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A Multiresidue Method for the Determination of Abamectin, Doramectin, Moxidectin, Ivermectin, Milbemectin A_3, and Milbemectin A_4 Residues in Bovine Muscle Using HPLC with Fluorescence Detection

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ABSTRACT

Abamectin, doramectin, moxidectin, ivermectin, milbemectin A_3 and milbemectin A_4 are similar macrocyclic lactone chemicals used as parasiticides or acaricides. A method using high performance liquid chromatography (HPLC) with fluorescence detection is presented for the simultaneous determination of the residue amounts of these compounds in bovine muscle. Samples are extracted using acetonitrile and cleaned up with solid phase extraction using a C18 column, followed by fluorescence-derivatized with 1-methylimidazole and trifluoroacetic anhydride in acetonitrile. The analogue was measured by HPLC with fluorescence detector at 365 nm excitation and 470 nm emission wavelengths. The limits of quantification are below the stipulated Taiwan Maximum Residue Limit for each compound. The recoveries of this method in bovine muscle ranged from 73.3 to 110%, with a RSD from 2.11 to 16.57%. The detection limit of those 6 compounds in bovine muscle was 5 ppb. No any above compounds were detected in 50 samples of bovine muscle tested. Therefore, the developed method can be used for rapid screen of macrocyclic lactones in bovine muscle.

Key words: macrocyclic lactone, high performance liquid chromatography, abamectin, doramectin, moxidectin, ivermectin, milbemectin A_3, milbemectin A_4

INTRODUCTION

Macrocyclic lactone, an antibiotic, is the fermentation product of *Actinomycetes*, genus *Streptomyces*, in soil. It can be classified into two groups: avermectins and milbemycins. The difference between these two is that avermectins have a disaccharide oxy residue at C_{13}, while milbemycins do not. Currently, domestic animal drugs with residue standards include abamectin, ivermectin, doramectin, moxidectin, and eprinomectin[(1)]; pesticides with safety tolerance specifications include abamectin, and mibemectin[(2)] (Figure 1).

Macrocyclic lactone is a strong pesticide for cow, sheep, pig, and horse. It presents good efficacy not only in killing interior nematodes, but also exterior arthropods. It is also named endectocides[(3)]. Its effective dose is very low, 0.2~0.5 mg/kg for animals. Studies showed that even macrocyclic lactone does not show any mutagenicity and carcinogenicity, but embryotoxicity might be possible[(4)]. According to MARTINDALE, the Extra Pharmacopoeia, ivermectin may induce mild Mazzotti reaction, including fever, pruritus, arthralgia, myalgia, postural hypotension, oedema, lymphadenopathy, gastrointestinal symptoms, soar throat, cough, and headache[(5)].

In the January 8, 2003, announcement of The Department of Health (DOH), Executive Yuan, Taiwan, Food Code No 0900002580[(1)], the "Residual Limit of Animal Drugs" listed macrocyclic lactone, but without its analysis methodology. In this study, we tried to establish a multi-residue HPLC method for analyzing such a compound. Meanwhile, milbemectin, structurally in the milbemycins group, was initially found with acaricidal effect. Its anthelmintic effect was not discovered until avermectins were analyzed[(6)]. According to current regulation,

Table. 1. Tolerance of abamectin, doramectin, ivermectin and moxidectin in Taiwan[(1)]

Compound	Species	Tissue/ product	Tolerance (ppm)
Abamectin	Cattle	Muscle and fat	0.1
		Kidney	0.05
Doramectin	Cattle	Muscle	0.01
	Swine		0.035
Ivermectin	Cattle	Liver	0.01
		Fat	0.04
	Swine, goat, sheep	Liver	0.015
	and poultry	Fat	0.02
	Cattle	Milk	0.01
Moxidectin	Cattle and deer	Muscle	0.02
	Sheep		0.05
	Cattle, sheep and deer	Liver	0.1
		Kidney	0.05

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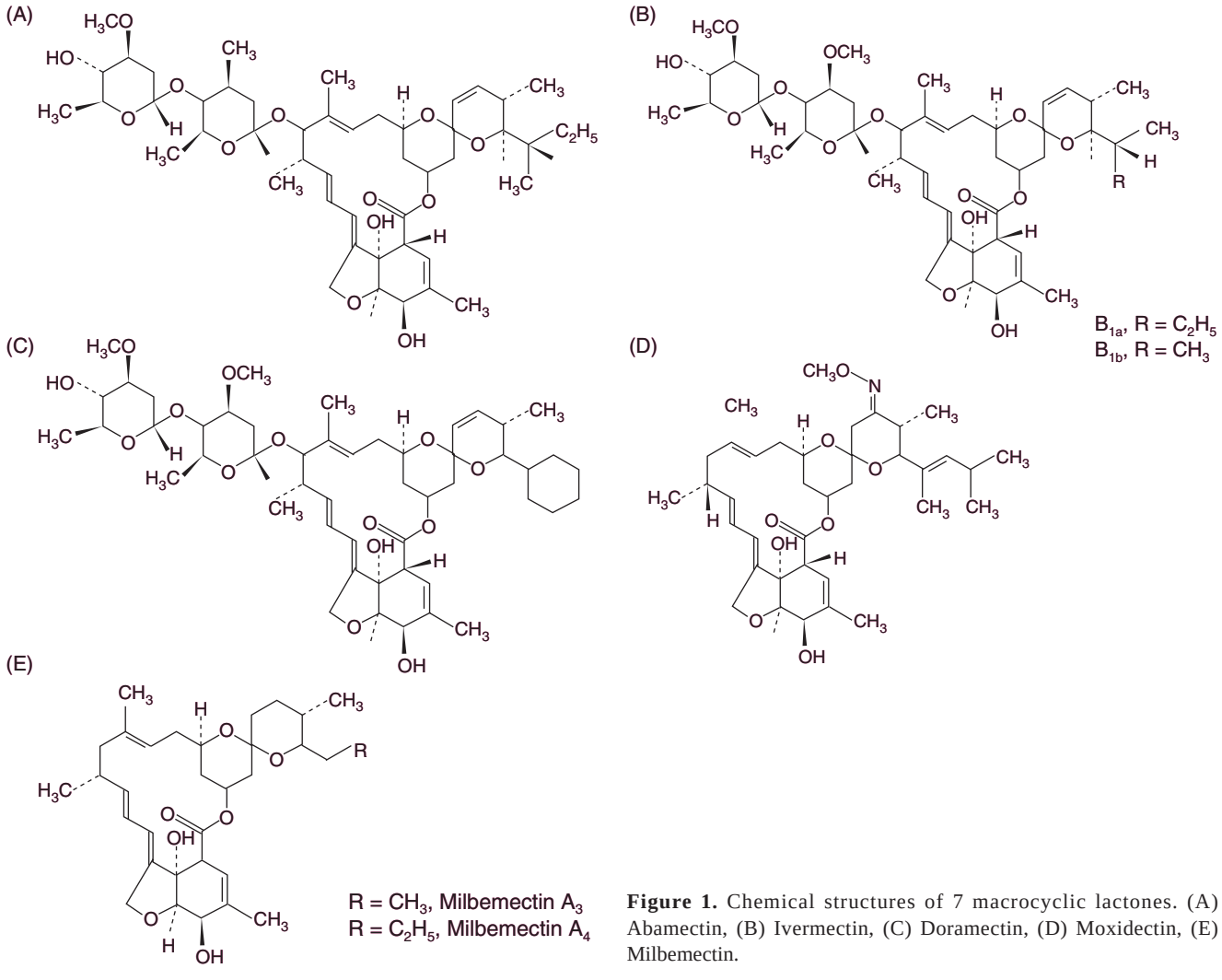

Figure 1. Chemical structures of 7 macrocyclic lactones. (A) Abamectin, (B) Ivermectin, (C) Doramectin, (D) Moxidectin, (E) Milbemectin.

Table 2. Tolerance of abamectin and milbemectin in Taiwan[2]

Compound	Group of crop	Tolerance (ppm)
Abamectin	Leaf vegetables with small leaves	0.05
	Small berries	0.01
	Leaf vegetables with wrapped leaves	0.02
	Melons	0.02
	Fruit vegetables	0.01
	Citrus	0.01
	Root vegetables	0.01
Milbemectin	Large berries	0.2
	Small berries	0.2
	Melons	0.2
	Melon vegetables	0.2
	Fruit vegetables	0.2
	Teas	2.0
	Pome	0.2

milbemectin is not allowed in the muscle, fat, kidney, liver, milk or egg of poultry and meat, but is allowed as an anthelmitic in vegetables[2] (Table 2). Due to structure similarity, milbemectin was also analyzed in this study.

Comparing the analysis methods in the reviewed papers (Table 3), we modified Roudaut's HPLC method, which was published in 1998, for the simultaneous quantification of abamectin, doramectin, ivermectin and moxidectin residues[4]. Two milbemycins, i.e., milbemectin A_3 and milbemectin A_4, were included in the analysis. We tried to establish an optimal derivatization and HPLC condition, not only for the analysis of the above mentioned 4 components, but also for analyzing milbemectin. Milbemectin, though popularly used in western countries, has not been imported to Taiwan due to its high price. There are about 60 thousand tons of fresh or frozen beef imported into Taiwan each year. In this study, a rapid and convenient multiresidue method for the analysis of bovine muscle contamination was established in order to ensure public health and monitor food safety in preparation after Taiwan joined the World Trade Organization.

MATERIALS AND METHODS

I. *Materials*

(I) *Samples*

Fifty refrigerated and frozen bovine muscle samples,

Table 3. Summary of 3 multiresidue methods using HPLC for macrocyclic lactones

Method	Roudaut (1998)[4]	Sher *et al*. (2000)[7]	Yoshii *et al*. (2000)[8]
Column	Lichrospher 100RP-18E 4 × 125 mm 35˚C	Zorbax ODS C18 4.6 × 150 mm RT*	Wakosil -3C18HG, 4.6 × 150 mm 50˚C
Mobile phase	Acetonitrile/water (94/6, v/v) Isocratic	Methanol/water (97/3, v/v) Isocratic	Acetonitrile/H_2O Gradient
Elution sequence of compounds	1. moxidectin 2. abamectin 3. doramectin 4. ivermectin	1. eprinomectin 2. moxidectin 3. abamectin 4. doramectin 5. ivermectin	1. milbemectin A_3 2. milbemectin A_4 3. abamectin 4. ivermectin
Limits of quantification	7.5 ppb	10 ppb	0.1 - 0.3 ppt**
Derivatization reaction	RT	65˚C, 90 min	Cooling box
Time of chromatogram	ca. 20 min	ca. 10 min	ca. 30 min
Mean of recovery	77.8 - 89.9% from bovine muscle	71.9 - 84.4% from beef liver	ca. 80 - 110% from crops

*RT: room temperature.
**Detection limits.

such as rib eye, rib finger, round and flank, were purchased from domestic traditional markets and supermarkets in mid-Taiwan, including Taichung county, Taichung city, Changhua county, Nantou county. Other samples were imports from Australia, U.S.A., Canada, and Netherlands. The samples used in the recovery test and sensitivity test were pre-screened to be free from containing tested components.

(II) *Reagents*

Methanol and acetonitrile, HPLC grade, were from LAB SCAN (Bangkok, Thailand). 1-Methylimidazole and trifluoracetic anhydride (99%) were from Aldirich (Milwaukee, WI, U.S.A.). Acetonitrile anhydrous (max. 0.005% H_2O), glacial acetic acid and triethylamine were from Merck (Darmstadt, Germany). Sodium sulfate anhydrous was from FSA (Loughborough, England).

(III) *Reference standards*

(1) Abamectin: from Chem-Service (Pennsylvania, U.S.A.), potency 3% B(l_a)ii - 91% B(l_b)i.
(2) Doramectin: from Pfizer Co. (Nagoya, Japan), potency 91.9%.
(3) Moxidectin: from Cyanide Co. (Princeton, NJ, U.S.A.), potency 98.2%.
(4) Ivermectin Bl_a: from Sigma Co. (St. Louis, MO, U.S.A.).
(5) Milbemectin A_3 (98.7% potency) and milbemectin A_4 (99.6% potency): from Hayashi Pure Chemical Industries Ltd. (Osaka, Japan).

(IV) *Equipments*

(1) HPLC: Shimadzu LC-10AT_{vp}.
  a. Detector: fluorescence detector, Shimadzu RF-535.
  b. Column: Merck Lichrospher 100RP-18E, 5 µm (125 × 4 mm), precolumn with same material.
  c. Auto-sampler: Shimadzu SIL-10A.
  d. Computer integrating software: SISC-LAB[32], Model 9724-2, 1250 minivolt in full scale, 10^{24} resolution, standard signal generator with CNS and ASTM certificates.
(2) Homogenizer: SMT Process Homogenizer, Japan.
(3) Centrifuge: Hettich Universal 30 F.
(4) Vortex mixer: IKA works MS1 minishaker.
(5) Oven: Memmert ULM - 500.
(6) Others:
  a. C18 solid-phase cartridge: Waters C18 cartridge, 100 mg, 1 cm^3.
  b. Filter paper: 0.45 µm, nylon.
  c. LC sample tube: 200 µL.
  d. Brown sample tube: 1.8 mL, screw-capped.

(V) *Preparation of mobile phase*

Acetonitrile (940 mL) was added with 60 mL of water. When gently mixed, degassed with sonication, and filtration, the filtrate was used as the mobile phase.

(VI) *Preparation of derivatization reagent*

The derivatization reagent was freshly prepared with anhydrous and light protected conditions.

Derivatization reagent (1): 1-Methylimidazole and acetonitrile anhydrous mixed gently (1:1, v/v).

Derivatization reagent (2): Trifluoracetic anhydride and acetonitrile anhydrous mixed gently (1:1, v/v).

(VII) *Analysis condition of HPLC*

Fluorescence detector: agitation wavelength 365 nm, emission wavelength 470 nm.

Mobile phase: According to Method I. (V)

Flow rate: 1.2 mL/min.

II. *Methods*

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(I) *Standard curve*

1. Preparation of standard solutions

Accurately weighted abamectin, doramectin, moxidectin, ivermectin, milbemectina A_3, and milbemectin A_4, were individually placed into brown volumetric bottles. The concentration was adjusted to 0.05 mg/mL with acetonitrile, and these served as the stock standard solutions. Adequate amounts of each solution was mixed with anhydrous acetonitrile, diluted in concentration to 3, 2, 1, 0.5, 0.25, 0.1, 0.05, 0.04, 0.025, 0.02 and 0.01 ppm, and these served as the mixed working standard solutions. The solutions were stored at -20˚C.

2. Derivatization reaction

The whole procedure was carried out at anhydrous and light-protected conditions. When 0.1 mL of above mixed working standard solution was individually placed in a capped brown sample bottle, 0.2 mL of derivatization reagent (1) was added. Vortex mixing for 2 min, 0.2 mL of derivatization reagent (2) was added. Vortex mixing for 1 min, 10 µL of glacial acetic acid was added. After vortex mixing for 1 min, placed in a 60˚C sand bath for 30 min, and then in a 0˚C ice bath for 5 min, the solution was filtrated with 0.45 µm filter and ready for HPLC analysis.

3. Plotting the standard curve

After derivatization reaction, the individually mixed working standard solution was placed into HPLC sample tube. Individual sample was analyzed in triplicate with HPLC under the above mentioned condition. The standard curves were plotted with the mean of the area under curve (AUC).

(II) *Preparation of sample solutions*

1. Extraction and purification

One gram of ground bovine muscle, in centrifuge tube, was mixed with 10 mL of polypropylene. After Vortex mixing with 0.1 mL of methanol and 1 mL of acetonitrile for 1 min, centrifuged at 4,800 rpm/min for 10 min, the supernatant was placed into a brown tube. The pellet was mixed with 0.5 mL of acetonitrile, centrifuged under the same condition, and the supernatant was combined with the previous one. After mixing gently with 2 mL of water, the supernatant was loaded into the C18 solid-phase cartridge at the flow rate of 0.2 mL/min. The cartridge was previously activated by 5 mL of acetonitrile, and 5 mL of acetonitrile/water (3/7, v/v, containing 0.1% triethylamine). The brown tube was washed with 1 mL of acetonitrile/water (3/7, v/v, containing 0.1% triethylamine), and passed through the C18 solid-phase cartridge for 1 min. The sample solution was extracted with 1 mL of acetonitrile/water (9/1, v/v), collected in a tube with 0.3 g of sodium sulfate anhydrous. After mixing, centrifuged at 4,800 rpm/min for 5 min, the supernatant was placed in a brown tube, air-dried with nitrogen, and 0.1 mL of anhydrous acetonitrile was added to elute for the derivatization reaction.

2. Recovery test

One gram of ground bovine muscle, in centrifuge tube, was mixed with 10 mL of polypropylene. Mixed working standard solution (0.1 mL) at the concentration of 3, 2, 1, 0.5, 0.3, 0.2, 0.1, and 0.05 ppm was mixed and left standing for 15 min. The sample was extracted, purified and derivatized according to the above method, then placed into sample tubes. Each sample was auto-injected into HPLC and analyzed in triplicate.

(III) *Determination of the limit of detection (LOD)*

A series of low-concentration of mixed working standard solution in 0.05, 0.04, 0.025, 0.02, and 0.01 ppm were prepared and derivatized as described in the above mentioned method. Each sample solution (0.1mL) was auto-injected into HPLC and analyzed in triplicate.

According to the ICH (International Conference on Harmonization) Q2A guideline[9], Validation Operation of Analytical Method (DOH), the linear regression of each standard solution was plotted by peak area vs concentration. The individual slope (S) and mean standard deviation (σ) were analyzed and the LOD was calculated by the equation:

$$LOQ = 3.3 \times \sigma / S$$

(IV) *Determination of the limit of quantitation (LOQ)*

Using the LOD method, the LOQ was calculated by the equation:

$$LOQ = 10 \times \sigma / S$$

(V) *Precision evaluation*

The recovery tests in triplicate were conducted by different personnel at different times and statistically analyzed with Microsoft Excel 7.0 and Statistical Analysis System (SAS) in the workstation of the Education Bureau. Covariant analysis with ANOVA, the recovery rate, the standard deviation and coefficient of variance were calculated individually.

RESULTS AND DISCUSSION

In this study, we tried to analyze 6 macrocyclic lactones, including milbemectin A_3, moxidectin, milbemectin A_4, abamectin, doramectin, and ivermectin, at the same time with a HPLC equipped with fluorescent detector. The individual influence factor on the results is discussed below.

I. Methodology Development

(I) *The factors that influence the peak height of the derivatives*

Table 4. Comparisons of peak areas of the macrocyclic lactones (100 ppb) at different temperatures after 30 min derivatization

Temp.	Mean peak area ($\times 10^3$)*					
(˚C)	Milbemectin A_3	Milbemectin A_4	Moxidectin	Abamectin	Doramectin	Ivermectin
25	375.5 ± 12.3^{b}**	231.6 ± 10.3^{b}	282.5 ± 09.8^{b}	188.0 ± 5.4^{b}	184.7 ± 1.9^{d}	198.0 ± 5.7^{b}
50	401.5 ± 13.2^{b}	245.5 ± 11.2^{b}	299.6 ± 10.4^{ab}	188.0 ± 5.4^{b}	195.0 ± 2.0^{c}	198.0 ± 5.6^{b}
60	494.4 ± 16.2^{a}	284.2 ± 11.9^{a}	312.5 ± 10.8^{a}	203.0 ± 5.8^{a}	205.3 ± 2.1^{b}	211.2 ± 6.1^{a}
65	505.5 ± 16.6^{a}	294.4 ± 11.8^{a}	325.3 ± 11.3^{a}	210.5 ± 6.0^{a}	225.8 ± 2.3^{a}	211.6 ± 6.6^{a}

* Data are mean ± standard deviation, n = 3.

**Values in a column with the different superscripts are significantly different ($P < 0.05$).

Results in Table 4 indicate the variation of peak area of each derivative when derivatation was performed under different temperatures. Derivatives of abamectin, doramectin, moxidectin and ivermectin at 60˚C increased 6~10%, compared to those at 25˚C. The results are similar to the study of Sher *et. al.*[7]. The peak areas of the derivatives of milbemectin (including milbemectin A_3 and milbemectin A_4) increased after 24 hr when derivitized at room temperature. This indicates the derivatization reaction is very slow at room temperature, and temperature increases could accelerate the reaction. Instant heat release was observed when the two derivatization reagents were added, raising the temperature to 45˚C. Therefore, increasing the temperature to 50˚C does not significantly change the peak area. When at 60˚C and 65˚C, the peak area of milbemectin increased significantly compared room temperature results. Because the boiling point of acetonitrile is 81˚C, too much heat could cause vaporization of acetonitrile and affect final concentration of the derivatives. It is considered that 60˚C is the appropriate temperature. Four hours after the derivatization reagents were added, the peak areas of derivatives of abamectin, doramectin and ivermectin decreased. To improve the stability of derivatives, we tried to change the pH condition. After derivatization reagents were added, 10μL of glacial acetic acid was added to acidify the derivatization condition. When 100 ppb of mixed standard solution was placed under the derivatization condition, the variation in peak area was less than 4% in 12 hr. It is possible that pH change stabilized the derivatives. Reaching the same conclusions as of Martin *et al.*[10], the addition of glacial acetic acid can stabilize derivatives of abamectin, doramectin, moxidectin and ivermectin. Also, in our study, acetic acid can also stabilize the derivatives of milbemectin A_3 and milbemectin A_4. It is worth to address that, during derivatization, anhydrous condition and light protection should be maintained because trifluoroacetic andydride could be hydrolyzed and interfere the peak height of chromatogram.

(II) *Linear relationship of standard curves*

Triplicate of derivatization and chromatography with mixed working standard solution at 3 different concentrations is shown in Table 5. The results lead to linear regression. As indicated by the results, the correlation coefficient (r^2) of milbemectin A_3, moxidectin, milbemectin A_4, abamectin, doramectin and ivermectin are 0.9994, 0.9992, 0.9995, 0.9990, 0.9995 and 0.9995, respectively. All of them are above 0.9990. For milbemectin A_3, moxidectin, milbemectin and abamectin, good linearship was shown between 1-300 ppb. For doramectin and ivermectin, the linear curve can also be shown between 2-300 ppb.

Table 5. The linear regression of standard anthelmintic drugs obtained at various concentrations

Compound Concentration (ppb)	r^2	Slope	Intercept
Milbemectin A_3 (1-300)	0.9994	+5029.3	-4701.9
Moxidectin (1-300)	0.9992	+3228.0	+4181.2
Milbemectin A_4 (1-300)	0.9995	+2918.1	-3196.5
Abamectin (1-300)	0.9990	+2089.3	-2202.4
Doramectin (2-300)	0.9995	+2118.6	-2610.2
Ivermectin (2-300)	0.9995	+2157.1	-2780.6

(III) *LOD and LOQ of reference standard*

As indicated in Table 6, different low concentrations of mixed working standard solutions were prepared in 1, 2, 2.5, 4 and 5 ppb. After derivatization and chromatography in triplicate, the mean, SD, slop of regression curve and intercept of regression can be calculated. According to the equation, theoretical LOD and LOQ of milbemectin A_3, moxidectin, milbemectin A_4, abamectin, doramectin and ivermectin were calculated. The LODs of each compound are 0.42, 0.21, 0.44, 0.44, 0.39 and 0.33 ppb, respectively. The LOQs of each compound are 1.27, 0.64, 1.34, 1.32, 1.19 and 1.00 ppb, respectively.

(IV) *Extraction and purification of spiked samples*

Taiwan is a small country with lot of people. The farm lands are usually small. Most of the beef consumption relies on importation. Due to large demand for beef and that macrocyclic lactones are popularly used in western countries[6], we chose beef as the study subject. Acetonitrile is used as the extraction solvent and the modified Roudaut's[4] method is used for the purification of bovine muscle samples.

The 6 macrocyclic lactones in this study are easily

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Table 6. The LOD & LOQ of standard Anthelmintic drugs calculated by the standard deviation regressed at various concentrations

Compound concentration (ppb)	Mean of standard deviation (σ)	Slope	Intercept	LOD* (ppb)	LOQ** (ppb)
Milbemectin A_3 (1-5)	588.6	4621.2	425.79	0.42	1.27
Moxidectin (1-5)	198.0	3070.8	-151.77	0.21	0.64
Milbemectin A4 (1-5)	363.3	2706.9	40.49	0.44	1.34
Abamectin (1-5)	272.1	2055.7	-464.26	0.44	1.32
Doramectin (2-5)	235.0	1977.8	-466.50	0.39	1.19
Ivermectin (2-5)	202.6	2031.6	-980.36	0.33	1.00

*LOD = $3.3 \times \sigma$ / S.
**LOQ = $10 \times \sigma$ / S.

decayed by light, heat and acid, nearly insoluble in water but easily dissolve in methanol, methylene chloride, isopropyl alcohol, acetonitrile, etc. Based on Roudaut's method, we used acetonitrile as the extractant because less solvent can be used and a more efficient extraction could be obtained, compared to Lori's method[11], where methylene chloride/acetone (1/1, v/v) was used as the extractant.

According to the purification procedure described in Roudaut's method, acetonitrile (~3 mL) should be mixed with 7 mL of water before purification by loading into a solid phase extraction cartridge (C18). In reality, the recovery rate is very low and it's time-consuming when loading samples into the cartridge. If we reduce water to equal the volume of extractant (2-3 mL) before loading samples into a cartridge, the recovery rate increases. The low recovery rate may be resulting from small chances for compound interaction with elements in the cartridge due to too much water. Such viewpoint matches the theory in the Waters Sep-Pak Cartridges Care and Use Manual[12] .

In addition, after sample extract was loaded, the solid-phase cartridge should be washed with 1 mL of acetonitrile/water (7/3, v/v) before extraction, according to Roudaut's method. But in reality, we obtained the same results if cartridge was extracted directly with acetonitrile/water (9/1, v/v).

II. *Methodology Evaluation*

(I) *The detection limit of methodology*

According to Waters[13], the sensitivity of HPLC detector, i.e., the S/N value (signal-to-noise ratio, the ratio of peak signal to noise), is usually at 2/1 or 3/1. If S/N value of LOQ reaches 10/1, it indicates good precision and accuracy. When the mixed working standard solutions with theoretical values of LOD and LOQ were prepared, i.e., 0.5 ppb and 1.5 ppb, and analyzed in triplicate, the respective S/N values were observed. At 0.5 ppb, the S/N values of milbemectin A_3, moxidectin, and milbemectin A_4 were higher than 8; while abamectin, doramectin, and ivermectin were less than 3. At 1.5 ppb, the S/N value of each compound was higher than 5. In recovery test, the bovine muscle samples were spiked with 2 ppb and 5 ppb mixed working standard solutions. At 2 ppb, the S/N values of milbemectin A_3, moxidectin, milbemectin A_4 and abamectin were higher than 4; while at 5 ppb, higher than 10. At 2 ppb, the S/N values of doramectin and ivermectin were higher than 3; while at 5 ppb, higher than 8. The method that we developed obtained LOD at 2 ppb and LOQ at 5 ppb, compared with Roudaut's method with LOQ at 7.5 ppb.

Table 7. The recoveries and relative standard deviation of macrocyclic lactones from spiked bovine muscle (n=3)

Compound	Theoretical concentration (μg/kg)	Recovery (%) (mean ± SD)	Relative standard deviation (%)
Milbemectin A_3	300	90.0 ± 6.9	7.70
	100	93.4 ± 6.7	7.13
	20	94.0 ± 8.4	8.95
	5	96.9 ± 10.6	10.93
Moxidectin	300	79.8 ± 6.8	8.59
	100	85.6 ± 6.7	7.89
	20	81.3 ± 4.3	5.30
	5	86.7 ± 9.0	10.41
Milbemectin A_4	300	86.3 ± 5.5	6.51
	100	93.1 ± 8.0	8.63
	20	82.0 ± 1.7	2.11
	5	100.7 ± 9.0	8.96
Abamectin	300	86.0 ± 5.0	5.88
	100	85.3 ± 3.5	4.13
	20	82.7 ± 3.8	4.62
	5	90.0 ± 13.1	14.57
Doramectin	300	81.8 ± 6.5	7.99
	100	89.9 ± 14.9	16.57
	20	97.3 ± 5.1	5.27
	5	92.7 ± 14.1	15.31
Ivermectin	300	79.2 ± 5.2	6.53
	100	81.7 ± 3.8	4.76
	20	80.8 ± 3.6	4.56
	5	98.7 ± 11.0	11.16

(II) *Recovery test and precision*

Figure 2 shows the HPLC chromatogram of mixed standard solutions with a good specificity of the developed method. Table 7 also shows a good precision of the developed method. The recovery rate and relative standard deviation (% RSD) of individual component at various concentrations were analyzed in triplicate by different personnel at different times using the above mentioned addition, extraction, derivatization and chromatographic procedures. When 300 ppb was spiked, the recovery rate of milbemectin A_3, moxidectin, milbemectin A_4, abamectin,

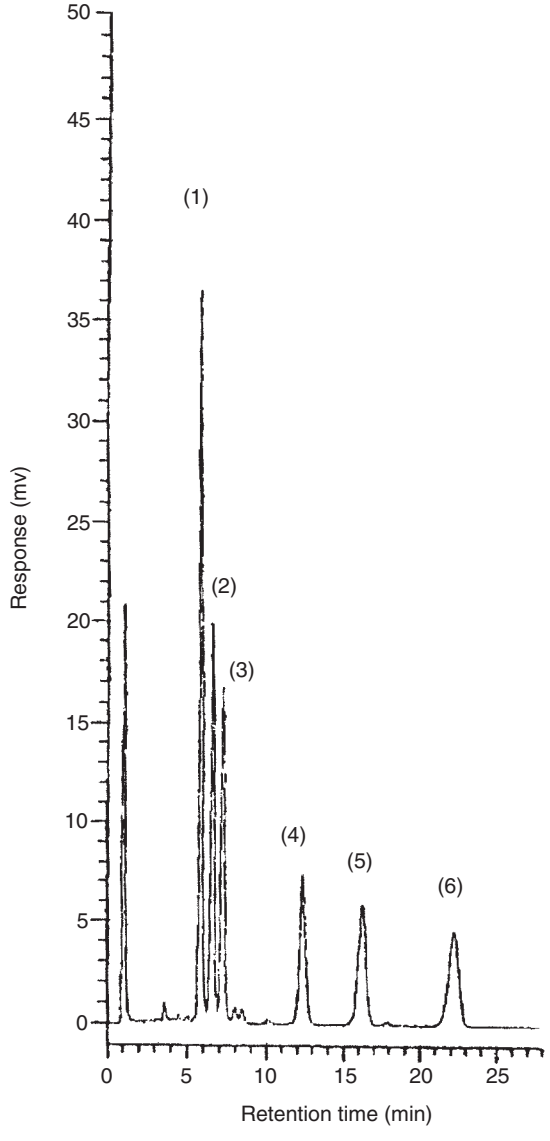

Figure 2. HPLC chromatogram of mixed standard solutions (100 ppb). (1). Milbemectin A_3; (2). Moxidectin; (3). Milbemectin A_4; (4). Abamectin; (5). Doramectin; (6). Ivermectin.
Column: Merck Lichrospher 100RP-18E, 5 μm, 125 × 4 mm.
Mobile phase: Acetonitrile/H_2O (94/6, v/v).
Fluorescence detector: Ex 365 nm, Em 470 nm.
Flow rate: 1.2 mL/min.

doramectin, and ivermectin was 82.3-95.7%, 72.3-85.7%, 80.9-92.1%, 82.5-91.8%, 76.5-89.1% and 74.1-84.5%, respectively. When 100 ppb was spiked, the recovery rate of milbemectin A_3, moxidectin, milbemectin A_4, abamectin, doramectin, and ivermectin was 86.6-93.7%, 78.9-92.4%, 94.0-100.7%, 81.6-88.6%, 73.3-102.1% and 77.3-84.6%, respectively. When 20 ppb was spiked, the recovery rate of milbemectin A_3, moxidectin, milbemectin A_4, abamectin, doramectin, and ivermectin was 84.5-100.5%, 77.5-86.0%, 80.0-83.0%, 78.5-86.0%, 93.0-103.0% and 78.0-85.0%, respectively. When 5 ppb was spiked, the recovery rate of milbemectin A_3, moxidectin, milbemectin A_4, abamectin, doramectin, and ivermectin was milbemectin A_3, moxidectin, milbemectin A_4, abamectin, doramectin, ivermectin was 88.4-123%, 78.0-96.0%, 92.0-110.0%, 78.0-104.0%, 80.0-108.0% and 86.0-106.0%, respectively. The relative standard deviations (% RSD) of these studies were all less than 20. The result corresponded to the Horwitz equation in Codex's report[14], when the concentration was at 10-8 (10 ppb), the % RSD should be less than 32. When 10 ppb was spiked in bovine muscle and Roudaut's method was applied, the mean recovery rate of abamectin, moxidectin, doramectin, and ivermectin was in the range of 77.8-89.9%.

(III) *Analysis of commercial bovine muscle samples*

In August 2002, we collected a total of 50 bovine muscle samples, 5 domestic samples from mid-Taiwan traditional markets and supermarkets, including Taichung county, Taichung city, Changhua county, and Nantou county, and 45 imported samples from USA, Australia, Canada, and Netherlands. When analyzed according to the above mentioned extraction, derivatization and chromatographic procedures, we did not identify any traces of milbemectin A_3, moxidectin, milbemectin A_4, abamectin, doramectin and ivermectin.

CONCLUSIONS

In conclusion, the linear regression coefficients (r^2) were all higher than 0.9990, which indicated that good linear correlation and good precision could be obtained for milbemectin A_3, moxidectin, milbemectin A_4, and abamectin at the concentration of 1-300 ppb and doramectin and ivermectin at 2-300 ppb. The LOQ is 5 ppb, far less than the bovine muscle residue tolerance in "The Residual Limit of Animal Drugs". The method is applicable for the quantification of these residues. The modified Roudaut method can separate and quantify structurally similar milbemectin A_3 and milbemectin A_4.

Besides cattle, abamectin, doramectin, moxidectin and ivermectin were also used in the raising of swine, deer, goat, sheep and pigs. Therefore, the residue tolerances of liver, kidney, milk, fat, muscle or egg in these products were identified in "The Residual Limit of Animal Drug", at the range of 5-100 ppb. Since abamectin and milbemectin were also allowed as anthelmintic drugs in planting vegetables, including leaf vegetables with small leaves, large and small berries, leaf vegetables with wrapped leaves, melons, fruit vegetables, citrus, root vegetables, melon vegetables, pomes, and teas, the residue tolerance in the DOH's "Safety Tolerance of Pesticide Residue" was at the range of 10-2000 ppb. They are quantifiable by using the derivatization and chromatographic method developed in this study. The sample extraction and recovery need further evaluation.

The method we developed, unlike mass chromatography that can further identify chemical structures, has advantages in rapid extraction, less organic solvent used, simple equipments, stable results after derivatization, and the ability to quantify residues at low detection limits. The

method is suitable for the rapid screening of a lot of samples in general laboratories.

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