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Quantitative Determination of Ivermectin in Raw Milk Using Positive ESI LC-MS/MS

MEENAKSHI DAHIYA, SHANTA SEN, KIRAN LAMBA,
MANJEET AGGARWAL and R. K. KHANDAL*

Shriram Institute for Industrial Research
University Road, Delhi-110007, India
rkhandal@shriraminstitute.org

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Abstract: Ivermectin, a veterinary drug, is commonly used endectocide for animal husbandry. The drug is available in the form of subcutaneous or topical formulations. Its application may cause accumulation of its residues into the animal tissues, which ultimately find their way into the food products, such as milk and meat products. In order to determine the residues of ivermectin in milk, a comparatively simple, sensitive and rapid method was developed and validated using LC-MS/MS. The MRM transitions corresponding to *m/z* 892.71>569.6, 892.71>551.5 and 892.71>307.3 were used for the purpose of quantification and evaluation of other parameters of the method. The limit of detection of the method was found to be 0.1 µg/kg and the limit of quantitation was calculated as 0.2 µg/kg. The method was found to be linear in the range of 1.0 ng/mL to 100.0 ng/mL with correlation coefficient of 0.9992 for pure calibration curve and 0.9990 for the matrix- matched calibration curve. The recoveries of ivermectin from the spiked samples of raw milk were found between 85 to 105%.

Keywords: Liquid chromatography, Mass spectrometry, Ivermectin, Milk.

Introduction

Ivermectin[1] B_{1a} (Figure 1), a broad spectrum antiparasitic veterinary drug derived from the bacterium *Streptomyces avermitilis,* is a member of the macrocyclic lactone class of endectocides, commonly referred to as avermectins. All the drugs belonging to the class are used for controlling helminthes and ectoparasities in animals[2-3].

Ivermectin is available in the form of subcutaneous and topical formulations and is used in the doses of 0.2 and 0.5 mg/kg body weight[4-5]. All avermectins are highly lipophilic and tend to accumulate in fat tissues, which act as a reservoir, contributing to their long-term persistence in the body[6].

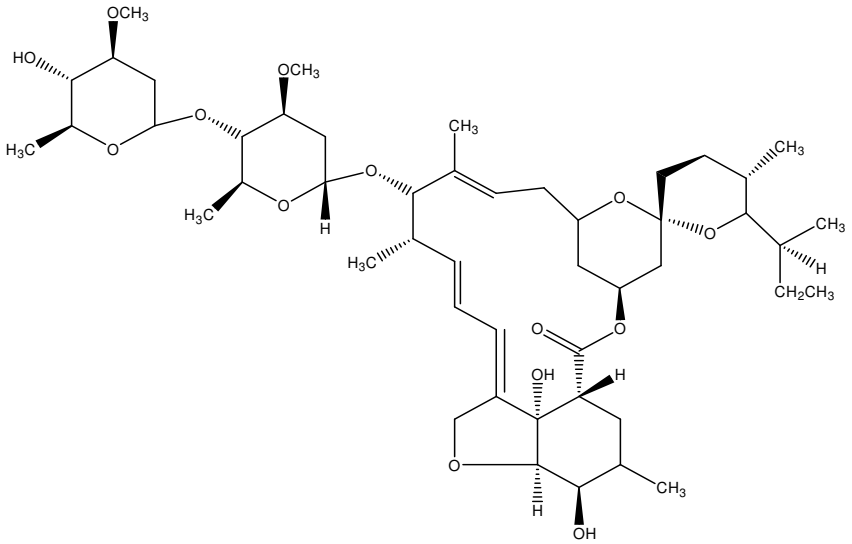

Figure 1. Structure of ivermectin B_{1a} ($C_{48}H_{74}O_{14}$)

Ivermectin residues may be found in various products of animal origin like milk and meat[7]. In a recent study in Brazil, ivermectin residues between 2 to 10 ppb were found in 17.8% of milk samples purchased from retail markets[8]. Since the residues of ivermectin are responsible for several health hazards, it therefore becomes essential that the residues be strictly regulated from food safety point of view[9]. Maximum residue levels for ivermectin residues in food products have been fixed by the various regulatory authorities: Joint Expert Committee of Food Additives and Contaminants (JECFA) has recommended a temporary MRL of 10 ppb for ivermectin in milk[10], a provisional acceptable residue (PAR) of 20 ppb for ivermectin in milk has been proposed in the United States[11], EU has fixed MRL value of 10 ppb for milk. Since 2009, the EU regulatory agencies have incorporated the limits of residues of ivermectin in milk for all their imports of milk and milk products from other countries. Accordingly, residue monitoring is being executed at the national level in India for both milk and the various milk products for export purposes. Therefore, in order to determine ivermectin residues present at such low levels in the various food products of animal origin, a few methods have been reported as per the published literature, most of them being for the simultaneous determination of residues of ivermectin[12-14] and other endectocides of avermectin group using LC-MS/MS in negative ionization mode. A method has been reported for the determination of ivermectin using atmospheric pressure chemical ionization (APCI) using the positive ionization mode[15]. All the reported methods involve the use of exhaustive extraction procedures.

The present paper describes an analytical method developed for determination of residues of ivermectin in milk using LC-MS/MS with ESI in positive ionization mode, which can easily be adapted by the laboratories in India and other parts of the world. The aim was to develop a method that involved a simple and less time-consuming extraction procedure. To begin with, the already developed methods as reported in the literature were tried mainly with the objective that they could be made simpler. But however, simplifying the extraction procedure resulted in loss of sensitivity of the method. On the other hand, if the extraction procedure was kept same as per the published methods, very low detection levels were observed. Assuming that the variation in the extraction procedure made the method unsuitable in negative ionization mode, the efforts were made to determine the

ivermectin content by using positive ionization mode for the analysis. In fact, results obtained in the positive ionization mode were found to be much better than that obtained in the negative ionization mode. Using positive ionization mode, intense signals were observed that enabled the detection of ivermectin to still lower levels, as compared to that reported using the negative ionization mode *i.e.* 0.1 µg/kg against the reported value 0.25 µg /kg[12]. The method has the potential to be the preferred one by the facilities in the developing world. Moreover, this may pave way for simplification of other methods for residual avermectins.

Experimental

Reference standard of ivermectin B_{1a} with purity of > 99% was purchased from Sigma Aldrich. Acetonitrile, water and methanol (liquid chromatographic grade) were purchased from Merck Specialties Private Limited. Ammonium formate (analytical reagent grade) was purchased from Loba Chem Private Limited. Formic acid and *n*-hexane (analytical reagent grade) was purchased from S.D Fine chem. Limited. Anhydrous sodium sulphate was purchased from Rankem chem. limited.

Instrumentation

LC-MS/MS system:Waters 2695 series Alliance quaternary liquid chromatographic system (Waters, USA) with a Triple Quadrupole Mass Spectrometer, Quattro micro API (Micro mass, UK) equipped with a electro spray interface and masslynx 4.1 software (Micro mass) for data acquisition and processing was used. The instrument was provided with a 120-vial capacity sample management system.

Separation and analysis was carried out on Waters X Terra MS C-18, 5 µm and 2.1 x 100 mm column. Balance with readability of 0.01 mg and capacity of 180 g, Afcoset 3200, Mettler toddler was used. Model-Spinix (Tarsons Products Pvt Ltd) vortex was used Syringe filters of pore size 0.2 mm and 0.45 mm, with diameter of 25 mm (Advanced Microdevices Private Limited) was used. Disposable 50 mL conical centrifuge tubes with screw caps (Tarsons Products Pvt Ltd) was used. Rapid Vap (Labconco Corporation) nitrogen evaporator was used. The extracts were centrifuged by using a high-speed refrigerated centrifuge, the rotor head was suitable for eight 50 mL sample tubes (Remi Sales and Engineering Ltd).

Milk samples

Two samples of milk containing 7.0% and 3.5% of fat were obtained from local milk processors and were initially tested for the presence of ivermectin before freezing and storage at -20 °C. Sample no. 1 and 2 contain 7.0% and 3.5% fat respectively.

Method

Preparation of standard solution

Approximately 10.0±0.1 mg ivermectin reference standard was accurately weighed into a 100 mL volumetric flask and dissolved and made to volume using methanol. This gave a stock solution of 100 µg/mL. The solution was stored at 2-8 °C. From the stock solution 1 mL of aliquot was taken and diluted to 100 mL to give a standard solution of ivermectin having a concentration of 1 µg/mL. The solutions were stored at 2° to 8 °C.

Preparation of calibration standard solutions

From the standard solution having concentration of 1 µg/mL, appropriate aliquots were taken and further diluted with methanol so as to give a series of calibration standard

solutions having ivermectin concentration of 1, 2.5, 5.0, 10, 25, 50 and 100 ng/mL respectively. All solutions were stored at 2 ° to 8 °C.

Preparation of matrix- matched calibration standard solutions

For the matrix-matched calibration curve seven portions of 5.0±0.1 g sample of milk were taken separately in seven individual centrifuge tubes and extracted using the procedure as given under the heading sample preparation. The individual extracts obtained were evaporated to dryness and reconstituted using ivermectin solutions containing 1, 2.5, 5.0, 10, 25, 50 and 100 ng/mL respectively, so as to give matrix matched calibrations standards having concentrations 0.2, 0.5, 1.0, 2.0, 5.0, 10, 20 µg/kg.

Preparation of mobile phase

The mobile phase was prepared by mixing two solutions *i.e*. A and B in the ratio of 10:90 (A: B) and filtered through 0.45-micron filter membrane using the Millipore filtration unit. Solution A (Ammonium formate 5 mM) prepared by dissolving 158 mg of ammonium formate in 500 mL volumetric flask using water and solution B (0.1% formic acid in methanol) prepared by adding 0.5 mL of formic acid in 500 mL of methanol.

Preparation of sample

Approximately 5.0±0.1 g of the liquid milk sample equilibrated at room temperature was taken in the centrifuge tube and extracted with 10 mL of 50:50 mixtures of acetonitrile and methanol using vortex mixer. The solution was then centrifuged at ambient temperature for 10 minutes at 7000 rpm followed again by centrifugation at 4 °C at 7000 rpm for further 10 minutes. The supernatant layer was collected in a dry separating funnel. The residue was extracted using the same process twice. The combined organic solvent from all the three extractions was passed through anhydrous sodium sulphate and washed with n-hexane saturated with acetonitrile. This solvent was then evaporated to dryness under the stream of nitrogen and the dried extract was re-dissolved in 1ml methanol before injecting into LC-MS/MS.

Liquid Chromatography- Mass Spectrometry (LC-MS-MS) conditions

Column

The separation of ivermectin was carried out using X Terra MS C-18 column (2.1 mm × 100 mm; 5 µm) and mobile phase comprising of A: 5 mM ammonium formate; B: 0.1% formic acid in methanol; (A:B-10:90 in the isocratic mode). The LC column was set at 50 °C.

ESI Interface

Optimal parameters of the ESI interface were optimized by infusing 100 ng/mL standard solution of ivermectin in the mobile phase using a Harvard syringe pump. LC-MS/MS determination was performed by operating the mass spectrometer in positive ionisation mode.

Typical MS settings

Capillary voltage (kV): 3.5; cone voltage (V): 20; source temperature (°C): 120; dessolvation temperature (°C): 450. Mass spectra was acquired over a scan range of *m/z* from 100 Da to1000 Da for MS/MS mode, product ion scan mass spectra of protonated molecules of ivermectin was acquired in the mass range of 200 Da to1000 Da. Three different characteristic fragments *i.e*. 892.71>569.6, 892.71>551.5 and 892.71>307.3 were monitored for ivermectin in multiple reaction monitoring mode (MRM) using a dwell time of 50 milli seconds and collision energy of 18 (V) was used.

Results and Discussion

Liquid chromatographic separation

A comparatively simple, sensitive and an accurate method was developed for the determination of ivermectin residues in raw milk using positive ESI LC-MS/MS. Using the chromatographic conditions as mentioned above, a well resolved peak for ivermectin was obtained within two minutes of the injection in the positive ionization mode. Optimum separation was achieved using 5 mM ammonium formate (A) and 0.1% formic acid in methanol (B) in the ratio 10:90 as mobile phase.

Extraction procedure

For the extraction of ivermectin from the raw milk samples, a simplified extraction procedure was developed as compared to the ones in the existing analytical methods reported[12,14]. The previous methods[12] have reported the use of tris buffer and SPE techniques for sample cleanup which not only makes the sample preparation cumbersome but the method was also proned to errors resulting in low recoveries and accuracy of the results. Based upon the past experience of the authors, the extraction method was thereby simplified as has been described above. Since ivermectin is soluble in solvents like methanol and acetonitrile, a combination of methanol and acetonitrile was taken for extracting the residues of ivermectin from milk samples. Any fat components which might have been coextracted along with the ivermectin residues were washed off with *n*-hexane saturated with acetonitrile. The extract was dried off under nitrogen and the dried extract was dissolved in 1 mL methanol and injected into LC-MS/MS.

Mass spectrometery

For the purpose of evaluating the fragment ions and the intensity of the signals, the reference standard solution of ivermectin was infused using both positive and negative ESI mode of the mass spectrometer detector. The results showed that the signals for both positive and negative mode were comparable and either of the modes could be used for the purpose of development of method for determination of residues of ivermectin in milk. But, when the same solution was passed through the liquid chromatographic column using the reported aqueous solution of triethyl amine as the mobile phase in negative ionisation mode[12], although all ions 873.3>229.25 and 873.3>567.03 were distinctly observed, the signal response was poor. Hence the same was tried using mobile phase comprising of ammonium formate in the positive ionization mode which produced highly intense signal so as to detect the ivermectin residues up to the concentration levels of 0.1 μg/kg. The result obtained using 1.0 μg/mL solution of ivermectin in both positive and the negative ESI mode is tabulated (Table 1).

Table 1. Intensity of response of the MS detector for various concentrations of standard ivermectin solutions examined under *+ve* ESI mode and *–ve* ESI mode

Concentration of ivermectin, ng/mL	Response (area count)	
	Positive ESI mode	Negative ESI mode
1000	1101623	13088
500	550488	7480
250	286899	3569
100	112631	1263
50	55474	Not detected
25	30369	Not detected
10	13503	Not detected
5	6960	Not detected
2.5	3206	Not detected
1.0	1327	Not detected

Mass spectrum of ivermectin using both ESI-MS and ESI-MS/MS is given in Figure 2. From the mass spectrometric data, it can be seen that the parent component *i.e*. ivermectin shows a molecular mass of 892.7 instead of 874.5 as per the molecular structure. The explanation lies in the fact that parent component, which is ivermectin with the mass 874.5, gets ammoniated in the presence of ammonium formate used in the mobile phase and the same gets fragmented as per the scheme given in Figure 3.

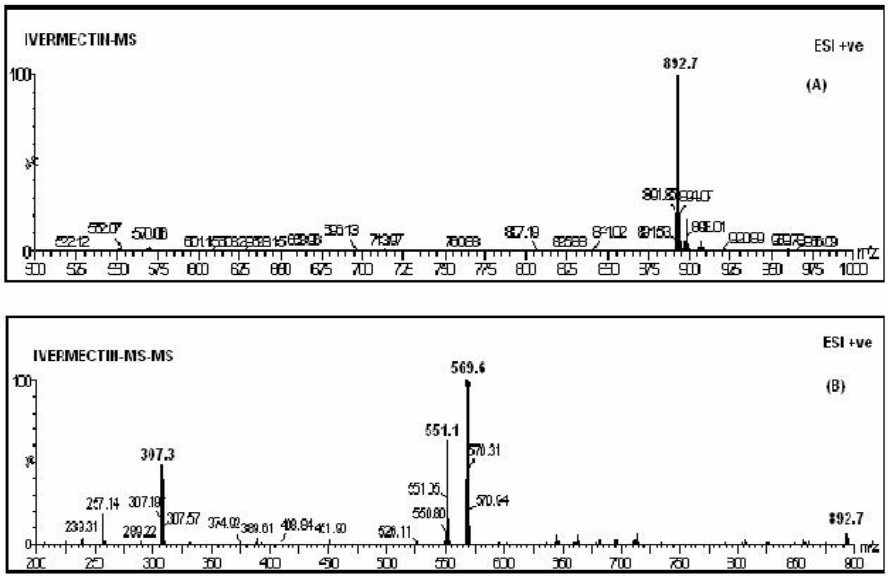

Figure 2. Mass spectrum of ivermectin using *+ve* ESI-MS (A) and *+ve* ESI-MS-MS (B)

Here it may be noted that the fragment ions detected, match exactly with the reported data[15] for ivermectin in the positive ionization mode (Table 2). For the purpose of developing and validating the method the three most distinct ions *i.e.* 569.6, 551.5 and 307.3 have been used.

Table 2. Fragment ions produced using *+ve* ESI mode and *–ve* ESI mode for the confirmation of ivermectin.

Compound	Mode	Ion 1	Ion 2	Ion 3	Ion 4	Ion 5
Ivermectin	*+ve* ESI	892.7	569.6	551.5	307.3	567.3
Ivermectin	*-ve* ESI	873.3	229.25	567.03	Not Detected	Not Detected

LC-MS-MS identification criteria for ivermectin

In spite of the fact that extraction procedure was simplified compared to the ones already reported earlier, it was observed that the extracts were free from any interference due to the presence of any other components in the matrix. Although the method gives good specificity, it is nevertheless important to establish criteria that can be used as the basis for rigorous identification. From the replicate analysis of milk samples spiked at different concentration levels *i.e*. 1.0 µg/kg, 2.5 µg/kg and 5.0 µg/kg, the ion ratios for the ions *m/z* 569.6/551.5 and *m/z* 551.5/307.3 were found within the acceptable range of ±20% against the predicted ion ratio of 5.90 and 2.0 respectively (Table 3). 569.6 was taken as a quantifier ion and 551.5 was taken as a qualifier ion. This is well within the acceptable limits. Combining this ion ratio criteria with the requirements of the analyte to fall within the retention time window of 1.8±0.5% (1.8±0.09= 1.71-1.89) minutes, provided the basis for identification criteria.

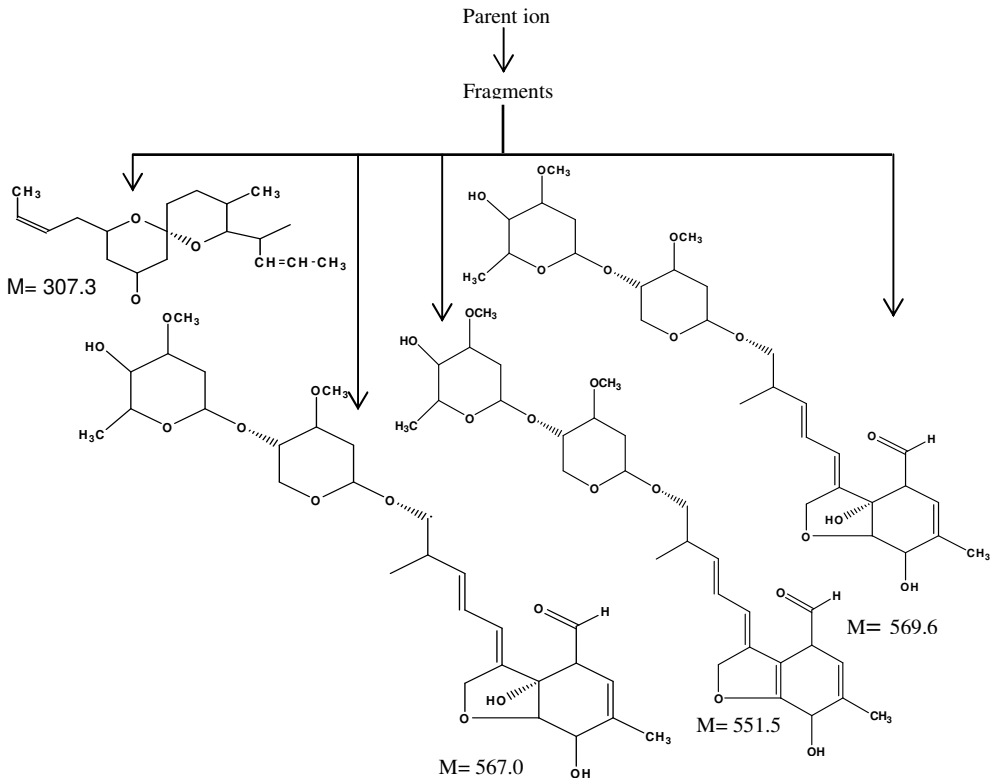

Figure 3. Fragmented ions of ivermectin

Table 3. Ion ratios of fragment ions predicted *vis a vis* observed in *+ve* ESI mode

Concentration of ivermectin, ng/mL	Ion ratio of *m/z* 569.6/551.5				Ion ratio of *m/z* 551.5/307.3			
	Predicted	Observed	Mean	% RSD	Predicted	Observed	Mean	% RSD
1.0	5.9	6.19 5.91 6.08 5.60	5.78	5.78	2.0	1.71 1.75 1.85 2.00	1.92	10.30
2.5	5.9	5.66 5.25 5.24 5.41 5.59 5.75 5.78 6.04	5.63	4.97	2.0	2.11 2.15 1.80 1.85 1.96 2.10 2.21 2.22	2.02	9.0
5.0	5.9	5.35 5.42 5.66 5.67 6.07 6.19	5.72	5.76	2.0	1.75 1.85 1.90 1.98 1.74 2.20	1.90	8.94

Method Performance Characteristics

The method was validated as per the international union of pure and applied chemistry (IUPAC) and Eurachem guidelines[16].

Linearity

Seven calibration standards evenly spread over the concentration range of interest and encompassing the concentration levels reflecting EU regulatory limits were analyzed. The calibration standards were run in triplicate. The calibration curve prepared using the pure standards were found to be linear in the range of 1 ng/mL to 100 ng/mL with correlation coefficient of 0.9992. Linearity of the matrix- matched calibration standards in the concentration range of 0.2 µg/kg to 20 µg/kg and at the same concentration levels as that for the calibration standards was also evaluated in triplicate. The calibration curve for the matrix-matched standards was also found to be linear with correlation coefficient of 0.9990. The matrix effect was investigated by comparing standards in solvent with matrix-matched standards at different concentration levels. The relative response (Response matrix matched/ Response solvent) for the concentration levels of 1.0 ng/mL, 2.5 ng/mL, 5.0 ng/mL, 10 ng/mL, 25 ng/mL, 50 ng/mL and 100 ng/mL in solvent with respect to 0.2 µg/kg, 0.5 µg/kg, 1.0 µg/kg, 2.0 µg/kg, 5.0 µg/kg, 10 µg/kg and 20 µg/kg for milk was found to be 0.9850, 0.9950, 0.9952, 0.9982, 0.9965, 0.9910 and 0.9976 respectively. Since the data indicates that the matrix does not significantly suppress or enhance the response of the instrument, therefore, for all the calculation purposes pure calibration curve was used because of its simpler and easier operability.

Specificity

The chromatographic interferences from the milk samples were investigated by comparing the chromatograms of blank and the spiked samples. For this purpose, samples were prepared using the same procedure as mentioned earlier and the specificity of the method was measured. It was found that the presence of interferences did not have any effect on the quantitative results of the analyte of interest thus providing reliability of the LC-MS/MS method for determination of ivermectin Figure 4 and 5.

Precision

Precision studies were carried out for both intra-day and inter-day repeatability and reproducibility (Table 4). Three spiked samples of milk at different concentration levels *i.e.* 1.0 µg/kg, 2.5 µg/kg and 5.0 µg/kg respectively were injected seven times on the same day and the same number of times on three subsequent days by three different analysts. The low %RSD value obtained for intra-day and inter-day variation within the acceptable norms showed that the proposed method is precise and can be adopted for analysis.

Table 4. Intra-day and Inter-day precision data for the proposed method for ivermectin residues in two samples of milk

Conc. of ivermectin µg/kg	Sample	Day 1		Day 2		Day 3		Intra-assay	
		Ivermectin concn obtained µg/kg, n=7	% RSD	Ivermectin concn obtained µg/kg, n=7	% RSD	Ivermectin concn obtained µg/kg, n=7	% RSD	Ivermectin concn obtained µg/kg, n=7	% RSD
1.0	1	1.05	3.72	1.04	3.55	1.10	3.65	1.22	3.62
	2	1.10	3.56	1.02	3.48	0.99	3.38	1.04	3.55
2.5	1	2.51	2.82	2.52	2.76	2.47	2.92	2.51	2.71
	2	2.49	2.75	2.53	2.86	2.51	2.85	2.49	2.82
5.0	1	5.10	2.25	5.15	2.42	5.11	2.2	5.05	2.35
	2	5.08	2.31	5.10	2.34	5.05	2.6	5.10	2.27

Milk sample no. 1 contains 7.0% fat, Milk sample no. 2 contains 3.5% fat

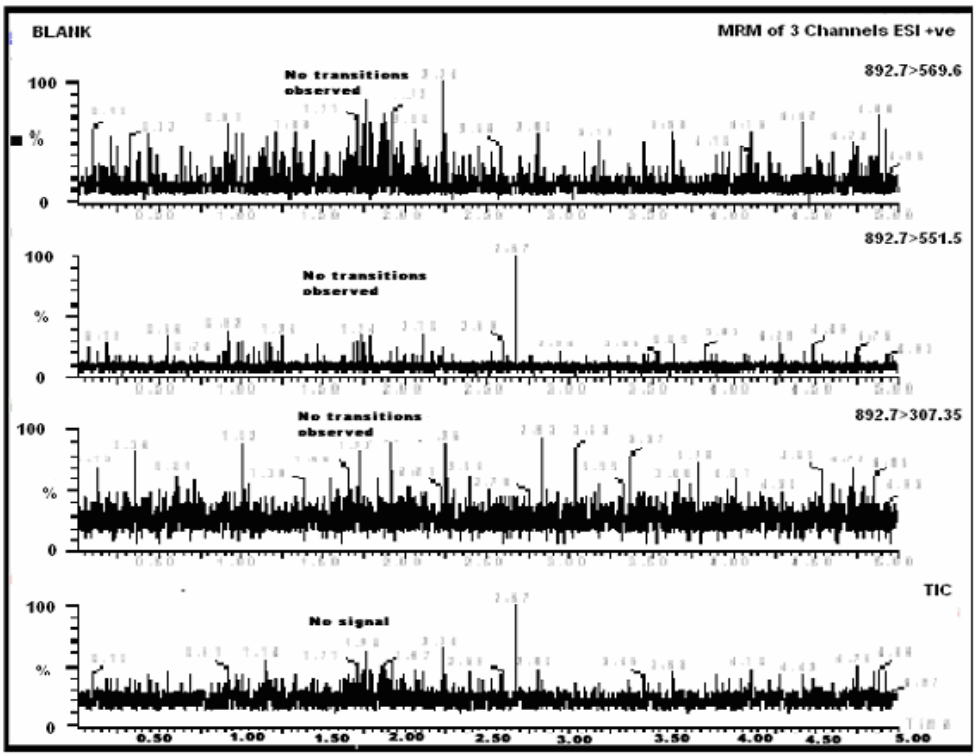

Figure 4. MRM transitions of control sample of milk showing absence of ivermectin

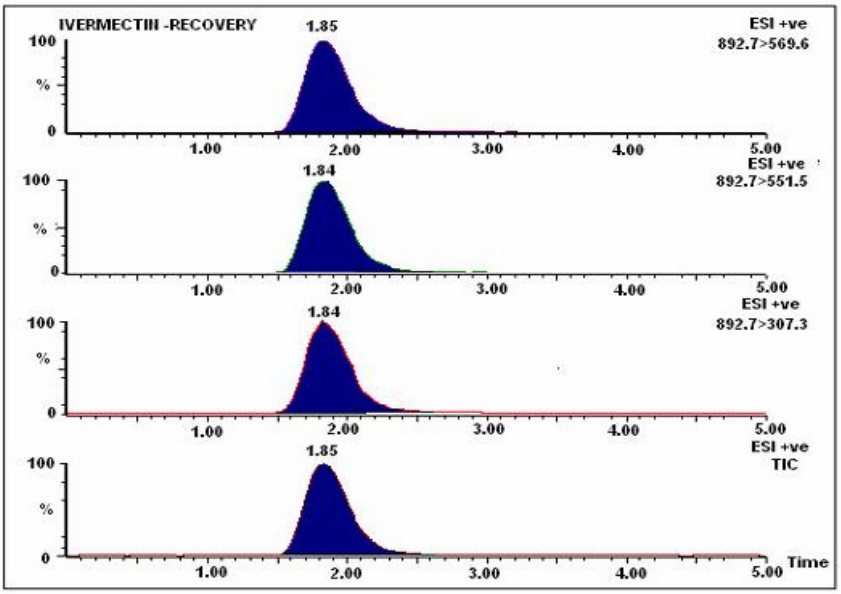

Figure 5. MRM transitions showing Ivermectin from analysis of spiked samples of milk (Ivermectin concentration at 5.0 µg/kg).

Accuracy

The recoveries (Table 5) of ivermectin in spiked samples were calculated to study the effect of matrix on the determination of ivermectin. The recovery studies were carried out at five different concentrations. For this five different portions of pre-analyzed milk samples were spiked with 1.0 µg/kg, 2.5µg/kg, 5µg/kg, 10 µg/kg and 20 µg/kg respectively in triplicate on three different days and then extracted and determined by the same method as mentioned earlier. The recoveries of ivermectin from the milk samples were found in the range of 85% to 105%.

Table 5. Percent recovery of ivermectin from two different milk samples analyzed on different days (n=3).

Spiking level, µg/kg	Sample No	Day 1		Day 2		Day 3	
		Amount calculated, µg/kg	% Recovery	Amount calculated, µg/kg	% Recovery	Amount calculated, µg/kg	% Recovery
1.0	1	0.85	85.0	0.86	86.0	0.85	85.0
	2	0.84	84	0.85	85	0.84	84
2.5	1	2.26	90.4	2.24	89.6	2.30	92.0
	2	2.24	89.6	2.21	88.4	2.28	91.2
5.0	1	4.88	97.6	4.90	98.0	4.80	96.0
	2	4.85	97	4.84	96.8	4.86	97.2
10.0	1	9.78	97.8	9.11	91.1	9.85	98.5
	2	9.68	96.8	9.25	92.5	9.55	95.5
20.0	1	20.8	104.0	19.8	99.0	20.2	101.0
	2	19.9	99.5	20.1	100.5	19.6	98.0

Milk sample no 1 contains 7.0% fat, Milk sample no 2 contains 3.5 % fat.

Robustness

Robustness of the method was determined by analyzing the same set of spiked samples (*i.e.* samples spiked at concentration levels of 1.0 µg/kg, 5.0 µg/kg and 20 µg/kg) under different parameters; such as same column chemistry from different manufacturers, different analysts, and different injection volumes. The method was found to be robust even with small changes in analytical conditions: change in flow rate (± 0.05 mL/min), a change in column temperature (± 5 °C), use of same column from different manufacturer (waters C18 column and Varian C-18). Under all of these conditions, the analytical values of the spiked samples were not affected and it was in accordance with the actual values.

Limit of detection (LOD) and limit of quantitation (LOQ)

LOD was determined by considering signal to noise (S/N) ratio of 3:1 for the strongest mass transition with respect to the background noise obtained from the blank sample whereas LOQ was determined similarly by considering signal to noise ratio (S/N) ratio of 6:1. Based upon the mean noise level for the ten injections each of the matrix blank of two milk samples, lowest detection limit of the instrument was calculated as 0.1 µg/kg and confirmed using standard solutions with concentration of 0.1 µg/kg and the lowest concentration levels that could be quantified with reproducible values was determined as 0.2 µg/kg and further confirmed by injecting matrix matched standard solution of ivermectin having concentration of 0.2 µg/kg. The level of detection and the level of quantitation of the method were calculated, taking into account the sample weight and the dilution factors: in this case the concentration factor since the dried extract contains 5 g of sample was made to 1 mL.

Conclusion

The developed method using positive ESI LC-MS/MS allows the detection, quantitation and confirmation of ivermectin in raw milk present at trace levels with high precision, accuracy and sensitivity by using simple extraction procedure. In spite of using a simplified extraction procedure, no interferences were observed from the matrix components during the determination of ivermectin residues. The method can be used for the routine analysis of ivermectin residues in milk with added advantages of speed and economy. The method can also be tried for ivermectin content in other animal products like meat and poultry.

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References

1. The Merck Index; Ivermectin, Fourteen Edition, **52** 48, 908.
2. Campbell W C, *New Zeal Vet J.,* 1981**, 29**, 174-178.
3. Vuik J, *J Chromatogr*., 1991, **553**, 299.
4. Toutain P L, Campan M, Galtier P and Alvinerie M, *J Vet Pharmacol Ther.,* 1988, **11**, 288-291.
5. Alvinerie M, Sutra J F, Galtier P and Mage C, *Res Vet Sci.,* 1999, **67**, 229-232.
6. Schenck F J and Lagman L H, *J AOAC Int.,* 1999, **75**, 747-750.
7. Bassissi M F, Alvinerie M and Lespine A, *Comp Biochem Physiol Part C: Toxicol Pharmacol.*, 2004, **138**, 437.
8. Lobato V, Rath S and Reyes F G R, *Food Addit Contam.,* 2006, **23(7)**, 668-673.
9. Koesukwiwat U, Jayanta S and Leepipatpiboon N, *J Chromatogr A,* 2007**, 1140**, 147-156.
10. Joint FAO/WHO Expert Committee on Food Additives (JECFA). Residues of some veterinary drugs in food animals (FNP 41-14) FAO Food and Nutrition Paper. WHO. 2002, p. 44.
11. Baynes R E, Martin-Jimenez T, Craigmill A L and Riviere J E, *Regul Toxicol Pharmacol.,* 1999, **29**,287-299.
12. David A Durden and Janice Wotske*, J AOAC Int.,* 2009, **92(2)**, 580-596.
13. Roberts Sheridan and Lucille Desjardins, *J AOAC Int*., 2006, **89(4)**, 1088-1094.
14. Alan L Chicoine, David A. Durden, George MacNaughton and Patricia M. Dowling, Can Vet J., 2007, 48(8), 836-838.
15. United States Department of Agriculture Food Safety and Inspection Service, Office of Public Health Science. SOP No. CLG-AVR1.01, 2003, 7-17.
16. Thompson M, Ellision S L R and Wood R, *Pure Appl Chem*., 2002, **74(5)**, 835-855.