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Development and validation of a method for the determination of sub-additive levels of virginiamycin in compound animal feeds by liquid chromatography

Chris A. J. Hajee, Hans (J.) A. van Rhijn,* Johan J. P. Lasaroms, Henk J. Keukens† and Jacob de Jong

State Institute for Quality Control of Agricultural Products (RIKILT), Bornsesteeg 45, P.O. Box 230, 6700 AE Wageningen, The Netherlands. E-mail: j.a.vanrhijn@rikilt.wag-ur.nl

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A method for the detection of virginiamycin M1 as a marker compound of virginiamycin at sub-additive level in pig, calf, piglet, sow, poultry, cattle and laying hen feeds was developed and validated. Both UV detection at 230 nm and MS detection were applied. Virginiamycin M1 was extracted from animal feeds with ethyl acetate after wetting of the feed with water followed by clean-up on Sep-Pak silica gel and OASIS HLB cartridges. Analysis of extracts was carried out on an Inertsil ODS-2 column with acetonitrile–water–formic acid as the mobile phase and UV detection at 230 nm. The limit of quantification (LOQ) of the method was 2.7 mg kg^{-1}. The proposed method was validated at a target species dependent minimum required performance limit (MRPL), at 2MRPL and at 5MRPL levels in pig, calf, piglet, sow, poultry, cattle and laying hen feeds. Recoveries at target species dependent MRPL levels ranged from 38 to 67%, within-day repeatabilities from 7 to 19% and within-laboratory reproducibilities from 13 to 27%. The proposed UV method is primarily suitable for screening purposes at subadditive levels, but semi-quantitative data can also be produced. Three MS detection modes (ion-source CID, full MS and MS^2) were tested as an alternative and/or extension to UV detection. The selectivity and sensitivity of both LC-MS^2 and LC-MS were much better than those of UV detection at 230 nm.

Introduction

Virginiamycin is a cyclic polypeptide antibiotic produced by *Streptomyces virginiae* consisting of several compounds, some of which are structurally related.[1] The two main compounds in this complex mixture, commonly referred to as virginiamycin M1 and virginiamycin S1, exhibit a maximum synergistic antimicrobial effect at M1: S1 = 67 : 33.[2]

Until July 1999, the use of virginiamycin as a feed additive to improve feed conversion was allowed in the European Union (EU).[3] Additive levels ranged from 5 to 50 mg kg^{-1} microbiological activity (m.a.), depending on the target species of the feed. The official EU method for virginiamycin in feeding stuffs[4] is based on microbiological principles and has been developed for the control of virginiamycin additive levels. This method permits detection down to 2 mg kg^{-1} (m.a.) and the possibilities of distinguishing virginiamycin from other microbiologically active compounds are limited, notably if a feeding stuff contains combinations of antibiotics. Microbiology-based multi-analyte detection methods that include virginiamycin have also been described.[5–7] Additionally, instrumental methods for the specific determination of virginiamycin at additive levels in premixes and animal feeds have been described.[8,9] These methods are capable of determining both virginiamycin M1 and virginiamycin S1 and use the synergy curve to calculate the virginiamycin concentration (m.a.).

Since July 1999, the use of virginiamycin as a feed additive has been banned in the EU.[10] In order to allow adequate control of possible illegal use of virginimycin, improved methods of analysis, preferably instrumental, are required which are suitable for the determination of virginiamycin at sub-additive levels in feeding stuffs. No such methods were available at the date of the ban.

An instrumental method was developed that was aimed at adequate monitoring of the ban at sub-additive levels down to 1 mg kg^{-1} (m.a.). This was done within the framework of a project supported by the EU (contract No. SMT4-CT98-2216) entitled 'Development and validation of HPLC methods for the official control of coccidiostats and antibiotics used as feed additives' (acronym: CANFAS). Although more convenient detection techniques may be used, the use of LC-UV detection was considered mandatory to allow easy transfer of the method between laboratories of EU member states.

This paper describes the development and within-laboratory validation of an instrumental method for the detection of virginiamycin M1 as a marker compound for the presence of virginiamycin in pig, piglet, calf, sow, poultry, cattle and laying hen feeds. Additionally, results from preliminary tests with LC-MS detection are presented.

Experimental

Chemicals and reagents

All chemicals and solvents were of at least analytical-reagent grade and were purchased from local commercial suppliers. Acetonitrile, methanol, ethyl acetate, hexane, formic acid (100%), ammonium acetate and anhydrous sodium sulfate were obtained from Merck (Darmstadt, Germany). HPLC quality water (conductivity $< 10\ M\Omega\ cm^2$) was prepared with a Milli-Q water purification system (Millipore, St. Louis, MO, USA). Sep-Pak Classic silica gel, 690 mg, and OASIS HLB, 60 mg per 3 mL, solid phase extraction (SPE) cartridges were purchased from Waters (St. Louis, MO, USA).

† Present address: Laboratory of the National Inspection Service of Livestock and Meat, Bornsesteeg 41, P.O. Box 144, 6700 AC Wageningen, The Netherlands.

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Reference standard solutions

Pfizer (Pharmaceutical Group, Antibiotics plant, Rixensart, Belgium) kindly supplied a virginiamycin reference standard mixture, batch 1252/T/V1-921014. The reference standard mixture, containing 57.2% virginiamycin M1 and 19.2% virginiamycin S1, had a certified microbiological potency of 2220 μg mg^{-1}. Stock standard solutions [200 μg mL^{-1} (m.a.)] of the virginiamycin reference mix standard were prepared in methanol taking account of the purity and the certified microbiological potency of the reference standard material. A freshly prepared stock standard solution was ultrasonicated for 5 min prior to use. The stock standard solutions are stable for at least 1 month when stored in a refrigerator below 8 °C.

Diluted stock standard solutions [20 μg mL^{-1} (m.a.)] were prepared, by 10-fold dilution with methanol. Standard solutions in the range 0.5–20 μg mL^{-1} (m.a.) were prepared fresh each day by evaporation to dryness of appropriate volumes (diluted) of stock solution and reconstitution in the HPLC mobile phase. The standard solutions were mixed vigorously and ultrasonicated for 5 min. A 100 μL aliquot was used for HPLC analysis.

Animal feeds

All compound animal feeds were ground in a mill equipped with a 1 mm screen. The entire feed sample was mixed thoroughly after milling.

During the optimisation experiments, a standard pig feed proved by the official EU microbiological assay[4] to be virginiamycin free was used as a blank feed. A large series of feed samples collected from EU member states was available for validation purposes.

Feed was spiked with virginiamycin in the range 2.2–20 mg kg^{-1} (m.a.) by addition of an appropriate volume of virginiamycin stock standard solution to 5 g feed test portions.

Instrumentation

Chromatography was performed on an Inertsil 5 μm ODS-2 analytical column (200 × 3.0 mm id) (Chrompack, Middelburg, the Netherlands) protected by a pellicular RP-18 guard column (10 × 2.1 mm id) (Chrompack). The chromatographic system consisted of a Model 231-401 autosampler (Gilson, Middleton, WI, USA), a Model P580 HPLC pump (Gynkotek, Germering Munich, Germany), a Model 785A HPLC monitor (Applied Biosystems, Foster City, CA, USA), a Model 996 photodiode array (PDA) detector (Waters), a PE Nelson 900 Series interface for data acquisition (Perkin-Elmer, Foster City, CA, USA) and Turbochrom Client/Server data processing software (Perkin-Elmer) for integration of chromatographic peaks. The mobile phase, acetonitrile–water–formic acid (350 + 650 + 3 v/v/v), was pumped at a flow rate of 0.6 mL min^{-1} and UV detection at 230 nm was used.

An LCQ ion trap LC-MS^n system (Thermoquest, San Jose, CA, USA) equipped with an atmospheric pressure chemical ionisation (APCI) interface operated at standard settings was used to assess its ability to detect virginiamycin M1 at sub-additive levels in animal feed.

Sample preparation

Extraction. Approximately 5 g of compound animal feed are weighed accurately into a 50 mL polypropylene (PP) tube. Water (5 mL) is added and the tube is capped and vigorously shaken manually to disperse the water homogeneously into the feed. The water should be taken up completely by and distributed evenly in the feed (critical). Ethyl acetate (20 mL) is added and the tube is capped and vigorously shaken manually. The feed layer should be loosely distributed in the ethyl acetate layer (critical). The sample is ultrasonicated for 30 min in an ultrasonic bath. The tube is shaken manually twice (at 10 and 20 min after the initiation of ultrasonication) during this ultrasonication period. The tube is centrifuged at 3000*g* at ambient temperature for 10 min.

From the ethyl acetate supernatant, an aliquot (10 mL) is transferred into a clean 50 mL PP tube. Approximately 5 g of anhydrous sodium sulfate and 10 mL of hexane are added and the tube is capped and vigorously shaken manually. The tube is centrifuged at 3000*g* at ambient temperature for 10 min.

Sample purification. A 690 mg Sep-Pak silica gel SPE cartridge is placed on a vacuum manifold–SPE column processor and conditioned with 2.5 mL of anhydrous ethyl acetate–hexane (1 + 1 v/v, dried over anhydrous sodium sulfate). The clear extract supernatant is carefully decanted on to and passed through the conditioned Sep-Pak cartridge. Do not allow the cartridge to run dry. The solvent flow rate should not exceed 2 mL min^{-1}. The sodium sulfate pellet is rinsed by addition of 5 mL of anhydrous hexane–ethyl acetate (1 + 1 v/v) to the tube and mixing on a vortex mixer. The tube is centrifuged at 3000*g* at ambient temperature for 10 min. The rinse liquid is carefully decanted on to and passed through the conditioned Sep-Pak cartridge. After all the liquid has passed through the Sep-Pak silica cartridge, the latter is allowed to run dry (vacuum or pressure). The Sep-Pak cartridge is washed with 2 mL of acetonitrile, allowed to run dry and dried by application of full vacuum for 30 min.

A 60 mg OASIS HLB cartridge (3 mL) is placed on a vacuum manifold–SPE column processor and successively activated with 1 mL of methanol and conditioned with 1 mL of ammonium acetate buffer (pH 4). The dried Sep-Pak cartridge is placed on top of a conditioned OASIS HLB cartridge. The Sep-Pak cartridge is eluted with 5 mL of methanol–ammonium acetate buffer (pH 4) (1 + 3 v/v) whilst allowing the eluate to pass directly through the conditioned OASIS HLB cartridge. The solvent flow rate should not exceed 2 mL min^{-1}. The Sep-Pak cartridge is disconnected and discarded. The OASIS HLB cartridge is washed with 2.5 mL of methanol–water (1 + 1 v/v) and subsequently allowed to run dry by applying vacuum. The OASIS HLB cartridge is eluted with two 2.5 mL volumes of ethyl acetate and the eluates are collected and combined in a clean 14 mL PP tube.

The tube is placed in an evaporation station set at 50 °C and the solvent is evaporated with a flow of dry nitrogen gas. The residual matter is reconstituted in 0.5 mL of HPLC mobile phase, acetonitrile–water–formic acid (350 + 650 + 3 v/v/v), mixed vigorously and ultrasonicated for 5 min. A 100 μL aliquot of this sample extract is used for HPLC analysis.

Method validation

Limit of quantification (LOQ). An extensive series (n = 26) of supposedly virginiamycin-free feeding stuffs originating from several EU member states for different animal species was analysed according to the described method. The response at the retention time of virginiamycin M1 in the chromatograms of these feeds was determined. The LOQ for the developed method was calculated as the content in feed derived from a response value calculated from the average background responses of the blanks plus six times the standard deviation of the average response.

Within-laboratory validation experiments. Within-day repeatability and within-laboratory reproducibility were assessed by replicate analyses on three days (six replicates on day

1 and three replicates on days 2 and 3). The tests were carried out for seven types of feedingstuffs (pig, calf, piglet, sow, poultry, cattle and laying hen feed) at the minimum required performance limit (MRPL) level. Within-day and between-day repeatability at MRPL levels were assessed using unbalanced one-way analysis of variance (ANOVA). Within-day and between-day variances were summed to calculate the within-laboratory reproducibility. Within-day repeatability was also tested at 2MRPL and 5MRPL levels with at least four replicates.

Interference tests. A set of components consisting of all allowed additives in compound animal feeds belonging to the categories of antibiotics, coccidiostats and growth promoters and some frequently used antimicrobial veterinary drugs were tested for interference in the HPLC method for virginiamycin. A 100 μL aliquot of a 5 μg mL^{-1} standard solution of these components was injected into the HPLC system. This corresponds to a 1 mg kg^{-1} level in feed processed according to the described method.

Photodiode array detection

A commercial poultry feed sample spiked with virginiamycin at MRPL 2.2 mg kg^{-1} (m.a.) and a commercial laying hen feed sample spiked with virginiamycin at target MRPL 4 mg kg^{-1} (m.a.) were processed according to the described method. A 100 μL aliquot of the extract solution was injected into the HPLC system equipped with a Model 996 PDA detector. Extracted UV spectra corresponding to virginiamycin M1 (range 200–350 nm) from the 3D PDA chromatograms of a virginiamycin calibration standard of 5 μg mL^{-1} and of the spiked feeds were compared. PDA software was used to calculate the match angle, a numerical measure of the similarity of the UV spectra obtained from the virginiamycin M1 calibration standard and from the spiked feeds. Similarity of UV spectra is considered large enough below a match angle of 10 to justify a conclusion of UV spectra being from the same component.

LC-MS analysis

Aliquots of sample extracts obtained with the proposed method were injected into the described chromatographic separation system coupled to a Thermoquest LCQ LC-MS system. The LC-MS system was equipped with an APCI interface that was operated at standard settings. Three modes of MS detection were studied: LC-MS, LC in-source collision-induced-dissociation (CID)-MS and LC-MS^n.

Results and discussion

Virginiamycin additive levels in feed are historically based on microbiological potency compared with the first industry-produced virginiamycin preparation.[9] Potencies of later-produced virginiamycin preparations are generally expressed relative to this original standard. The original batch had the best purity attainable at that time and was given a certified microbial potency of 100%. Since then, the production of virginiamycin preparations has been improved considerably, resulting in higher chemical purities and improved antimicrobial synergy. As a consequence, microbiological potencies of these preparations exceed 100% compared with the original batch. The reference standard mixture used during this study had a certified microbiological potency of 2220 μg mg^{-1} (= 220%). Thus, a feed spiked at 1 mg kg^{-1} (w/w) with this virginiamycin standard is considered, based on its microbiological potency, to have a virginiamycin additive level of 2.2 mg kg^{-1} (m.a.).

Method development

A primary extraction of the standard pig feed with methanol and phosphate buffer (pH 8) followed by clean-up with ion exchange chromatography and microbiological detection[7] has been described. The suitability of these primary extraction conditions and some variations herein were tested.

Primary extraction of 5 g of feed with methanol and aqueous buffer was tested. To facilitate the exclusion of fat and apolar (matrix) components from the extract, primary extraction in the presence of hexane was also tested (hexane layer discarded). The primary methanol–buffer extract was applied to OASIS HLB after dilution with aqueous buffer. Preliminary repeatability, linearity and blank sample tests were performed and showed the presence of many peaks in the chromatograms. These made identification of virginiamycin M1 with UV detection at 230 nm unreliable. Therefore, this concept for the extraction and clean-up of virginiamycin from animal feeds was abandoned.

Alternatively, a primary extraction of the standard pig feed with ethyl acetate in the presence of 0.1 M EDTA–McIlvaine buffer (pH 4) showed high recoveries for virginiamycin M1 but many interfering peaks related to the feed matrix in the chromatograms recorded at 230 nm. Further clean-up proved necessary. Other primary extraction solvents, *e.g.*, dichloromethane, methanol and acetonitrile, were tested. Only ethyl acetate and methanol showed acceptable results regarding recovered virginiamycin M1.

Extraction of feed with ethyl acetate or methanol after wetting with water was tested. Wetting/swelling of feed with water has been used before in our laboratory to enhance accessibility in the feed for the extraction solvent and thus recovery of an analyte through increased contact surface during the primary extraction. Both ethyl acetate and methanol showed good results with respect to recovered virginiamycin M1. Ethyl acetate was adopted as the extraction solvent of choice because its extraction efficiency was better. Also, as will be shown, no evaporation is necessary before the following clean-up.

A feed extract in ethyl acetate was shown to be too polar to allow retention of virginiamycin M1 on Sep-Pak silica gel SPE columns. Addition of hexane to the ethyl acetate extract decreases the polarity and enhances the retention of virginiamycin M1 on silica gel. Addition of 10 mL of hexane to a 10 mL ethyl acetate extract aliquot proved to be sufficient to allow complete retention of virginiamycin M1 to Sep-Pak silica. Addition of larger volumes of hexane also resulted in complete retention of virginiamycin M1 of Sep-Pak silica but increased the total volume (possible breakthrough of virginiamycin M1) and decreased the polarity of the solution (possible retention of polar matrix constituents).

Drying of the ethyl acetate–hexane (1 + 1 v/v) extract with sodium sulfate before application to the Sep-Pak silica gel clean-up markedly improved virginiamycin M1 recovery and precision.

The results of suitability tests for solvents or solutions to serve as wash solvent or elution solvent for the Sep-Pak silica gel SPE are presented in Table 1.

Methanol–0.1 M EDTA–McIlvaine buffer (pH 4) (1 + 3 v/v) and acetonitrile–0.1 M EDTA–McIlvaine buffer (pH 4) (1 + 3 v/v) resulted in complete elution of virginiamycin M1 from Sep-Pak silica gel. Chromatograms recorded at 230 nm of spiked feed extracts obtained in this way, however, still showed many interfering peaks.

Additional clean-up on a different SPE material was considered. A novel SPE material, OASIS HLB (Waters) with presumed favourable characteristics for tandem SPE clean-up was tested.

Results of suitability tests on solvents and solutions to serve as loading, wash or elution solvent for the OASIS HLB SPE are presented in Table 1. The conclusion drawn from these data is that methanol–0.1 M ammonium acetate (pH 4) (1 + 3 v/v) is the

most suitable for both effective elution from silica gel and complete retention on OASIS HLB. This solution did not suffer from crystallisation of salts as does the EDTA–McIlvaine buffer mixture.

Methanol–water (1 + 1 v/v) was adopted as a wash solvent. This induced a loss of a minor fraction (5%) of the virginiamycin M1 applied to the OASIS HLB cartridge.

Methanol, acetonitrile and water-saturated ethyl acetate proved to be acceptable solvents for elution of virginiamycin M1 from an OASIS HLB cartridge with respect to recovered virginiamycin M1. Because methanol and acetonitrile elution resulted in more matrix-related chromatographic peaks than ethyl acetate elution, ethyl acetate was adopted as the elution solvent for the OASIS cartridge.

A 2 mL acetonitrile wash step of the SepPak silica gel cartridge before virginiamycin M1 transfer to OASIS HLB was introduced to prevent or decrease co-eluting matrix-related interferences in chromatograms of spiked feeds, despite a slight loss of virginiamycin M1 by elution.

A graphical representation of the final procedure that was validated is shown in Fig. 1.

Method validation

The primary target of the method validation was the detection of virginiamycin M1 as a marker component for virginiamycin and quantification was considered to be of secondary importance. Overlays of chromatograms from a representative blank and a spiked feed at the MRPL level for all seven feed types tested are shown in Fig. 2. The 230 nm UV chromatograms of all seven blank feed type extracts show many responses. The virginiamycin M1 peak, nonetheless, is sufficiently separated from adjacent peaks to be clearly identifiable in each of the chromatograms of feed type extracts spiked at the relevant target MRPL level.

Determination of LOQ. An extensive series (n = 26) of supposedly virginiamycin-free feeding stuffs for different animal species originating from several EU member states was analysed according to the developed method. Relatively high background responses, compared with the other feeding stuffs, at the retention time of virginiamycin M1 were found with four of the 26 supposedly virginiamycin-free feeding stuffs. The highest background response found for a supposedly virginiamycin-free feeding stuff corresponded to 0.8 mg kg^{-1} m.a. (= 0.4 mg kg^{-1} w/w).

The LOQ of the method was calculated to be 2.76 mg kg^{-1} (m.a.) (= 1.25 mg kg^{-1} w/w) if all 26 supposedly virginiamycin-free feeding stuffs were taken into account. The four feeding stuffs showing relatively high background responses might be left out on the basis of suspected presence of virginiamycin. In that case, the LOQ would be calculated as 1.42 mg kg^{-1} (m.a.) (= 0.65 mg kg^{-1} w/w). Both the lower average background response and the smaller standard deviation contribute to the significant lowering of the LOQ value in this case.

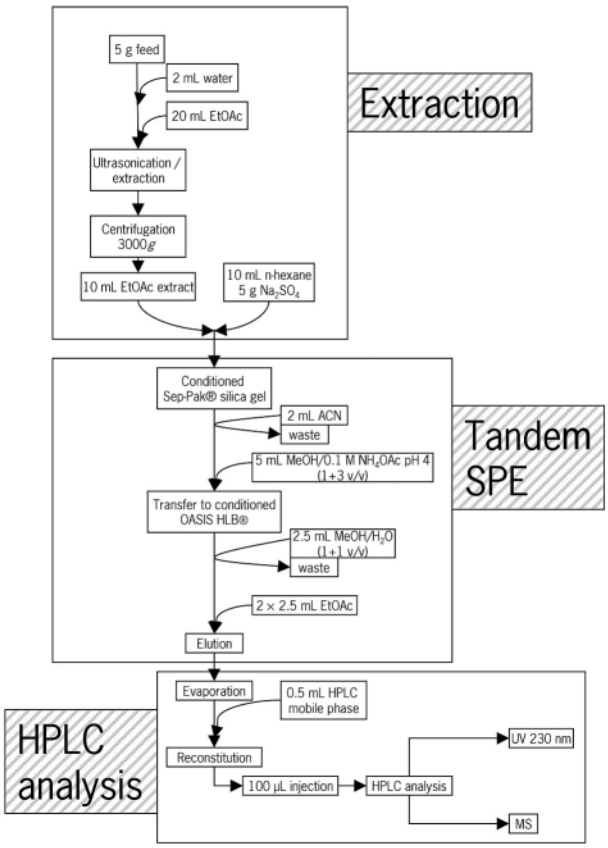

Fig. 1 Schematic representation of the sample preparation procedure developed for the determination of virginiamycin M1 as marker component for virginiamycin in compound animal feed. Abbreviations: EtOAc, ethyl acetate; ACN, acetonitrile; MeOH, methanol; NH_4OAc, ammonium acetate.

Table 1 Summary of suitability characteristics of the tested solvents and solutions as loading, wash and/or elution solvent during SPE. Retention on and elution from 690 mg Sep Pak silica gel cartridges and 60 mg OASIS HLB cartridges of virginiamycin M1 were tested

	Silica gel cartridge[a]		OASIS HLB cartridge[a]	
Solvent/solution	Wash	Elution	Wash	Elution
0.1 M EDTA–McIlvaine buffer (pH 4)	+	−		
0.1 M acetate buffer (pH 3.5)	+	−		
Acetonitrile–water (1 + 1)	+/−	+/−		
Acetonitrile–water (1 + 3)	+/−	+/−		
Acetonitrile–EDTA–McIlvaine buffer (1 + 3)	−	+	+/−	+/−
Methanol–EDTA–McIlvaine buffer (1 + 3)	−	+	+	−
Methanol–acetate buffer (1 + 3)	−	+	+	−
Methanol–water 1 : 1			+	−
Water–saturated ethyl acetate			−	+
Ethyl acetate			+/−	+/−
Acetone	−	+/−		
Methanol	−	+/−	−	+
Acetonitrile	+	−	−	+
Formic acid–methanol (1 + 99)	+/−	+/−	+/−	+/−
Formic acid–acetonitrile (1 + 99)	+	−	+/−	+/−
Formic acid–ethyl acetate			+/−	−

[a] + = Suitable ; +/− = moderately suitable; − = not suitable.

Thus, using UV detection at 230 nm it appeared not to be possible to achieve an additive level of 1 mg kg^{-1} (= 0.45 mg kg^{-1} w/w) for feeding stuffs for pigs, calves, piglets and poultry corresponding to one-fifth of the formerly permitted lowest additive level. The primary target for the method validation was the detection of virginiamycin M1. Because the use of UV detection was required, given the intended transfer of the method to other laboratories within the EU, and because two independent SPE clean-up and isolation steps were already incorporated in the method, there appeared to be no scope for lowering the LOQ level. Consequently, the lowest level that was validated for feeding stuffs for pigs, calves, piglets and poultry was set at 2.2 mg kg^{-1} m.a. (= 1 mg kg^{-1} w/w). This Minimum Required Performance Limit (MRPL) level is in between the two estimated LOQ levels mentioned above.

Within-laboratory reproducibility. Average virginiamycin M1 recoveries, within-day repeatabilities and within-laboratory reproducibility at the indicated MRPL levels for each feed type are presented in Table 2. For calf, piglet, sow, poultry and cattle feed, single observations/replicates were considered an outlier on the basis of a recovery clearly above 100%. These outliers were not taken into account in the statistical calculations. Within-day repeatabilities (RSDs) of the method at the tested MRPL level over three days were below 20% for all seven tested feed types, ranging from 7% (poultry) to 19% (cattle, calves). High within-day repeatabilities are attributed to single replicate values being deviant whilst others were more in line with the average value. The average recoveries of virginiamycin M1 at the MRPL level ranged from 37% for cattle feed to 67% for piglet feed. The within-laboratory reproducibility of the

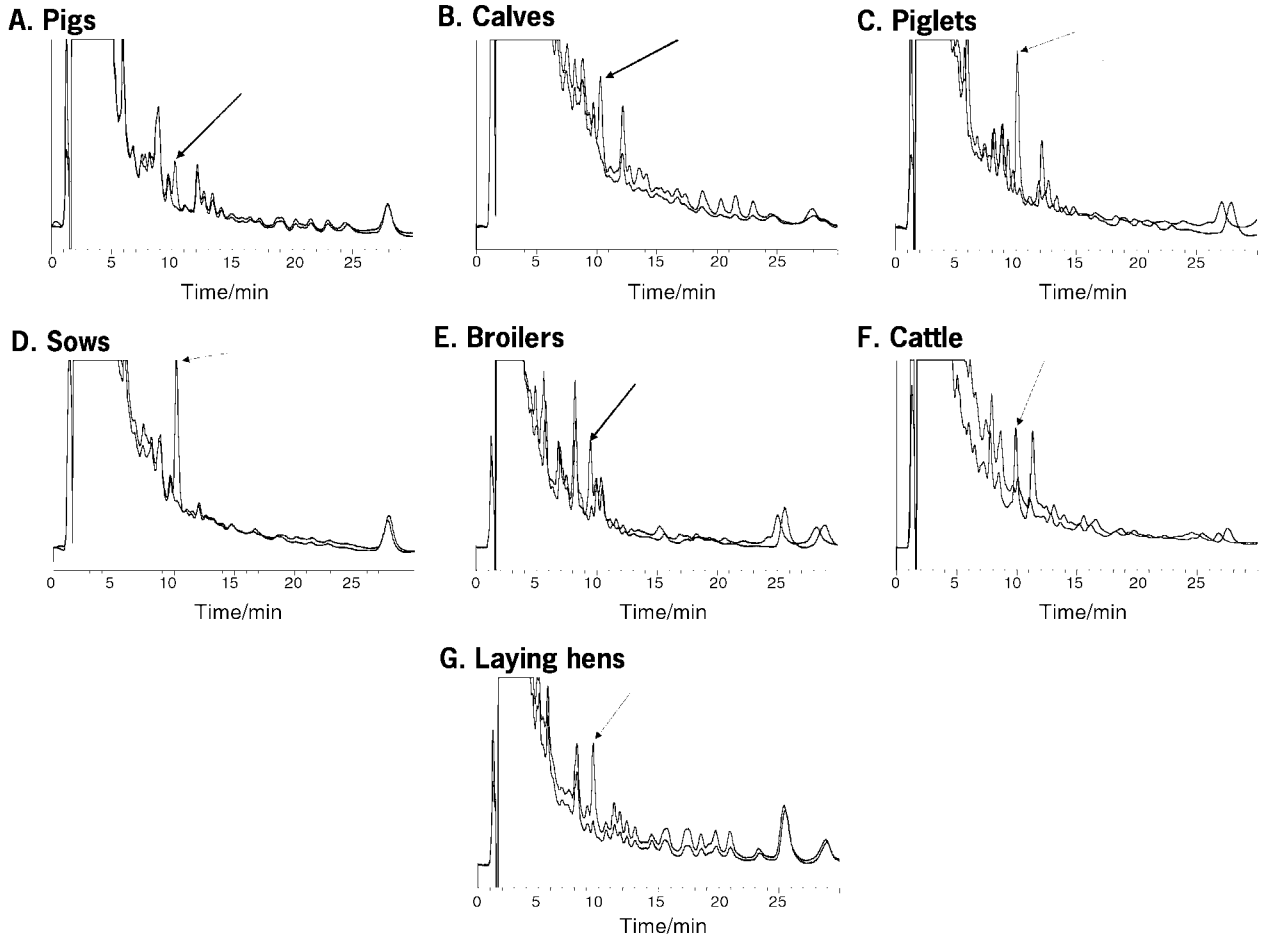

Fig. 2 Overlays of chromatograms from a representative blank and a feed spiked with virginiamycin at the MRPL level for (A) pig feed at 2.2 mg kg^{-1} (m.a.); (B) calf feed at 2.2 mg kg^{-1} (m.a.); (C) piglet feed at 2.2 mg kg^{-1} (m.a.); (D) sow feed at 4 mg kg^{-1} (m.a.); (E) poultry feed at 2.2 mg kg^{-1} (m.a.); (F) cattle feed at 3 mg kg^{-1} (m.a.); and (G) laying hen feed at 4 mg kg^{-1} (m.a.). Arrows indicate position of virginiamycin M1 peak in the chromatograms.

Table 2 Average recoveries, within-day repeatabilities and within-laboratory reproducibilities of virginiamycin M1 from feed type groups with the proposed method at the MRPL level over three days. Within-day repeatability and within-laboratory reproducibility were calculated as RSD with one-way ANOVA

Feed type	MRPL level/ mg kg^{-1}	Measured content overall average/ mg kg^{-1}	Recovery (%)	Within-day repeatability	Within-laboratory reproducibility (%)
Pig	2.2	1.07	49 (n = 9)	8–12[a]	—[a]
Calf	2.2	1.47	67 (n = 11)	19	19
Piglet	2.2	1.44	65 (n = 11)	13	12
Sow	4	2.60	65 (n = 11)	10	13
Poultry	2.2	1.31	60 (n = 11)	7	16
Cattle	3	1.27	42 (n = 11)	19	23
Laying hen	3	1.12	38 (n = 12)	8	27

[a] Results from only two days available.

method at the tested MRPL level over three days was acceptable below 20% for four out of the seven feed type groups and above 20% for cattle (23%) and laying hen (27%) feeds. The variability in recovery and repeatability for each feed type group indicate that the matrix composition is an important factor for the performance of the method. Average virginiamycin M1 recoveries and within-day repeatabilities at the 2MRPL and 5MRPL levels for each feed type are presented in Table 3. These data show a similar pattern to the MRPL data in Table 2. Recoveries are variable, repeatabilities are relatively high and matrix (= feed) composition appeared to be a critical factor.

The validation data should, therefore, be interpreted in the light of the intended use of the developed method. The method is primarily intended for screening purposes and the production of semi-quantitative data is considered of secondary interest. Detection of virginiamycin M1 as a marker for the presence of virginiamycin at or above the MRPL level in the different feeding stuffs was always possible in each of the spiked replicates analysed with the method. Consequently, no false-negatives for spiked feed were found. The areas of the peak corresponding to virginiamycin M1 of the spiked samples at selected MRPL levels were at least twice the peak area found for the supposedly virginiamycin-free feeding stuff showing the highest background signal at the retention time of virginiamycin M1. This justifies the use of the developed method for screening for the presence of virginiamycin in feeding stuffs above 2.2 mg kg^{-1} m.a. (= 1 mg kg^{-1} w/w) with virginiamycin M1 as the marker component. Only an indicative value of the actual virginiamycin content may be reported using this method.

Selectivity/interference test. Results of the interference tests are presented in Table 4. None of the tested components co-eluted with virginiamycin M1 at a retention time of 9.5 min. Flumequine, at ~8 min, and robenidine, at ~8.5 min, eluted closest to virginiamycin M1. However, the resolution of the peaks of these components and the peak of virginiamycin M1 was sufficient to allow differentiation.

Table 3 Average recoveries and repeatabilities (RSD in parentheses) of virginiamycin M1 from feed type groups with the proposed method at the 2MRPL and 5MRPL levels

Feed type	MRPL level/ mg kg^{-1}	Recovery (%) 2MRPL	5MRPL
Pig	2.2	47 (13%)	71 (4%)
Calf	2.2	68 (7%)	70 (4%)
Piglet	2.2	64 (11%)	69 (4%)
Sow	4	65 (9%)	59 (22%)
Poultry	2.2	62 (6%)	59 (23%)
Cattle	3	31 (16%)	40 (26%)
Laying hen	3	51 (9%)	51 (9%)

Photodiode array detection

The extracted UV spectra corresponding to virginiamycin M1 (range 200–350 nm) from the PDA chromatograms of a virginiamycin calibration standard at 5 μg mL^{-1} and of two spiked feeds are shown in Fig. 3. It was possible to obtain justified conclusions, based on a match angle below 10, of the presence of virginiamycin M1 in both tested spiked feed samples. A poultry feed sample spiked at 2.2 mg kg^{-1} (= 1 mg kg^{-1} w/w) resulted in a match angle of 8.1 and a laying hen feed sample spiked at 4 mg kg^{-1} (= 1.8 mg kg^{-1} w/w) in a match angle of 6.6.

LC-MS analysis

The results of pilot experiments with piglet feed extracts at the MRPL level of 2.2 mg kg^{-1} (m.a.) are presented in Fig. 4. The MS detection modes applied were, in order of sophistication, full MS, CID-MS and MS^2. Examples are presented of an LC-MS mass chromatogram of the protonated parent molecule, of an LC in-source CID-MS mass chromatogram of the fragment ion at *m/z* 355 and of an LC-MS^2 mass chromatogram of the transition *m/z* 526 → 355 of virginiamycin in piglet feed. With all three modes of LC-MS virginiamycin M1 was readily detected. As an illustration of the signal-to-noise (S/N) ratio, in each mass chromatogram a 10-fold enlargement section of the

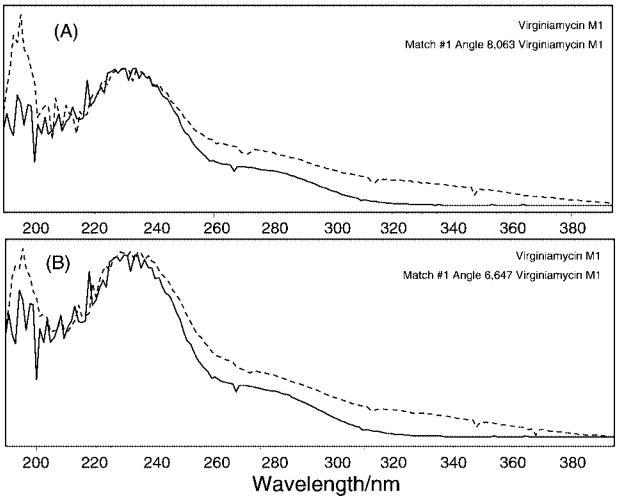

Fig. 3 PDA overlay spectra showing a direct comparison of the UV spectrum of the chromatographic peaks corresponding to virginiamycin M1 of a standard and two different feeds spiked with virginiamycin. (A) PDA overlay spectra of a calibration standard at 5 μg mL^{-1} virginiamycin and a broiler feed spiked with virginiamycin at 2.2 mg kg^{-1} (m.a.). (B) PDA overlay spectra of a calibration standard at 5 μg mL^{-1} virginiamycin and a laying hen feed spiked with virginiamycin at 4 mg kg^{-1} (m.a.).

Table 4 Components subjected to interference testing in the virginiamycin HPLC analysis method. The corresponding retention times (t_R) of the peaks, if found, are given in parentheses after the component name

Reference compound	Virginiamycin M1 (t_R ≈ 9.5 min)
Antibiotics, coccidiostats, *etc.*	Bacitracin, spiramycin, flavofosfolipol, tylosine, monensin, salinomycin, avilamycin (t_R ≈ 39 min), amprolium, meticlopindol, decoquinaat, lasolacide, halofuginon, narasin, nicarbazin (t_R ≈ 51 min), nifursol (t_R ≈ 55 min), maduramycin, diclazuril, robenidine (t_R ≈ 8.5 min), arpronicide, dinitolmide (t_R ≈ 4 min), penicillin
Sulfonamides	Sulfanilamide, sulfadimethoxine (t_R ≈ 5 min), sulfamethazine (t_R ≈ 3 min), sulfadoxine (t_R ≈ 3.5 min), sulfachlorpyrazine (t_R ≈ 5 min), sulfaquinoxaline (t_R ≈ 5 min), sulfachlorpyridazine (t_R ≈ 3.5 min)
Tetracyclines	Oxytetracycline (t_R ≈ 2 min), tetracycline (t_R ≈ 2 min), chlortetracycline (t_R ≈ 2 min), doxycycline (t_R ≈ 2 min)
Quinolones	Enrofloxacin, ciprofloxacin, flumequine (t_R ≈ 8 min)
Nitrofurans	Nitrofurantoin (t_R ≈ 2-4 min), nitrofurazone (t_R ≈ 2.5 min), furazolidone (t_R ≈ 3.5 min), furaltadone (t_R ≈ 2 min)
Nitroimidazoles	Dimetridazole (t_R ≈ 2 min), ronidazole (t_R ≈ 2 min), ipronidazole (t_R ≈ 4 min)
Growth promoters	Carbadox, olaquindox (t_R ≈ 41 min)
Other compounds	Trimethoprim, dapson (t_R ≈ 3 min)

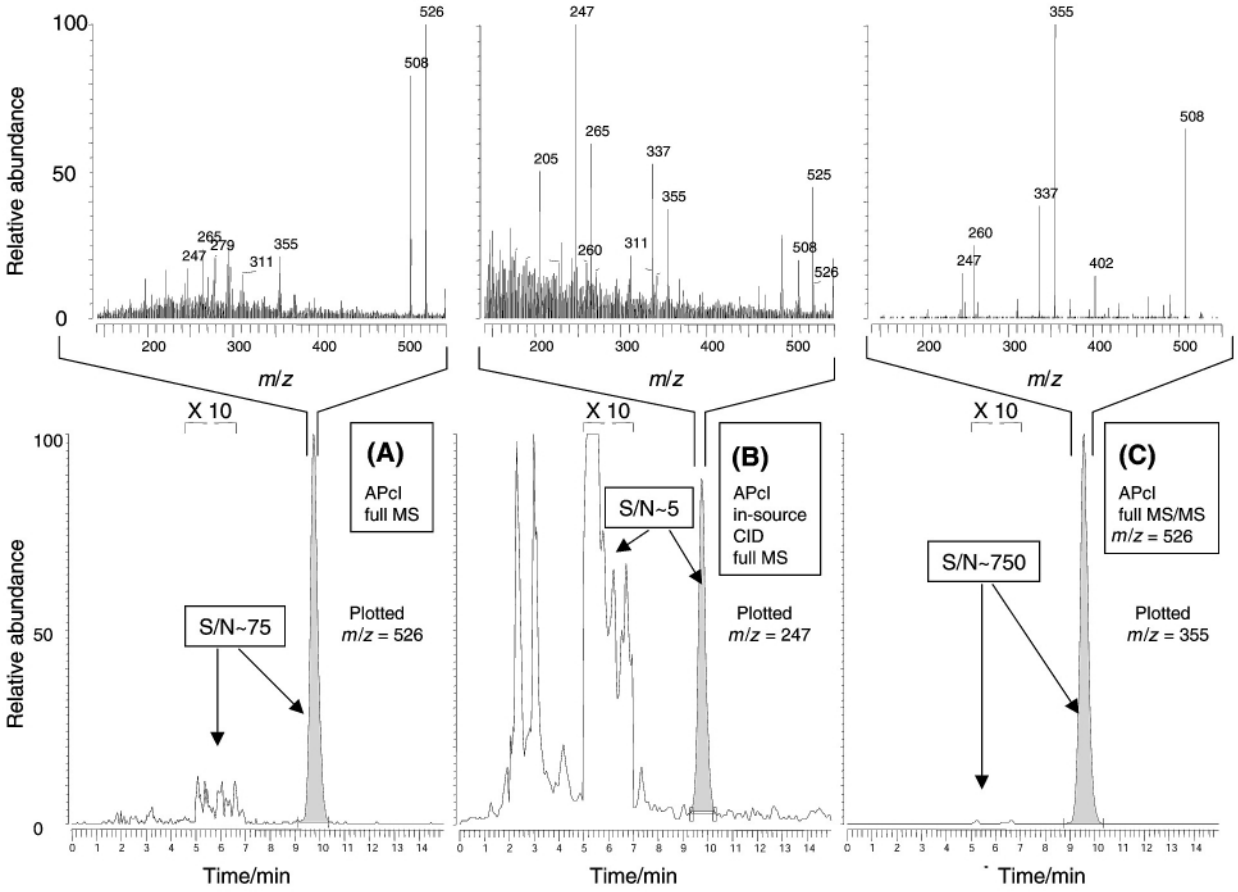

Fig. 4 Mass chromatograms and mass spectra of virginiamycin M1 peak in an extract of piglet feed spiked at MRPL 2.2 mg kg^{-1} (m.a.) with virginiamycin obtained after injection into an LC system coupled to MS detection in (A) LC-MS mode, (B) LC-in-source CID-MS mode and (C) LC-MS^2 mode. The most abundant ion is plotted in these mass chromatograms.

noise from 5–7 min is shown. As expected, the selectivity of LC-MS was much better than that of UV detection at 230 nm showing an S/N ratio of ~75 at the MRPL level in piglet feed.

In-source CID-MS detection was comparable in sensitivity and selectivity to LC-UV, showing an S/N ratio of ~5 at the MRPL level in piglet feed. Considering the large number of peaks in the 230 nm UV chromatograms, it is clear that many components entering the source of the mass spectrometer, are fractionated there and detected in the mass analyser. This may explain the unfavourable S/N ratio in the mass chromatogram.

As expected, the selectivity and sensitivity of LC-MS^2 were much better than those of LC-MS detection. The plotted transition in the MS^2 mode in Fig. 4 was of the molecular ion $[M + H]^+$ from *m*/*z* 526 to 355. Injection volumes as small as 10 μL, compared to 100 μL in the LC-UV method, could be used. Despite the smaller injection volume, an S/N ratio of ~750 resulted, which is more than 100-fold better at the selected MRPL level in feed than with either UV detection at 230 nm or in-source CID-MS detection.

A draft revision of Commission Decision 93/256/EC[11] aims to lay down criteria for the confirmatory analysis of contaminants with MS techniques. The assignment of identification points based on measured diagnostic ions using a range of MS techniques is proposed. Possible diagnostic ions for virginiamycin M1 confirmation in the LC-MS^2 mode are the daughter ions from CID fragmentation of the molecular ion $[M + H]^+$ of *m*/*z* 526 at *m*/*z* 508, 355, 337, 260 and 247. Future work may address the suitability of these fragmentations for confirmatory purposes in more detail because it is beyond the scope of this paper. The preliminary results obtained using LC-MS^2 indicate that a very specific and sensitive assay for virginiamycin M1 may be developed.

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