Detection of macrocyclic lactones in porcine liver, meat and fish tissues using LC-APCI-MS-MS

Herlinde Noppe, Karolien Verheyden, Julie Vanden Bussche, Klaas Wille,
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Herlinde Noppe, Karolien Verheyden, Julie Vanden Bussche, Klaas Wille, Hubert de Brabander. Detection of macrocyclic lactones in porcine liver, meat and fish tissues using LC-APCI-MS-MS. Food Additives and Contaminants, 2009, 26 (09), pp.1232-1238. 10.1080/02652030903046674 . hal-00573884

HAL Id: hal-00573884

<https://hal.science/hal-00573884>

Submitted on 5 Mar 2011

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Detection of macrocyclic lactones in porcine liver, meat and fish tissues using LC-APCI-MS-MS

Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2008-398.R2
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	16-May-2009
Complete List of Authors:	Noppe, Herlinde; Ghent University, Veterinary Public Health and Food Safety Verheyden, Karolien; Ghent University, Veterinary Public Health and Food Safety Vanden Bussche, Julie; Ghent University, Veterinary Public Health and Food Safety Wille, Klaas; Ghent University, Veterinary Public Health and Food Safety De Brabander, Hubert; Ghent University, Veterinary Public Health and Food Safety
Methods/Techniques:	Chromatography - LC/MS
Additives/Contaminants:	Drug residues - ivermectin
Food Types:	Animal products – meat, Fish

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Manuscripts

Answer to the comments of the referees:

1) *Follow the SI unit.*

This is adapted in the manuscript.

2) *Results and discussion (part 3.2.4. Calibration Curve) Your data were between 0,59 and 0,97. I doubt how to count of recovery, precision, precision, CCa and CCb. According to the CODEX guideline, correlation coefficients is over 0,95. I recommend you have to retest calibration curves.*

Calibration curves are not retested. And this for the following reasons:

- 1) Method validation was carried out according to the EUROPEAN CRITERIA 2002/657/EC which are the criteria for detecting residues of veterinary drugs in matrices of animal origin. Moreover, this validation study was carried out in a laboratory with a large experience in the analytics of veterinary and environmental chemicals in various matrices of animal and environmental origin. Above this, this laboratory works conform the EN ISO/IEC 17025 and the criteria of BELAC which is the Belgian Accreditation Body. As such, CODEX guidelines were not used and to the opinion of the authors, these guidelines are in general the same than the 2002/657/EC guidelines.
- 2) Linearity was evaluated through the repeated analysis of 3 calibration points (0.5, 1 and 2 times the MRL value (more than 50 samples). In the manuscript it was mentioned that the 'obtained R² values were between 0,59 and 0,97 indicating that selamectin suits as IS, but that a better internal standard (f.i. deuterated) can lead to better results. Nevertheless, up to today, no deuterated MLs are available'. This all indicated that the different possibilities were considered. But to our knowledge and based on the current available literature concerning the analytical approaches for the MLs considered no better alternatives are available so far.
- 3) Additionally, from the MLs considered, only a few compounds have an MRL depending on the animal matrix. For the other compounds considered, a laboratory MRL was set as low as possible e.g. 10 µg/kg. These levels are not legal, although we try to determine the MLs considered as low as possible. One should also be aware that, when MLs are abused, concentration levels that will be determined will be in a concentration range much higher than 10 µg/kg. As such, these R² levels were not considered as problematic to the quality of our validation study.

3) *Title of the paper – ‘coupled to multiple mass spectrometry’ is cumbersome and talking ‘edible matrices of animal origin’ tends not to lead one to think fish are included. I suggest using LC/MS/MS in the title and saying ‘porcine liver, meat and fish tissues’.*

This suggestion is adapted in the manuscript.

4) *Your use of ML as an abbreviation gets confusing when used in the same sentence as MRL. Maybe not necessary to use ML at all.*

This suggestion is adapted in the manuscript.

5) *No experiments were carried out with ‘incurred tissues, which would be the definitive proof of the validity of the method. Whilst the authors explain that no CRMs were available, they have said nothing about the failure to analyse incurred samples and what that might mean in terms of fully testing the methodology e.g. extraction step.*

In section 2.5. Validation study, it was described that the whole study was based on the analysis of ‘incurred’ samples. ‘Method validation for detection of targeted macrocyclic lactones in different edible matrices 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 and decision ($CC\alpha$) and detection ($CC\beta$) limits. For these, blank matrix samples were fortified with a standard solution of a mixture of the considered macrocyclic lactones’.

Moreover, in section 2.4. Quality assurance it was described that the quantitative analysis of the routine samples is based on analysis of incurred samples. ‘For quantitative analysis, calibration standards were spiked at MRL level in blank matrix.

Moreover, the quality control of the routine analysis was carried out using ‘incurred’ samples in the same matrix. When using this analytical method for routine analysis, quality control of the method was performed by analysis of blank and spiked samples at MRL level, together with every series of samples. At regular times, secondary quality control, using blind fortified samples, was carried out within the routine analysis.

6) *Table 3 reports recoveries and these are discussed in the text. However, it was not clear to me whether the reported recoveries were after correction using selamectin IS. Please explain how the use of selamectin works for recovery correction.*

This was explained in the Quality Assurance section (2.4.) of the manuscript. ‘To every sample selamectin was added 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 in order to correct for extraction losses. This compound was considered as a very suitable

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internal standard because in Belgium, this avermectin is only registered for treatment of cats and dogs (BCFI-VET, 2007). For quantitative analysis, calibration standards (of which the construction is also included in the manuscript) were spiked at MRL level in blank matrix. When using this analytical method for routine analysis, quality control of the method was performed by analysis of blank and spiked samples at MRL level, together with every series of samples. At regular times, secondary quality control, using blind fortified samples, was carried out within the routine analysis.'

Concentrations were calculated using the area ratio of the response of the analyte to the response of selamectine. This is the general way of using an Internal Standard and included in the 2002/657/EC criteria.

7) *Figure 2 needs to be improved. Please delete all the writing which has been printed from the data system.*

Figure 2 is optimized.

Detection of macrocyclic lactones in porcine liver, meat and fish tissues using LC-APCI-MS-MS

HERLINDE NOPPE, KAROLIEN VERHEYDEN, JULIE VANDEN BUSSCHE, KLAAS WILLE & HUBERT DE BRABANDER

Ghent University, Faculty of Veterinary Medicine, Department of Veterinary Public Health and Food Safety, Research Group of Veterinary Zoonoses, Salisburylaan 133, 9820 Merelbeke, Belgium

Correspondence to:

Prof. Dr. Hubert De Brabander

Ghent University, Faculty of Veterinary Medicine,

Dept. of Veterinary Public Health and Food Safety, Laboratory of Chemical Analysis,

Salisburylaan 133

B-9820 Merelbeke

Belgium

Hubert.DeBrabander@UGent.be

Copyright and page proofs to:

Dr. Ir. Herlinde Noppe

Ghent University, Faculty of Veterinary Medicine,

Dept. of Veterinary Public Health and Food Safety, Laboratory of Chemical Analysis,

Salisburylaan 133

B-9820 Merelbeke

Belgium

Herlinde.Noppe@UGent.be

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Abstract

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. Besides the linear response (R² between 59 and 97%), good repeatability (CV between 20 and 35%), reproducibility (CV between 20 and 35%) and detection (CC_α) and quantification (CC_β) limits were obtained for all compounds in all matrices considered.

Keywords

macrocyclic lactones, avermectins, milbemycins, liver, meat, LC-MSⁿ

1. 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 a widespread use in veterinary medicine for both endo- and ectoparasitic infections (Howells & Sauer, 2001, Daeseleire et al., 2004, Sheridan & 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 are defined depending 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 establishes therefore 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 described that liver and fat are the most suitable for residue analysis and that the parent drugs are the most suitable marker residues (Daeseleire et al., 2004, Danaher et al., 2006).

In recent years, there has been a growing concern about the presence of residues of veterinary drugs in edible matrices of animal origin. Consequently, there was a need for continuous development of improved multi-residue methods. Within this perspective, 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. Also the milbemycin moxidectin (MOX), structurally similar to the group of the avermectins but lacks the disaccharide group was considered (Fig. 1). Secondly, this quantitative multi-residue

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3 66 method was evaluated and validated according to the European Criteria 2002/657 concerning
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5 67 the performance of analytical methods and the interpretation of results.
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10 69 **2. Materials and methods**

15 71 *2.1. Reagents and chemicals*

17 72
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20 73 Solvents and reagents, purchased from Across Organics (Fairlawn, NJ, USA) or VWR
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22 74 (Merck, Darmstadt, Germany) were of analytical grade when used for extraction and of
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24 75 HPLC-grade for LC-MS-MS application. Triethylamin (TEA) was purchased from VWR
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26 76 (Merck, Darmstadt, Germany). The avermectins (AVMs) Abamectin (ABA, purity 97.1%),
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28 77 doramectin (DORA, purity 84.6%), emamectin-benzoate (EMA, purity 99.1%) and
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30 78 eprinomectin (EPRINO, purity 100%) were obtained from Sigma-Aldrich (St-Louis, MO,
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32 79 USA). Ivermectin (IVER, purity 90%) was purchased from VWR (Dr. Ehrenstorfer). The
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34 80 milbemycin (MM) moxidectin (MOXI, purity 99.3%) and the internal standard selamectin
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36 81 (SELA, 95.1%), were kindly provided by the Scientific Institute for Public Health (Brussels,
37
38 82 Belgium). The chemical structures of the compounds considered are shown in Fig. 1.
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40 83 Individual and composite working standards were prepared by appropriate dilution of the
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42 84 standard stock solutions. For each compound considered, stock solutions of 1 µg µl⁻¹ were
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44 85 prepared in ethanol. For eprinomectin (100 ng µL⁻¹) acetonitrile was used. The stock solution
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46 86 of selamectin was prepared in a mixture of acetonitrile and methanol (95:5). All stock
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48 87 solutions were stored at -20°C in the dark. Eprinomectin was stored at 4°C. Working standard
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50 88 and internal standard solutions used for spiking were prepared by appropriate dilution in
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52 89 ethanol and renewed every month following the quality assurance criteria of the laboratory
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54 90 (EN-17025).

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92 *2.2. Extraction and clean-up*

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94 Extraction of the samples was based on the previously described method of Hou et al. 2007.

95 In short, 2.5 g of homogenized tissue was extracted with 8 mL acetonitrile. After mixing and

96 centrifugation at 7000 g, 25 mL water and 40 µL triethylamin (TEA) was added. C₁₈ SPE

97 (Bond Elute 6 mL, 500 mg, Varian Inc, Palo Alto, CA, USA) cartridges were conditioned

98 with 5 mL acetonitrile and subsequently with 5 mL of a mixture of acetonitrile and 0.1%

99 aqueous TEA (2:3). Elution was performed using 2 times 2 mL acetonitrile. Extracts were

100 subsequently evaporated to dryness under nitrogen (40°C) and reconstituted by sequential

101 addition of 100 µL 0.1% TEA in AcN and 50 µL 0.1% aqueous TEA.

103 *2.3. Chromatographic Instrumentation*

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105 The HPLC apparatus consisted of a P4000 quaternary pump and an AS3000 autosampler

106 (Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic separation was achieved

107 using reversed phase chromatography with gradient elution using a Symmetry[®] C₁₈ (5 µm, 2.1

108 x 150 mm, Waters, Milford, USA). The mobile phase consisted of a mixture of (A) 0.05%

109 TEA in acetonitrile and (B) 0.05% TEA in water. A linear gradient of 0.3 mL min⁻¹ was run:

110 70% A:30% B for 7 min, increasing to 100% A over 10 min (hold 7 min) and finally back to

111 initial conditions for 11 min. Total run time is 35 minutes. Sample tray temperature was set at

112 10°C. Injection volume for the samples was set at 30 µL. Detection of the analytes was

113 carried out with an LCQ Deca Ion Trap (Thermo Fisher Scientific, Waltham, MA, USA)

114 equipped with an atmospheric interface (APCI) operating in the negative MS-MS ion mode.

115 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, Waltham, MA, USA). All data were further analysed using Microsoft® Excel (Microsoft Corporation, USA).

2.4. 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 every sample selamectin was added at a concentration of 20 $\mu\text{g kg}^{-1}$ (50 μL of a spiking solution of 1 ng μL^{-1}) prior to extraction in order to correct for extraction losses. This compound was considered as a very suitable internal standard because in Belgium, this avermectin is only registered for treatment of cats and dogs (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 of the method was performed by analysis of blank and spiked samples at MRL level, together with every series of samples. At regular times, secondary quality control, using blind fortified samples, was carried out within the routine analysis.

2.5. Validation procedure

Method validation for detection of targeted macrocyclic lactones in different edible matrices 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 and

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3 140 decision ($CC\alpha$) and detection ($CC\beta$) limits. For these, blank matrix samples were fortified
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5 141 with a standard solution of a mixture of the considered macrocyclic lactones.
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8 142 The analytical method was validated at MRL level for porcine liver, bovine meat and fish
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10 143 tissue. For the non-MRL compounds, validation was performed at a concentration of 10 μg
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12 144 kg^{-1} , unless otherwise mentioned.
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15 145 16 17 146 **3. Results and discussion** 18 19

20 147 21 22 148 *3.1. Performance of the LC-MS-MS method* 23 24

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27 150 Up to now, several analytical methods for the detection of residues of **macrocyclic lactones** in
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29 151 edible tissues and milk have been described (Scarano et al., 1998, Howells & Sauer, 2001,
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31 152 Turnipseed et al., 2005, Durden et al., 2007, Hou et al., 2007). These methods are usually
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33 153 based on separation by liquid chromatography and fluorescence detection (Danaher et al.,
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35 154 2006). Moreover, most described methods are single-residue, although multi-residue methods
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37 155 are preferred for surveillance of residues in edible matrices to achieve the inspection services.
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39 156 To enable robust confirmatory analysis, unambiguous identification and quantification of
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41 157 residues of these compounds is necessary. Therefore, detection using mass spectrometry was
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43 158 preferred above fluorescence detection in this study because of the gain in specificity and
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45 159 selectivity when dealing with complex matrices and the structural information on the resolved
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47 160 peaks. Additionally, no time-consuming and labour-intensive derivatization step is needed in
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49 161 comparison with the fluorescence detection.
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53 162 As can be seen in table 2 and figure 2 and as been described by Durden et al. (2007), all
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55 163 **macrocyclic lactones** considered produced the $[M-H]^-$ ion by using the chromatographic
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57 164 conditions as described above. The absence of interferences in the separation of the targeted
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compounds suggests a high specificity of the chromatographic method and a good selectivity of the extraction procedure. As such, the above described analytical approach was considered suitable for screening as well as for confirmatory analysis.

3.2. Full in-house method validation: Macrocyclic lactones in porcine liver

3.2.1. Maximum Residue Level (MRL)

For porcine liver, a Maximum Residue Level (MRL) of 50 µg kg⁻¹ and 100 µg kg⁻¹ is set for doramectin and ivermectin respectively (see Table 1). For all other macrocyclic lactones considered in porcine liver no specific MRLs were formulated. Consequently, the latter were considered as forbidden substances and as such a laboratory MRL of 10 µg kg⁻¹ was set.

3.2.2. Specificity

As one of the general requirements of EC/2002/657 is the investigation of the occurrence of interfering compounds, specificity/selectivity was evaluated through the analysis of blank matrix samples fortified with the macrocyclic lactones considered separately at the MRL concentration level. As can be seen in figure 2, the specificity of the chromatographic method was demonstrated since no significant peaks with a signal to noise ratio of 3 or more were observed at the retention times of the targeted macrocyclic lactones by using LC-APCI-MS-MS as described above.

3.2.3. Selectivity

Conform European Criteria 2002/657, analytes are identified on the basis of their relative retention time and the ion ratio of the precursor and product ions in the obtained 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% in comparison with the spike or standard mixture was allowed. Furthermore, to interpret the data of the chromatographic analysis a system of identification points (IPs) was used. For doramectin and ivermectin a minimum of three IPs (depending on the used chromatographic technique) and the obtained number of ions as described in EC/2002/657 were taken in account. For the other targeted **macrocyclic lactones**, abamectin, emamectin, eprinomectin and moxidectin, no official MRL value was set. Consequently, these compounds were considered as forbidden and four IPs are needed.

3.2.4. 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. The linearity was also proved in matrix using repeated analysis of 3 calibration points in the concentration range of 0.5, 1 and 2 times the MRL value. Correlation coefficients were between 0.59 and 0.97 indicating that selamectin suits as internal standard but possibly a better internal standard (f.i. deuterated) can lead to better results. Nevertheless, up to today, no deuterated **macrocyclic lactones** are available.

3.2.5. Recovery

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216 Since no Certified Reference Material was available, trueness of the measurements was

217 assessed by fortifying blank liver tissue with 0.5, 1 and 2 times the MRL concentration of

218 ivermectin and doramectin in 6 replicates and on 3 different days. For the non-MRL

219 compounds, samples were fortified at $10 \mu\text{g kg}^{-1}$ except for emamectin being fortified at 25

220 $\mu\text{g kg}^{-1}$. As can be noticed in table 3, all calculated recoveries fulfill the criterion that recovery

221 should range from 80 to 110% for a mass fraction of, or greater than, $10 \mu\text{g kg}^{-1}$. The obtained

222 recoveries were also within the same order of magnitude as those reported in literature for the

223 same matrix (Roudaut et al., 1998, Rudik et al., 2002).

225 3.2.6. Precision

227 To evaluate the precision of the method, repeatability and within-laboratory reproducibility

228 were determined.

229 Repeatability was evaluated by calculating the coefficients of variation (CV) using the data of

230 2 series of 6 replicates of fortified samples of an identical matrix at 0.5, 1 and 2 times MRL or

231 at $10 \mu\text{g kg}^{-1}$ for the non-MRL compounds. These analyses were carried out on two different

232 occasions by the same analyst and under repeatable conditions. According to the Horwitz

233 (HW) equation, the CV for the repeated analysis of fortified samples at a mass fraction of 10

234 and $200 \mu\text{g kg}^{-1}$ should not exceed 32% and 20% respectively. For all **macrocyclic lactones**

235 considered good repeatability was obtained since all calculated CVs were lower, or in the

236 same order of magnitude, in comparison with the calculated values with the HW equation.

237 Because no proficiency tests were available for **macrocyclic lactones** in matrices of animal

238 origin, for evaluation of the reproducibility only the within-reproducibility was considered.

239 To this end, 3 series of 6 replicates of fortified samples at 0.5, 1 and 2 times MRL or at $10 \mu\text{g}$

kg⁻¹ for the non-MRL compounds were analysed by different operators and on different days. The calculated CVs for both different operators, different days of analysis and different concentrations were all between 20 and 35%, approaching values of the same order of the CVs calculated using the HW equation (Table 3).

3.2.7. Decision limit (CC α) and detection capability (CC β)

The decision limit (CC α), which is the concentration level at and above which the sample is non-compliant with 95% certainty, was calculated for the macrocyclic lactones considered. For the MRL compounds ivermectin and doramectin, this value was defined as the mean concentration of the spiked blank samples at MRL concentration 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.

3.2.8. Compound stability

Experiments were also conducted on the stability of the considered compounds in solvent, in matrix during storage and/or sample preparation as requested 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

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3 265 degradation of these compounds the tray temperature of the autosampler of the
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10 268 problematic. However, future experiments on the stability of the targeted macrocyclic
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12 269 lactones in solvents/different matrices will be conducted.
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17 271 3.2.9. Ruggedness
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21 273 In order to determine the ruggedness of the analytical method, pre-investigative experiments
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23 274 were carried out by changing factors of the sample pre-treatment, clean-up and
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25 275 chromatographic analysis. For this purpose, different extraction cartridges (Isolute C₁₈, SEP
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27 276 PAK VAC C₁₈ and Bond Elute C₁₈) were tested and checked. Also different extraction
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29 277 solvents and extraction volumes were evaluated. In addition to this, a lot of parameters of the
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31 278 chromatographic analysis, e.g. injection volume, liquid phases, chromatographic columns and
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33 279 solvent gradient programs were tested. The parameters as described in the Materials and
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35 280 Methods section were preferred. Further experiments on applicability and ruggedness will be
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37 281 carried out during the use of the developed analytical method in routine analysis. So far, this
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39 282 method is a semi-quantitative method. Quantification of the obtained residues was performed
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41 283 based on an MRL spike in matrix to compensate for matrix effects.
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50 285 3.3. Method validation: MLs in bovine meat tissue
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54 287 For the validation of bovine meat 6 tissue samples were fortified with the MRL concentration
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56 288 of doramectin 10 µg kg⁻¹, eprinomectin 50 µg kg⁻¹ and moxidectin 50 µg kg⁻¹ (see also table
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58 289 1). All other targeted macrocyclic lactones were considered as forbidden and were added at 10
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$\mu\text{g kg}^{-1}$. Specificity/selectivity was evaluated by the analysis of blank matrix and fortified samples similar as described for the liver samples. Also for this matrix, no significant interferences were demonstrated using the described method. Also identification and quantification of the targeted compounds was carried out similarly as described above. Recoveries were assessed through the analysis of fortified blank samples at 10 or 50 $\mu\text{g kg}^{-1}$, depending on the compound. The recoveries of the compounds considered were all 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. As can be seen in table 3, 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 of the same order of magnitude in comparison with the CV calculated using the HW equation. Finally, the decision limit $CC\alpha$ and the detection capability $CC\beta$ were calculated as described above. The calculated $CC\alpha$ -values for respectively eprinomectin, doramectin and moxidectin were 69, 13 and 74 $\mu\text{g kg}^{-1}$ respectively. $CC\beta$ values were 89, 16 and 99 $\mu\text{g kg}^{-1}$ respectively. $CC\beta$ values for the compounds without a specified MRL were set at 10 $\mu\text{g kg}^{-1}$.

3.4. Validation for fish tissue

The above described method was also applied to fish tissue (salmon). For this purpose, 6 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 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

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3 315 fish tissue, the criteria described in EC/2002/657 concerning relative retention time and ion
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5 316 ratio were used as well.
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8 317 Similar to liver and meat the trueness of the analysis of **macrocyclic lactones** in fish tissue
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10 318 was assessed by determining the recovery of known additions of the considered analytes to
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12 319 blank matrix. Based on the measurements (n=6), all recoveries obtained were satisfactory,
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14 320 ranging between 80 and 110% for a mass fraction of, or greater than, 10 µg kg⁻¹.
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17 321 As can be seen in table 5, CVs at area ratio level for the repeated analysis at respectively 10
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19 322 and 100 µg kg⁻¹ should not exceed 32 and 23% according to the HW equation. However, only
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21 323 moxidectin fits this criterion. It was observed that fish tissue is a dirty matrix, depending on
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23 324 the fat content of the tissue. These observations led to the conclusion that for fish another
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25 325 internal standard or a deuterated compound might have to be considered.
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28 326 Finally, the decision limit CC_α and the detection capability CC_β were calculated as described
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30 327 above. For emamectin the CC_α and CC_β were respectively 122 and 194 µg kg⁻¹. CC_β values
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32 328 for the compounds without a specified MRL were set at 10 µg kg⁻¹.
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4. 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 obtained showed that the simultaneous determination of 5 avermectins (abamectin, doramectin, emamectin, eprinomectin, ivermectin) and 1 milbemycin (moxidectin) in porcine liver, as well as in bovine meat and fish tissue was enabled combining liquid chromatography with a high specific mass spectrometric detection technique. Moreover, the reported method meets the European requirements. The use of a suitable sample preparation method and internal standard (selamectin), together with an optimized chromatographic analysis, allowed to reach good recovery, repeatability, reproducibility, precision and detection limits. Besides that, it needs to be pointed out that this method was not only developed for different macrocyclic lactones in different matrices of animal origin, but that it was performed with a very sensitive and specific determination technique, mass spectrometry, and this in contrast with the fluorescence detection reported before.

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Acknowledgement

The authors are grateful to M. Naessens and E. Laverge for their assistance in experimental work and the skilful operation of the LC-MS-MS apparatus.

References

BCFI-vet (2007). www.bcfi-vet.be. Last update 12/09/2007.

Commission Decision (2002/657/EC). Implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Official Journal of the European Communities L221 8-36.

Daeseleire E., Mortier L., Delahaut P. , Proceedings of the Euroresidue V conference, Noordwijkerhout, The Netherlands (2004) 422.

Danaher M., Howells L.C., Crooks S.R.H., Cerkvénik-Flajs V., O’Keeffe M. 2006. Review of methodology for the determination of macrocyclic lactone residues in biological matrices. Journal of Chromatography B 844: 175-203.

Durden D.A. 2007. Positive and negative electrospray LC-MS-MS methods for quantification of the antiparasitic endectocide drugs, abamectin, doramectin, emamectin, eprinomectin, ivermectin, moxidectin and selamectin in milk. Journal of Chromatography B 850:134-147.

Hou X.L., Wu Y., Shen J., Wang L., Ding S. 2007. Multiresidue analysis of avermectins in bovine liver and muscle by liquid chromatography-fluorescence detector. Chromatographia 65: 77-80.

Howells L., Sauer M.J. 2001. Multi-residue analysis of avermectins and moxidectin by ion-trap LC-MSn. The Analyst 126: 155-160.

- 368 Roudaut B. 1998. Multiresidue method for the determination of avermectin and moxidectin
369 residues in liver using HPLC with fluorescence detection. *The Analyst* 123: 2541-2544.
- 370 Rudik I. , Cummings M.R., Poppenga R.H. 2002. Isolation and multiresidue detection of
371 macrolide endectocides present in animal matrices. *Journal of Veterinary Diagnostic*
372 *Investigation* 14: 295-302.
- 373 Scarano G., Esposito H., Grasso L., Soprano V., Oliviero G. 1998. Use of automated solid-
374 phase extraction equipment for the determination of ivermectin residues in animal liver by
375 HPLC. *The Analyst* 123: 2551-2553.
- 376 Sheridan R., Desjardins L. 2006. Determination of abamectin, doramectin, emamectin,
377 eprinomectin, ivermectin and moxidectin in milk by liquid chromatography electrospray
378 tandem mass spectrometry. *Journal of AOAC International* 89: 1088-1094.
- 379 Turnipseed S.B., Roybal J.E., Andersen W.C., Kuck L.R. 2005. Analysis of avermectin and
380 moxidectin residues in milk by liquid chromatography-tandem mass spectrometry using an
381 atmospheric pressure chemical ionization/atmospheric pressure photoionization source.
382 *Analytica Chimica Acta* 529: 159-165.

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

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Table 1. MRL levels for **macrocyclic lactones** in different matrices.

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

Table 2. Precursor and product ions (m/z), isolation width and collision energy for the **macrocyclic lactones** considered (internal standard is marked in italic, 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	481, <u>591</u> , 815
EMA	1008 (886)	884.5	2.5	35	565, 802, 840, <u>848</u> , 866
EPRINO	914.14	912.5	2.5	35	<u>565</u> , 802, 830
IVER	875.10	874.0	4.0	35	<u>567</u> , 585, 791
MOX	639.82	638.4	3.0	34	313, 528, <u>594</u> , 602, 620
<i>SELA</i>	769.96	769.3	2.5	33	606, <u>624</u> , 723, 750

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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, 1 and 2 MRL concentration)