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Positive and negative electrospray LC–MS–MS methods for quantitation of the antiparasitic endectocide drugs, abamectin, doramectin, emamectin, eprinomectin, ivermectin, moxidectin and selamectin in milk

David A. Durden *

Canadian Food Inspection Agency, 3650 36 St. NW, Calgary, Alta., Canada T2L 2L1

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Abstract

Avermectin endectocides are used for the treatment of cattle against a variety of nematode and arthropod parasites, and consequently may appear in milk after normal or off-label use. The compounds abamectin, doramectin, and ivermectin, contain only C, H and O and may be expected to be detected by LC–MS in negative ion mode. The others contain nitrogen in addition and would be expected to be preferentially ionized in positive mode. The use of positive ion and negative ion methods with electrospray LC–MS–MS were compared. Using negative ion the compounds abamectin, doramectin, ivermectin, emamectin, eprinomectin, and moxidectin gave a curvilinear response and were quantified in raw milk by LC–MS–MS with a triethylamine–acetonitrile buffer over the concentration range 1–60 ppb (μg/kg) using selamectin as the internal standard. The limits of detection (LOD) were between 0.19 ppb (doramectin) and 0.38 ppb (emamectin). The compounds gave maximum sensitivity with positive ionisation from a formic acid–ammonium formate–acetonitrile buffer and were detected in milk (LC–MS–MS) also with a curvilinear response over the range 0.5–60 ppb. Although the positive ion signals were larger, with somewhat lower limits of detection (LOD between 0.06 ppb (doramectin) and 0.32 ppb (moxidectin) the negative ion procedure gave a more linear response and more consistent results. Comparison of spiked samples in the range 2–50 ppb showed a high degree of correlation between the two methods.

Keywords: Avermectin; Endectocide; Milk; LC–MS–MS; Electrospray ionization; Liquid chromatography; Tandem mass spectrometry; Residue analysis; Abamectin; Doramectin; Emamectin; Eprinomectin; Ivermectin; Moxidectin; Selamectin

1. Introduction

Macrolide endectocides, Fig. 1, are active against a wide variety of mammalian internal and external (endo- and ecto-) parasites such as nematodes, heart, lung, round, and intestinal worms, arthropods, mites, lice, flees, ticks etc [1]. They have very high potencies and consequently are convenient for treatment of agricultural animals. These macrocyclic lactone compounds consist of two subgroups: the avermectins, which include abamectin, doramectin, ivermectin, emamectin, eprinomectin and selamectin, and which have saccharide substituents at position C13, and the milbemycins (also called nemodectins), of which moxidectin is the representative here, and which do not have saccharide substituents. Avermectins were originally isolated from cultures of *Streptomyces avermitilis* and the milbemycins were isolated from *S. cyanogrise or S. hygroscopicus*. Both groups were found to have potent insecticidal properties. Abamectin is the commercialised major pair of isomers of avermectin itself, and ivermectin is the cis-hydrogenated product of abamectin (at the 22,23 position). In both compounds, the major isomer, B1a, contains a 2-butyl group, and the minor isomer (B1b) an isopropyl group attached to the carbon at the 25 position [2]. Many new avermectins have been synthesised to improve both the anti-parasitic efficacy and to change their pharmacokinetic profiles. Doramectin was biosynthesised from mutant *S. avermitilis* with a cyclohexyl group at the 25 position [3]. These three avermectins contain only C, H and O atoms (Fig. 1). Further modifications of the

* Tel.: +1 403 299 7612; fax: +1 403 221 3293.
E-mail address: durdend@inspection.gc.ca.

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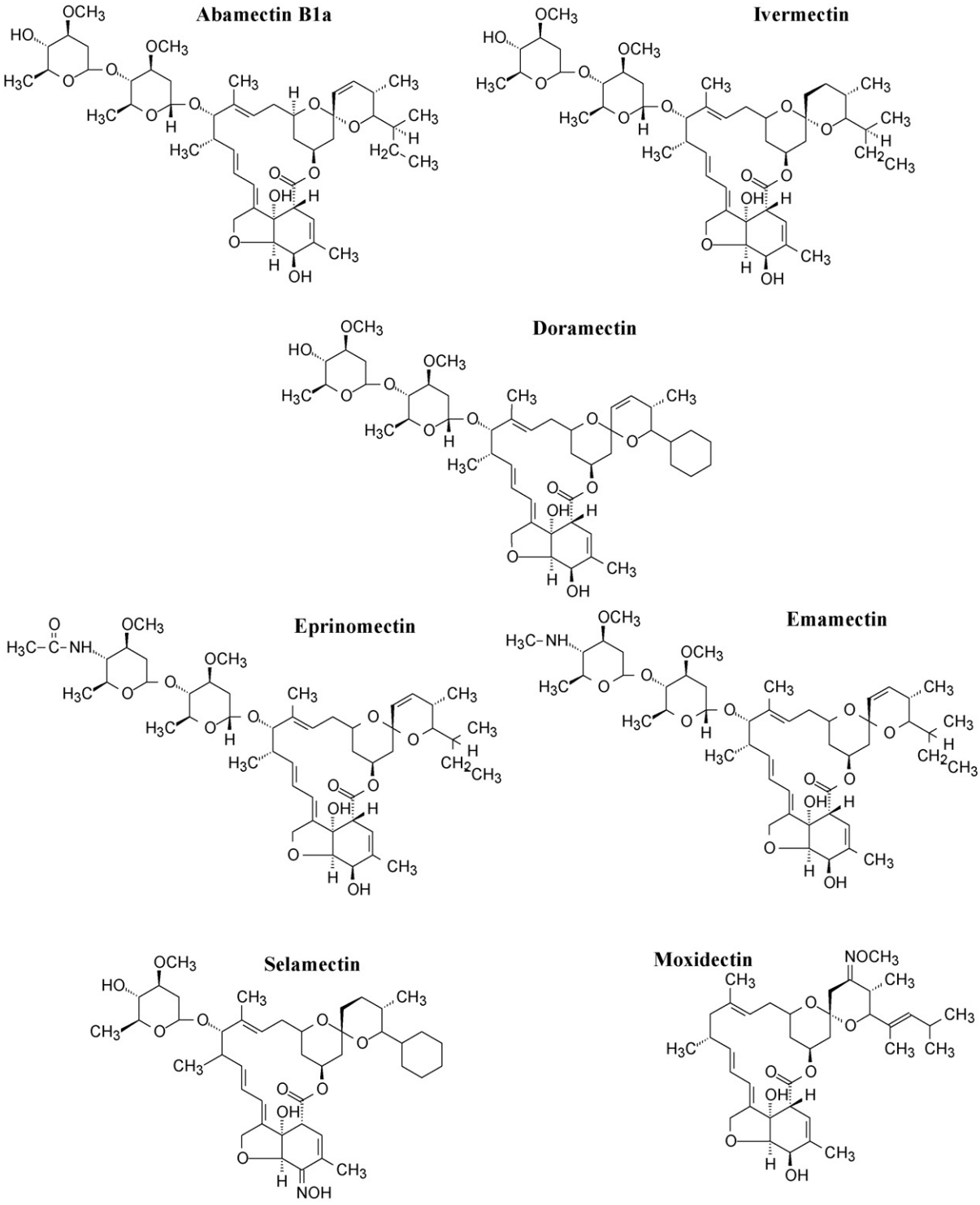

Fig. 1. Structures of avermectins.

avermectin and nemodectin structures have been obtained by introducing groups containing nitrogen atoms to obtain different insecticidal properties, and reduce meat and milk withdrawal times. Thus various OH groups were replaced by: in emamectin, a methylamino group, in eprinomectin, an acetylamino group, in selamectin an oxime and in moxidectin a methoxime group. The compounds are registered for use in cattle and other food animals, for fish farming or for pet animals only. For example, in Canada, abamectin, doramectin, eprinomectin, ivermectin and moxidectin are used to treat food animals: cattle, swine, sheep, bison, deer and reindeer against nematodes and arthropods and for mastitis; emamectin is used to control sea lice in fish farms and selamectin is used for the treatment of pets against heart and round worms. Of this group, only eprinomectin and moxidectin

are permitted for use with dairy cattle, with no milk withholding time. Eprinomectin was designed to exhibit a low milk/plasma ratio [4], and moxidectin is less toxic with a larger acceptable daily intake (ADI). Eprinomectin has an (administrative) MRL of 20 ppb in Canada, and MRLs of 20 ppb in the EU and 12 ppb in the USA. The marker residue is the B1a isomer. Moxidectin has a MRL value of 40 ppb for the USA and EU. The avermectins are also used for control of mites in fruits and vegetables. Although they are considered safe with low toxicity towards mammals, misuse or extra-label use may produce food levels in violation of zero tolerance or MRL levels. Thus a number of methods for quantitation and verification of avermectin and milbemycin endectocides have been developed.

In many laboratories the macrolide endectocides are screened in a variety of fluids and tissues using HPLC with fluorescence detection. The fluorophor is formed by dehydration of the fused hexahydrobenzofuran ring system to a conjugated benzofuran system with a strong acid such as trifluoroacetic acid in the presence of an organic base such as methylimidazole [2,5–9].

Mass spectrometry, with either particle beam negative ion chemical ionization or thermospray, was originally used to confirm the presence of ivermectin [10] or moxidectin [11], after quantitation by the LC-fluorescence method since the MS response was not linear [10]. Use of positive ion electrospray ionization (ESI) for quantitation has been plagued by the tendency of the avermectins to form sodium adduct ions $[M+Na]^+$ [12–15]. Monitoring of the $[M+Na]^+$ ion tended to produce a very non linear response, which was attributed to traces of sodium present in the extracts from the matrix or the method [12,15]. Using positive electrospray ionization and the $[M+Na]^+$ ion it was possible to get a linear relative response when abamectin was used as the internal standard for quantitation of ivermectin. Presumably these close analogues respond equally to the presence of sodium in the extracts and the non linear responses cancel. However, this method was limited to only one analyte, and presumed the absence of abamectin in the samples. To overcome the sodium adduct and linearity problems, several methods have been reported using LC–MS with atmospheric pressure chemical ionization (APCI) with positive ion detection in which either the $[M+H]^+$ [12], the $[M+NH_4]^+$ [16], or fragment [17] ions were monitored. Linear calibration curves were obtained using APCI with negative ion $[M-H]^-$ monitoring [15,18,19]. Second order polynomial calibration curves were found to be more appropriate for quantitation using the $[M-H]^-$ ions formed by negative ion APCI with a wide range of analyte concentrations.

The aim of this work was twofold. Firstly to examine the different conditions for analysis of a wide variety of avermectins using electrospray ionization with both positive and negative ions, and secondly compare the results obtained when quantifying the avermectins in milk. Although eprinomectin and moxidectin are the only avermectins permitted for use with dairy cattle, the other compounds were included since off-label use of various avermectins with dairy cattle does occur [20]. Selamectin was chosen as the internal standard as it is unlikely to be used in food producing animals.

2. Materials

Water was purified to ASTM type II (MQ water) with a Milli Q system (Millipore, Billerica, MA). Acetonitrile (ACN), hexane and methanol (MeOH), HPLC grade were purchased from Caledon Laboratories, Georgetown, ON, triethylamine (TEA), Analar, from BDH Inc., Toronto, ON, formic acid (98%) EM Science from VWR International Canada, Edmonton, AB, and Sylon CT, Trizma base and Trizma HCl from Sigma–Aldrich Canada, Oakville, ON. Polyethylene glycols, PEG-400, PEG-600, PEG-1000 and PEG-1500 and ammonium formate and ammonium acetate were also purchased from Sigma–Aldrich. SPE tubes were Varian BondElut (Cat 1210-2052), with adapters (Supelco) and 20 ml reservoirs (Chromatographic Specialities, Brockville, ON). Disposable culture tubes, 16 mm × 100 mm, were either silanized with Sylon-CT according to the package instructions, washed with methanol and hexane and dried, or purchased pre-silanized (CTS-16100, level 2) from VWR International. Polypropylene centrifuge tubes, 50 mL suitable for up to $9000 \times g$ (Cat. 21008-240) were obtained from VWR International. Filter vials were (Whatman Mini-UniPrep), 12 mm × 32 mm, with PVDF filters.

Drugs: abamectin 89% B1a (Chem Services Inc, Supelco), emamectin benzoate, 82.27% B1a, Pestanal grade (Riedel-de-Haen), ivermectin 93% B1a, were all purchased from Sigma–Aldrich, Canada; doramectin, 91.9% B1, and selamectin, 96.4%, were kindly provided by Pfizer, UK, eprinomectin 92.6% B1a was provided by Merial Ltd, and moxidectin, 98.2%, by Cyanamid Canada (now Wyeth).

Standard solutions were prepared as follows. Somewhat greater than 6.25 mg of each compound, the actual amount being calculated to correct for purity, was weighed and dissolved in 50 ml of ACN:H_2O 50:50. The stock concentrations were about 125 ppm. One millilitre aliquots of each were pipetted into a second 50 ml volumetric flask and taken to volume in 50% ACN. This solution (1/50 stock) was diluted twice to 1/500 and 1/5000. The internal standard stock was diluted 1/100. These were the working solutions.

Other solutions were prepared as follows: formic acid, nominal 1% (0.26 M) was prepared by diluting 10.0 mL 98% formic acid to 1000 mL with MQ water. Formic acid 1% with 3 mM ammonium formate was prepared by adding 189 mg ammonium formate to the above before dilution. Triethylamine, 10 mM, was prepared by diluting 1400 μl TEA to 1000 mL with MQ water. Tris buffer, nominal pH 8, used for extraction was prepared by adding 5.38 g Trizma base to 8.88 g Trizma HCl and diluting to 2000 mL with MQ water. The measured pH at 21 to 22 °C was 8.14 ± 0.05 (Orion 320 meter with calomel electrode). The SPE conditioning solution (ACN: Tris pH 8 30:70 v/v) was prepared by mixing 300 mL ACN with 700 mL pH 8 Tris buffer, the SPE wash solution (ACN: water 50:50 v/v) was prepared by combining 500 mL ACN with 500 mL MQ water and the SPE elution solution (ACN: TEA 99.9:0.1 v/v) was prepared by diluting 1.00 mL TEA to 1000 mL with ACN. The calibration solution was made using 5 mg PEG-400, 7.5 mg PEG-600, 25 mg PEG-1000 and 25 mg PEG-1500 dissolved in 100 ml 50% acetonitrile, 50% water containing 2 mmol ammonium acetate.

Instrumentation: Waters Alliance 2695 connected to a Micromass Quattro Ultima MS–MS via a 6 way multiport valve. The instrument was operated under Masslynx software, Versions 3.5 or 4.0. Collision gas was Argon and desolvation gas was Nitrogen. The mass spectrometer was initially calibrated using sodium–cesium iodide solutions and later with the mixture of polyethyleneglycols and ammonium acetate over the range 70–1200 Da. The instrument was operated as follows: positive ion: 3.2 kV, hexapole one 10 V, source 130 °C, desolvation gas 280 °C, cone gas 175 l/h, desolvation gas 610 l/h, collision gas 2.5×10^{-3} mB and detector 450 V; negative ion: −3.5 kV, hexapole one 0 V, detector 600 V and other parameters were the same. For spectra, the resolution was set to about 0.6 Da FWHM ('LM' and 'HM' resolution settings 15) and for MRM to 1.0 Da FWHM ('LM' and 'LM' resolution settings 13) with Q1 and Q2 ion energies set to 0.5 and 3 V, respectively. For spectra, the entrance voltage, collision energy and exit voltages were set to 50, 1, 50, respectively and for MRM entrance voltage and exit voltages were set to −2 and 1, respectively and the ions, collision energies (CE) and cone voltages set according to Tables 1 and 2.

HPLC column: Waters Xterra RP18, 3.5 micron, 3 mm × 100 mm with 3 mm × 20 mm RP18 guard column. Isocratic HPLC buffers used were: for negative ion ES, buffer A, ACN at 75%, 10 mM triethylamine in water at 25%, and for positive ion ES, either buffer B, ACN at 75%, 1% formic acid at 10%, water at 15%, or buffer C, ACN at 75%, 1% formic acid (260 mM) with 3 mM ammonium formate at 10% water at 15%. To reduce evaporation of the 10 mM triethylamine into the headspace and thus minimize any change in concentration during a set of analyses, the solution was prepared weekly and 0.25 in. polypropylene beads (Small Parts Inc., Miami Lakes FL), which floated, were added to the reservoir which was maintained with minimum headspace. In all cases the flow rate was 350 μl/min, and 35 μl (positive ion) or 70 μl (negative

Table 1
Multiple reaction monitoring (MRM) settings for negative ion MS–MS analysis of avermectins

Compound	Parent ion (Da)	Identity	Daughter ion (Da)	Collision energy (V)	Cone voltage	Dwell time (ms)	Retention times (min)
Abamectin	871.40	$(M-H)^-$	565.20 229.10	26 36	70	100 100	4.31
Doramectin	897.50	$(M-H)^-$	591.30 229.10	26 35	70	100 100	5.05
Emamectin	884.50	$(M-H)^-$	565.20 242.10	26 30	70	100 100	4.49
Eprinomectin	912.50	$(M-H)^-$	565.20 270.00	26 30	70	100 100	3.63
Ivermectin	873.40	$(M-H)^-$	567.20 229.10	26 30	70	100 100	5.92
Moxidectin	638.30	$(M-H)^-$	528.30 247.10	20 30	70	100 100	6.25
Selamectin	768.40	$(M-H)^-$	750.20 722.20	24 24	70	100 100	6.60

Table 2
Multiple reaction monitoring (MRM) settings for positive ion MS–MS analysis of avermectins

Compound	Parent ion (Da)	Identity	Daughter ion (Da)	Collision energy (V)	Cone voltage	Dwell time (ms)	Retention times (min)
Abamectin	890.50	MNH_4^+	567.20 305.20	16 20	20	100 100	4.34
Doramectin	916.50	MNH_4^+	593.70 331.30	16 25	20	100 100	5.08
Emamectin	886.45	MH^+	302.12 158.05	30 35	20	100 100	2.76
Eprinomectin	914.45	MH^+	330.10 186.10	15 20	20	100 100	3.66
Ivermectin	892.50	MNH_4^+	569.30 307.20	16 25	20	100 100	5.98
Moxidectin	640.30	MH^+	528.20 498.20	10 12	20	100 100	6.25
Selamectin	770.40	MH^+	626.30 158.05	16 22	20	100 100	6.66

ion) was injected. Column oven temperature was maintained at 35 °C.

2.1. Samples

Various batches of raw milk were obtained from a local milk processor by a Canadian Food Inspection Agency inspector in 4 l polyethylene containers. Each was portioned into 100.0 ± 0.1 g aliquots and frozen at −20 °C.

3. Method

Milk was thawed, either overnight in a refrigerator (4 °C) or in the water bath, until it was at least at room temperature (20 − 22 °C) and then mixed by gently shaking. Aliquots (5.00 ml) were pipetted into 50 ml polypropylene centrifuge tubes using a digital macropipettor with polypropylene tips. Method matrix calibration standard curves were used for this procedure and run in parallel to the spiked and unknown samples. Standards were added to 5 ml milk at nominal concentrations of 0, 0.3, 1, 3, 10, 30, and 60 or 100 ppb, for the negative ion method. Standards at 0.1 ppb were also added for the positive ion analyses. Other samples, for recovery calculations, were spiked at 0.2 (for LOD calculations), 0.5, 2, 5, 20, and 50 ppb. Internal standard was added at 60 ppb to all tubes except the matrix blanks. The tubes were capped, vortexed briefly, and left for approximately 20 min to allow the compounds to be thoroughly mixed. To each tube was added 15 ml ACN. The tubes were capped, mixed (vortex), and left at 40 °C ± 1 °C for 5 min in a circulating water bath (Boekel Scientific). The tubes were shaken for 10 min (Eberbach shaker on low) and then centrifuged at 3200 × *g* (Beckman Coulter Allegra 6, 3750 rpm) for 15 min. The supernatants were filtered into new 50 mL PP tubes either through filter paper (Whatman No 41, 9 cm) by gravity or through Pall AP4529 glass fibre/GxF syringe filters using a SPE vacuum manifold with slight suction to empty the filter units. To the supernatant was added 24 ml of pH 8 Tris buffer with mixing, and the mixture was applied to the SPE tubes. If the buffer was added from a bottle top dispenser adequate mixing occurred. The SPE tubes had previously been fitted with 20 ml reservoirs and adapters and conditioned with 5 ml ACN followed by 10 ml of SPE conditioning buffer. The tubes were allowed to drain by gravity in all cases. After the supernatents had drained through the SPE tubes, assisted with a small amount of suction when necessary, the tubes were washed with 10 ml ACN:H_2O (v/v 50:50), and 'dried' by suction for 10 s at −0.3 to −0.4 bar. The endectocides were eluted with 10 ml ACN containing 0.1% TEA into 16 mm × 100 mm silanized disposable glass test tubes. The eluents were then dried under nitrogen (N-Evap) at 60 °C. The test tubes were cooled, and the residue dissolved in 500 ul ACN:H_2O (50:50). The tubes were vortexed, centrifuged at 1750 rpm for 30 s (clinical centrifuge) and the contents transferred, using polypropylene pipette tips into filter vials (Whatman Mini-UniPrep, 12 mm × 32 mm, with PVDF filters). The contents were analyzed by LC–MS–MS sequentially under both negative and positive ion ES conditions.

3.1. Spectra

The standard stock solutions were diluted 1:4 with ACN: H_2O (v/v 50:50), and initially, negative ion or positive ion spectra were recorded using a syringe pump connected directly to the electrospray probe with a variety of buffers at 10 ul/min. From the observed results for positive ions, either the acetonitrile–water–formic acid buffer or the acetonitrile–formic acid–ammonium formate buffer was chosen, and positive ion spectra were repeated with the syringe pump (10 ul/min) connected into the HPLC outlet with a PEEK tee, and with a main buffer flow rate of 300 ul/min. Product ion spectra were recorded at several collision energies, and the signal intensities plotted to obtain the optimum voltage for each product transition. The two most abundant product ions of the marker residue for each compound (the B1 or B1a isomer) were selected for MRM, and other MS parameters were then optimized for maximum signal. Similarly, negative ion electrospray spectra were obtained again with the syringe pump 'teed' into a flow of 300 ul/min of the acetonitrile–triethylamine buffer. After LC conditions were established, the spectra were repeated by injection on the column. In this way the parent ions could be verified as those obtained from the syringe pump injection and that the same adduct ions were formed.

3.2. Multiple reaction monitoring

Parent and product ion masses were calculated from the elemental compositions. The optimum collision energies were obtained as described above. The conditions are shown in Tables 1 and 2.

3.3. LC–MS–MS analysis and quantitation

In order to obtain the most reproducible and maximized signals and reduce cross-over effects from other types of analyses, the inner and outer cone assemblies of the Quattro Ultima were cleaned before each set of samples with a mixture of methanol, water, formic acid (45:45:10) in an ultrasonic cleaner. For each batch of samples the following order of injection was followed. Pure (chemical) standards in ACN: water (50:50 v/v) were injected for one hour followed by milk matrix blanks. The method matrix standard curve samples were then followed by the unknown or spiked milk samples and finally the method matrix standard curve samples were re-injected. The column and HPLC were then washed with ACN. Calibration curves were generated by the Quanlynx (Mass lynx 4.0) software using a quadratic polynomial and 1/x weighting. A linear function was acceptable over a narrower range, i.e., 0–10 ppb. The limits of detection were obtained by injecting milk samples that had been spiked at levels which gave signal to noise (S/N) values approximately 3:1 (0.2–0.5 ppb), and calculated according to Miller and Miller, [21] (Table 3). The lower values of repeat measures of the standard curve, 0 to 10 ppb, were used to calculate CCα and CCβ according to document ISO 11843-2:2000 (E), Section 5.2 (Table 4).

Table 3
Limits of detection of avermectins in negative and positive MS modes

Compound	Negative ion				Positive ion				Positive vs negative
	LOD 0.2 ppb spike (ppb)	LOD 0.5 ppb spike (ppb)	Pooled[a] (ppb)	LOQ (ppb)	LOD 0.2 ppb spike (ppb)	LOD 0.5 ppb spike (ppb)	Pooled (ppb)	LOQ (ppb)	
Abamectin	0.21	0.27	0.25	0.63	0.15	0.29	0.25	0.45	
Doramectin	0.21	0.17	0.19	0.57	0.06	0.26	[a]	0.18	Sig[b]
Emamectin	0.35	0.39	0.38	0.85	0.19	0.14	0.16	0.57	Sig[c]
Eprinomectin	0.21	0.50	[a]	0.63	0.10	0.27	[a]	0.30	Sig[b]
Ivermectin	0.26	0.36	0.33	0.72	0.23	0.22	0.23	0.69	
Moxidectin	0.28	0.41	0.37	0.84	0.31	0.32	0.32	0.93	

[a] If the variances of the data spiked at the 0.2 and 0.5 ppb levels were found to be not different (*F*-test), the pooled LOD was calculated, otherwise, not calculated, and the 0.2 ppb value is used.
[b] The variances of the positive to negative data were found to be significantly different for both the 0.2 and 0.5 ppb spikes.
[c] The variances of the positive to negative data were found to be significantly different for only the 0.5 ppb spikes.

Table 4
Calculations of CCα and CCβ of avermectins in negative and positive mode

Compound	Negative ion		Positive ion	
	CCα (ppb)	CCβ (ppb)	CCα (ppb)	CCβ(ppb)
Abamectin	0.24	0.49	0.13	0.26
Doramectin	0.29	0.58	0.26	0.52
Emamectin	0.24	0.48	0.47	0.94
Eprinomectin	0.24	0.49	0.31	0.63
Ivermectin	0.46	0.92	0.23	0.46
Moxidectin	0.36	0.72	0.29	0.58

4. Results

4.1. Negative ion spectra

The negative ion spectra were dominated by the $[M-H]^-$ pseudo-molecular ions of each compound. An adduct ion with increased mass of 62 Da is present in most of the spectra (from the flow injection analysis) and at present cannot be explained, but perhaps is due to an impurity in triethylamine. When the spectra were recorded after separation by the HPLC column, the adducts became $[M-H]^- + 46$ DA which is perhaps due to formate. All of the spectra, except that of selamectin exhibit a loss of 110 Da, which was hypothesised by Howells and Sauer [19] to be loss of the neutral fragment dimethylpyranose ($C_7H_{10}O$). This is a collisional loss which occurs in the cone entrance of the mass spectrometer. Fragmentation of the $[M-H]^-$ ions in the collision cell (Fig. 2), generally follows the scheme given by Howell and Sauer [19] using the ion trap mass spectrometer, but with some differences. The major fragment of abamectin, ivermectin, doramectin, emamectin and eprinomectin is, as before [19], the loss of the disaccharide neutral to leave the macrocyclic lactone ion, which is used as the primary ion for quantitation. Loss of 82 Da, the neutral pyranose fragment or loss of 110, the dimethylpyranose fragment [19], were not as significant in the collision cell of the Quattro Ultima. Consequently, smaller structurally significant ions which arise from fragmentation of the oleandrose portion were used for the confirmation ion, for abamectin, ivermectin, doramectin, emamectin and eprinomectin. The concentration of

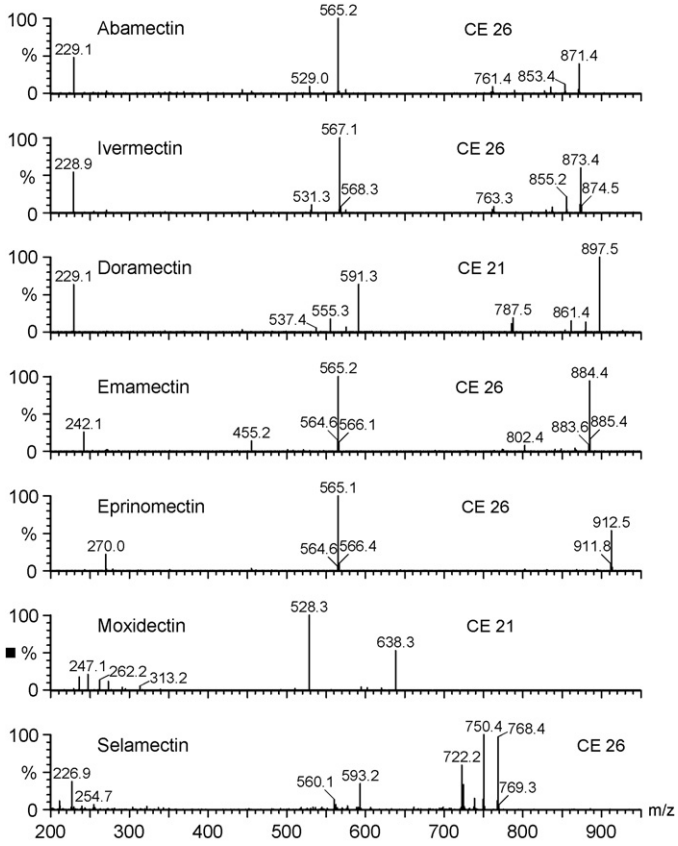

Fig. 2. Negative ion collision spectra of avermectins: product ions of $[M-H]^-$ precursors of abamectin *m/z* 871, ivermectin *m/z* 873, doramectin *m/z* 897, emamectin *m/z* 884, eprinomectin *m/z* 912, moxidectin, 638 and selamectin *m/z* 768.

triethylamine was found to be optimum at 1 mM in the final buffer (Fig. 3)

4.2. Positive Ion Spectra

When the HPLC buffer contained only acetonitrile or methanol and water and formic acid, the positive spectra of moxidectin, for example, exhibited an $[M+H]^+$ pseudo-molecular ion, which was accompanied by a series of

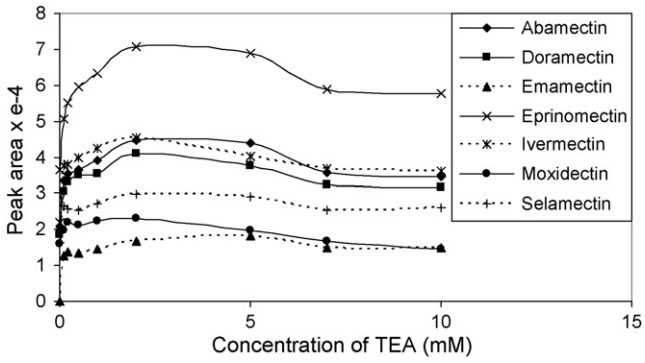

Fig. 3. Effect of triethylamine concentration on negative ion signal intensity.

adduct ions (i.e. $[M+Na]^+$, $[M+K]^+$, $[M+Na+CH_3OH]^+$, $[M+Na+CH_3CN]^+$ etc.) with relative abundances which were not completely dependent upon the composition of the buffer. In the spectra of the three compounds that contain only C, H and O, abamectin, doramectin and ivermectin, the $[M+Na]^+$ adduct ions predominated, with $[M+K]^+$, and $[M+Na+CH_3OH]^+$, or $[M+Na+CH_3CN]^+$ in much lower abundance. The $[M+H]^+$ ion was in very low abundance, and not useful as a characteristic ion. The nitrogen containing compounds, emamectin, eprinomectin and selamectin exhibited mainly MH^+ ions with the sodium and potassium adducts in much lower abundance. For all compounds, the Na adducts and Na plus solvent adducts were most predominant when the organic component of the buffer was MeOH and least when it was CH_3CN, and thus the latter was used as the organic component of the HPLC buffer. It was observed that the sodium adducts of abamectin, doramectin, and ivermectin, were very stable and high collision energies were required for fragmentation and that even with high collision energies few product ions, characteristic of the compound, were observed. This was especially the case with abamectin as the $[M+Na]^+$ ion tended to disappear as the collision energy was increased without any new ions being detected. The fragment probably was Na^+, whose mass was below the calibration range. Similar observations for adduct ions of ivermectin and moxidectin have previously been reported [12,22]. It was also observed that the predominance of the $[M+Na]^+$ ions was maximized after calibration with the sodium–cesium iodide mixture and that the ratio of $[M+Na]^+/[M+H]^+$ ions decreased over several weeks. Similarly the calibration curves using the Na adduct ions were very non-linear with a strong positive curvature that was not reproducible. One can assume this behaviour has been observed previously, as Stout et al. [12] commented that in fortified extracts the relative intensity of the $[M+Na]^+$ ion increased markedly compared to the ratio observed for standards and attributed it to ubiquitous traces of sodium in the extracts. Similarly Wu et al. [15], observed the formation of the $[M+Na]^+$, $[M+K]^+$ adducts and observed that it was difficult to obtain correct results when Na^+ and K^+ were present. Finally in many of the methods in which positive ion MS was used for confirmation, it appears that quantitation was undertaken using LC with fluorescence detection as there is no mention of the linearity of the MS method ([10,14,16,18]. Because of this non-linearity, the positive ion method was initially abandoned and the negative ion method developed. A similar conclusion was reached by Wu et al. [15].

From early experiments, however, it was clear that a positive ion method might give greater sensitivity than a negative ion method and the ESI-MS conditions were re-evaluated. It seemed possible that the $[M+Na]^+$ adduct might be replaced by a more amenable adduct, $[M+NH_4]^+$, by addition of ammonium formate or ammonium acetate to the buffer [12,16]. Consequently it was decided to minimize the use of sodium in the method

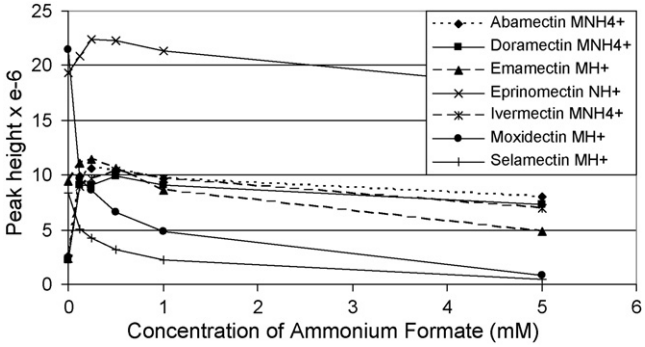

Fig. 4. Effect off ammonium ion concentration on positive ion signal intensities.

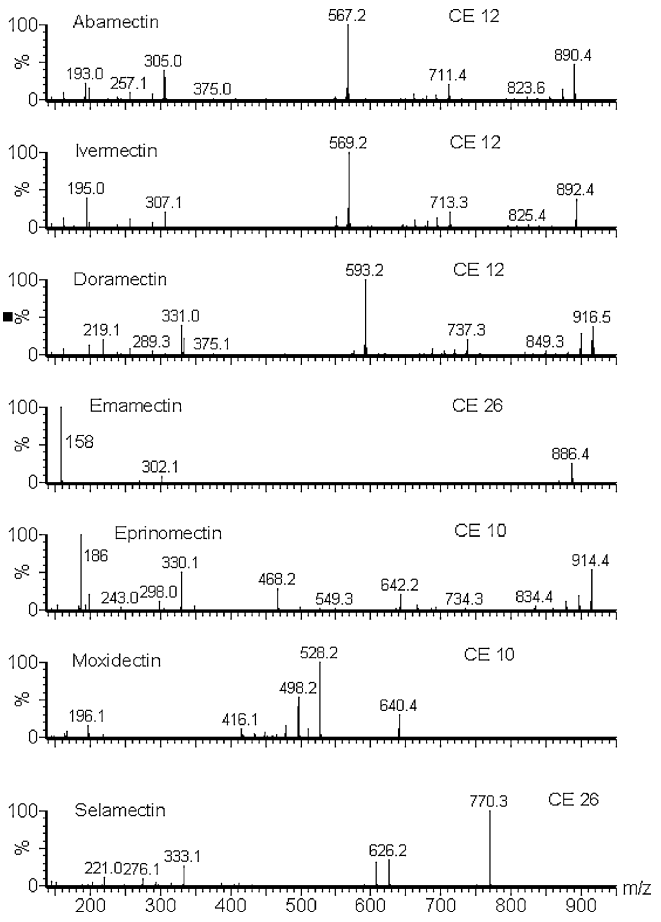

Fig. 5. Positive collision ion spectra of avermectins: product ions of $[M+NH_4]^+$ precursors of abamectin *m/z* 890, ivermectin *m/z* 892, doramectin *m/z* 916, and $[M+H]^+$ precursors of emamectin *m/z* 886, eprinomectin *m/z* 914, moxidectin, 640 and selamectin *m/z* 770.

and the instrument was calibrated with the polyethylene glycol solution instead of NaCsI.

The relative signals intensities of $[M + NH_4]^+$, $[M + Na]^+$ and $[M + H]^+$ were found to depend upon the concentration of ammonium formate added to the formic acid 'buffer'. By 'titrating' the amount of ammonium it was possible to optimize the relative amounts of these ions to maximize either the MH^+ or $[M + NH_4]^+$ ions (Fig. 4). When even a small amount of ammonium formate was added, the signal for the $[M + NH_4]^+$ adduct in the spectra of the CHO compounds, abamectin, doramectin and ivermectin, became much greater than that of the $[M + Na]^+$ adduct. For the nitrogen containing compounds, emamectin, eprinomectin, moxidectin and selamectin, the $[M + H]^+$ ion predominated over $[M + NH_4]^+$, which only became significant at very much greater concentrations of ammonium formate. Similar observations were made by Ali et al. [16]. The signals for abamectin, doramectin, emamectin, eprinomectin and ivermectin, either $[M + H]^+$ or $[M + NH_4]^+$ were observed to be maximum in the range 0.2–0.5 mM in the buffer, i.e. 2–5 mM in the 1% formic acid. Unfortunately, the $[M + H]^+$ ions of moxidectin and selamectin decreased rapidly with increasing ammonium concentrations (Fig. 4). Thus a compromise of 0.3 mM ammonium formate was chosen. The $[M + H]^+$ or $[M + NH_4]^+$ ions, therefore, made ideal parent ions, characteristic of each of the compounds, and which were relatively easy to fragment (Fig. 5).

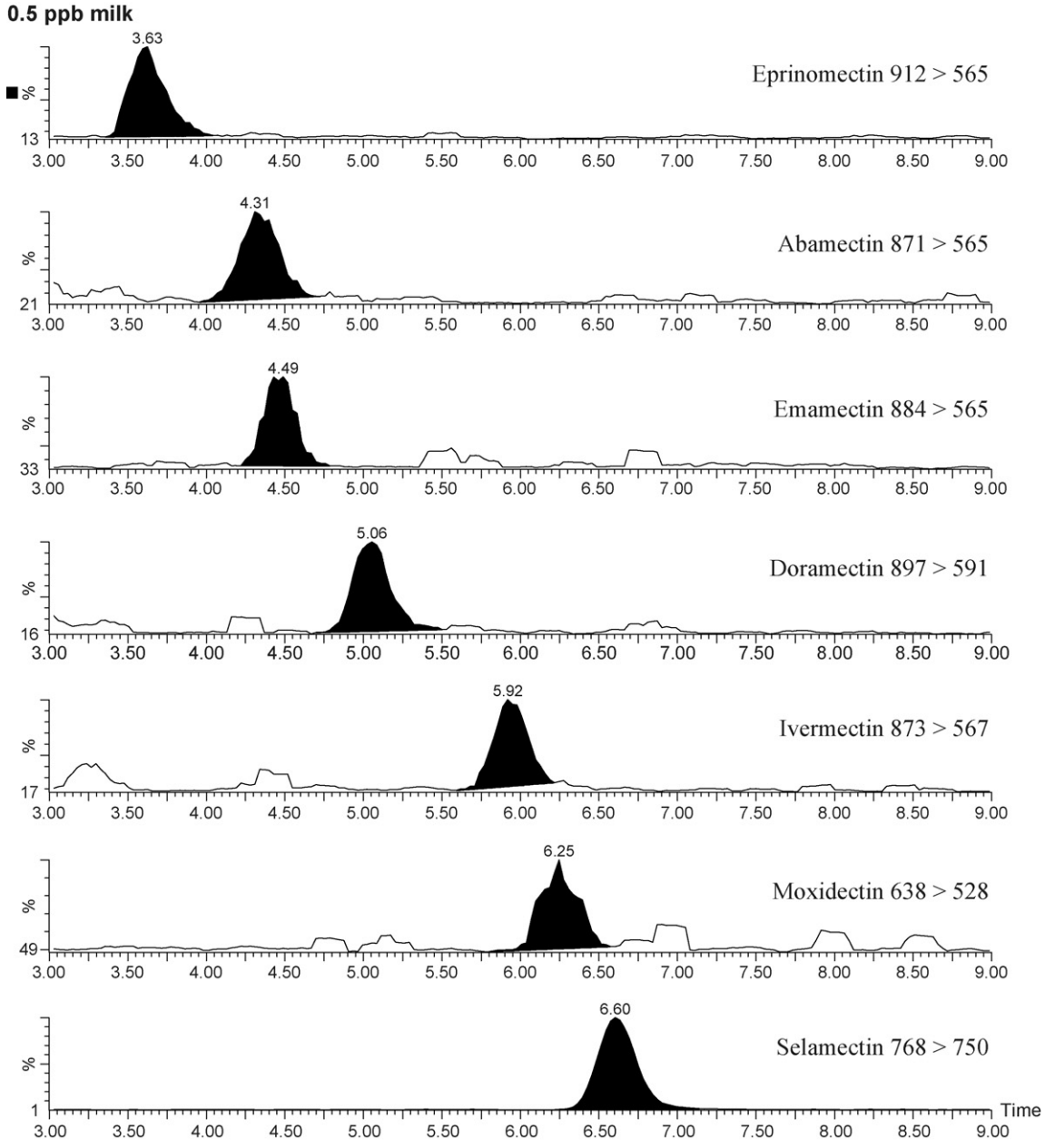

Fig. 6. Negative ion chromatograms of the avermectins spiked into raw milk at 0.5 ppb.

4.3. Calibration

Both positive and negative ion calibration curves exhibited basically linear responses. The residual plots, however, indicated a slightly negative curvature. Although many reports in which electrospray or atmospheric pressure chemical (APCI) ionization list linear calibration functions [17,13,15], others report the use of second order polynomials gave the better results [19]. A variety of theoretical models have been developed to explain the electrospray process. Such a model [23], predicts a negatively curving polynomial at increasing concentration.

One set of calibrations values, randomly chosen, were examined for the effects of different treatments: quadratic versus linear, various weighting factors (none, $1/x$, $1/x^2$, $1/y$, $1/y^2$) [24], and internal versus external standard. It was observed that a quadratic calibration function with $1/x$ weighting and internal standard gave the best results. Selamectin was chosen for the internal standard as it is designed for treatment of pet animals and is less likely to be used in food producing animals. If it were to be suspected as present in a milk sample, it could be quantified either by the external standard method or by using one of the other avermectins as internal standard.

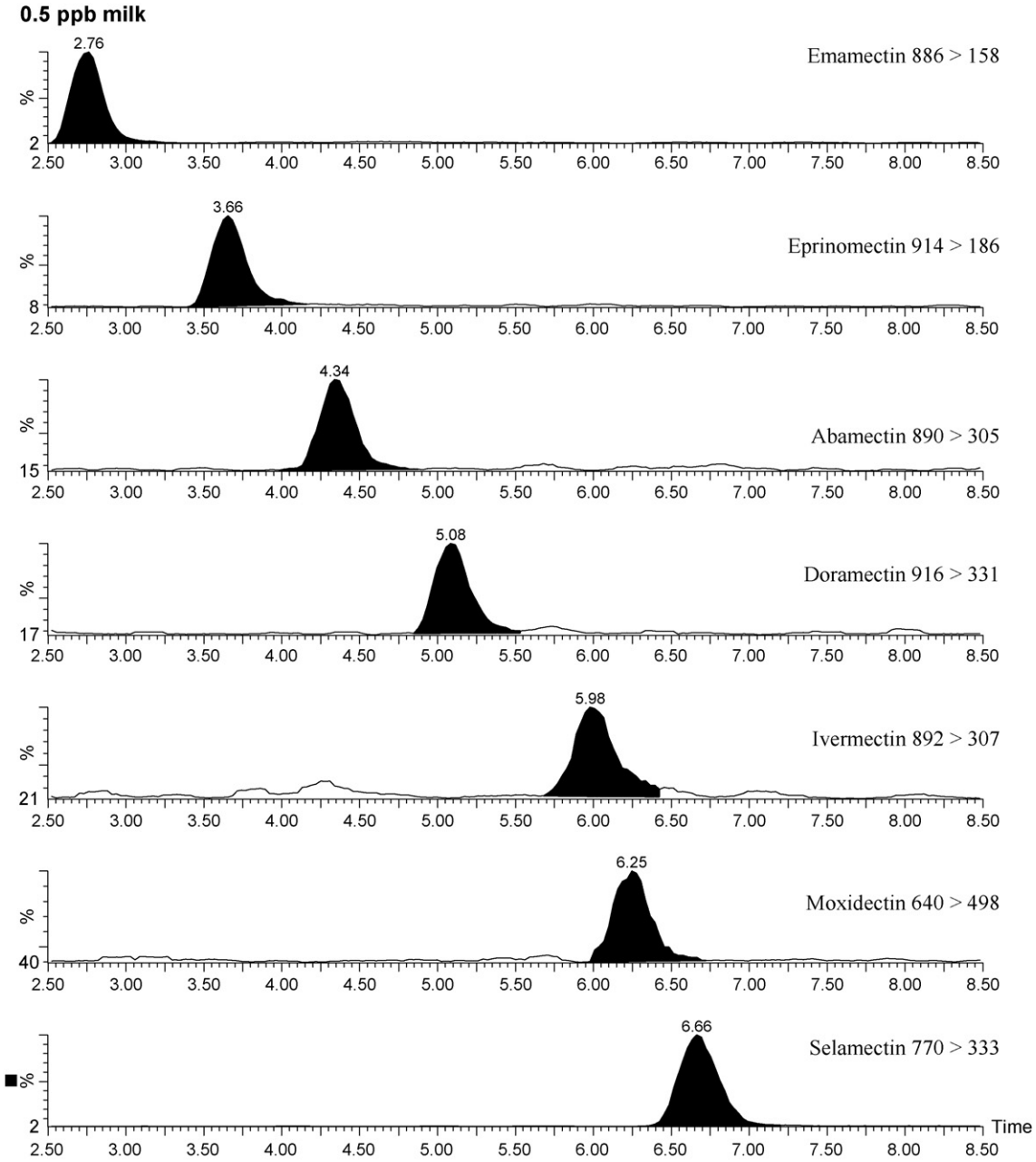

Fig. 7. Positive ion chromatograms of the avermectins spiked into raw milk at 0.5 ppb.

Table 5
Recovery of avermectins: within-day repeat measures

Compound	Amount added (ppb)	Negative ion						Positive ion					
		Amount calculated (ppb)	SD	Recovery %	RSD	Ion ratio Lo/Hi	SD	Amount calculated (ppb)	SD	Recovery %	RSD	Ion ratio Lo/Hi	SD
Abamectin	0.5	0.54	0.11	107.3	20.1	0.77[a]	0.18	0.54	0.11	108.6	20.3	0.39	0.07
	2	2.17	0.09	108.6	4.2	0.65	0.10	2.07	0.23	103.5	11.0	0.43	0.04
	5	5.24	0.53	104.8	10.1	0.61	0.04	5.09	0.30	101.9	5.9	0.44	0.02
	20	19.83	0.82	99.1	4.1	0.62	0.05	20.98	0.55	104.9	2.6	0.43	0.01
	50	48.87	1.90	97.7	3.9	0.64	0.03	47.83	2.94	95.7	6.1	0.43	0.00
Doramectin	0.5	0.51	0.03	101.3	6.8	0.59	0.05	0.54	0.12	108.9	22.7	0.32	0.01
	2	2.09	0.07	104.4	3.4	0.62	0.08	2.23	0.04	111.4	1.8	0.33	0.01
	5	5.32	0.33	106.4	6.2	0.64	0.03	5.37	0.70	107.5	13.0	0.33	0.01
	20	19.45	1.06	97.3	5.5	0.63	0.03	21.08	0.55	105.4	2.6	0.34	0.01
	50	49.76	0.90	99.5	1.8	0.63	0.01	47.89	3.14	95.8	6.6	0.35	0.02
Emamectin	0.5	0.32	0.11	64.9	33.2	0.52[a]	0.09	0.48	0.01	96.8	2.6	0.047	0.002
	2	2.17	0.13	108.3	6.1	0.34	0.02	2.02	0.16	101.1	8.1	0.049	0.001
	5	5.40	0.46	108.0	8.5	0.39	0.03	5.42	0.69	108.4	12.7	0.047	0.001
	20	20.19	1.82	101.0	9.0	0.37	0.02	22.54	2.49	112.7	11.1	0.046	0.002
	50	48.45	1.03	96.9	2.1	0.39	0.02	50.77	5.42	101.5	10.7	0.046	0.001
Eprinomectin	0.5	0.58	0.08	116.0	13.6	0.31	0.05	0.55	0.03	109.3	6.2	0.31	0.01
	2	1.83	0.36	91.7	19.9	0.32	0.05	1.88	0.34	94.0	18.1	0.30	0.02
	5	4.96	0.70	99.1	14.1	0.31	0.04	5.04	0.72	100.8	14.3	0.29	0.02
	20	18.76	2.85	93.8	15.2	0.33	0.01	20.94	2.23	104.7	10.6	0.30	0.00
	50	45.72	5.03	91.4	11.0	0.33	0.01	46.48	7.33	93.0	15.8	0.30	0.01
Ivermectin	0.5	0.51	0.11	101.3	21.0	0.39	0.09	0.51	0.04	101.3	8.0	0.67	0.15
	2	1.93	0.17	96.5	8.7	0.45	0.06	2.19	0.10	109.7	4.7	0.69	0.04
	5	5.07	0.08	101.4	1.5	0.42	0.03	5.59	0.87	111.7	15.6	0.67	0.05
	20	19.67	1.63	98.4	8.3	0.42	0.01	20.96	0.67	104.8	3.2	0.64	0.01
	50	49.29	1.83	98.6	3.7	0.43	0.00	51.25	4.31	102.5	8.4	0.64	0.00
Moxidectin	0.5	0.58	0.17	116.7	29.6	0.33	0.27	0.56	0.09	111.7	16.1	0.71	0.14
	2	2.40	0.21	120.1	8.8	0.22	0.11	2.22	0.15	111.2	6.6	0.69	0.05
	5	5.28	0.86	105.7	16.2	0.24	0.05	5.17	1.06	103.4	20.5	0.78	0.10
	20	23.07	1.59	115.3	6.9	0.20	0.01	19.82	1.59	99.1	8.0	0.74	0.02
	50	53.98	3.40	108.0	6.3	0.22	0.02	49.31	3.47	98.6	7.0	0.69	0.02

[a] ratio outside limit.

4.4. Extraction

The milk in this study came primarily from Holstein cattle, which are used as the primary milk producers in North America. Holstein milk contains approximately 3.6% fat, 3.2% protein and 4.7% lactic acid [25]. The protein was precipitated by warm acetonitrile, and removed by centrifugation. Sodium chloride was not added to the acetonitrile–water solution, as is conventionally done to generate a bilayer, because of the problems with positive ions described above. The acetonitrile solutions were filtered to reduce chance of the SPE tubes becoming plugged, either with Whatman No 41 filter paper or with Pall glass fibre/GxF Acrodisc filters as these were found to have no differences on recoveries. On the other hand when AP4523 (PVDF/GxF) filters were compared to the No 41 filter paper, small but significant decreases in recovery were observed for eprinomectin, moxidectin and selamectin. Since the avermectins are very fat soluble, the fat was not extracted at this point to prevent loss by co-extraction. This lipophilicity of the avermectins was used in the SPE process. To avoid drying down the solvent, the acetonitrile was diluted with buffer to the point that the avermectins preferentially bound to the C18 layer. The SPE columns were washed with dilute acetonitrile to remove fat and other compounds. Elution with 100% acetonitrile, broke the binding to the SPE phase, and addition of triethylamine prevented binding to other surfaces [16,19]. A wash with hexane was added after SPE to remove eluted fat. After removal of the solvent the analytes were then redissolved in 50% acetonitrile in water to provide solvent focusing in the 73% acetonitrile HPLC buffer. Silanized tubes were used for the extracts and drying step as plain glass tubes caused erratic results.

4.5. Relative sensitivity

The positive ion signals were considerably larger than the negative ion signals. When the signals of the most intense transitions were compared, the senstivity increased by factors ranging from 6 for selamectin to 550 for emamectin. The negative ion signals for all compounds were fairly similar. However the emamectin positive signal was very much larger than the positive ion signals of the other compounds. Since the ion intensity was so large for Emamectin, due to the very efficient formation of the $[M+H]^+$ ion, the detector gain was reduced by a factor of 4.5 for positive ions, and the maximum concentration limited to 60 ppb (instead of the originally proposed 100 ppb) to prevent detector saturation and yet be able to run the samples under both polarities.

4.6. Chromatography

Isocratic conditions were chosen for both the negative assay and the positive assay. With the acetonitrile content of 73% in the HPLC buffer, the retention times were almost identical for most of the compounds with the basic and acidic buffers. Only the retention time of emamectin changed, with a much shorter retention time being measured under the acidic positive ion conditions. The multiport valve was used to divert the flow to waste during the solvent front and after the compounds eluted.

4.7. Limits of detection and quantitation

The limits of detection and quantitation (LOQ) were measured two ways. Initially, estimates of the levels at which the raw signal to noise was about 3:1 was determined from spiked milk samples at low levels. These values were generally between 0.2 and 0.5 ppb. Spikes at these two levels were run with each batch of samples and compiled over several weeks. The limit of detection was calculated as: LOD = blank + 3.28 × standard deviation of the spikes, where 3.28 is the t value at which both types I and II errors are less than 5% [21]. The variances were compared for the 0.2 ppb group and the 0.5 ppb group. If they were not different (F test), a pooled LOD was calculated. This assumes a normal distribution and constant standard deviation as the low concentration spike approaches zero. When they were different, the LOD was based upon the 0.2 ppb values. The LOQ was then determined as LOQ = blank + 10 × standard deviation of the spikes. This data is shown in Table 3.

The limits of detection and quantitation were also calculated from the calculated values of the spikes in the calibration curves as CCα and CCβ (Table 4) following the European model as described in the ISO document 11843-2:2000 (E). The formulae are those under case one i.e. assumption of homoscedacity i.e constant standard deviation. The values are similar to the values calculated by the first method. The CCα and CCβ values were also calculated using the weighted heteroscedastic model, and were found to be lower than those in Table 4. Unfortunately they represented concentrations at which the signal to noise would be <=3, and thus not usable.

Figs. 6 and 7 demonstrate the sensitivity of the method. The avermectins were spiked into raw milk at concentrations of 0.5 ppb which are close to the LOQ values and the same sample was analysed firstly in negative ion mode with 70 μl injected and then later in positive ion mode with 35 ul injected and the detector gain reduced. The chromatograms were subjected to smoothing with a seven point moving average, which normally is used during quantitation.

4.8. Within day precision

The within-day (intra-assay) precision and repeatability with $n = 3$ was initially studied over the range 0.5–50 ppb (Table 5). It must be remembered that the 0.5 ppb values are at, or below, the measured limits of quantitation determined either as LOQ (Table 3) or as CCβ (Table 4), and thus RSD values greater than 20% may be expected. For the 2–50 ppb values, in the negative ion mode, the precision, presented as percent recovery, ranges from 96 to 120% The relative standard deviations (RSD) for abamectin, doramectin, emamectin and ivermectin were generally below 10%, while moxidectin had somewhat larger RSD and eprinomectin the largest RSD. In positive ion similar results were observed. Again eprinomectin had the greatest variation with seconded by emamectin with RSDs somewhat greater than 10%. It is interesting to note that the high mass spectrometric

Table 6
Recovery of avermectins: between-day repeat measures

Compound	Amount added (ppb)	Negative ion				Positive ion			
		Amount calculated (ppb)	SD	Recovery %	RSD	Amount calculated (ppb)	SD	Recovery %	RSD
Abamectin	0.5	0.55	0.14	110.7	24.8	0.53	0.12	106.1	21.8
	2	1.96	0.11	97.9	5.6	1.98	0.06	99.2	3.2
	5	5.08	0.26	101.7	5.2	4.94	0.27	98.8	5.4
	20	20.40	0.96	102.0	4.7	20.61	0.96	103.0	4.7
	50	49.18	1.44	98.4	2.9	50.09	2.82	100.2	5.6
Doramectin	0.5	0.52	0.04	104.2	7.5	0.51	0.04	101.8	7.9
	2	2.00	0.20	99.8	10.2	1.99	0.14	99.7	7.1
	5	4.75	0.21	95.0	4.5	5.08	0.26	101.5	5.2
	20	20.32	1.27	101.6	6.3	20.30	0.80	101.5	3.9
	50	48.03	0.46	96.1	1.0	50.69	1.33	101.4	2.6
Emamectin	0.5	0.52	0.02	103.9	3.6	0.53	0.02	105.6	3.3
	2	2.06	0.21	103.2	10.3	1.80	0.13	90.1	7.1
	5	5.13	0.67	102.6	13.0	4.72	0.07	94.5	1.5
	20	20.31	0.98	101.6	4.8	20.08	3.10	100.4	15.5
	50	48.61	2.28	97.2	4.7	54.84	3.12	109.7	5.7
Eprinomectin	0.5	0.35	0.21	70.1	60.0	0.47	0.07	94.9	15.3
	2	1.92	0.23	96.1	12.0	2.03	0.37	101.7	18.0
	5	5.05	0.27	101.1	5.3	5.19	0.93	103.8	17.9
	20	19.97	1.74	99.8	8.7	22.21	3.20	111.1	14.4
	50	47.91	2.37	95.8	4.9	53.15	3.87	106.3	7.3
Ivermectin	0.5	0.40	0.01	79.2	1.8	0.62	0.00	123.8	0.7
	2	2.12	0.12	106.1	5.5	2.07	0.41	103.4	19.8
	5	5.27	0.44	105.4	8.4	5.38	1.22	107.6	22.7
	20	21.28	0.41	106.4	1.9	21.46	2.64	107.3	12.3
	50	51.91	3.31	103.8	6.4	51.07	3.89	102.1	7.6
Moxidectin	0.5	0.50	0.10	99.9	19.6	0.59	0.02	118.3	3.7
	2	2.10	0.11	104.9	5.3	2.07	0.39	103.7	18.8
	5	5.04	0.51	100.8	10.2	5.70	1.44	113.9	25.2
	20	20.01	1.81	100.0	9.1	21.63	1.25	108.2	5.8
	50	49.70	0.72	99.4	1.5	47.16	4.43	94.3	9.4

sensitivity for emamectin in positive ion mode did not translate into lower RSD values, as might be expected, and thus one could assume the major contribution to the variation must be due to the extraction process. The ion ratios were compared to the average ion ratio (outliers removed by Grubbs test) obtained from the matrix matched standard curve for each experiment (Table 5). In all cases for the 2 to 50 ppb spikes the ion ratios were within the ratio limits set by the European Union Commission Decision 2002/657/EC guidelines. The ion ratios for the 0.5 ppb spikes were also within the limits except those for abamectin and eprinomectin in negative ion mode.

4.9. Between day precision

The results for the between-day (intermediate) precision with $n = 5$ are presented in Table 6. The recoveries for the 2–50 ppb spikes, in negative ion mode, are similar to those in the within-day results, ranging from 95 to 106%, with RSD again mostly below 10%. As expected the recoveries and variation of the 0.5 ppb level are higher. Again the positive ion results, excluding the 0.5 ppb values, although similar to the negative ion results exhibit somewhat less precision (90.1% to 113.9%) with RSD from 2.6% to 25.2%. The results for eprinomectin, ivermectin, and moxidectin exhibited the greatest variations in positive ion mode. The tolerances of the ion ratios for each experiment were all within the European Union Commission Decision 2002/657/EC guidelines.

4.10. Correlation between positive ion results and negative ion results

The results of the individual samples analysed in both negative ion mode and positive ion mode showed excellent correlations between the positive and negative ion results for abamectin, doramectin, emamectin, eprinomectin, ivermectin and moxidectin (correlation coefficients, r^2 of 0.9964, 0.9983, 0.9879, 0.9801, 0.9904 and 0.9895, respectively).

5. Conclusions

The two methods presented are suitable for the quantitation and verification of the presence of avermectins in raw milk

over the concentration range of approximately 0.5 ppb up to 60 ppb. They enable the detection of avermectins that have no permitted tolerance levels, abamectin, doramectin, emamectin, and ivermectin at sub ppb concentrations. The calibration range also covers the range for those with permitted MRL levels in the appropriate jurisdictions, eprinomectin (12 ppb or 20 ppb) and moxidectin (40 ppb). The problems experienced by previous methods for positive ions, in which the response exhibited a strong positively curved response with increasing concentration, have been overcome by careful modification of the buffer with ammonium formate. Although the negative ion method demonstrates somewhat lower sensitivities than does the positive ion method, the reproducibility is somewhat greater and this method has been chosen for use in our laboratory.

Acknowledgements

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References

[1] W.L. Shoop, H. Mrozik, M.H. Fisher, Vet. Parasitol. 59 (1995) 139.
[2] J.W. Tolan, P. Eskola, D.W. Fink, H. Mrozik, L.A. Zimmerman, J. Chromatogr. 190 (1980) 367.
[3] A.C. Goudie, N.A. Evans, K.A. Gration, B.F. Bishop, S.P. Gibson, K.S. Holdom, B. Kaye, S.R. Wicks, D. Lewis, A.J. Weatherley, et al., Vet. Parasitol. 49 (1993) 5.
[4] W.L. Shoop, P. DeMontigny, D.W. Fink, J.B. Williams, J.R. Egerton, H. Mrozik, M.H. Fisher, B.J. Skelly, M.J. Turner, Int. J. Parasitol. 26 (1996) 1227.
[5] J.V. Pivnichny, J.S. Shim, L.A. Zimmerman, J. Pharm. Sci. 72 (1983) 1447.
[6] B. Roudaut, Analyst 123 (1998) 2541.
[7] M.S. Ali, T. Sun, G.E. McLeroy, E.T. Phillippo, J. AOAC Int. 83 (2000) 31.
[8] F.J. Schenck, L.H. Lagman, J. AOAC Int. 82 (1999) 1340.
[9] M. Danaher, M. O'Keeffe, J.D. Glennon, L. Howells, Analyst 126 (2001) 576.
[10] D.N. Heller, F.J. Schenck, Biol. Mass Spectrom. 22 (1993) 184.
[11] A. Khunachak, A.R. Dacunha, S.J. Stout, J. AOAC Int. 76 (1993) 1230.
[12] S.J. Stout, E. Wickremesinhe, A.R. daCunha, A. Khunachak, J. AOAC Int. 83 (2000) 1446.
[13] A.I. Valenzuela, M.J. Redondo, Y. Pico, G. Font, J. Chromatogr. A 871 (2000) 57.
[14] K. Yoshii, A. Kaihara, Y. Tsumura, S. Ishimitsu, Y. Tonogai, J. Chromatogr. A 896 (2000) 75.
[15] Z. Wu, J. Li, L. Zhu, H. Luo, X. Xu, J. Chromatogr. B Biomed. Sci. Appl. 755 (2001) 361.
[16] M.S. Ali, T. Sun, G.E. McLeroy, E.T. Phillippo, J. AOAC Int. 83 (2000) 39.
[17] I. Rudik, M.R. Cummings, R.H. Poppenga, J. Vet. Diagn. Invest. 14 (2002) 295.
[18] S.B. Turnipseed, J.E. Roybal, H.S. Rupp, S.A. Gonzales, A.P. Pfenning, J.A. Hurlbut, Rapid Commun. Mass Spectrom. 13 (1999) 493.
[19] L. Howells, M.J. Sauer, Analyst 126 (2001) 155.
[20] R.E. Baynes, T. Martin-Jimenez, A.L. Craigmill, J.E. Riviere, Regul. Toxicol. Pharmacol. 29 (1999) 287.
[21] J.N. Miller, J.C. Miller, Statistics and Chemometrics for Analytical Chemistry, Prentice Hall, Harlow, 2000.
[22] S. Croubels, S. De Baere, M. Cherlet, P. De Backer, J. Mass Spectrom. 37 (2002) 840.
[23] C.G. Enke, Anal. Chem. 69 (1997) 4885.
[24] A.M. Almeida, M.M. Castel-Branco, A.C. Falcao, J. Chromatogr. B 774 (2002) 215.
[25] R.G. Jensen, Handbook of Milk Composition, Academic Press, San Diego, 1995.