

Detection of macrocyclic lactones in porcine liver, meat and fish tissue using LC–APCI–MS–MS

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A selective and sensitive method for the simultaneous determination of five avermectins (abamectin, ivermectin, doramectin, emamectin and eprinomectin) and one milbemycin (moxidectin) in porcine liver, bovine meat and fish tissue was developed. The method involved extraction with acetonitrile and purification by C₁₈ solid-phase extraction. Detection was carried out using liquid chromatography coupled to multiple mass spectrometry (LC–MS²) equipped with APCI in the negative mode. This method was validated according to the requirements of Commission Decision EC/2002/657 (Implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Off J Eur Commun. L221: 8–36.). In addition to the linear response (R^2 between 59 and 97%), good repeatability (CV between 20 and 35%), reproducibility (CV between 20 and 35%) and detection ($CC\alpha$) and quantification ($CC\beta$) limits were obtained for all compounds in all matrices considered.

Keywords: chromatography; LC/MS; drug residues; ivermectin; animal products; meat; fish

Introduction

Avermectins (AVMs) and milbemycins (MMs) belong to the family of the macrocyclic lactones and are natural fermentation products of *Streptomyces* cultures. These anti-parasitic agents have widespread use in veterinary medicine for both endo- and ectoparasitic infections (Howells and Sauer 2001; Daeseleire et al. 2004; Sheridan and Desjardins 2006). They can be administered to cattle via injection, pour-on or via bolus formulation. It has been proposed that their neurotoxic action is based on an interaction with the receptor channels for inhibitory neurotransmitters (Rudik et al. 2002; Danaher et al. 2006).

Specific withdrawal periods have been defined which depend on the macrocyclic lactone, the treated animal and the formulation. To protect consumer's health, the European Union requires that the use of these veterinary drugs and their withdrawal periods are evaluated and it has established maximum residue limits (MRLs) for these compounds in specific edible matrices, i.e. muscle, fat, kidney, liver and milk (Table 1). The metabolism of macrocyclic lactones in animal tissues is well investigated. It has been reported that liver and fat are most suitable for residue analysis and that the parent drugs are the most appropriate marker residues (Daeseleire et al. 2004; Danaher et al. 2006).

In recent years, there has been a growing concern about the presence of veterinary drug residues in edible matrices of animal origin. Consequently, there is a need for continuous development of improved multi-residue methods. Within this in mind, the objective of this study was, firstly, the development of an analytical approach for the quantitative detection of residues of five avermectins: ivermectin (IVM), doramectin (DOR), eprinomectin (EPR), abamectin (ABA) and emamectin (EMA) in edible matrices of animal origin. In addition, the milbemycin, moxidectin (MOX), structurally similar to the group of avermectins but lacking the disaccharide group, was considered (Figure 1). Secondly, this quantitative multi-residue method was evaluated and validated according to the European Criteria 2002/657 concerning the performance of analytical methods and interpretation of results.

Materials and methods

Reagents and chemicals

Solvents and reagents, purchased from Across Organics (Fairlawn, NJ, USA) or VWR (Darmstadt, Germany), were of analytical-grade when used for extraction and of HPLC-grade for LC–MS–MS application. Triethylamin (TEA) was purchased from VWR (Darmstadt, Germany). The avermectins

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Table 1. MRL levels for macrocyclic lactones in various matrices.

Compounds	Species	MRL ($\mu\text{g kg}^{-1}$)	
Abamectin	Bovine	10	Fat
		20	Liver
Doramectin	Bovine	10	Muscle
		150	Fat
		100	Liver
		30	Kidney
	Porcine	20	Muscle
		100	Fat
		50	Liver
		30	Kidney
Emamectin	Fish	100	
Eprinomectin	Bovine	50	Muscle
		250	Fat
		1500	Liver
		300	Kidney
Ivermectin	Mammalian	100	Fat
		100	Liver
		100	Kidney
		50	Muscle
Moxidectin	Bovine	500	Fat
		100	Liver
		50	Kidney

(AVMs), abamectin (ABA, purity 97.1%), doramectin (DORA, purity 84.6%), emamectin-benzoate (EMA, purity 99.1%) and eprinomectin (EPRINO, purity 100%), were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ivermectin (IVER, purity 90%) was purchased from Dr Ehrenstorfer (Ausberg, Germany). The milbemycin (MM), moxidectin (MOXI, purity 99.3%), and the internal standard, selamectin (SELA, 95.1%), were kindly provided by the Scientific Institute for Public Health (Brussels, Belgium). The chemical structures of the compounds considered are shown in Figure 1. Individual and composite working standards were prepared by appropriate dilution of the standard stock solutions. For each compound considered, stock solutions of $1 \mu\text{g}\mu\text{l}^{-1}$ were prepared in ethanol. For eprinomectin ($100 \text{ ng}\mu\text{l}^{-1}$), acetonitrile was used. The stock solution of selamectin was prepared in a mixture of acetonitrile and methanol (95:5, v/v). All stock solutions were stored at -20°C in the dark. Eprinomectin was stored at 4°C . Working standard and internal standard solutions used for spiking were prepared by appropriate dilution in ethanol and renewed every month following the quality assurance criteria of the laboratory (EN-17025).

Extraction and clean-up

Extraction of the samples was based on the previously described method of Hou et al. (2007). In short, 2.5 g of homogenised tissue was extracted with 8 ml acetonitrile. After mixing and centrifugation at 7000 g, 25 ml

water and 40 μl triethylamine (TEA) was added. C_{18} SPE (Bond Elute 6 ml, 500 mg; Varian Inc., Palo Alto, CA, USA) cartridges were conditioned with 5 ml acetonitrile (AcN) and subsequently with 5 ml of a mixture of acetonitrile and 0.1% aqueous TEA (2:3, v/v). Elution was performed using $2 \times 2 \text{ ml}$ acetonitrile. Extracts were subsequently evaporated to dryness under nitrogen (40°C) and reconstituted by sequential addition of 100 μl 0.1% TEA in AcN and 50 μl 0.1% aqueous TEA.

Chromatographic instrumentation

The HPLC apparatus consisted of a P4000 quaternary pump and an AS3000 autosampler (Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic separation was achieved using reversed-phase chromatography with gradient elution using a Symmetry[®] C_{18} ($5 \mu\text{m}$, $2.1 \times 150 \text{ mm}$, Waters, Milford, MA, USA). The mobile-phase consisted of a mixture of (A) 0.05% TEA in acetonitrile and (B) 0.05% TEA in water. A linear gradient of 0.3 ml min^{-1} was run: 70% A:30% B for 7 min, increasing to 100% A over 10 min (hold 7 min) and, finally, back to initial conditions for 11 min. Total run time was 35 min. Sample tray temperature was set at 10°C . Injection volume for the samples was set at 30 μl . Detection of the analytes was carried out with an LCQ Deca Ion Trap (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an atmospheric interface (APCI) operating in the negative MS–MS ion mode. Diagnostic ions and collision energies are described in Table 2. Activation Q (frequency to fragment ions) was set at the default value of 0.25. Data acquisition was carried out using Xcalibur 1.3. software (Thermo Fisher Scientific). All data were further analysed using Microsoft[®] Excel (Microsoft Corporation, USA).

Quality assurance

Prior to and after sample analysis, a standard mixture (20 ng on column) of the macrocyclic lactones of interest was injected to check the performance conditions of the LC–APCI–MS–MS instrument. To correct for extraction losses, selamectin was added to every sample at a concentration of $20 \mu\text{g kg}^{-1}$ (50 μl of a spiking solution of $1 \text{ ng}\mu\text{l}^{-1}$) prior to extraction. This compound was considered a suitable internal standard as, in Belgium, it is registered for treatment of cats and dogs only (BCFI-VET, 2007).

For quantitative analysis, calibration standards were spiked at MRL level in blank matrix. When using this analytical method for routine analysis, quality control was performed by analysis of blank and spiked samples at MRL level, together with each series of samples. At regular intervals, secondary quality control, using blind fortified samples, was carried out within the routine analysis.

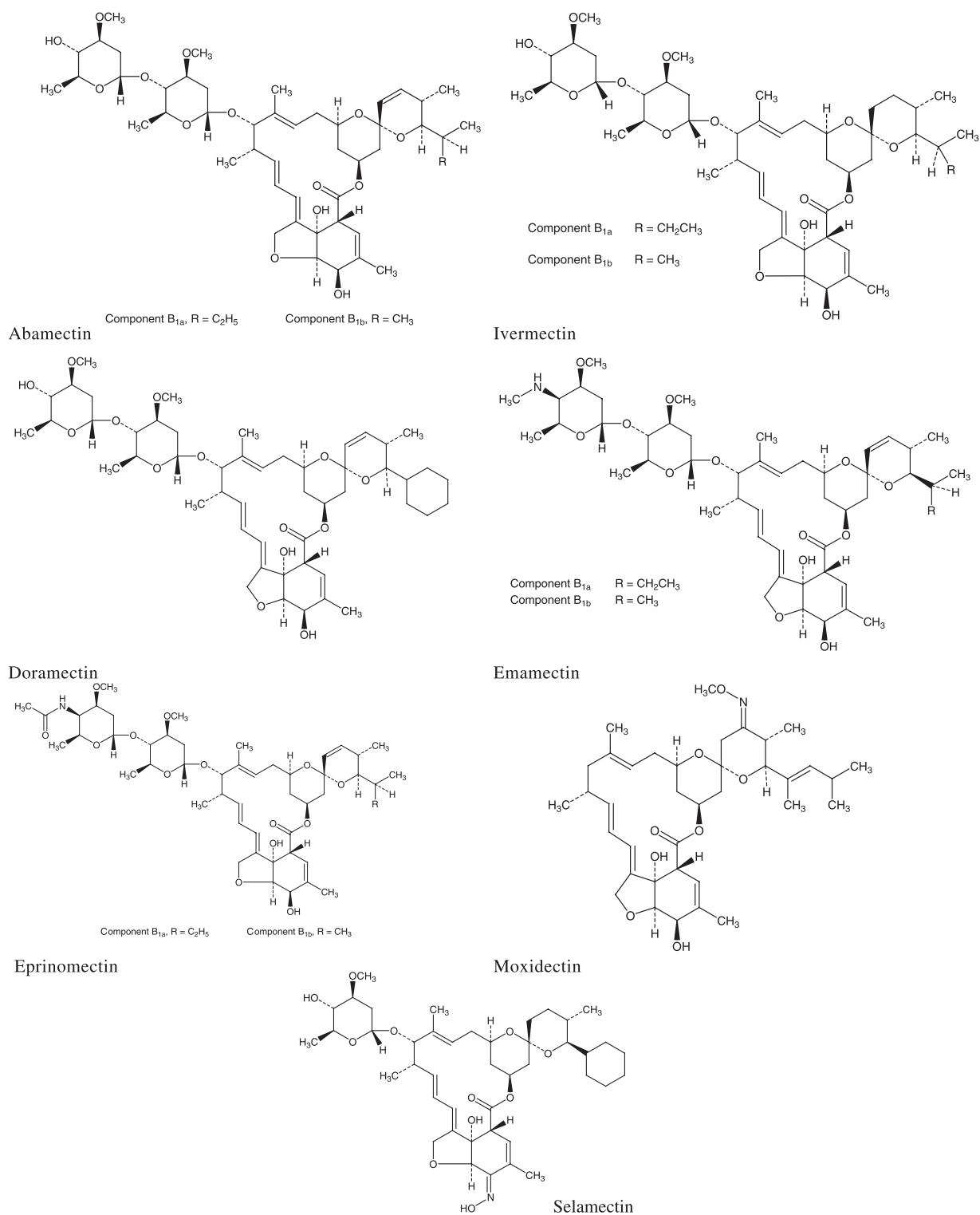


Figure 1. Chemical structures of the avermectins: ivermectin (IVM), doramectin (DOR), eprinomectin (EPR), abamectin (ABA), emamectin (EMA), selamectin (SEL) and the milbemycin, moxidectin (MOX).

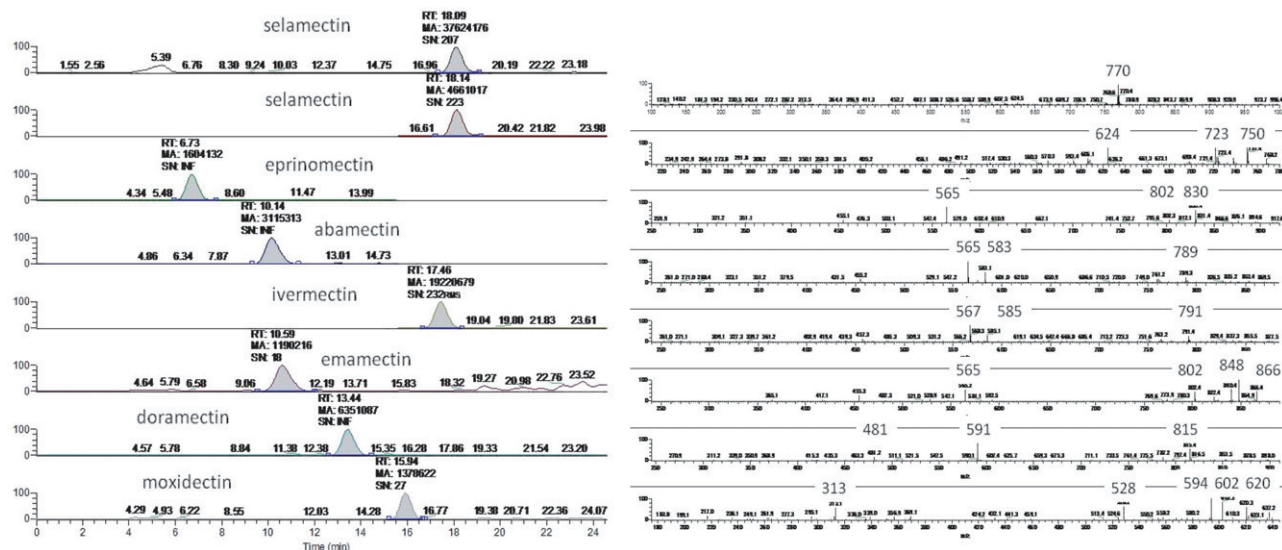
Validation procedure

Method validation for detection of targeted macrocyclic lactones was carried out according to the European Criteria 2002/657 for detecting residues of veterinary drug substances in matrices of animal origin.

The examined validation parameters were selectivity/specificity, linearity, trueness/recovery, applicability/ruggedness/stability, decision limits (CC α) and detection capability (CC β). For these, blank matrix samples were fortified with a standard solution of a mixture of the considered macrocyclic lactones.

Table 2. Precursor and product ions (m/z), isolation width and collision energy for the macrocyclic lactones considered (internal standard is in *italics*; most abundant product ion is underlined).

Macrocyclic lactone	Molecular weight	Precursor ion (m/z)	Isolation width	Collision energy (eV)	Product ions (m/z)
ABA	873.11	872.0	4.0	35	<u>565</u> , 583, 789
DORA	899.11	897.5	2.5	34	<u>481</u> , <u>591</u> , 815
EMA	1008 (886)	884.5	2.5	35	565, <u>802</u> , 840, <u>848</u> , 866
EPRINO	914.14	912.5	2.5	35	565, 802, 830
IVER	875.10	874.0	4.0	35	<u>567</u> , 585, 791
MOX	639.82	638.4	3.0	34	<u>313</u> , 528, 594, 602, 620
SELA	769.96	769.3	2.5	33	606, <u>624</u> , <u>723</u> , 750

Figure 2. Chromatograms (shaded zones=peak area) of selamectin ($20 \mu\text{g kg}^{-1}$), eprinomectin ($10 \mu\text{g kg}^{-1}$), abamectin ($10 \mu\text{g kg}^{-1}$), ivermectin ($100 \mu\text{g kg}^{-1}$), emamectin ($50 \mu\text{g kg}^{-1}$), doramectin ($50 \mu\text{g kg}^{-1}$), moxidectin ($10 \mu\text{g kg}^{-1}$) spiked in liver tissue.

The analytical method was validated at the MRL level for porcine liver, bovine meat and fish tissue. For non-MRL compounds, validation was performed at a concentration of $10 \mu\text{g kg}^{-1}$, unless otherwise mentioned.

Results and discussion

Performance of the LC-MS-MS method

To date, several analytical methods for the detection of residues of macrocyclic lactones in edible tissues and milk have been described (Scarano et al. 1998; Howells and Sauer 2001; Turnipseed et al. 2005; Durden 2007; Hou et al. 2007). These methods are usually based on separation by liquid chromatography and fluorescence detection (Danaher et al. 2006). Moreover, most described methods are single-residue, although multi-residue methods are preferred for surveillance of residues in edible matrices by the inspection services. To enable robust confirmatory analysis, unambiguous identification and quantification of these residues is necessary. Therefore, in this study, mass spectrometry was preferred to fluorescence detection due to the gain

in specificity and selectivity when dealing with complex matrices and the structural information on the resolved peaks. Additionally, compared to fluorescence detection, no time-consuming and labour-intensive derivatisation step is required.

As can be seen in Table 2 and Figure 2 and described by Durden (2007), all macrocyclic lactones considered produced the $[M-H]^-$ ion using the chromatographic conditions described above. The absence of interferences in the separation of the targeted compounds suggests a high specificity of the chromatographic method and a good selectivity of the extraction procedure. Thus, the analytical approach was considered suitable for screening as well as confirmatory analysis.

Full in-house method validation: macrocyclic lactones in porcine liver

Maximum residue level

For porcine liver, a maximum residue level (MRL) of 50 and $100 \mu\text{g kg}^{-1}$ is set for doramectin and ivermectin, respectively (Table 1). For all other macrocyclic

lactones considered in porcine liver, no specific MRLs were formulated. Consequently, these were considered forbidden substances and, as such, a laboratory MRL of $10\text{ }\mu\text{g kg}^{-1}$ was set.

Specificity

Investigation of the occurrence of interfering compounds is one of the general requirements of EC/2002/657; therefore, specificity/selectivity was evaluated via analysis of blank matrix samples fortified separately with the various macrocyclic lactones at MRL concentrations. As can be seen in Figure 2, specificity was demonstrated since no significant peaks with an S/N ratios of 3 or more were observed at the retention times of the targeted macrocyclic lactones using LC-APCI-MS-MS, as described above.

Selectivity

Conforming to European Criteria 2002/657, analytes are identified on the basis of their relative retention time and ion ratio of the precursor and product ions in the acquired spectrum after multiple MS analysis of the pseudo-molecular ion $[M-H]^-$. Precursor and product ions of each analyte are given in Table 2. For the relative retention time, a deviation of 2.5% compared with the spiked or standard mixture was allowed. Furthermore, to interpret the chromatographic data, a system of identification points (IPs) was used. For doramectin and ivermectin, a minimum of three IPs (depending on the chromatographic technique applied) and the acquired number of ions, as described in EC/2002/657, were taken into account. For the other targeted macrocyclic lactones, abamectin, emamectin, eprinomectin and moxidectin, no official MRL value has been set. Consequently, these compounds were considered as forbidden and four IPs are required.

Calibration curves

The linearity of the LC-APCI-MS-MS response for the MRL compounds, doramectin and ivermectin, was evaluated by injections of a dilution series of standard mixture (10 ng up to 100 ng on column, triple injection). All calibration curves were forced through the origin and linearly fitted. Correlation coefficients were between 0.83 and 0.97. Linearity was also proven in matrix using repeated analysis of three calibration points in the concentration range 0.5, 1 and 2 times the MRL value. Correlation coefficients were between 0.59 and 0.97, indicating that selamectin is a suitable internal standard but better results could possibly be achieved with another internal standard (f.i. deuterated); however, to the best of our knowledge, no deuterated macrocyclic lactones are currently available.

Recovery

Since no certified reference material was available, trueness of the measurements was assessed by fortifying blank liver tissue with 0.5, 1 and 2 times the MRL concentration of ivermectin and doramectin in six replicates and on three different days. For non-MRL compounds, samples were fortified at $10\text{ }\mu\text{g kg}^{-1}$, except for emamectin being fortified at $25\text{ }\mu\text{g kg}^{-1}$. Table 3 demonstrates that all calculated recoveries fulfil the criterion that recovery should range from 80 to 110% for a mass fraction of, or greater than, $10\text{ }\mu\text{g kg}^{-1}$. The obtained recoveries were also within the same order of magnitude as those reported in literature for the same matrix (Roudaut 1998; Rudik et al. 2002).

Precision

To evaluate the precision of the method, repeatability and within-laboratory reproducibility were determined.

Repeatability was evaluated by calculating the coefficients of variation (CV) using the data from two series of six replicates of fortified samples of an identical matrix at 0.5, 1 and 2 times MRL or at $10\text{ }\mu\text{g kg}^{-1}$ for the non-MRL compounds. These analyses were carried out on two different occasions by the same analyst and under repeatable conditions. According to the Horwitz (HW) equation, the CV for the repeated analysis of fortified samples at a mass fraction of 10 and $200\text{ }\mu\text{g kg}^{-1}$ should not exceed 32 and 20%, respectively. For all macrocyclic lactones considered, good repeatability was obtained since all calculated CVs were lower or in the same order of magnitude as the calculated values with the HW equation.

Because no proficiency tests are available for macrocyclic lactones in matrices of animal origin, only the within-reproducibility was considered for evaluation of reproducibility. Thus, three series of six replicates of fortified samples at 0.5, 1 and 2 times MRL or at $10\text{ }\mu\text{g kg}^{-1}$ for the non-MRL compounds were analysed by different operators and on different days. The calculated CVs for both operators, different days of analysis and various concentrations were all between 20 and 35%, approaching values in the same order of the CVs calculated using the HW equation (Table 3).

Decision limit ($CC\alpha$) and detection capability ($CC\beta$)

The decision limit ($CC\alpha$), which is the concentration level at and above which the sample is non-compliant with 95% certainty, was calculated for specific macrocyclic lactones. For the MRL compounds, ivermectin and doramectin, this value was defined as the mean

Table 3. Recovery (%), repeatability (CV%), precision (CV%), decision limit (CC α) and detection capability (CC β) ($\mu\text{g kg}^{-1}$) of the developed method for porcine liver ($n=54$), bovine meat ($n=6$) and fish tissue ($n=6$).

	Recovery (%)	Precision (%)	Precision (CV%)			Liver	
			Liver	Meat	Fish	CC α ($\mu\text{g kg}^{-1}$)	CC β ($\mu\text{g kg}^{-1}$)
EPRINO	93	26–34	32	24	42	–	10
ABA	99	22–35	31	25	43	–	10
IVM	90–101*	18–20	20	19	33	122	144
EMA	100	20–25	21	29	44	–	25
DORA	82–98*	21–28	29	19	40	71	92
MOXI	103	24–37	32	30	30	–	10

*At 0.5 \times , 1 \times and 2 \times MRL concentration.

concentration of the spiked blank samples at the MRL level plus 1.64 times the corresponding standard deviation at this concentration. The detection capability (CC β), which is the smallest concentration that can be detected with 95% certainty, was calculated as the CC α plus 1.64 times the above-mentioned standard deviation. For the non-MRL compounds, which only require a qualitative validation, the CC β was set at $10 \mu\text{g kg}^{-1}$ (for emamectin $25 \mu\text{g kg}^{-1}$). The CC α values for the two MRL compounds ivermectin and doramectin were 122 and $71 \mu\text{g kg}^{-1}$, respectively. CC β values for these compounds were calculated as 144 and $92 \mu\text{g kg}^{-1}$, respectively.

Compound stability

Experiments were also conducted on the stability of the considered compounds in solvent, in matrix during storage and/or sample preparation, as required in EC/2002/657. Based on preliminary experiments, it was observed that ivermectin and abamectin degrade easily. For this reason, working solutions are renewed every month. It was also observed that, to avoid degradation of these compounds, the tray temperature of the autosampler of the chromatographic devices should be kept at 10°C . Because samples were extracted as soon as possible and, if not frozen at $-20 \pm 1^\circ\text{C}$, this parameter was not considered problematic. However, future experiments on the stability of the targeted macrocyclic lactones in solvents/different matrices will be conducted.

Ruggedness

To determine the ruggedness of the analytical method, pre-investigative experiments were carried out by varying factors of sample pre-treatment, clean-up and chromatographic analysis. Thus, different extraction cartridges (Isolute C₁₈, SEP PAK VAC C₁₈ and Bond Elute C₁₈) were tested and checked. Also, different extraction solvents and extraction volumes were evaluated. In addition, various chromatographic parameters, e.g. injection volume, liquid phases,

chromatographic columns and solvent gradient programs, were tested. Nevertheless, the parameters described in the section 'Materials and methods' were preferred. Further monitoring of applicability and ruggedness will be carried out during routine use of the developed analytical method. As yet, the method is a semi-quantitative method only. Quantification of the obtained residues was performed based on an MRL spike to compensate for matrix effects.

Method validation: macrocyclic lactones in bovine meat tissue

For the validation of bovine meat, six tissue samples were fortified with the MRL concentration of doramectin ($10 \mu\text{g kg}^{-1}$), eprinomectin ($50 \mu\text{g kg}^{-1}$) and moxidectin ($50 \mu\text{g kg}^{-1}$) (see also Table 1). All other targeted macrocyclic lactones were considered as forbidden and were added at $10 \mu\text{g kg}^{-1}$. Specificity/selectivity was evaluated by the analysis of blank matrix and fortified samples, as described for liver samples. For this matrix, no significant interferences were demonstrated using the described method. Identification and quantification of the targeted compounds was carried out as described above.

Recoveries were assessed via the analysis of fortified blank samples at 10 or $50 \mu\text{g kg}^{-1}$, depending on the compound. Recoveries of specific compounds were satisfactory, ranging between 80 and 110% for a mass fraction of, or bigger than, $10 \mu\text{g kg}^{-1}$. According to this observation, it can be concluded that the method as developed for liver can be applied to meat samples. Table 3 demonstrates that CVs for the repeated analysis at, respectively, 10 and $100 \mu\text{g kg}^{-1}$ should not exceed 32 and 23% according to the HW equation. For abamectin, this value is slightly exceeded, but the obtained CV was the same order of magnitude as the CV calculated using the HW equation.

Finally, the decision limit CC α and the detection capability CC β were calculated as described above. The calculated CC α values for eprinomectin,

doramectin and moxidectin were 69, 13 and 74 $\mu\text{g kg}^{-1}$, respectively, while $\text{CC}\beta$ values were 89, 16 and 99 $\mu\text{g kg}^{-1}$ respectively. $\text{CC}\beta$ values for the compounds without a specified MRL were set at 10 $\mu\text{g kg}^{-1}$.

Validation for fish tissue

The above-described method was also applied to fish tissue (salmon). Thus, six samples were fortified with the MRL concentration of emamectin (100 $\mu\text{g kg}^{-1}$). All other macrocyclic lactones considered were added at a concentration of 10 $\mu\text{g kg}^{-1}$, which was set as a laboratory MRL.

Specificity and selectivity were evaluated as described above for liver and meat. No significant interferences were observed. For the identification of the macrocyclic lactones in fish tissue, the criteria described in EC/2002/657 concerning relative retention time and ion ratio were used.

Similar to liver and meat, the trueness of the analysis of macrocyclic lactones in fish tissue was assessed by determining the recovery of known additions of the considered analytes to a blank matrix. Based on the measurements ($n=6$), all recoveries were satisfactory, ranging between 80 and 110% for a mass fraction of, or greater than, 10 $\mu\text{g kg}^{-1}$.

Table 3 also demonstrates that CVs at area ratio level for repeated analysis at, respectively, 10 and 100 $\mu\text{g kg}^{-1}$ should not exceed 32 and 23% according to the HW equation. However, only moxidectin fits this criterion. It was observed that fish tissue is a dirty matrix, dependent on the fat content of the tissue. These observations suggest that, for fish, another internal standard or a deuterated compound needs to be considered.

Finally, the decision limit $\text{CC}\alpha$ and the detection capability $\text{CC}\beta$ were calculated as described above. For emamectin, the $\text{CC}\alpha$ and $\text{CC}\beta$ were, respectively, 122 and 194 $\mu\text{g kg}^{-1}$. $\text{CC}\beta$ values for the compounds without a specified MRL were set at 10 $\mu\text{g kg}^{-1}$.

Conclusion

The described method was designed for multi-residue analysis of macrocyclic lactones in different matrices of animal origin within the framework of residue analysis. The results showed that the simultaneous determination of five avermectins (abamectin, doramectin, emamectin, eprinomectin, ivermectin) and one milbemycin (moxidectin) in porcine liver, bovine meat and fish tissue was possible, combining liquid chromatography with a highly specific mass spectrometric detection technique. Moreover, the reported method meets European requirements. The use of a suitable sample preparation method and internal standard (selamectin), together with an optimised chromatographic

analysis, offered good recovery, repeatability, reproducibility, precision and detection limits. It should be noted that this method was developed for different macrocyclic lactones in various matrices of animal origin using mass spectrometry, a very sensitive and specific determination technique, in contrast to fluorescence detection, as applied previously.

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