data were recorded and the concentrations of any ionophores found were calculated with reference to the corresponding standards. The standard (100 ng/ml) was equivalent to 40 ng/g in tissue.

3. Results and discussion

The positive-ion full-scan electrospray spectra for each of the three ionophores are shown in Fig. 2. The most prominent ions in each case are those of the sodium complexes (M+23) at *m/z* 693, 773 and 787 for monensin, salinomycin and narasin, respectively, with the water adducts at M+18 also apparent. No molecular ions are present in any of the spectra and this reflects the high affinity of the ionophores to complex with cations, particularly sodium. In this assay, we have used the M+Na ions as the diagnostic ions for single-ion monitoring and for quantitation purposes and have shown them to be reproducible (see later). With LC–EMS it is sometimes possible to

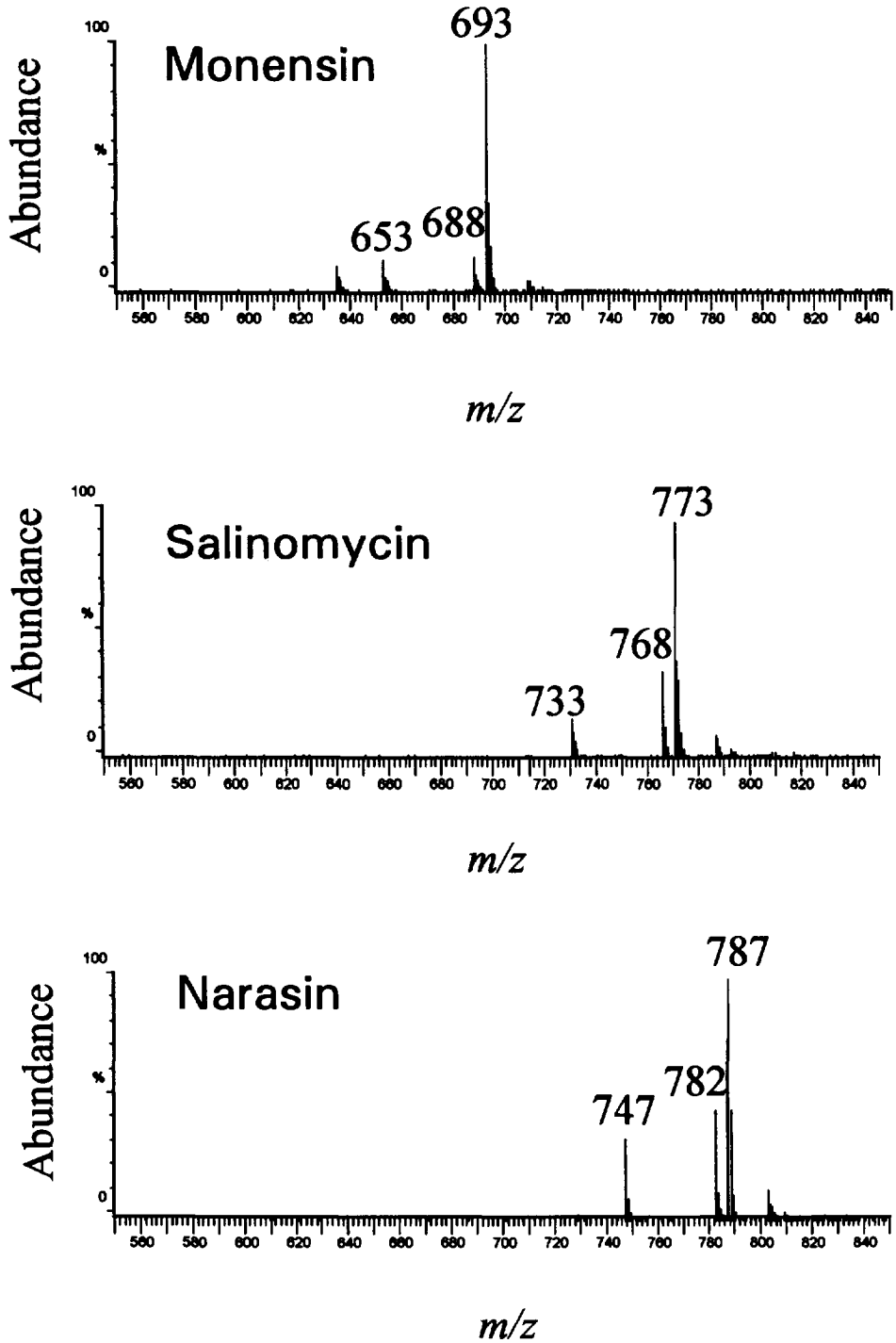

Fig. 2. Positive-ion electrospray spectra of monensin, salinomycin and narasin.

increase the fragmentation pattern by increasing the voltage on the fragmentation cone of the instrument. This enables ion ratio measurements to be used for confirmation purposes. In this instance we found that this mainly increased the intensity of the water adducts, but the ratios of the M+18 ions to the

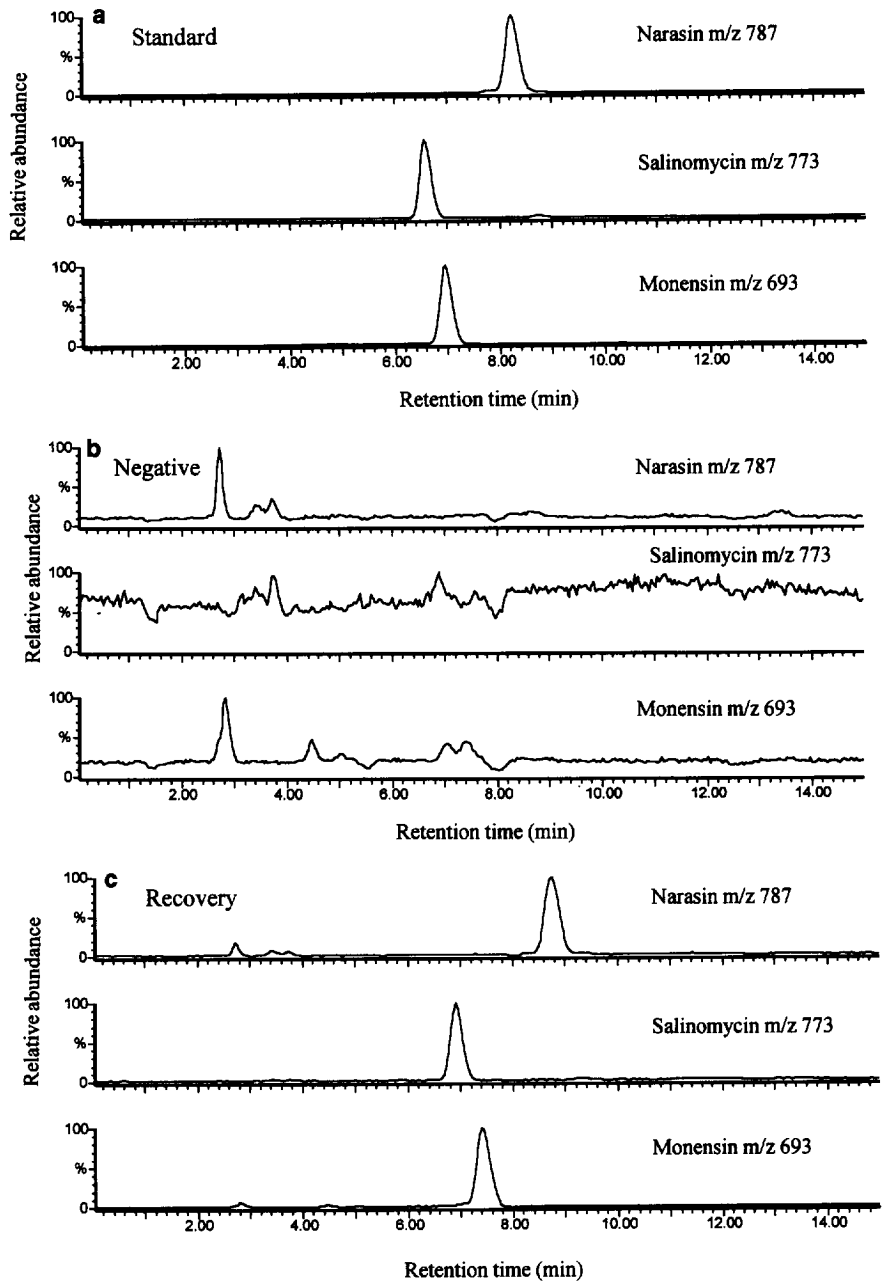

Fig. 3. (A) Single-ion chromatograms from a 20-μl injection of a mixed standard containing 100 ng/ml monensin, salinomycin and narasin. (B) Single-ion chromatograms from a 20-μl injection of a negative muscle extract. (C) Single-ion chromatograms from a 20-μl injection of a muscle extract spiked with 20 ng/g of monensin, salinomycin and narasin.

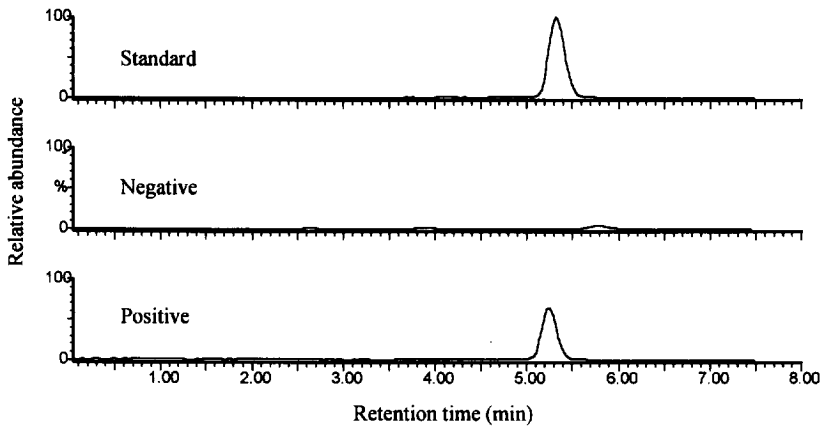

Fig. 4. Single-ion chromatograms at *m/z* 693 (monensin) for a standard, a negative egg extract and an extract of egg estimated to contain 25 ng/g incurred monensin.

M+23 ions were not reproducible, so could not be used for ion ratio measurements.

Single-ion chromatograms from a mixed standard (100 ng/ml), a negative muscle extract and an extract from muscle spiked with 20 ng/g of each of the three ionophores are shown in Fig. 3. The chromatograms are clean and free of interference from other compounds in the vicinity of the retention times of the ionophores. Chromatograms from liver extracts (not shown) were similar.

Single-ion chromatograms at *m/z* 693 (monensin) for a standard, negative egg extract and an extract of egg from a chicken which had been fed on meal medicated with monensin are shown in Fig. 4. The egg was estimated to contain 25 ng/g monensin.

The relatively simple extraction and clean-up procedure used in the assay enables samples to be analysed quickly and more elaborate techniques are not required. The procedure differs from that used for lasalocid [10] where this was best extracted from samples under acidic conditions. With monensin, salinomycin and narasin, best results were obtained by extracting the ionophores from tissue or eggs without buffering. Initially, acetonitrile was used for the first extraction step of the assay, but a broad interfering peak appeared on some of the chromatograms after about 50 min into a run. This peak was eliminated by changing to methanol and was probably due to a lipid component which was extractable with acetonitrile but not methanol. This was followed by a solvent partitioning step into toluene–hexane after the addition of 0.1 *M* sodium hydroxide to the extract. This resulted in better recoveries and cleaner chromatograms than diluting the extracts in water alone.

The intra- and inter-assay variations and recovery values for the assay are shown in Table 1, Table 2 and Table 3. Muscle and liver samples were spiked with 20 and 40 ng/g and eggs with 5, 10, and 20 ng/g of each of the ionophores and analysed five times each on three different days. The overall recoveries ranged from 77 to 113% and the overall C.V. values ranged from 4.4 to 15%. As with any MS quantitative assay, these figures could probably be improved by the use of deuterated internal standards, but at the present time these are not available.

The linearity of the assay was determined by running a series of standards with concentrations ranging from 10 to 400 ng/ml for each of the three ionophores, on three different occasions. These were equivalent to 4–160 ng/g in tissue or eggs. The assay was linear up to the highest concentrations tested, with linear regression coefficients (r) ranging from 0.992 to 1.000 for each of the three ionophores over the three occasions.

The detection limit of the assay is dependent mainly on the sensitivity of the LC–EMS instrument towards the ionophores. This could be influenced by such factors as the cleanliness of the source, the position of the electrospray probe, the composition of the mobile phase and the flow-rate of mobile phase into the source. Cleaning the source after

Table 1
Reproducibility and mean recovery values for muscle and liver samples spiked with 20 ng/g monensin (Mon), salinomycin (Sal) and narasin (Nar) (five replicates were analysed on each of three days)

	Muscle			Liver		
	Mon	Sal	Nar	Mon	Sal	Nar
Day 1						
Mean (ng/g)	16.8	20.6	19.4	18.7	19.0	18.2
S.D.	0.78	0.84	0.38	2.76	0.42	0.47
C.V. (%)	4.6	4.1	2.0	14.7	2.2	2.6
Recovery (%)	84	103	97	93	95	91
Day 2						
Mean (ng/g)	16.8	20.8	19.9	17.3	16.3	16.6
S.D.	0.59	0.59	1.04	2.63	0.81	0.32
C.V. (%)	3.5	2.8	5.2	15.2	5.0	1.9
Recovery (%)	84	104	99	86	81	83
Day 3						
Mean (ng/g)	17.5	20.3	20.3	17.5	17.1	17.3
S.D.	1.70	2.56	1.11	3.08	0.67	0.34
C.V. (%)	9.7	12.6	5.5	17.6	3.9	2.0
Recovery (%)	87	102	101	87	85	86
Overall						
Mean (ng/g)	17.0	20.5	19.8	17.9	17.5	17.4
S.D.	1.09	1.49	0.93	2.69	1.31	0.77
C.V. (%)	6.4	7.3	4.7	15.0	7.5	4.4
Recovery (%)	85	102	99	89	87	87

Table 2
Reproducibility and mean recovery values for muscle and liver samples spiked with 40 ng/g monensin (Mon), salinomycin (Sal) and narasin (Nar) (five replicates were analysed on each of three days)

	Muscle			Liver		
	Mon	Sal	Nar	Mon	Sal	Nar
Day 1						
Mean (ng/g)	29.6	38.4	39.4	37.9	34.1	36.2
S.D.	3.36	2.54	3.66	3.32	1.69	2.13
C.V. (%)	11.3	6.6	9.3	8.8	5.0	5.9
Recovery (%)	74	96	99	95	85	90
Day 2						
Mean (ng/g)	29.4	37.2	35.8	35.0	29.9	32.6
S.D.	2.41	1.49	3.07	3.53	0.91	1.02
C.V. (%)	8.2	4.0	8.6	10.0	3.0	3.1
Recovery (%)	73	93	89	87	75	81
Day 3						
Mean (ng/g)	33.6	41.8	40.24	35.7	29.3	32.8
S.D.	1.19	1.43	1.33	3.15	1.90	0.76
C.V. (%)	3.5	3.4	3.3	8.8	6.5	2.3
Recovery (%)	84	104	101	89	73	82
Overall						
Mean (ng/g)	30.89	39.1	38.5	36.2	31.1	33.6
S.D.	3.06	2.67	3.32	3.33	2.64	1.58
C.V. (%)	9.9	6.8	8.6	9.2	8.5	4.7
Recovery (%)	77	98	96	90	78	84

Table 3
Reproducibility and mean recovery values for egg samples spiked with 5, 10 or 20 ng/g monensin (Mon), salinomycin (Sal) or narasin (Nar) (five replicates were analysed on each of three days)

Added	5 ng/g			10 ng/g			20 ng/g		
	Mon	Sal	Nar	Mon	Sal	Nar	Mon	Sal	Nar
Day 1									
Mean (ng/g)	5.4	4.8	4.1	10.9	8.8	8.2	22.2	19.0	19.1
S.D.	0.57	0.28	0.46	1.29	0.85	0.51	2.63	1.37	1.41
C.V. (%)	10.6	5.8	11.2	11.8	9.6	6.2	11.8	7.2	7.4
Recovery (%)	107	97	80	109	88	82	111	95	95
Day 2									
Mean (ng/g)	5.8	4.9	4.3	10.7	9.3	9.2	21.4	19.3	18.5
S.D.	0.40	0.28	0.26	0.43	0.60	0.61	0.86	0.71	0.90
C.V. (%)	6.9	5.7	6.1	4.0	6.4	6.6	4.0	3.7	4.9
Recovery (%)	117	98	85	107	93	92	107	96	93
Day 3									
Mean (ng/g)	5.2	4.9	4.2	10.9	10.2	9.5	22.6	17.9	18.5
S.D.	0.69	0.38	0.35	0.52	0.47	0.35	1.44	1.11	0.70
C.V. (%)	13.3	7.2	8.3	4.8	4.6	3.7	6.4	6.2	3.8
Recovery (%)	104	97	84	109	102	95	113	90	92
Overall									
Mean (ng/g)	5.5	4.9	4.2	10.9	9.5	9.0	22.1	18.7	18.7
S.D.	0.57	0.28	0.35	0.79	0.86	9.0	1.67	1.17	1.00
C.V. (%)	10.4	5.7	8.3	7.2	9.1	9.0	7.6	6.3	5.3
Recovery (%)	110	98	84	109	95	90	110	93	93

about every 100 sample analyses maintained good sensitivity and the position of the electrospray probe could easily be optimised using one of the solvent ions from the mobile phase. The addition of 0.1% (v/v) trifluoroacetic acid to the mobile phase maintained a low pH suitable for positive-ion EMS without causing significant ion-suppression effects and the addition of 10% (v/v) methanol increased the signal intensity by a factor of about 2. Tetrahydrofuran was included in the mobile phase to improve peak symmetry and prevent tailing. A flow-rate of 1 ml/min was used for the HPLC system and although all the effluent from column could be directed into the Megaflow probe of the EMS, it was found beneficial to split the flow using a low-volume T-piece so that only about 200 μl/min entered the source. This gave improved sensitivity, less contamination of the source and decreased ion-suppression effects. In practice it was therefore possible to achieve typical detection limits of 0.5–1 ng/g for each of the ionophores in tissue or eggs at a signal-to-noise ratio of 3:1. The limit of determination (i.e. the level at which the method can reliably quantitate the data, with a C.V. <30%) was determined as 2 ng/g for each of the ionophores.

4. Conclusions

The described assay offers a number of significant advantages over previously published methods for the detection and quantitation of monensin, salinomycin and narasin in tissues or eggs of domestic fowl. The detection limit of less than 1 ng/g approaches that of the ELISA assay [3] (0.2 ng/g), is an order of magnitude better than the most sensitive chemical assay using ADAM as fluorometric derivatising reagent and 25 times better than methods using vanillin. In addition, ADAM is relatively unstable and must be freshly synthesised at regular intervals (30 days). The extraction procedure

used for the fluorescence method is also more complex, requiring column chromatography as well as several liquid–liquid extraction steps. In contrast, the LC–EMS method requires only a simple clean-up procedure and no derivatisation. It also uses mass-related detection, recommended for confirmatory assays, with higher specificity than UV or fluorescence detection methods. The assay is therefore suitable for use both in confirmatory residue testing programmes and in the diagnosis of poisoning associated with the incorrect use of these ionophores. It also demonstrates the usefulness of LC–EMS as a routine tool for residue analyses, particularly for compounds which are difficult to measure by other means.

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