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Determination of ionophore coccidiostats in feeding stuffs by liquid chromatography–tandem mass spectrometry. Part II. Application to cross-contamination levels and non-targeted feed

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ABSTRACT

A fit to purpose multi-analyte method for the official control of six coccidiostats (monensin sodium, salinomycin sodium, narasin, lasalocid sodium, semduramicin sodium and maduramicin ammonium alpha) at cross-contamination concentration levels in poultry, cattle, pig and calf compound feed by liquid chromatography tandem mass spectrometry (LC–MS/MS) has been developed and in-house validated. The corresponding maximum levels have been recently introduced by European legislation. The method developed involved a simple extraction of the coccidiostats from the feed samples followed by centrifugation and filtration of the supernatants for all matrices. For calf feed an additional de-fattening step of the filtrated supernatants with n-hexane was necessary. The resulting supernatants were submitted to chromatographic analysis. The analytes were quantified by a modified approach of the standard additions technique applied to the extracts, hence allowing a workload comparable to matrix-matched standard calibration curves. A further simplification of this technique was reached by applying the same addition levels of the target analytes for different concentration ranging from 0.5× maximum level up to 2.5× maximum level (universal approach). The concentration independent intermediate precision expressed in terms of relative standard deviation varied between 3 and 12% (except for maduramicin ammonium alpha and semduramicin sodium up to 21%) and the recovery rates ranged from 80 to 111%, depending on the target analyte and matrix. The limits of detection (LOD) and limits of quantification (LOQ) were different for the various analyte/matrix/instrument combinations but all LOQs were in the 0.01–0.65 mg kg^{-1} range, hence well below the target concentrations of each analyte. Based on the obtained method performance characteristics the method is considered fit for the intended purpose.

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1. Introduction

Within the European Union feed additives are authorised according to Regulation (EC) No 1831/2003 [1] requiring various criteria to be fulfilled including the need of providing suitable methods of analysis for official control in feedingstuffs. Coccidiosis is a major disease in poultry as well as in many other hosts. Coccidiostats are the only anti-bacterial substances still authorised as feed additives [1,2] and constitute the main choice to fight against coccidiosis. The conditions of use are given in the respective Commission Regulations authorising the feed additive, specifying individually for each additive important aspects such as the target animal, the inclusion level of the active substance in the feed and – in the case of cocciodiostats – the duration of the period before slaughter (withdrawal period) when the use of these substances is prohibited.

Analytical methods for the determination of coccidiostats exist but they are either not sensitive enough for low level detection or single-analyte methods [3,4], or targeting at maximum four coccidostats [5–7], or require a derivatisation step [8], or focus on food [9–16], or environmental matrices [17–19]. To our knowledge, the only method published for determination of coccidiostats in feed with mass spectrometry detection was a MALDI-TOF-MS method targeting four coccidiostats in poultry feeds [20]. A significant improvement of the state of the art was recently achieved with the development and validation of a reliable high performance liquid chromatography (HPLC)–tandem mass spectrometry method allowing the simultaneous determination of the six authorised ionophore coccidiostats (monensin sodium, salinomycin sodium, narasin, lasalocid sodium, semduramicin sodium and maduramicin ammonium alpha) [21] at authorised level in target matrices. This method constitutes a valuable tool in the frame of official control.

However, it is well known that during the production of feed containing coccdiostats as feed additives, unavoidable carry-over of the coccidiostats from target feed to non-target feed occur when the same production lines are used. A too high concentration of coccid-

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iostats in non-target feed would harm non-target animal species. Additionally, as all anti-bacterial substances, coccidiostats may be a risk for human health because the presence of their residues in foodstuffs could cause toxic effects, directly in sensitive individuals and also indirectly because their widespread usage could be responsible for the promotion of resistant strains of bacteria. Recently, the EU legislation therefore addressed both concerns and established maximum limits for the unavoidable carry-over of coccidiostats and histomonostats [22].

The objective of this work was therefore to optimise our previous analytical methodology [21] and to validate it in order to answer the clear need for reliable analytical methods for the determination of ionophore coccidiostats at trace level in feed, thus allowing for enforcement of the maximum levels by chemical analysis. Liquid chromatography tandem mass spectrometry (LC–MS/MS) was used after a simpler sample preparation for the analysis. The method was developed and validated in four different feed matrices containing the target coccidiostats at concentrations varying from 0.5% to 6% of the authorised dose of the coccidiostats depending on the tested matrix.

2. Experimental

2.1. Reagents and solvents

All chemicals and solvents used were of analytical purity and suitable for HPLC.

Semduramicin sodium (SEM) was obtained from Phibro Animal Health (Fairfield, NJ, USA), maduramicin ammonium alpha (MAD) from Alpharma (Willow Island, USA), monensin sodium (MON), salinomycin sodium (SAL), narasin A (NAR A), lasalocid sodium (LAS) and nigericin (NIG) (used as internal standard for the LC–MS/MS measurement) were obtained from Sigma–Aldrich (St. Louis, MO, USA).

Acetonitrile HPLC grade (ACN), methyl isobutyl ketone (MIBK), methanol HPLC grade (MeOH) and n-hexane GC grade were from Merck Sciences (Darmstadt, Germany). Formic acid 98% was obtained from Fluka Chemie (Steinheim, Germany). Pure water (H_2O) (18.2 MΩ cm^{-1} quality) used for the preparation of all the aqueous solutions was obtained from a MilliQ Plus 185 System (Millipore, Molsheim, F).

2.2. Standard solutions and test samples

2.2.1. Standard solutions

A standard mixture solution containing narasin A, salinomycin sodium, monensin sodium, lasalocid sodium, semduramicin sodium and maduramicin ammonium alpha at 19 μg ml^{-1}, 19 μg ml^{-1}, 45 μg ml^{-1}, 15 μg ml^{-1}, 9 μg ml^{-1} and 2 μg ml^{-1} respectively, was prepared in acetonitrile and used for the spiking of the blank feed samples as described in Section 2.2.2 and to perform the standard additions as described in Section 2.4. A 10 μg ml^{-1} standard solution was also prepared for the internal standard nigericin in acetonitrile.

2.2.2. Test samples

The test materials obtained from the European FP5 project SIMBAG-FEED [23] were compound feeding stuffs for cattle, poultry, pig and calf containing typical ingredients using a realistic recipe. Prior to use, the absence of the target analytes was confirmed by chemical analysis. The test samples were prepared by spiking the respective blank feed sample with the mixture of the 6 coccidiostats dissolved in acetonitrile, in order to obtain the target concentrations of the analytes in feed at three different concentration levels, namely the target cross contamination level (level C2), 1/2C2 (level C1) and 2C2 (level C3) (see Table 1). The fortified samples were vortexed in order to distribute the analytes more homogeneously into the feed and left for 1 h to ensure a satisfactory penetration of the target ionophore coccidiostats into the feed matrix. The samples were prepared in triplicates for each matrix and for each concentration level.

2.3. LC–MS/MS conditions

All chromatographic and mass spectrometric measurements were performed using two different LC–MS/MS systems, with the aim of ensuring the transferability of the developed method. The first instrument used was a HPLC Waters Alliance 2690 quaternary solvent delivery system (Waters Corporation, Milford, MA, USA) coupled to a Quattro LC triple stage quadrupole mass spectrometer (Micromass Co., Manchester, UK) while the second was a ultra-HPLC Accela quaternary solvent delivery system coupled to a Quantum Ultra triple stage quadrupole mass spectrometer (Thermo Fisher Scientific Co., San Jose, USA).

2.3.1. Chromatography

The chromatographic separation conditions of the ionophore coccidiostats were almost identical to those used in our previous work [21] in both LC–MS/MS systems except for the injection volume which was 25 μl instead of 40 μl when the Quantum Ultra triple stage quadrupole mass spectrometer was used. The column used was a reverse phase Nucleosil® C18 (250 mm × 4.6 mm, 5 μm particle diameter) column from Alltech Associate Inc. (Lokeren, Belgium) equipped with a Nucleosil® C18 guard column 7.5 mm × 4.6 mm, 5 μm particle diameter from Alltech Associate Inc. (Lokeren, Belgium) and the separation was performed in isocratic conditions. The mobile phase was composed of a 94:6 (v/v) mixture of MeOH containing 0.1% formic acid and H_2O containing 0.1% formic acid. The chromatographic flow rate was 1.0 ml min^{-1} and a T-piece splitter was used between the chromatographic column and the mass spectrometer in order to only introduce 0.25 ml min^{-1} of effluent into the ion source. The column temper-

Table 1
Concentrations analysed for the different coccidiostats in pig, poultry, cattle and calf compound feed. C2 is the cross-contamination level defined as being the unavoidable carry-over concentration from target to non-target feed; C1 = 1/2C2, C3 = 2C2.

	Concentrations of coccidiostats analysed in each compound feed, in mg of active substance kg^{-1} feed											
	Poultry feed			Pig feed			Cattle feed			Calf feed		
	C1	C2	C3	C1	C2	C3	C1	C2	C3	C1	C2	C3
Nar	0.25	0.50	1.00	0.75	1.50	3.00	0.75	1.50	3.00	0.75	1.50	3.00
Las	0.63	1.25	2.50	1.88	3.75	7.50	0.63	1.25	2.50	0.63	1.25	2.50
Sem	0.13	0.25	0.50	0.38	0.75	1.50	0.38	0.75	1.50	0.38	0.75	1.50
Mad	0.03	0.05	0.10	0.08	0.15	0.30	0.08	0.15	0.30	0.08	0.15	0.30
Sal	0.25	0.50	1.00	0.75	1.50	3.00	0.75	1.50	3.00	0.75	1.50	3.00
Mon	0.60	1.20	2.40	1.80	3.60	7.20	0.60	1.20	2.40	1.80	3.60	7.20

Nar, narasin A; Las, lasalocid A sodium; Sem, semduramicin sodium; Mad, maduramicin ammonium alpha; Sal, salinomycin sodium; Mon, monensin A sodium.

Table 2
MS parameters for the MRM ESI positive mode acquisition for the target six ionophore coccidiostats and nigericin (internal standard).

Analyte	Precursor ion (m/z)	LC Quattro II			Thermo Quantum Ultra		
		Product ions (m/z)	Cone voltage (V)	Collision energy (eV)	Product ions (m/z)	Tube lens offset (V)	Collision energy (eV)
Sem	895.5	833.3 851.3	45	40 40	833.3 851.3	169	27 30
Mon	693.4	461.3 479.3	60	55 55	461.1 479.2	140	49 49
Las	613.3	377.3 359.3	50	40 40	377.1 358.9	135	34 34
Mad	939.5	877.3 719.3	35	40 60	877.3 719.1	165	28 64
Sal	773.5	430.9 531.0	60	50 45	430.9 531.0	157	46 40
Nig (IS)	747.5	729.3 703.3	55	45 55	703.3 729.3	144	52 38
Nar	787.5	431.3 531.3	65	50 45	431.0 530.9	177	45 39

Sem, semduramicin sodium; Mon, monensin A sodium; Las, lasalocid A sodium; Mad, maduramicin sodium; Sal, salinomycin sodium; Nar, narasin A sodium; Nig, nigericin sodium (IS).

ature was 25 °C and the sample temperature was kept at 4 °C by means of the thermostated carroussel of the LC autosampler.

2.3.2. *Mass spectrometry*

For the detection, the positive electrospray ionisation mode (ESI+) was used and the ions were monitored in the multiple reaction monitoring (MRM) mode.

For the Quattro LC triple stage quadrupole mass spectrometer, the conditions were identical to the ones used in our previous work [21].

For the Thermo Fisher LC–MS/MS instrument, the mass spectrometrical conditions were optimised using 5 μg ml^{-1} individual standard solutions of the coccidiostats in acetonitrile. For the tuning, automatic loop injection mode was used by direct injection of 5 μl of the individual standard solutions into the heated electrospray (H-ESI) ionization source, using a methanol:water (94:6, v/v) mixture containing 0.1% of formic acid at a flow rate of 0.25 ml min^{-1}. The electrospray voltage was set at +3.7 kV, the ion tube temperature was 350 °C. Nitrogen was used as sheat and auxiliary gas at pressures of 50 and 35 arbitrary units, respectively. Skimmer and capillary offsets were 10 and 35 V, respectively. The sweep gas was not used and heating of the H-ESI probe was disabled. Collision of precursor ions was performed by argon at a pressure of −1.5 m Torr. The scan (dwell) time was set at 50 ms for each transition and the acquisition was carried out at unit mass resolution. Four points were taken into account for the identification, earned by the parent ion and two fragment ions. Since the MRM mode was chosen in this method, two transitions were thus followed for confirmation of the identity of the analysed compound. The transitions for each analyte as well as the MS parameters are displayed in Table 2.

2.4. *Analytical procedures*

All target analytes were quantified applying the standard additions technique, in order to compensate for adverse effects of unspecific matrix components on the quantification of the target analytes by mass spectrometry. Therefore known amounts of the target analytes were added to *aliquots* of the extracts *prior* to the LC–MS/MS measurements.

2.4.1. *Sample preparation*

The protocol includes two solvents that can be alternatively used for the extraction of the samples, namely ACN and MIBK.

2.4.1.1. *Poultry, cattle and pig feed samples.* For the extraction, 5 g of fortified test sample were suspended in 40 ml of the extracting solvent (ACN or MIBK) and left in an ultrasonic bath (Branson 5510E-DTH, USA) for 0.5 h. The sample was afterwards transferred to a head-to-head agitation device (GFL 3018, Gesellschaft für Labortechnick mbH, Burgwedel, Germany) for 1 h. After 10 min of centrifugation of the sample at 1850 × *g* (Sigma 2-16KC, Germany), all the supernatant was filtered through a 0.2 μm Acrodisc® PSF syringe Nylon filter (Pall Europe Limited, UK). Finally, seven 2 ml-aliquots of supernatant per sample were taken for standard additions and labelled as S0a, S0b indicating aliquots of the sample extracts as such, and S1, S2, S3, S4 and S5 indicating aliquots of the extracts after addition of the target analytes at different concentrations. The target added concentrations for the latter solutions were 0.5C2, C2, 1.5C2, 2C2 and 2.5C2 respectively.

MIBK as extraction solvent. After the extraction step the seven aliquots were evaporated until dryness and afterwards fortified individually with 20 μl of the internal standard solution (Nigericin at 10 μg ml^{-1}). The fractions S1–S5 were additionally fortified with the appropriate volumes of the target analytes. All fractions S0–S5 were subsequently re-dissolved and made up to a final volume of 2 ml with acetonitrile. The homogenisation of the fractions was achieved through vortexing, sonicating and centrifuging at 1850 × *g*. Finally 800 μl of acetonitrile were added to 200 μl of the obtained final supernatants, hence applying a five-fold dilution, prior to LC–MS/MS analysis.

ACN as extraction solvent. To each of the 2 ml-aliquots solutions of the target analytes at the required increasing concentrations and the internal standard were added. The final five-fold dilution of samples prior to LC–MS/MS analysis was then ensured with acetonitrile.

2.4.1.2. *Calf feed samples.* Given the high fat content of calf feed samples, a de-fattening step with n-hexane was added after the extraction with MIBK or ACN and performed on the S0a, S0b, S1, S2, S3, S4 and S5 aliquots. The additional step consisted of adding 0.5 ml of n-hexane to each of the aliquots and vortexing for 30 s. The aliquots were then centrifuged for 10 min at 1850 × *g* and 200 μl from the lower layer (acetonitrile) were selectively taken for evap-

oration to dryness. Then the dry residue was dissolved in 1 ml of acetonitrile and was ready for LC–MS/MS analysis.

2.5. Method validation

Since there is no validation guideline specifically designed for the analysis of feedingstuffs, various internationally recognised guidelines [24,25] for single-laboratory validation were taken into account including Commission Decision 657/2002 [26]. The latter document is related to methods for the analysis of specific compounds in food matrices from animal origin. The validation of the method included the estimation of the limit of detection (LOD), limit of quantification (LOQ), precision under repeatability and intermediate conditions and the trueness. The concentration range included in this validation study was from 0.5% to 6% of the target authorisation level of the analytes, depending on the legal limits specified in European legislation [22]. The single-laboratory validation was first performed on poultry, pig, cattle and calf compound feeds using ACN as extraction solvent. However, due to the worldwide shortage of ACN, MIBK was tested as an alternative solvent and the single-laboratory validation was also performed with this extraction solvent on two non-target compound feeds, namely pig and calf feeds.

3. Results and discussion

3.1. Development/optimisation of the LC–MS/MS method

3.1.1. Sample preparation

The sample preparation differed from our previous work by mainly two key aspects. First, it was shown that the sample preparation could be simplified by removing the clean-up step on solid phase extraction cartridges. This simplification certainly led to an improvement of the recovery of the analytes.

A second major modification consisted of selecting exclusively the standard additions approach in order to cope with the adverse effects of matrix components on the quantification of the target analytes. Indeed, in our previous work [21], the two approaches, namely matrix-matched standard calibration curves and standard additions were successfully tested and validated. Calibration against matrix matched standards was excluded in this study due to the fact that identical blank feedingstuffs samples required to implement this approach are rarely available to official control laboratories. Also dilution of the sample extract into pure solvent may reduce matrix effects, but was not applicable in this study due to the low target concentration of the analytes.

When applying the classical standard additions technique, the analyte is added prior to the extraction of the samples, thereby leading to a significant increase of the work connected to anal-

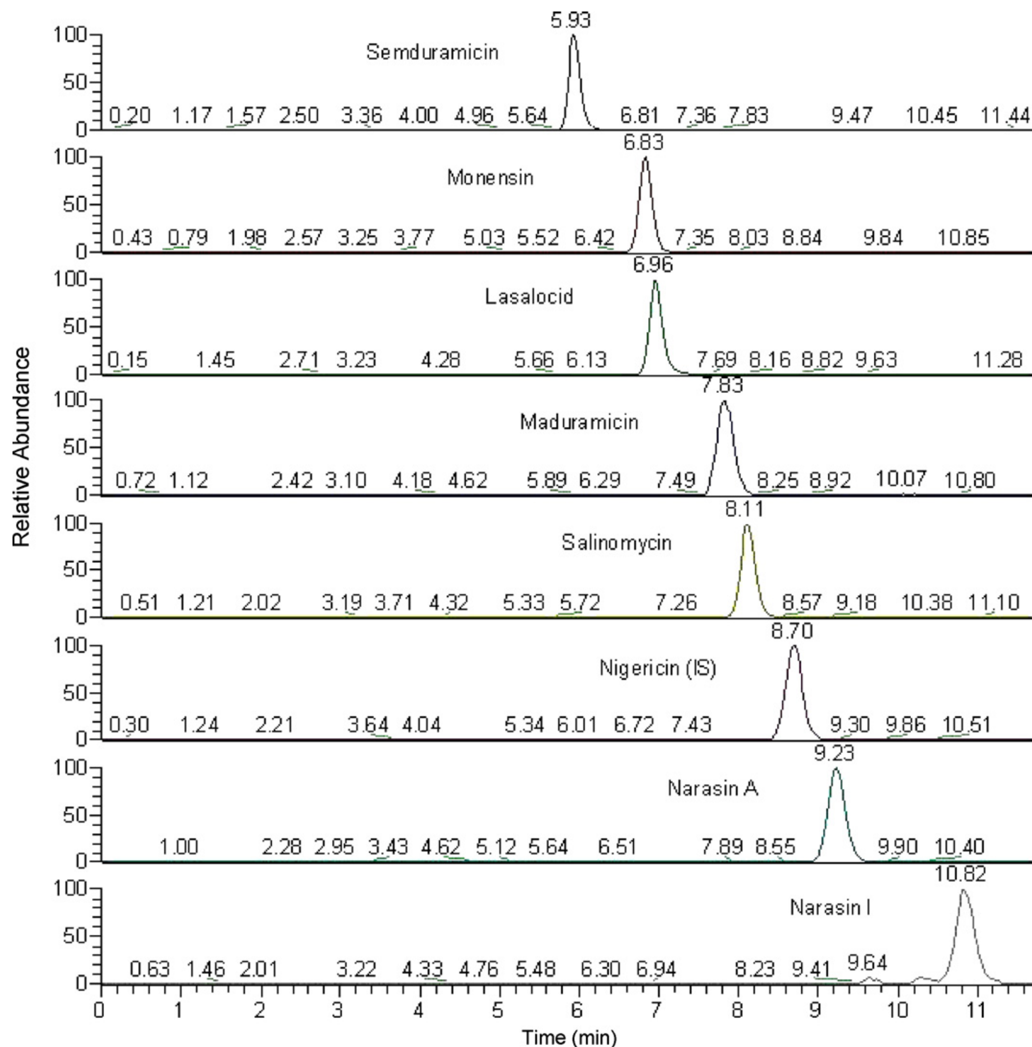

Fig. 1. LC–MS/MS separation of six ionophore coccidiostats in the S0 aliquot of a cattle feed sample containing the analytes at a concentration of C2 mg kg^{-1}. Narasin I is a minor form in Narasin and can also be separated by this method.

Table 3
Limits of detection (LODs) and of quantification (LOQs) for the six ionophore coccidiostats in poultry, pig, cattle and calf feed.

	Poultry feed		Pig feed		Cattle feed		Calf feed	
	LOD ($mg\,kg^{-1}$)	LOQ ($mg\,kg^{-1}$)	LOD ($mg\,kg^{-1}$)	LOQ ($mg\,kg^{-1}$)	LOD ($mg\,kg^{-1}$)	LOQ ($mg\,kg^{-1}$)	LOD ($mg\,kg^{-1}$)	LOQ ($mg\,kg^{-1}$)
Mad	0.002	0.01	0.01	0.04	0.01	0.03	0.01	0.03
Sem	0.01	0.04	0.05	0.14	0.03	0.10	0.03	0.09
Nar	0.01	0.05	0.04	0.14	0.01	0.08	0.02	0.09
Sal	0.01	0.05	0.03	0.17	0.01	0.08	0.02	0.10
Las	0.03	0.14	0.06	0.65	0.01	0.10	0.02	0.15
Mon	0.03	0.12	0.04	0.43	0.01	0.10	0.05	0.29

Note: The LOD is given by $((y_{S0}+3s)-a)/b$ and LOQ by$((y_{S0}+10s)-a)/b$ for each analyte, where y_{S0} is the area obtained for the S0 aliquot, s corresponds to the standard deviation of the injections of S0, a and b are respectively the slope and the intercept of the standard addition regression line. Mad, maduramicin ammonium alpha; Sem, semduramicin sodium; Nar, narasin A; Sal, salinomycin sodium; Las: lasalocid A sodium; Mon, monensin A sodium.

ysis of the samples. Since this drawback is often considered as impediment to use this technique, we proposed an approach in which the standard additions are performed after extraction of the original unknown sample. This was considered possible, since the major problems of the quantification of the analyte in this study are related to the ionisation in the MS, whereas problems related to the extraction of the analyte are shown to be minor. With this approach, the workload is significantly reduced and it leads to a matrix-matched calibration curve in each single matrix and per sample.

The other major development is the set up of the so-called universal approach for standard additions. Indeed, classically, the analysis of an unknown sample starts with a pre-assessment of the concentration of the target analytes in order to set the appropriate standard additions. In practice this approach would lead again to a very cumbersome methodology if it would be applied to all unknown samples to be analysed in routine. In this work, we demonstrated that over the complete concentration range, i.e. from half of the C2 to 2C2 and for each of the target analytes, the standard additions can be identical and following a so-called "universal" scheme 0.5C2, C2, 1.5C2, 2C2 and 2.5C2 independently of the actual concentration of the target analytes.

The performance of the standard additions technique was therefore evaluated at three concentration levels of the target analytes, namely C1, C2 and C3 (Table 1). For each analyte, the peak area ratios analyte (y) to internal standard of all aliquots S0a, S0b, S1, S2, S3, S4 and S5 were plotted versus the added concentrations (x) of the corresponding target analyte 0, 0.5C2, C2, 1.5C2, 2C2 and 2.5C2. Finally the regression line is extrapolated to $y=0$, obtaining the intercept with the x-axis indicating the actual initial concentration.

3.1.2. LC–MS/MS conditions

For the detection the electrospray positive ionisation mode (ESI+) was used and the ions were monitored in the multiple reaction monitoring (MRM) mode as previously done [21]. However, in this work, all conditions were also adapted to the use of a second instrument.

Four points were taken into account for the identification, earned by the parent ion and two fragment ions. Two transitions were thus followed for confirmation of the identity of the analysed compound while for quantification, only transition 1 (Table 1) was used.

A chromatogram of the LC–MS/MS analysis of a cattle feed sample at cross-contamination level C2 performed on the Thermo Fisher instrument is shown in Fig. 1.

Table 4
Results from the statistical evaluation for the target coccidiostats in poultry and cattle feed at the three analysed concentrations C1, C2 and C3..

	Poultry feed					Cattle feed				
	Mean value ($mg\,kg^{-1}$)	RR%	RSD_r %	$RSD_{Int.}$ (%)	H(r)	Mean value ($mg\,kg^{-1}$)	RR%	RSD_r %	$RSD_{Int.}$ (%)	H(r)
Mad	0.03	94	4.2	4.2	0.2	0.07	93	8.6	12.9	0.5
	0.05	96	10.4	14.6	0.6	0.15	98	6.1	6.8	0.3
	0.09	92	2.2	11.9	0.5	0.30	100	3.3	3.3	0.2
Sem	0.13	91	1.8	7.0	0.4	0.12	94	12.8	15.3	0.7
	0.24	95	15.1	18.9	1.0	0.36	95	3.5	6.7	0.4
	0.45	90	2.9	12.5	0.7	1.48	99	3.6	3.6	0.2
Nar	0.25	100	3.3	4.4	0.3	0.77	102	3.9	4.3	0.2
	0.49	99	2.0	2.8	0.2	1.48	98	2.3	2.4	0.2
	0.98	98	1.7	4.7	0.3	2.96	99	2.7	2.9	0.2
Sal	0.28	101	2.1	2.9	0.2	0.77	102	4.3	4.3	0.2
	0.49	99	2.6	2.6	0.2	1.49	99	2.1	2.5	0.2
	0.98	98	2.0	4.4	0.3	2.98	99	1.7	2.6	0.2
Las	0.68	108	3.4	3.4	0.2	0.65	104	3.4	5.8	0.3
	1.26	101	3.5	5.6	0.4	1.36	109	3.2	3.2	0.2
	2.61	104	3.2	8.2	0.6	3.00	120	5.9	6.2	0.5
Mon	0.62	104	2.4	3.1	0.2	0.62	103	4.0	4.0	0.2
	1.22	102	2.7	3.4	0.2	1.23	102	3.3	4.2	0.3
	2.39	100	2.6	3.4	0.2	2.62	109	3.0	3.3	0.3

Mad, maduramicin ammonium alpha; Sem, semduramicin sodium; Nar, narasin A; Sal, Salinomycin sodium; Las, lasalocid A sodium; Mon, monensin A sodium. RSD_r (%): relative standard deviation for repeatability; $RSD_{Int.}$ (%): relative standard deviation for intermediate precision. The mean value is calculated from the 54 results obtained for each analyte/concentration/matrix combination. RR (%), mean percentage recovery rate; H(r): Horrat value (r); extraction solvent, ACN.

Table 5
Results from the statistical evaluation for the target coccidiostats in pig and calf feed at the three analysed concentrations C1, C2 and C3.

	Pig feed					Calf feed				
	Mean value (mg kg^{-1})	RR%	RSD_r %	$RSD_{Int.}$ (%)	H(r)	Mean value (mg kg^{-1})	RR%	RSD_r %	$RSD_{Int.}$ (%)	H(r)
(a) Extraction solvent: ACN										
	0.08	103	10.3	16.8	0.7	0.07	90	4.4	5.9	0.3
Mad	0.16	106	6.9	11.4	0.5	0.14	96	2.1	4.2	0.2
	0.29	96	5.9	5.9	0.3	0.29	97	5.5	5.9	0.3
	0.36	95	10.4	20.9	1.1	0.37	97	8.8	10.9	0.6
Sem	0.75	101	9.0	10.5	0.6	0.71	95	2.7	2.9	0.2
	1.41	94	5. 9	5.9	0.4	1.43	95	4.8	4.8	0.3
	0.76	102	9.4	9.4	0.6	0.74	99	1.9	4.9	0.3
Nar	1.44	96	4.6	4.7	0.3	1.44	96	1.5	1.5	0.1
	2.93	98	3.8	4.9	0.4	2.83	94	4.3	4.3	0.3
	0.79	106	8.5	8.5	0.5	0.75	99	2.4	4.8	0.3
Sal	1.49	100	3.0	4.0	0.3	1.45	97	1.0	1.0	0.1
	2.96	99	2.7	3.0	0.2	2.86	95	4.0	4.0	0.3
	2.18	116	7.5	10.3	0.7	0.67	107	4.3	6.4	0.4
Las	4.00	107	4.5	11.3	0.9	1.34	107	2.6	4.4	0.3
	8.18	109	4.5	8.2	0.7	2.69	108	8.0	8.0	0.6
	2.06	114	7.7	9.7	0.7	1.90	105	4.8	5.8	0.4
Mon	3.79	105	3.0	3.0	0.2	3.66	102	2.8	2.8	0.2
	7.32	102	2.8	4.0	0.3	7.06	98	7.2	7.2	0.5
(b) Extraction solvent: MIBK										
	0.08	102	6.6	11.8	0.5	0.07	89	6.0	7.5	0.3
Mad	0.15	100	5.4	22.1	1.0	0.13	88	4.5	4.5	0.2
	0.27	89	6.4	8.3	0.4	0.29	95	10.9	10.9	0.6
	0.32	86	5.9	7.8	0.4	0.37	98	5.7	6.5	0.4
Sem	0.61	81	2.6	30.0	1.7	0.71	95	5.1	5.6	0.3
	1.16	77	10.7	14.5	0.9	1.36	91	5.4	6.6	0.4
	0.73	97	14.6	14.6	0.9	0.78	104	6.7	6.7	0.4
Nar	1.43	96	2.4	5.3	0.4	1.52	101	3.0	3.0	0.2
	2.97	99	4.4	6.3	0.5	2.96	99	2.1	2.3	0.2
	0.77	103	3.1	5.0	0.3	0.78	103	4.3	4.3	0.3
Sal	1.44	96	3.8	7.2	0.5	1.52	101	1.7	1.7	0.1
	2.95	98	4.1	6.1	0.5	3.00	100	2.2	2.2	0.2
	1.41	75	4.7	7.0	0.5	0.70	112	5.4	12.0	0.7
Las	2.69	72	5.8	8.6	0.6	1.30	104	8.4	8.4	0.5
	6.94	93	3.9	3.9	0.3	2.77	111	5.8	5.8	0.4
	1.89	105	3.9	8.7	0.6	1.88	104	7.3	8.3	0.6
Mon	3.53	98	2.7	8.3	0.6	3.68	102	3.8	3.8	0.3
	8.11	113	3.0	5.3	0.5	7.52	104	3.8	3.8	0.3

Mad, maduramicin ammonium alpha; Sem, semduramicin sodium; Nar, narasin A; Sal, Salinomycin sodium; Las, lasalocid A sodium; Mon, monensin A sodium. RSD_r (%): relative standard deviation for repeatability; $RSD_{Int.}$ (%): relative standard deviation for intermediate precision. The mean value is calculated from the 54 results obtained for each analyte/concentration/matrix combination. RR (%), mean percentage recovery rate; H(r): Horrat value (r).

3.2. Single-laboratory validation

3.2.1. Selectivity

All blank materials used were analysed following the optimised procedure and were shown not to contain any of the target ionophore coccidiostats or any interfering analyte.

3.2.2. Sensitivity

The limits of detection (LODs) and limits of quantification (LOQs) were determined using the standard additions regression lines established at half the lowest cross-contamination level of the target analytes in feed. In detail, three samples of each type of feed were individually fortified at a concentration of half the established cross-contamination level of the target analytes and subsequently subjected to the whole analytical procedure. Based on these measurements regression lines for each analyte were established which were then utilised to calculate the LODs and LOQs (Table 3) [27]. As shown in Table 3 all LOQs ranged from 0.01 to 0.65 mg kg^{-1} and were therefore well below the target C2 concentration for the respective analyte, demonstrating that the developed method is sensitive enough for the intended purpose.

3.2.3. Precision

The precision of the method was determined according to ISO standard 5725-3 [24] under different circumstances, namely repeatability conditions where the experiments were carried out on the same day and intermediate conditions where the experiments were distributed over different days. All experiments for one given compound feed type were conducted by the same operator using the same instrumentation. The experiments were carried out at three concentrations of the analyte in the feed, which were 0.5C2, C2 and 2C2. This exercise was applied for cattle, poultry, pig and calf feed, respectively. Fitness for purpose criteria for the precision were taken from Commission Decision 2002/657 [25], specifying that the intermediate precision values were considered acceptable when these value were not larger than the precision calculated by the Horwitz equation [28]. This condition was expressed by the HORRAT(r) value which is the ratio of the experimentally obtained

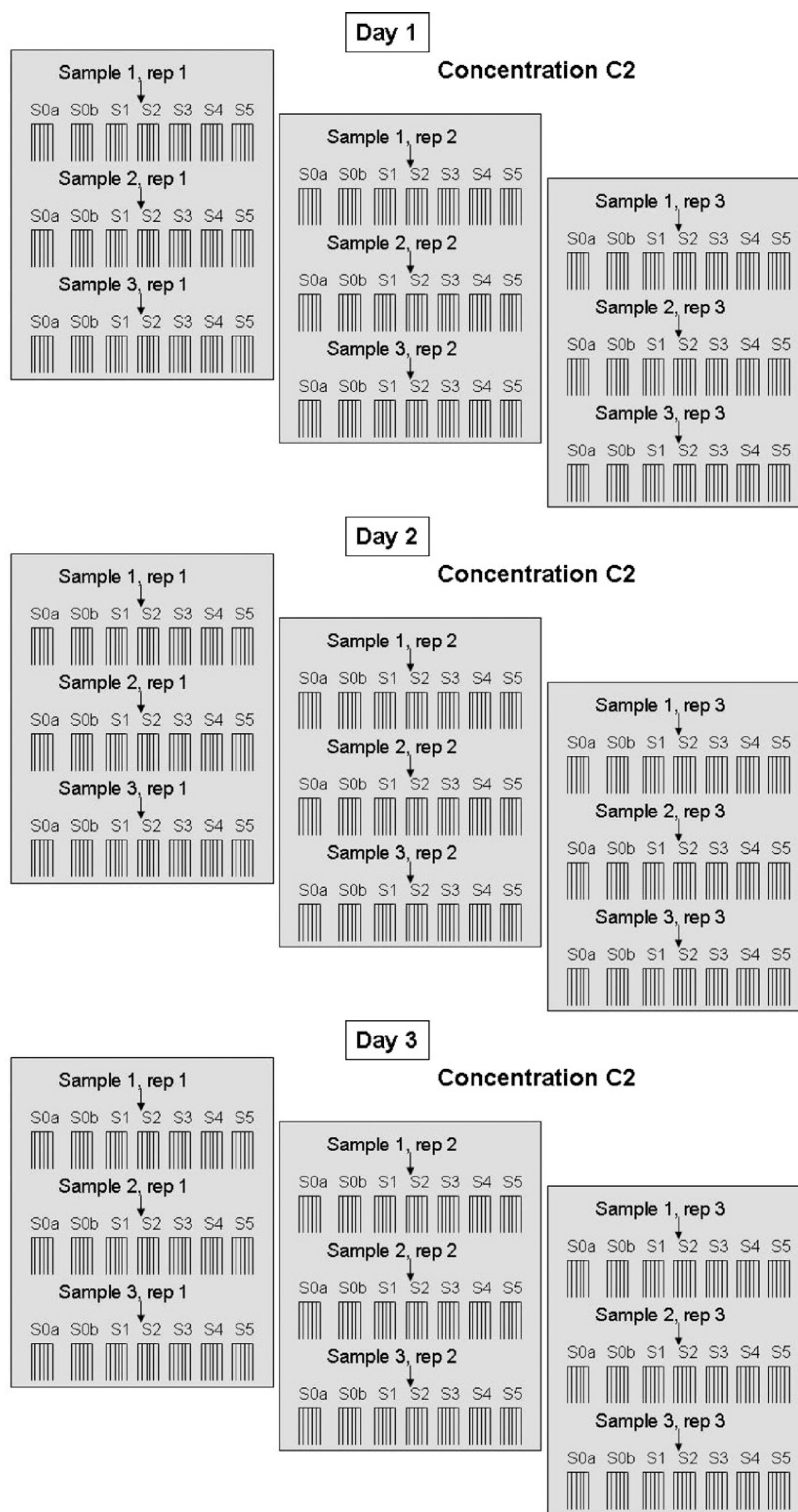

Fig. 2. Design of the experiments for calculating the precision and the trueness of the method. The scheme is identical for concentrations C1 and C3.

values of the standard deviation and the target standard deviation calculated by the Horwitz equation [28]. As a fitness for purpose criterion this value should be lower than 1.3. [29].

3.2.3.1. Statistical model. The experimental design is shown in Fig. 2. Nine samples containing the target analytes at the concentrations given in Table 1 for each compound feed type were distributed over 3 days, analysing three samples each day. Each aliquot was injected six times, obtaining 54 results for each analyte/concentration/matrix combination. Therefore, the experiment is a 3 factor-nested design as specified in the ISO standard [24]. The total variability of the analytical results can be attributed to three levels of variability as previously described in [21].

The between injection and between samples variability contribute to the precision under repeatability conditions, whereas

Table 6
Results from the statistical evaluation on the recovery rates pooled over the three concentrations C1, C2 and C3 for the six ionophore coccidiostats in the four tested feeds.

	Poultry feed		Pig feed		Cattle feed		Calf feed	
	RR (%)	$RSD_{Int.}$ (%)	RR (%)	$RSD_{Int.}$ (%)	RR (%)	$RSD_{Int.}$ (%)	RR (%)	$RSD_{Int.}$ (%)
(a) Extraction solvent: ACN								
Mad	94	13	101	13	96	6	96	6
Sem	93	16	96	14	94	6	94	6
Nar	100	4	99	7	98	4	98	4
Sal	100	4	101	6	99	4	99	4
Las	103	7	111	12	108	5	108	5
Mon	102	4	107	9	101	5	101	5

	Pig Feed		Calf Feed	
	RR (%)	$RSD_{Int.}$ (%)	RR (%)	$RSD_{Int.}$ (%)
(b) Extraction solvent: MIBK				
Mad	97	17	91	8
Sem	87	21	94	7
Nar	97	9	101	5
Sal	99	7	102	3
Las	80	12	109	11
Mon	105	11	104	7

Mad, maduramicin ammonium alpha; Sem, semduramicin sodium; Nar, narasin A; Sal, salinomycin sodium; Las, lasalocid A sodium; Mon, monensin A sodium. RR (%), mean percentage recovery rate of the recovery rates obtained on the three concentrations; $RSD_{Int.}$ (%), relative standard deviation for intermediate precision.

all components including the between days variability, give the intermediate precision.

The model that underlies the analysis of variance of the data collected by the nested design is that each of the measurement is defined as the sum of 3 variance components plus the true value (fixed quantity) [21].

For the calculation of the repeatability standard deviation and intermediate precision the analytical results were subjected to analysis of variance (ANOVA) using the software package MINITAB™ Statistical Software for Windows (version 15).

3.2.3.2. Results of the statistical assessment. The results of the statistical evaluation are shown in Table 4 for poultry and cattle feed, and in Table 5 for pig and calf feed respectively. The obtained relative standard deviation for repeatability varied from 1% to 15% (except for the less concentrated maduramicin ammonium alpha and semduramicin sodium in some feedingstuffs) and the relative standard deviation of intermediate precision ranged from 1% to 15% (except for the less concentrated maduramicin ammonium alpha and semduramicin sodium in some feedingstuffs), depending on the target analyte, concentration, extraction solvent and feedingstuff. However, in all cases the HORRAT(r) values were equal or below 1.3, thus indicating that the precision of the method fulfils the criteria.

3.2.3.3. Estimation of the precision of the method independent of the analyte concentration. As recommended by ISO standard 5725 [24] the data were also evaluated to establish whether a concentration independent precision of the method for each compound and matrix could be calculated. Plotting the absolute standard deviation of intermediate precision against the analyte concentration revealed a strong dependence, whereas the corresponding relative standard deviation was in general not much affected by the analyte concentration. Therefore all measured concentrations were first divided by the respective mean values given in Tables 4 and 5, in order to obtain normalised data. Since the normalised data do not depend anymore on the respective concentrations, they can be pooled to one data set per analyte and matrix. These data were subsequently subjected to statistical analysis. The obtained standard deviations of these normalised concentrations are identical to the *relative* standard deviations of the original measurements (i.e. absolute concentrations) and are shown in the aggregated Table 6. The statistical evaluation revealed satisfactory values for the intermediate precision that are independent of the concentration within the frame of this study.

3.2.4. Trueness estimated from the obtained recovery rates

The trueness of the method was calculated from the mean values of the results from all trials of the experimental design shown in Fig. 2 and expressed in terms of the recovery rate. The obtained values for the recovery rate are given in Table 4 for poultry and cattle feed, and in Table 5 for pig and calf feed. The values ranged from 88% to 120%, except for lasalocid sodium and semduramicin sodium in pig feed when MIBK was used as extracting solvent. In this latter case, the values ranged from 72% to 93% and 77% to 86% respectively. Given the complexity of the feed matrix, the low concentrations targeted and since the lower recovery rates were only obtained with the alternative solvent in one matrix, the obtained estimates were considered acceptable. Finally, since the recovery rates did not show dependence on the analyte concentration, recovery rates independent of the concentration were also calculated after normalisation of the data (Table 6). All recovery rates for all analytes were shown to be very satisfactory since the values ranged from 93% to 111% (with $RSD_{Int.}$ from 4% to 16%) in the four matrices when ACN was used as extracting solvent and from 80% to 109% ($RSD_{Int.}$ from 3% to 21%) when the extracting solvent was MIBK.

3.2.5. Comparison of the performance profile of the methods using ACN and MIBK

The statistical evaluation of the results obtained did not show any significant difference neither for the method precision (Table 5) nor for the trueness (Table 6) when one or the other extraction solvent was used. It can therefore be concluded that in both cases, the whole procedure is fit for the purpose and fulfils the set requirements.

4. Conclusion

A new LC–MS/MS method fit for the purpose of detection, confirmation and quantification of semduramicin sodium, maduramicin ammonium alpha, narasin, lasalocid sodium, salinomycin sodium and monensin sodium ionophore coccidiostats in poultry, pig, cattle and calf feed has been developed and single-laboratory validated. The quantification was carried out using a newly developed “universal” approach of the standard additions technique in order

to ensure the applicability of the methods in routine control of unknown materials for which blanks are not available, while limiting the workload usually linked to classical standard additions. The results of the validation study confirmed satisfactory values for the sensitivity, precision and trueness of the developed method for both instruments used. Therefore it was concluded that the developed method is fit for the purpose to be used in the frame of official control of the presence of the coccidiostats included in this study at trace level.

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