Determination of avermectin and milbemycin residues in bovine muscle by liquid chromatography-tandem mass spectrometry and fluorescence detection using solvent extraction and low temperature cleanup

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A B S T R A C T

A simple and inexpensive sample preparation method based on solvent extraction, followed by low temperature cleanup, was demonstrated to be applicable for the determination of avermectin and milbemycin residues in bovine muscle by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and liquid chromatography with fluorescence (LC-FL) detection. The analytical methodology was validated according to the Commission Decision 2002/657/EC, using LC-MS/MS for confirmatory and LC-FL for quantitative purposes. Mean recovery was between 88.9 and 100.7% in three distinct concentrations. The coefficient of variation for repeatability and within-laboratory reproducibility ranged from 0.78 to 5.1% and from 0.28 to 9.0%, respectively. Method precision led to satisfactory values of decision limits (CCα) and detection capabilities (CCβ). The proposed method has been applied in the Brazilian National Residue Control Plan since 2010 for the determination of avermectins and milbemycin residues in bovine muscle samples. A total of 760 samples were analyzed and none of them presented residues at concentrations above the permitted levels established by the more recently applied directives.

1. Introduction

Antiparasitic drugs are used worldwide for the treatment and prevention of parasitic diseases in food producing animals, and are particularly important for cattle raising in tropical regions where cattle graze on rangelands and are intensively affected by both endo and ectoparasitosis (Phillips, 2010). In Brazil, which is the world’s largest exporter of cattle meat (ABIEC, 2011), the avermectins ivermectin (IVR), abamectin (ABA), doramectin (DOR), eprinomectin (EPR), and the milbemycin moxidectin (MOX) are among the most common antiparasitic drugs administered to cattle, representing more than 44% of the total veterinary antiparasitic compounds commercialized in the country (SINDAN, 2012). This preference is probably a consequence of their extended endectocide activity and due to the slow elimination rates of these compounds in cattle. However, the longer persistence of these compounds in the cattle body may also lead to the presence of residues in foodstuff of animal origin, at levels above the maximum residue limits (MRL) established by different countries, causing undesirables risks of public health associated with their consumption.

In early 2010, a special attention was given to the occurrence of IVR residues in corned beef and similar products exported from Brazil to United States (US) and European Community (EC) at levels above the US tolerance of 10 μg kg⁻¹ (USDA-FAS, 2010). At that time, the EC had not established MRL for IVR in bovine muscle, however, due to this occurrence, an action level of 30 μg kg⁻¹ was adopted for non-injection site samples (Kaufmann, Butcher, Maden, Walker, & Widmer, 2011; RASFF, 2011). Additionally, it was recommended a provisional MRL of 1300 μg kg⁻¹ for injection site samples (EMA, 2011). Until early 2010, no MRL was established by Brazilian legislation for avermectin residues in muscle samples from both injection and non-injection sites. Although the exportation of these beef products was temporarily suspended by the Brazilian Ministry of Agriculture in May 2010, others 19 occurrences of IVR residues in beef products imported from Brazil were registered by the EC in 2010 (RASFF, 2011), and others 22 violations detected by the United States Food Safety and Inspection Service (FSIS) between March and June 2010 (USDA, 2010). In June 2010, a series of corrective actions were introduced by the Brazilian government to solve such problems, within which the inclusion of avermectins analysis in bovine muscle in the Brazilian National Residue Control Plan (NRCP), following the same concern of NRCP expansion and analytical coverage proposed elsewhere (Mauricio, Lins, & Alvarenga, 2009).
Residues of avermectins have been monitored by the Brazilian NRCP in liver and milk samples for more than thirteen years through LC-FL after derivatization with 1-methylimidazole/acetic anhydride and, more recently, through LC-MS/MS, using an atmospheric pressure chemical ionization (APCI) interface. In both methods, the residues are extracted with acetonitrile (ACN) and purified by solid phase extraction (SPE) cartridges with C18 before the chromatographic analyses, similarly to those methods reported in the literature for the same purposes in milk (Souza, Lima, Teodoro, & Junqueira, 2007; Turnispeed, Roybal, Andersen, & Kuck, 2005) and liver (Ali, Sun, McLeroy, & Phillipi, 2000; Souza et al., 2003) samples. Recently, we have applied an alternative sample preparation based on liquid–liquid extraction with low temperature purification (LTP) for avermectins analysis in milk (Rübensam, Barreto, Hoff, Kist, & Pizzolato, 2011). Methods based on this cleanup technique have been used predominantly for pesticides residues analysis in different matrices (Goulart, Queiroz, Neves, & Queiroz, 2008; Lentza-Rizos, Avramides, & Frédérique, 2001; Pinho, Neves, Queiroz, & Silvério, 2010a, 2010b). In these procedures, generally the extract is obtained adding organic solvent to the sample, and the cleanup is performed by freezing this extract at −20 °C. Under this condition, the interfering compounds are frozen, whereas analytes remains in the liquid phase and are subsequently separated. For avermectins analysis, additional procedures were introduced in this method, such as sodium chloride additions (salting-out effect) and extract centrifugation, in order to obtain an extract clear and relatively free of interfering compounds (eg. polar proteins). In its first application for veterinary drugs analysis, this method proved to be simple, easy, inexpensive, and adequate alternative for high-throughput analysis of a large number of samples per day. Based on these issues and considering the urgency for method validation, the application of LTP was extended to the extraction procedure of avermectins and milbemycin residues in bovine muscle samples.

The goal of the present work was to report the application of LTP in the analysis of avermectins and milbemycin residues in bovine muscle by LC-MS/MS and LC-FL methods validated according to the Decision 2002/657/EC (EC, 2002). The practical issues for the implementation of the proposed methods in the Brazilian NRCP are also addressed. To our knowledge, this is the first application of an LTP cleanup-based method for avermectins analysis in bovine muscle.

2. Material and methods

2.1. Chemicals

Ivermectin, eprinomectin, emamectin benzoate (EMA), doramectin, abamectin, and moxidectin standards with purity higher than 81% were purchased from Sigma–Aldrich (St. Louis, MO, USA), ACN and acetic acid with HPLC grade were from J.T. Baker (Phillipsburg, NJ, USA). Ammonium acetate was obtained from Mallinkrodt-Baker (Phillipsburg, NJ, USA). 1-Methylimidazole and trifluoroacetic anhydride with derivatization grade were obtained from Vetec (Rio de Janeiro, Brazil).

Individual stock solutions of 1.0 mg mL
-1 were prepared by dissolving 10 mg of the solid standard in 10 mL of ACN. The working solution was prepared by combining aliquots of each stock solution in ACN to obtain a final concentration of 1 μg mL
-1 for ABA, IVR and MOX, 1.5 μg mL
-1 for DOR and 2.0 μg mL
-1 for EPR in acetonitrile. EMA was used as surrogate standard and its working solution was prepared and stored in separate flask at 1.0 μg mL
-1 in ACN. All the standard solutions were stored at ~ 20 °C in a polypropylene tube.

2.2. LC-MS/MS analysis

LC-MS/MS analyses were performed on an Agilent 1100 series LC system (Santa Clara, CA, USA) equipped with a binary pump, a vacuum degasser, and an autosampler, coupled to an API 5000 triple quadrupole tandem mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionization source (ESI), operating in the positive mode. The ionspray voltage and the source temperature were set at ~4500 V and 500 °C, respectively. The ions were monitored using the multiple reaction-monitoring (MRM) mode for two transitions with a dwell time of 100 ms. The tandem MS conditions are reported in the Table 1. Separation was performed on a Phenomenex Luna C18 column (150 mm × 2.1 mm, i.d., 5 μm), preceded by a guard column (4 mm × 3 mm i.d.) with the same packing material (Phenomenex, Torrance, CA, USA), using a mobile phase consisting of water (A), ACN (B), and 50 mM ammonium acetate buffer pH 5 (C), in gradient mode, at flow rate of 0.2 mL min
-1. The gradient was programmed to start with a mobile phase composition of 50% A, 45% B, and 5% C, and then programmed to 95% B and 5% C after 2 min. This composition was maintained for 15 min before returning to the start condition. The injection volume was 10 μL. A divert valve was used to direct the eluent flow to waste for the first 3.5 min to help remove any matrix impurities from entering the MS/MS.

2.3. LC-FL analysis

LC-FL analyses were performed on a Shimadzu LC-20AT system (Kyoto, Japan) equipped with a SIL-HTc autosampler, and an RF-10 AXL fluorescence detector operating at 365 nm (excitation) and 470 nm (emission). The separation was achieved on a Phenomenex Luna C18 column (50 mm × 2.1 mm, i.d., 5 μm) preceded by a guard column (4 mm × 3 mm i.d.) with the same packing material (Phenomenex, Torrence, CA, US), using a mobile phase consisted of water (A) and ACN (B), in gradient mode, at flow rate of

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<th>Table 1 Mass spectrometry settings for MS/MS analysis of avermectins and milbemycin in positive mode.</th>
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<td><strong>Analytes</strong></td>
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Most abundant fragment ion; (RT) Retention time.
The derivatized solution was left at room temperature for 10 min and then transferred to a 50 mL centrifuge tube, evaporated to dryness at 12 h, in order to perform LTP. After this, the derivatized solution was left at room temperature for 10 min and then analyzed (5 μL) by LC-FL.

2.4. Sample preparation

Samples of bovine muscle (from different anatomical locations, excluding injection sites) were obtained from distinct regions of Brazil, according to the sampling program established in the Brazilian NRCP, by the Brazilian Federal Inspection Service, from July 2010 to December 2011, resulting in 760 samples. The samples with no residues of avermectins and milbemycin were used for calibration, quality control (QC), and validation purposes. Matrix-matched calibration curves were prepared in triplicate, adding 0, 2.5, 12.5, 25, 50, 75, and 100 μL of standard working solution with 25 μL of surrogate to 5 g of blank samples. QC samples were prepared at two different levels, one at MRL and the other at an arbitrary concentration, surrogate to 5 g of blank samples. QC samples were prepared at two distinct levels, one at MRL and the other at an arbitrary concentration of variation (CV%) and recovery, excluded for EPR. EMA was used as surrogate at 5 μg kg⁻¹ for EPR. For the calculation of method precision (in terms of intra- and inter-day precision, expressed as coefficient of variation CV%), and recovery, three lots of eighteen blank samples fortified at 0.5, 1 and 1.5 times the standard deviation to the VL value, using six aliquots at each spiked level, were prepared on three different days and were independently analyzed. CCx was determined analyzing 21 blank samples fortified at VL and adding 1.64 times the standard deviation to the VL value. CCx was determined analyzing 21 blank samples fortified at CCx concentration and adding 1.64 times the standard deviation to the CCx value. Linearity was performed studying the regression significance and the linearity deviation of the matrix-matched calibration curves by analysis of variance, considering a p < 0.05 as significant. The limit of detection was calculated by LOD = 3.3 s/S, and the limit of quantification was calculated by LOQ = 10 s/S, in which “s” was the standard deviation of the linear coefficient and “S” was the slope of the calibration curve.

3. Results and discussion

3.1. Optimization of sample preparation

ACN was the solvent of choice for the extraction method due to its high efficiency for extracting avermectins residues from different matrices, even in samples with high fat content (Danaher, Howells, Crooks, Cerkvenik-Flajs, & O’Keeffe, 2006). The optimization was performed evaluating the volume of solvent in relation to the samples amount and varying the contact time between sample and solvent. Both experiments were evaluated by analyte recovery average obtained from LC-FL analysis. The influence of these two variables on the extraction method is demonstrated in the Fig. 1. It was observed that adding 10 mL of ACN in 5 g of sample (corresponding to solvent/sample ratio of 2) showed to be more adequate to extract all the target compounds, resulting in recoveries higher than 90% with contact time of 10 min. It was also observed that the increase in the organic solvent volume (eg. solvent/sample ratio of 3) does not improve the analyte recovery, but can lead to an increase in the matrix co-extractives, which can cause analytical problems. In addition, considering that real samples may differ in fat content and can cause variations in the avermectins recoveries due to the high lipophilicity of these compounds, it was defined a contact time between solvent and sample of 20 min.

The cleanup efficiency of LTP procedure was optimized varying the freezing time of the extract, at −20 °C. As can be seen in the standard solutions at concentrations corresponding to validation levels (VL) of 10 μg kg⁻¹ for ABA, IVR, and MOX, 15 μg kg⁻¹ for DOR, and 20 μg kg⁻¹ for EPR. EMA was used as surrogate at 5 μg kg⁻¹. For the calculation of method precision (in terms of intra- and inter-day precision, expressed as coefficient of variation CV%), and recovery, three lots of eighteen blank samples fortified at 0.5, 1 and 1.5 times the VL value, using six aliquots at each spiked level, were prepared on three different days and were independently analyzed. CCx was determined analyzing 21 blank samples fortified at VL and adding 1.64 times the standard deviation to the VL value. CCx was determined analyzing 21 blank samples fortified at CCx concentration and adding 1.64 times the standard deviation to the CCx value. Linearity was performed studying the regression significance and the linearity deviation of the matrix-matched calibration curves by analysis of variance, considering a p < 0.05 as significant. The limit of detection was calculated by LOD = 3.3 s/S, and the limit of quantification was calculated by LOQ = 10 s/S, in which “s” was the standard deviation of the linear coefficient and “S” was the slope of the calibration curve.

2.5. Validation procedure

The validation of the methods using LC-MS/MS and LC-FL was carried out according to the European Commission decision 2002/657/EC (EC, 2002) for qualitative confirmation and quantitative screening purposes, respectively. The performance characteristics evaluated were selectivity, specificity, CCx (only for LC-MS/MS), CCx, precision (only for LC-FL), recovery (R), and stability. In addition, linearity and the detection and quantification limits were also determined for LC-FL method.

Analyte confirmation was performed based on the ion ratio criteria established in the 2002/657/EC guide for LC-MS/MS, in which the relative intensity of two transitions for each analyte shall correspond to those of the calibration standard, associated with analyte retention time. Selectivity and specificity were evaluated analyzing twenty different blank muscle samples with and without addition of analytes, and analyzing samples spiked with the

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Fig. 1. Effects of solvent-sample ratio and the contact time on the compounds extraction.
Fig. 2A, the freezing time that gives an extract relatively free of interference was 12 h. It was also observed that as lower the freezing time, higher is the presence of interfering compounds. The result was an increase in the base line of LC-FL analysis that hindered the correct peak integration at low concentrations (Fig. 2B).

Fig. 2B, the freezing time that gives an extract relatively free of interference was 12 h. It was also observed that as lower the freezing time, higher is the presence of interfering compounds. The result was an increase in the base line of LC-FL analysis that hindered the correct peak integration at low concentrations (Fig. 2B).

3.2. Validation

Commission Decision 2002/657/EC require validation around the MRL values for authorized drugs or at concentrations as low as possible for drugs without MRL (EC, 2002). The validation levels used in this work were based on Codex Alimentarius and EC MRL, and on US regulatory tolerance (USDA-FAS, 2010) because MRL for avermectins and milbemycin residues in bovine muscle was established by the Brazilian legislation in 2011 (BRASIL, 2011). Emamectin was selected as surrogate standard since it is an avermectin licensed only for treatment of sea-lice on farmed salmon and its presence in bovine tissues is unlikely. The surrogate standard was used in order to correct possible losses during the analytical procedure, resulting in a satisfactory calibration curves with no-significant deviation from linearity (p < 0.05) in the working range of 0–2 times VL levels, with determination coefficients ($R^2$) higher than 0.99, and a significant regression at 95% confidence level after performing analysis of variance.

The selectivity was evaluated analyzing an appropriate number of representative blank samples and check for any interference in and around the retention time of the target analytes. As shown in Figs. 2C and 3, no interfering compounds were observed in both LC-FL and LC-MS/MS methods. Specificity was determined for LC-MS/MS, analyzing blank samples with and without avermectins additions. As can see in Fig. 3, there is a compound at 9.6 min in the two extracted ion chromatograms of IVR. However no apparent interference was observed, since a chromatographic resolution higher than 2 was obtained.

The proposed sample preparation result in satisfactory analyte recovery, ranging from 88.9 to 100.7% for all analytes in three different concentrations assessed. Method precision was satisfactory with a CV% intra-day inferior of 5.4% and the CV% inter-day lower than 8.8% for all analytes, and are in compliance with the Commission Decision 2002/EC/657 requirements.

The analytical parameters CcA and CcB are important to estimate the level of confidence in the routine analytical results and are normally used to prevent the reporting of false positive and false negative findings, respectively (Kaufmann et al., 2011). Since these parameters were calculated based on the relatively low standard deviation from the precision data, values near to MRL established by the consulted legislation were obtained. The confirmation of the identities of avermectins and milbemycin residues, in terms of relative ion intensities, were calculated for each analyte and compared with those limits required by the Commission Decision. The relative ion intensities of all analytes were stable during the validation study and were in the range from 20 to 50% of base peak.
with a coefficient of variation below 17%, demonstrating accordance with EU requirements. In addition, the relative retention times of the analytes for LC-MS/MS and LC-FL analysis were below of 1.5% from a maximum tolerance of 2.5% required as performance criteria. Quantification and detection limits were also calculated and were similar to the results presented by Hou, Wu, Shen, Wang, and Ding (2007), who reported limits of detection between 0.5 and 1.0 μg kg⁻¹ and limits of quantification between 1.0 and 2.0 μg kg⁻¹ for avermectins in bovine tissues analyzed by LC-FL after extraction with ACN and cleanup on a C18 cartridges. All results from validation study were presented in the Table 2.

### 3.3. Method application

After validation, the proposed method was applied to the simultaneous analysis of ABA, DOR, EPR, IVR, and MOX residues in 760 real samples of bovine muscle obtained from the Brazilian monitoring programs during 2010 and 2011. Until 100 samples per batch were processed by only one analyst, using approximately 1000 mL of ACN less than traditional extraction procedures used for the same purpose (Chou, Lai, Chen, & Yen, 2004; Hernando, Suárez-Barcena, Bueno, García-Reyes, & Fernández-Alba, 2007; Xia et al., 2010). The results of avermectins and milbemycin analyses are shown in the Table 3.

Although 39.1% of the total samples analyzed presented avermectins or milbemycin residues between LOD and MRL levels (positive samples), none contained residues above the MRL established by the Brazilian legislation. A number of them presented two avermectins per sample, 23 samples with ABA and IVR, and 13 with DOR and IVR, probably due to the use of associated antiparasitic drugs. The highest concentration found was 8.9 μg kg⁻¹ of IVR. In addition, IVR was the most common residue detected (21.0%), followed by ABA (10.5%), DOR (6.4%), MOX (5.1%), and EPR (0.8%). The highest occurrence of IVR residues can be related to the high availability of this antiparasitic in the Brazilian veterinary market, representing more than 64% of the total veterinary drugs containing avermectins (SINDAN, 2012).

Recovery for QC samples, analyzed along the real samples, was similar to the values obtained in the validation study, varying from 87 to 102%. All positive samples were confirmed by LC-MS/MS considering the 2002/657/EC criteria.

### 4. Conclusions

Extraction of avermectins and milbemycin from bovine muscle showed to be applicable using solid–liquid extraction with low temperature purification. The proposed extraction method was considered simple, easy and suitable for processing a large volume of samples with low consumption of organic solvent. Avermectins and milbemycin residues were quantitated and confirmed by validated LC-fluorescence and LC-MS/MS methods, respectively. The performance characteristics determined by validation study were considered satisfactory and demonstrate the method suitability for the routine analysis in the Brazilian National Residue Control Plan. Based on the results from the Brazilian monitoring program and on a more comprehensive auditing process conducted by FSIS in September 2010, the resumption of meat products exports from Brazil to US was authorized by US government in December 2010. In addition, in December 2011, MAPA established new criteria for the use of avermectins in cattle, restricting the use of formulations with withdrawal time above 28 days, which represents the average withdrawal time recommended for formulations containing 1% of ivermectin.

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