DRUGS, COSMETICS, FORENSIC SCIENCES

Multiresidue Chromatographic Method for the Determination of Macrolide Residues in Muscle by High-Performance Liquid Chromatography with UV Detection

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A high-performance liquid chromatographic (HPLC) method for the simultaneous determination of tilmicosin, tylosin, spiramycin, and its major metabolite neospiramycin was developed that is suitable for porcine, bovine, and poultry muscles. Macrolide residues were extracted from muscle with acetonitrile, fat was removed by liquid–liquid extraction with isooctane, and the extract was then cleaned on Bond Elut C_{18} cartridges. The HPLC separation was performed on an Inertsil ODS3 C_{18} column (150 × 4 mm) with 0.05% trifluoroacetic acid–acetonitrile in a gradient mode. Two different chromatographic gradients were used for tilmicosin–tylosin and spiramycin–neospiramycin, and the detection wavelengths were 287 and 232 nm, respectively. The method was validated from ½ the maximum residue limit (MRL) to 4 times the MRL with pork muscle samples. Mean recoveries were 60, 63.5, 51, and 42% for tilmicosin, tylosin, spiramycin, and neospiramycin, respectively. The detection limits are 15 µg/kg for tilmicosin and tylosin, 30 µg/kg for spiramycin, and 25 µg/kg for neospiramycin. Linearity, precision, and accuracy of the method were also tested.

Macrolides belong to the family of macrocyclic antibiotics. They form a homogeneous class of antibiotics in terms of their chemical structure (macrocyclic lactone nucleus) and antibacterial spectrum. The 4 macrolides we have studied are 16-member-ring macrolides. These drugs are often used as feed additives for growth promotion, but they can also be used for therapeutic purposes because they have a wide range antibacterial spectrum. Their use can leave residues in edible products that not only can have direct toxic effects but also may lead to allergic reactions in consumers and to the development of resistant bacteria. Measures to monitor a certain number of residues of pharmacological substances in farm animals and in the fresh meat obtained from such animals are described in European Community (EC) Council Directive 96/23/EEC, and the criteria for routine methods to be used for this purpose are described in EC Commission Decision 93/256/EEC. Maximum residue limits (MRL) in muscle are 50 µg/kg for tilmicosin, 100 µg/kg for tylosin, and 200 and 300 µg/kg for the sum of spiramycin and neospiramycin in bovine and porcine muscle, respectively.

The bibliography in the field of macrolides residues is very poor. Chromatographic methods have already been described for the determination of macrolides residues by liquid chromatography with mass spectrometry (LC/MS), gas chromatography/MS (GC/MS), or LC with UV detection (LC–UV), but only a few were multiresidue methods (1–3). The LC–UV method described by Chan (3) included only tylosin and tilmicosin residues. Some monoresidue methods have been developed for the determination of tylosin (4–6) and spiramycin (7, 8). UV detection was chosen to develop a multiresidue method, but the UV absorption of spiramycin and neospiramycin at 232 nm involved an exhaustive cleanup of the samples to compensate the nonspecificity of this wavelength. Another alternative was to develop a method with a fluorimetric detection by using derivatization of the aldehyde function, but tilmicosin would have been then excluded. Therefore, the purpose of this work was to develop a simple and reliable multiresidue method for the simultaneous determination of tilmicosin, tylosin, spiramycin, and neospiramycin in muscle by using high-performance liquid chromatography (HPLC) with UV detection.

Experimental

Reagents

(**a**) *Solvents.*—Reagent grade methanol, acetonitrile, and isooctane (Merck, Nogent-Sur-Marne, France).

(**b**) *Water.*—Deionized.

(**c**) *Ammonium acetate and dipotassium hydrogen phosphate.*—Reagent grade (Merck).

(**d**) *5% Dimethyldichlorosylane in toluene.*—Supelco (Saint Quentin Fallavier, France).

(**e**) *Trifluoroacetic acid.*—UV grade (Merck).

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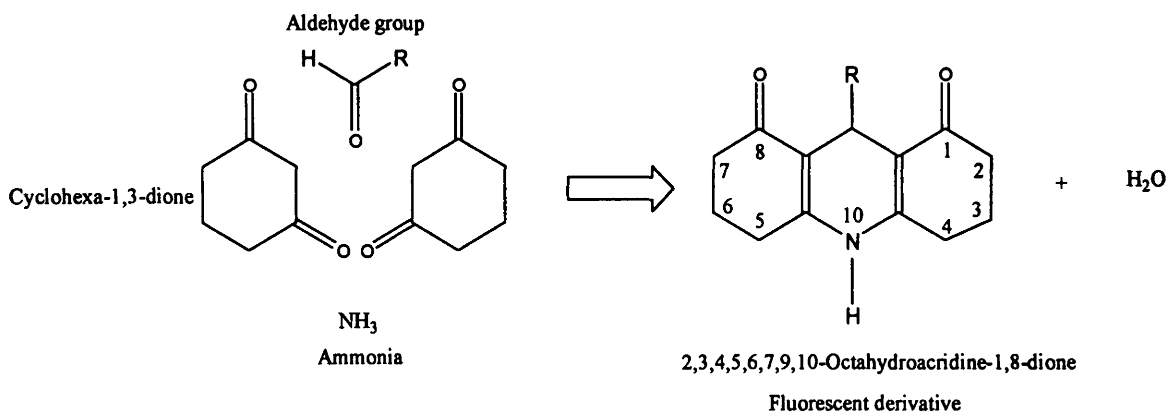

Figure 1. Reaction scheme of the formation of the fluorescent derivative among aldehyde group, ammonia, and cyclohexa-1,3-dione.

(f) *Elution solution.*—0.1M methanolic ammonium acetate solution.

(g) *0.2M buffer solution.*—Prepared by dissolving 34.84 g dipotassium hydrogen phosphate in 1 L deionized water.

(h) *LC mobile phase.*—0.05% trifluoroacetic acid in water–acetonitrile in a gradient mode at a flow rate of 0.7 mL/min.

Standards

(a) *Reference standards.*—Tilmicosin and tylosin were obtained from Eli Lilly and Co. (Saint Cloud, France), spiramycin from Rhône Merieux (Toulouse, France), and neospiramycin from Rhône Poulenc Rorer (Vitry sur Seine, France).

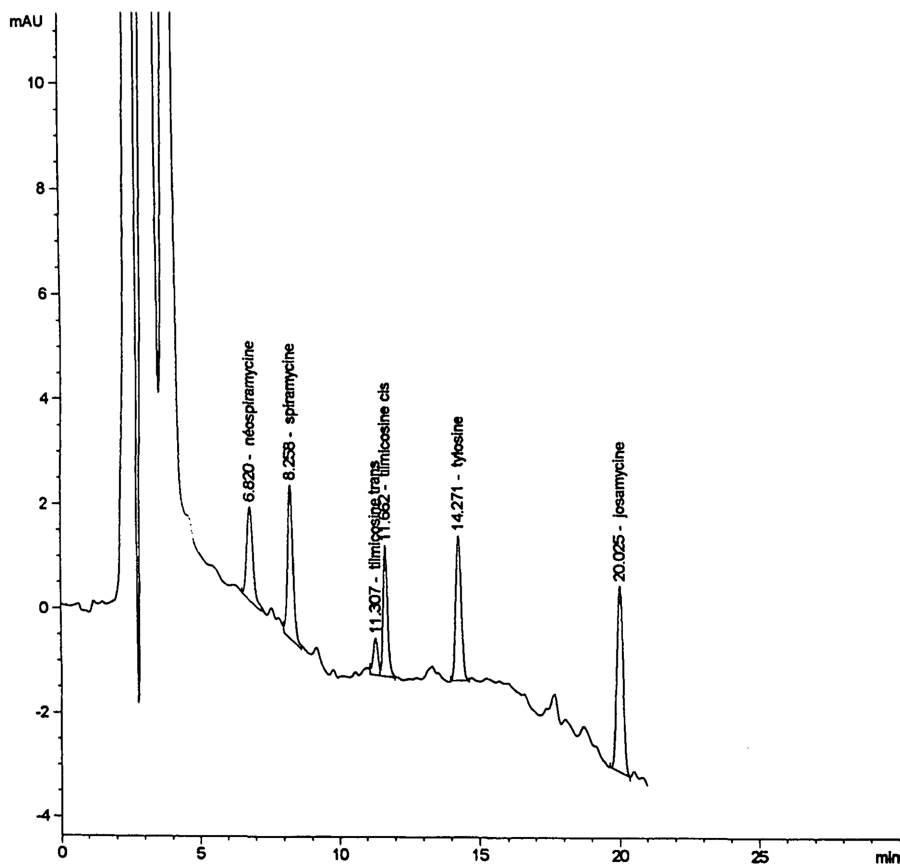

Figure 2. Typical liquid chromatogram example of pork muscle fortified with spiramycin, neospiramycin, tilmicosin, tylosin, and josamycin injected into a gradient mode. mAU, milli-absorbance units.

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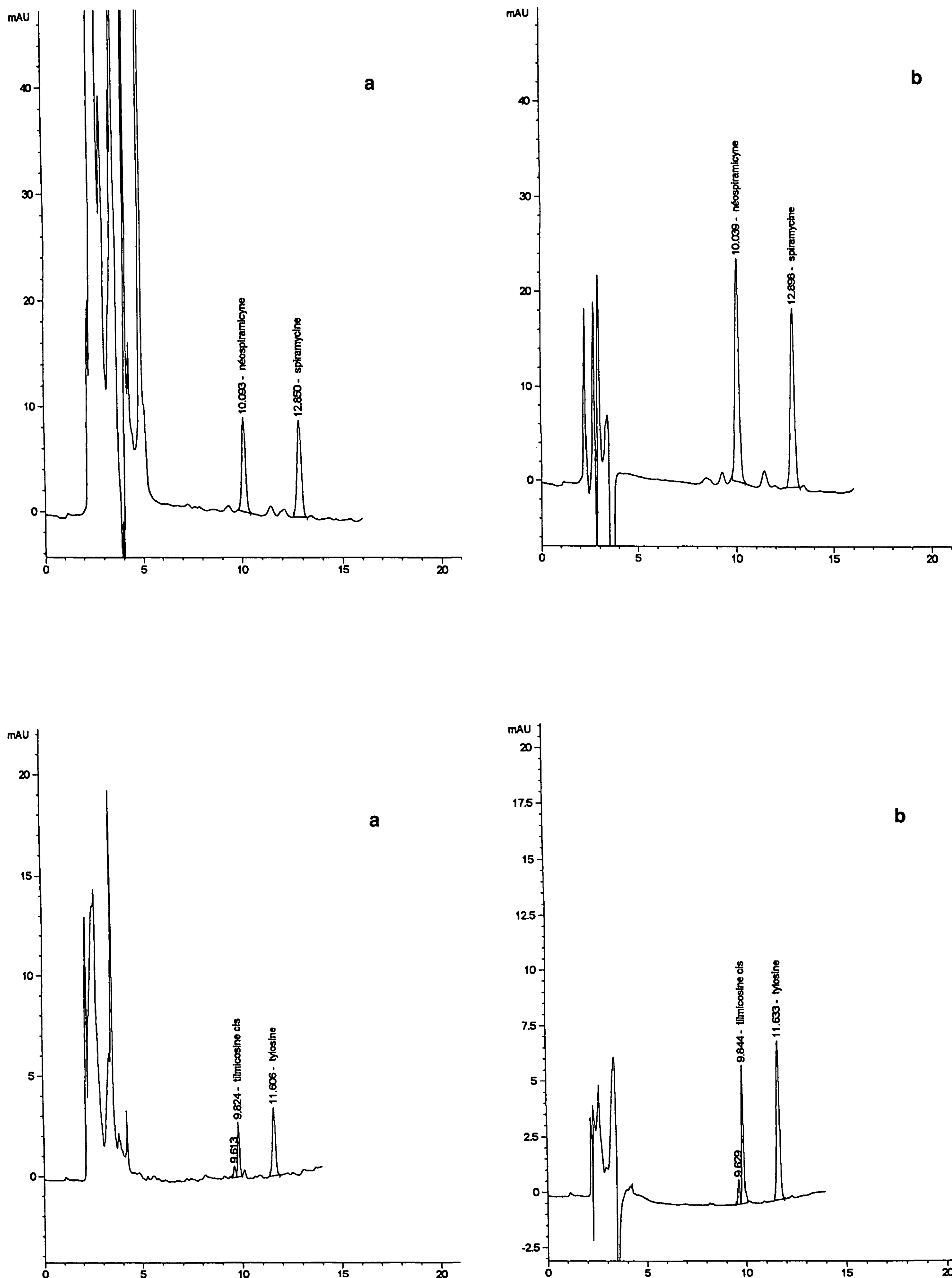

Figure 3. Typical liquid chromatogram examples of muscle samples spiked at the MRL level (a) and corresponding standards (b) injected with 2 different elution gradients. The top set of chromatograms are neospiramycin and spiramycin, and the bottom set of chromatograms are tilmicosine and tylosin.

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Table 1. Recovery results[a]

Compound	1/2 MRL[b]	MRL	2 MRL	4 MRL
Tilmicosin	61.5	59.9	58.2	44.1
Tylosin	64.6	64.2	63.0	52.2
Neospiramycin	43.0	39.5	41.5	57.2
Spiramycin	51.8	47.4	52.4	61.0

[a] Values are given in %.
[b] MRL, maximum residue limit.

(**b**) *Individual stock solutions*.—Four methanolic solutions containing 1 mg/mL of each standard were prepared. For tilmicosin, a solution containing 1 mg/mL *cis*-tilmicosin was prepared. Solutions were stored below –15°C.

(**c**) *Intermediate standard solution*.—A methanolic intermediate solution was prepared by diluting 0.5 mL tilmicosin, 1 mL tylosin, 2 mL spiramycin, and 2 mL neospiramycin in 20 mL with methanol. The concentrations obtained were 25, 50, 100, 100, and 100 μg/mL, respectively. This solution was stored below –15°C.

(**d**) *Calibration standards*.—One milliliter of the intermediate standard solution was transferred to flasks of 25, 50, 100, and 200 mL and diluted with dipotassium hydrogen phosphate buffer to obtain 4 mixed standard solutions of (*1*) 0.125 μg/mL tilmicosin, 0.25 μg/mL tylosin, and 0.5 μg/mL spiramycin and neospiramycin; (*2*) 0.25 μg/mL tilmicosin, 0.5 μg/mL tylosin, and 1 μg/mL spiramycin and neospiramycin; (*3*) 0.5 μg/mL tilmicosin, 1 μg/mL tylosin, and 2 μg/mL spiramycin and neospiramycin; (*4*) 1 μg/mL tilmicosin, 2 μg/mL tylosin, and 4 μg/mL spiramycin and neospiramycin. These solutions were stored in a freezer (ca 4°C).

(**e**) *Spiking solution*.—Four milliliters of intermediate solution was diluted in 200 mL deionized water to obtain a solution containing 0.5 μg/mL *cis*-tilmicosin, 1 μg/mL tylosin, and 2 μg/mL spiramycin and neospiramycin.

(**f**) *Spiked control samples*.—Spiked muscle samples were prepared by adding 500 μL spiking solution to 5 g control muscle (free of macrolides) to obtain spiking levels equal to the MRL for each macrolide.

Apparatus

(**a**) *Solid-phase extraction (SPE) columns*.—Bond Elut C_{18} (500 mg, 3 or 6 mL; Varian, Les Ulis, France), 3 mL cartridges were used for porcine and bovine muscle samples, and 6 mL cartridges were used for poultry muscle samples.

(**b**) *SPE manifold*.—Supelco.

(**c**) *Filters*.—Millex HV13 filters (0.45 μm, 13 mm id) (Millipore, Saint Quentin Yvelines, France).

(**d**) *Analytical column*.—ODS3 column (150 × 4 mm id, containing C_{18} Inertsil, 5 μm particle size; Interchim, Montluçon, France) and Lichrospher 100RP18-e guard column (4 × 4 mm).

(**e**) *Liquid chromatograph*.—Series 1050 quaternary gradient pump, Series 1050 autosampler, Series 1050 UV-Vis detector, and HPLC 2D Chemstation software (Hewlett-Packard, Les Ulis, France).

Preparation of Sample

Five grams of grinded muscle was weighed into a polypropylene centrifuge tube. Ten milliliters acetonitrile was added, the mixture was homogenized, and then stirred for 10 min at 100 rpm with the rotary stirrer. Ten milliliters isooctane was added and stirred again for 5 min at a slow speed of ca 30 rpm (to avoid emulsions) with the rotary stirrer. Centrifugation was performed for 10 min at 4000 × *g*. The upper layer (isooctane) was removed, and 8 mL of the lower layer was pipetted and diluted with 50 mL deionized water.

SPE Column Cleanup

A 50 mL reservoir was connected, and the Bond Elut cartridge was activated with 1 mL dimethyldichlorosylane followed by 10 mL methanol and 10 mL deionized water. The sample extract was loaded and pulled through the cartridge at a flow rate of no more than 2 drops/s. The cartridge was not allowed to dry at this step. The cartridge was flushed with 10 mL deionized water and then dried for 2 min under vacuum. Elution was performed successively with 1 and 0.5 mL 0.1M methanolic ammonium solution into a previously weighed polypropylene tube. One-half milliliter dipotassium hydrogen phosphate buffer was added and the solution was evaporated to a volume below or equal to 0.5 mL. Phosphate buffer was added until the total content weight is 1 g. The sam-

Table 2. Repeatability and within-laboratory reproducibility coefficients of variation[a]

	Tilmicosin		Tylosin		Neospiramycin		Spiramycin	
Content	Repeatability	Within-laboratory reproducibility	Repeatability	Within-laboratory reproducibility	Repeatability	Within-laboratory reproducibility	Repeatability	Within-laboratory reproducibility
1/2 MRL	16.4	16.4	6.3	10.5	9.9	11.9	9.7	14.5
MRL	12.1	15.9	4.8	9.3	9.4	15.7	7.6	15.1
2 MRL	5.9	7.7	3.8	10.3	7.4	10.5	5.5	10.4
4 MRL	8.6	12.9	3.0	10.3	6.6	15.5	6.7	13.9

[a] Values are given in %.

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Table 3. Method accuracy for the determination of macrolide residues in muscle

Macrolide	Content	Accuracy, %
Tilmicosin	1/2 MRL	3.822
	MRL	1.223
	2 MRL	−1.6357
	4 MRL	−3.4097
Tylosin	1/2 MRL	2.185
	MRL	1.68269
	2 MRL	−0.325
	4 MRL	−3.40288
Neospiramycin	1/2 MRL	2.396
	MRL	−5.98
	2 MRL	−1.134
	4 MRL	4.98
Spiramycin	1/2 MRL	1.744
	MRL	−6.897
	2 MRL	2.77
	4 MRL	2.479

ples were mixed and filtered through 0.45 μm filters before a 100 μL volume was injected into the LC system.

Chromatographic Determination

One hundred microliters of each standard, spiked sample extract, and test sample extract were injected. Tilmicosin and tylosin are detected at 287 nm. Spiramycin and neospiramycin are detected at 232 nm.

(**a**) *Elution gradient for tilmicosin and tylosin*.—(*1*) 0 min, acetonitrile–0.05% trifluoroacetic acid (29 + 71); (*2*) 11 min, acetonitrile–0.05% trifluoroacetic acid (45.5 + 54.5); (*3*) 11.5 min, acetonitrile–0.05% trifluoroacetic acid (50 + 50); (*4*) 14 min, acetonitrile–0.05% trifluoroacetic acid (50 + 50); (*5*) posttime, 6 min; (*6*) flow rate, 0.7 mL/min.

(**b**) *Elution gradient for spiramycin and neospiramycin*.—(*1*) 0 min, acetonitrile–0.05% trifluoroacetic acid (20 + 80); (*2*) 12 min, acetonitrile–0.05% trifluoroacetic acid (27.5 + 72.5); (*3*) 13 min, acetonitrile–0.05% trifluoroacetic acid (50 + 50); (*4*) 16 min, acetonitrile–0.05% trifluoroacetic acid (50 + 50); (*5*) posttime, 5 min; (*6*) flow rate, 0.7 mL/min.

Results and Discussion

Development

During development, we first tried a derivatization reaction with cyclohexa-1,3-dione (CHD). CHD is a specific fluorogenic reagent of the aldehyde function that was already used for the derivatization of josamycin by Leroy et al. (9). In the presence of ammonia, CHD reacts with the aldehyde group to give a heterocyclic structure with fluorescence properties (Figure 1). Therefore, this reagent was used with spiramycin, neospiramycin, josamycin, and tylosin but not with tilmicosin, which does not contain any aldehyde group. The results were satisfactory only with josamycin. The reaction with tylosin and spiramycin gave so many peaks that it was almost impossible to identify each fragment. It was then decided to continue the development with a UV detection method, which allowed the inclusion of tilmicosin.

Because the mobile phase is composed of trifluoroacetic acid and acetonitrile, the calibration standards were first diluted in trifluoroacetic acid solution. However, problems of stability were observed with spiramycin. This compound gave neospiramycin by acidic hydrolysis, and therefore calibration standard solution was then prepared with dipotassium hydrogen phosphate (pH ≈ 9.3).

A steeply eluting gradient was first determined to analyze the 5 compounds, but this gradient involved drifting baseline, particularly for the extracts (Figure 2), and it was sometimes difficult to have a precise integration of the peaks. Two different gradients were then used: one for tilmicosin, tylosin, and josamycin (UV detection at 287 and 232 nm) and another for neospiramycin and spiramycin (UV detection at 232 nm). After validation of this method for all 5 compounds, josamycin

Table 4. Homogeneity study of compounds used in the stability study

Compound	Source of variation	Sum of squares	df	Mean square	F^a
Spiramycin	Between samples	3055.40	4.00	763.85	2.01
	Analytical	1903.00	5.00	380.60	
	Total	4958.40	9.00	1144.45	
Tilmicosin	Between samples	22061.40	4.00	5515.35	0.87
	Analytical	31795.00	5.00	6359.00	
	Total	53856.40	9.00	11874.35	
Tylosin	Between samples	672697.48	4.00	168174.37	1.84
	Analytical	456278.17	5.00	91255.63	
	Total	1128975.65	9.00	259430.00	

[a] Critical value of F = 5, 19 (p = 0.05, df1 = 4, df2 = 5); df, degrees of freedom. There are no significant differences between samples.

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was excluded because of poor results in terms of repeatability and reproducibility. A specific method for josamycin will need to be used.

Assay Validation

Chromatograms obtained with this method are presented in Figure 3. The method was validated in the range from 25 to 200 µg/kg for tilmicosin, 50 to 400 µg/kg for tylosin, and 150 to 1200 µg/kg for spiramycin and neospiramycin. The validation was performed with pork muscle samples, and it was also verified that this method can be used with bovine and poultry muscle. Because the poultry muscle samples sometimes became plugged during SPE, Bond Elut C_{18} (500 mg, 6 mL) can be used. It was verified that the recoveries were not altered by using 6 mL instead of 3 mL cartridges. The limit of detection (LOD) was calculated as the apparent content corresponding to the value of the mean plus 3 times the standard deviation for the blank determinations (20 representative blank samples).

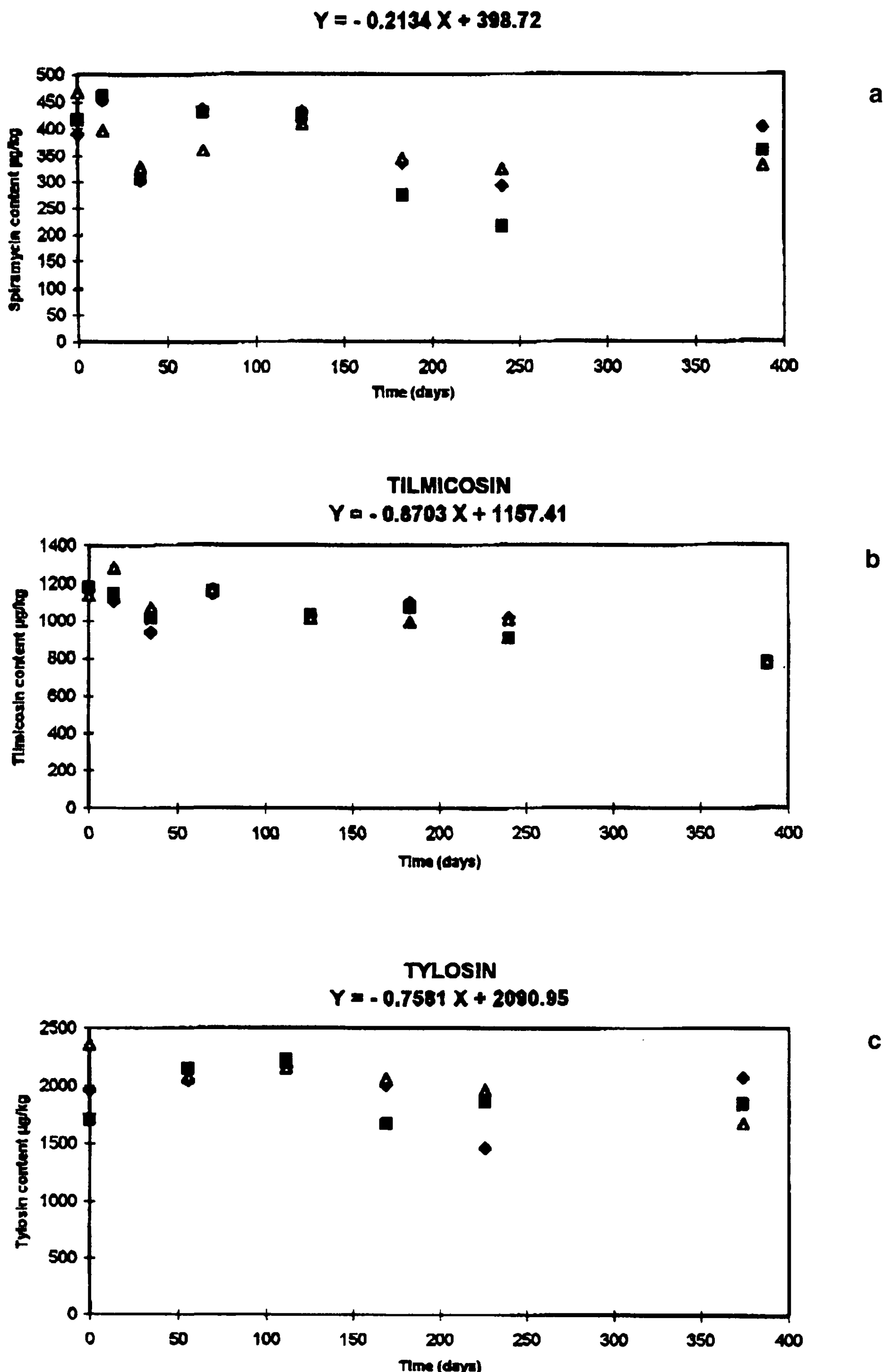

Figure 4. Stability study of spiramycin (a), tilmicosin (b), and tylosin (c) incurred muscle samples stored at –20°C.

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Table 5. ANOVA table derived from the stability studies of spiramycin, tilmicosin, and tylosin

Source of variation	Sum of squares	df	Mean square	*F*
		Spiramycin[a]		
Linear regression	16967.0	1.0	16967.0	
Residual	84262.1	22.0	3830.1	4.4
		Tilmicosin[b]		
Linear regression	282139.0	1.0	282139.0	
Residual	117338.0	22.0	5333.5	52.9
		Tylosin[c]		
Linear regression	152696.0	1.0	152696.0	
Residual	750300.0	16.0	46893.8	3.3

[a] Critical value of $F = 4.30$ ($p = 0.05$, df 1 = 1, df 2 = 22).
[b] Critical value of $F = 4.30$ ($p = 0.05$, df 1 = 1, df 2 = 22).
[c] Critical value of $F = 4.49$ ($p = 0.05$, df 1 = 1, df 2 = 16).

These LODs are 15 μg/kg for tilmicosin and tylosin, 30 μg/kg for spiramycin, and 25 μg/kg for neospiramycin.

Twelve samples per concentration were tested during the validation. Linear calibration curves were obtained from ½ MRL to 4 MRL. During extraction, an 8 mL aliquot of the 10 mL supernatant was taken, and a correction factor of 10/8 was applied to the raw data before the estimated concentrations were calculated.

Recoveries averaged 60% for tilmicosin, 63.5% for tylosin, 51% for spiramycin, and 42.5% for neospiramycin. Table 1 summarizes the recoveries of each drug for different levels of spiking. Student's *t*-test proved that recoveries are independent of the concentrations. These recoveries were low compared with monoresidue methods, particularly for neospiramycin. However, it is clear that it was not possible to obtain high recoveries with a method that must be suitable to several substances residues. Furthermore, the 232 nm UV detection required an exhaustive cleaning of the samples, which involved a decrease of the recoveries.

Precision and accuracy of the method were tested and gave results in accordance with EC Commission Decision 93/256/EEC (Tables 2 and 3).

Suitability of the Method to Incurred Samples

It was important to study incurred samples as well as spiked samples to get information about the metabolism of macrolides. The incurred samples can then be used for stability studies. Four pigs were ordered, and 3 of them were treated with macrolides. The fourth one was slaughtered without any treatment to obtain blank samples for comparison. Spiramycin and tylosin were injected by intramuscular route, and tilmicosin was added to the feed. The pigs were then slaughtered at different times, and the biceps femoris and gluteal muscle were then minced, sampled, and stored at about –20°C. The samples were then analyzed with the previously described method. No particular metabolites were detected for spiramycin and tilmicosin. The quantity of neospiramycin observed was minimal compared with that of spiramycin. A small second peak was observed with tylosin but was not present in all samples. Further studies will be performed by LC/MS to ascertain more information concerning this compound.

A homogeneity study was then made before the stability study was started. The homogeneity was determined for each material according to the *International Harmonized Protocol for Proficiency Testing of (Chemical) Analytical Laboratories* (10) by comparing the sampling variance with the analytical variance. The results are given in Table 4. There was no significant differences between samples, and therefore the material was considered sufficiently homogeneous for the purpose of a stability study. The results of the stability study are given in Figures 4a–c. A statistical test was performed for each analyte to verify whether the slope of the linear regression line was significantly different from 0. Results are given in Table 5. No significant slope was detected with the results concerning tylosin. For spiramycin and tylosin, slopes of the linear regression lines were significantly different from 0, indicating that the compounds may be not stable when stored at –20°C. The stability study will be continued for at least 1 year.

Conclusion

This method allows simultaneous determination of tilmicosin, tylosin, spiramycin, and neospiramycin between ½ and 4 MRL. It was validated according to EC Commission Decision 93/256/EEC. The suitability to incurred samples was verified, and this method was determined to be very reliable.

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