Application of QuEChERS - High Performance Liquid Chromatography with Postcolumn Fluorescence Derivatization (HPLC-FLD) method to analyze Eprinomectin B1a residues from a pour-on conditioning in bovine edible tissues

Abstract: A QuEChERS in house method for determining the marker residue of eprinomectin (eprinomectin B1a) by HPLC-FLD in bovine tissues and milk provided from treated animals was developed and applied. Briefly: all samples were extracted with acetonitrile using a dispersive SPE purification stage. The ascertained detection limits were 1 µg kg⁻¹ and the quantification limits 2 µg kg⁻¹. Recoveries on tissue samples fortified in the range of 10 µg kg⁻¹ to 200 µg kg⁻¹ were from 80.0% to 87.2%, with variation coefficients between 2.7% to 10.6%. The confirmation of residues in the purified extracts was made by LC-MS/MS after separation on an XTerra MS C₁₈ (10 cm × 2.1 mm, 3.5 µm) column with a mobile phase of acetonitrile / formic acid 0.1% (97:3, v/v) at a flow rate of 0.2 mL min⁻¹ and MRM monitoring of three characteristic ions (m/z 896.1, m/z 467.9 and m/z 329.9), resulting from the fragmentation of molecular ions [M-H]⁺ (m/z 914.6) of eprinomectin and the comparison of the abundance ratio of fragmented ions was obtained in the booth, sample and standard at comparative concentrations. In conclusion, this method has proven its advantage and versatility as a routine drug residues analysis method.

Keywords: Eprinomectin B1a, QuEChERS HPLC-FLD, Bovine edible tissues, Residues

1 Introduction

1.1. Eprinomectin

Eprinomectin is a semi-synthetic compound belonging to avermectins, a Macrocyclic Lactones (ML) class, derived from abamectin by replacing the hydroxy group at C₄ with the acetamide group, tested in 1996 and introduced in therapy in 1997 [3,9].

From a chemical point of view, eprinomectin is a mixture of two related compounds: eprinomectin B₁₄ (in a proportion of 90%) and eprinomectin B₁₅ (in proportion of 10%). As structure, they differ only by an alkyl group in position C₂₅: ethyl for B₁₄ and methyl for B₁₅ [1-3].

From a pharmacological point of view, the two related compounds have basically identical activities and belong to pharmacological group B (of the substances for which there are limits on the marker residue of eprinomectin B₁₄ in tissues extracted from treated animals). Because of the very low residues, eprinomectin can be considered as a choice substance in the fight against the anthelmintics toxicity and resistance, in this respect, this molecule...
being widely used in large ruminants. According with Commission Regulation (EU) No 37/2010, Eprinomectin for use in bovine is included in Annex II [2,4,5].

Eprinomectin has a broad antiparasitic spectrum, with a good action against three great classes of parasites: nematodes, insects and mites. Due to reduced risk of residues in tissues and organs until today, eprinomectin is the only endectocide approved in EU for topical use (e.g. pour-on) in fattening cattle category, and so far, the only representative of the ML group that can be introduced in the national strategic programs against endo and ecto-parasites, [2,6-9].

Also it is important to mention that, among its group, eprinomectin proved to have the lowest deleterious impact to environment [10,11].

Pharmacologic activity of eprinomectin is linked by its selective interaction with chloride ion transition channels mediated by glutamate in invertebrate (parasite) nervous and muscle cells. By the hyperpolarisation of the nervous or muscle cell, this will lead to an increased permeability to chloride ions in cell membrane followed by parasites’ paralysis and death. In addition, eprinomectin intensifies the release of GABA (gamma amino butyric acid) by presynaptic neurons, acting as neurotransmitter centres inhibitor and stimulation blocker of the adjacent neurons (in nematodes), or the muscle fiber (in arthropods). Differently, in the mammals, chloride ion transition channels mediated by glutamate have not been identified and, therefore, they will not transit the blood-brain barrier [2,3,6,9,12].

The ML comparative studies accomplished in lab animals, revealed that eprinomectin disposition in mice is controlled mainly by P-glycoprotein efflux, this finding being considered as a useful predictor of in vivo kinetic behaviour for this drug [13]. It was ascertained that after topical application in cattle, eprinomectin is absorbed through the skin at a rate of 21–36% of the dose, usually in the first seven days, continuing in a lesser extent up to 21 days. Eprinomectin is strongly linked to bovine plasma protein (more than 99%), peak eprinomectin concentrations identifiable in plasma (47–109 μg L⁻¹) being achieved generally to 2–5 days after administration [2,14-16].

Eprinomectin is accumulated especially in the liver (metabolising organ) and, in small amounts, in kidneys, fat and muscles. So far, it is known that in adult cattle species, after topical administrations of eprinomectin B₁a (500 μg kg⁻¹ BW) single doses, drug maximum plasma peaks (9–12 μg L⁻¹) were recorded on day 2 and 3 after application, followed by a significant decrease of plasma levels (2 μg L⁻¹) until day seven after applications [2,6,16,17].

In the case of calves, after the topical administrations of the same doses, of 500 μg kg⁻¹ BW, the highest eprinomectin B₁a plasma concentrations were recorded between days 4 (116 μg L⁻¹) and day 8 (136 μg L⁻¹) after applications, then plasma levels decreased to value 77.2 μg L⁻¹ on day 10 and respectively to 49.6 μg L⁻¹, on day 14 post administrations [2,9].

The main eprinomectin excretion route is by faeces, this being as 17.0 to 19.8% of the initial dose (and about 30% of the bioavailable dose) and only a small part is urinary excreted; about 0.35% (meaning less than 0.5% of bioavailable dose). The amount of eprinomectin excreted by milk is representing 0.32–0.54% of the initial dose (about 1% of bioavailable dose), in its unchanged chemical form [2,7,9,18].

From the studies accomplished with radioactive marked eprinomectin, it resulted that the predominant residue is eprinomectin B₁a which represents between 85–95% of the total residue in the liver, muscle and milk. Its counterpart, eprinomectin B₁b, it was found only in 7.6–8.4% of the total residue. Additionally, five to seven minor metabolites were found, (with a contribution of 1–2% to the total residue) [1,2,15,18].

### 1.2 About eprinomectin residues & analytical methods

The EMA-CVMP (European Medicines Agency - Committee for Medicinal Products for Veterinary Use) toxicological studies included in the Summary report for eprinomectin [4], revealed an Acceptable Daily Intake (ADI) value of 0–10 μg kg⁻¹ BW which is equivalent to 600 μg total residue (meaning eprinomectin including metabolites) for a 60 kg person. ADI value is based on a NOEL value (level at which no adverse effects are observed) of 1.0 mg kg⁻¹ BW per day and a safety factor of 100. Based on these considerations and the average daily food consumption by a person of 60 kg were established maximum residue limits of eprinomectin maker as: 50 μg kg⁻¹ for muscle, 100 μg kg⁻¹ for fat, 1500 μg kg⁻¹ for liver, 300 μg kg⁻¹ for the kidneys and 20 μg kg⁻¹ for milk, which is less than 50% of total residues (expressed as eprinomectin) in the amount of 600 mg ADI (Table 1).

The related to eprinomectin analytical techniques described so far, differ essentially by: solvents used in the extraction of derivatives and the extract purification mode (like: condensation with trifluoroacetic anhydride and N-methylimidazole [19]; addition of acetic acid to produce acid fluorescent derivatives [20]; acetonitrile extracts purified by using C₈ solid-phase extraction
A QuEChERS-HPLC-FLD method for Eprinomectin residues

cartridge [21,22]; use of sodium chloride for extraction [23]; acetonitrile extraction of samples followed by salting with MgSO₄:NaCl [24]; liquid-liquid extraction by partitioning into acetonitrile with subsequent solvent exchange into methanol-water etc. [8].

Today, attempts for different residues detection applications are tested to conceive more and more efficient and rapid methods. Currently the methods for determining residues of eprinomectin, and other compounds from the ML class are focusing mainly on the extraction and purification techniques of the analytes by SPE (Solid Phase Extraction) and derivatives quantification by HPLC methodology with fluorescence detection (FLD), generally achieved with 1-methylimidazole and trifluoroacetic anhydride [6,20-22].

In this respect, based on literature consulted, our study aim is to develop an Quick Easy Cheap Effective Rugged Safe (QuEChERS) in house method, in terms of derivatization, for determining residues of eprinomectin, and other compounds from the ML class are focusing mainly on the extraction and purification techniques of the analytes by SPE (Solid Phase Extraction) and derivatives quantification by HPLC methodology with fluorescence detection (FLD), generally achieved with 1-methylimidazole and trifluoroacetic anhydride [6,20-22].

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2 Materials and methods

The study was performed according to the GLP principles and quality requirements of Directives 2004/9/EC and 2004/10/EC. Method validation was done according to the requirements of the Annex to Directive 2002/657/EC and is based on:

– the method of determination of eprinomectin B₁₅₆ residues in animal tissues and milk by HPLC with fluorometric detection (HPLC-FLD) [25-27],
– validation and confirmation by LC-MS/MS – as described in the specific procedures [25-27].

2.1 Animals

All animals used in the experiment were not previously treated with any antiparasitic drug. The animals were housed appropriate to their species’ welfare and weight category, the water, concentrated feed and hay provided, being of very good quality and given without restraint. The residue depletion studies of eprinomectin B₁₅₆ has been carried out on beef cattle (250–350 kg weight, average: 280 kg) and dairy cattle (445–490 kg weight, average: 460 kg). The experiment on beef cattle, included one untreated Control group (C) (2 animals) and an Experimental group (E) (18 animals) and for dairy cattle experiment (M) a group of 8 animals was formed, from which milk samples were taken before and after the treatment, every 12 hours for 12 days.

2.2 Treatments and sampling

The product administered was a pour on Romanian eprinomectin; a topic antiparasitic veterinary conditioning used to cattle. The treatments were made individually for each animal after the pour-on technique, meaning a unique administration of antiparasitic liquid, poured onto the animal’s back, from withers to tail head, in the form of a narrow stripe (from where eprinomectin is absorbed through the cutaneous layer to give a systemic and efficient antiparasitary effect).

The dose administered to the experimental group (E) was of 0.5 mg kg⁻¹ BW, meaning 1 mL 10⁻³ kg⁻¹ BW, to each treated animal, (1 mL of used pour-on product containing: 5 mg eprinomectin active substance).

After treatments, the animals were sacrificed on days: 1, 3, 7, 14, 21 and respectively 28, from the veterinary conditioning application, and the edible tissues indicated by the Regulation EC 2377/2010, namely: muscle, kidney, liver, fat and dorsal muscles were sampled at a minimum of 50 grams per sample, respecting the experimental scheme presented in Table 2.

As control tissues, samples from untreated animals were used, animals being sacrificed in day 1. Samples that were not analyzed immediately were frozen at -18°C and stored until the analysis.

2.3 Standards, Reagents

The following standard and reagents were used:

– CRS standard eprinomectin (USP 1237752, 300 mg)
– HPLC acetonitrile and methanol,

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<table>
<thead>
<tr>
<th>Active substance</th>
<th>Marker residue</th>
<th>Species</th>
<th>MRL</th>
<th>Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eprinomectin</td>
<td>Eprinomectin B₁₅₆</td>
<td>Bovine</td>
<td>50 µg kg⁻¹</td>
<td>Muscle</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100 µg kg⁻¹</td>
<td>Fat</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1500 µg kg⁻¹</td>
<td>Liver</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>300 µg kg⁻¹</td>
<td>Kidney</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>20 µg kg⁻¹</td>
<td>Milk</td>
</tr>
</tbody>
</table>

Table 1: The EMA-CVMP accepted values for MRL for bovine edible tissues and milk after EMEA/MRL/114/96 Final [4].
– LiChroprep® RP-18 (25–40 µm) (Merck, Germany)
– anhydrous magnesium sulphate (MgSO4),
– sodium chloride (NaCl) and glacial acetic acid (Panreac Quimica, Spain),
– N-methylimidazole and trifluoroacetic anhydride (Sigma-Aldrich, Germany).
– Ultrapure water (conductivity: 0.055 mS cm−1) was obtained with the Ultra Clear TWF Series (Siemens water Technologies, Germany).

2.4 Instrumentation

The following instrumentation and accessories were used:
– Samples were weighted with a Kern ABJ 220-4M model analytical balance (Kern & Sohn GmbH, Germany).
– Refrigerated Centrifuge model IEC Centra MP-4R Bench-model (Labequip Ltd., Canada).
– Samples were homogenized using a Moulinette type mixer (Moulinex Inc.).
– Ultrapure water was obtained with a SG Water system, (Siemens GmbH, Germany).
– Vortex stirring was accomplished with Ultra-Turrax T25, (model IK444 Werke GmbH, Germany).
– Evaporation to dryness was accomplished with TurboVap® LV (Caliper Life Sciences, USA) under a stream of nitrogen.
– For HPLC analysis, a Waters 2695, connected to a Waters 2475 multi-wavelength fluorescence detector system (Waters Alliance, USA) was used.
– For LC-MS/MS it was used a Waters Micromass Quattro Micro API Tandem Quadrupole Mass Spectrometer LC-MS/MS System (Waters Alliance, USA) (mass Range: 2–2000 m/z) operated in ESI mode, Edwards E2M28 vacuum pump, (with Pentium IV PC and Mass Lynx 4.0 software).

2.5 Extraction and purification

Frozen samples were allowed to thaw at room temperature. When they were still cold, the tissues were cut into small pieces and homogenized using a mixer. A known amount of the homogenised sample (10 g) was transferred to a centrifuge tube, where acetonitrile (10 mL) was added followed by a mixture of anhydrous MgSO4:NaCl solution (4:1) (5 g) and immediately stirred vigorously for 1 minute, in order to prevent formation of agglomerates during the MgSO4 hydration.

After centrifugation at 4000 rpm for 10 min, the supernatant was transferred to a clean centrifuge tube containing MgSO4 (1.5 g) and C18 (0.5 g). After vortex stirring (1 minute) and centrifugation (4000 rpm for 10 minutes) a part of the supernatant was transferred (1–5 mL) in a test tube, and evaporated to dryness under a stream of nitrogen with the water bath set at 60°C.

2.6 Derivatization of eprinomectin

It was performed as described initially by Danaher et al., (2001) and improved by Jiang et al., (2007), using N-methylimidazole, trifluoroacetic anhydride and glacial acetic acid in acetonitrile [20,28].
Preparation of reagents for derivatization consisted of preparation of a solution of trifluoroacetic anhydride in acetonitrile (1/2, v/v) and of a solution of N-methylimidazole in acetonitrile (1/1, v/v). These two reagents were prepared on the day of use and may stand for several hours at the ambient temperature.

The preparation of standard derivatized solutions and calibration was accomplished by transferring in 8 glass tubes of: 5, 10, 50, 100, 250, 500, 750 and 1000 µL of ethalon solution (1.0 µg mL⁻¹), corresponding to: 0.005, 0.01, 0.05, 0.10, 0.25, 0.50, 0.75 and respectively 1.0 µg of eprinomectin B₁a what in working conditions corresponds to: 2, 10, 20, 50, 100, 150 and respectively 200 µg kg⁻¹.

After evaporation to dryness accomplished with TurboVap® at 60°C, to the residue will be added 200 (± 10) µL solution of N-methylimidazole and stirred for 10 seconds to the vortex. Then 300 (± 15) µL trifluoroacetic anhydride solution was added and stirred again for 10 seconds to the vortex.

According to Jiang et al., (2007), to shorten the reaction time, an amount of 50 (± 5) µL glacial acetic acid was added finally and stirred for the last 10 seconds. After this, the sealed derivatization tube was maintained at 65°C for 30 minutes and consecutively was cooled in a refrigerator for 3 minutes and the allowed to stand in the dark for 10 minutes before the HPLC injection.

Anhydrous conditions are imperative to avoid hydrolysis of C₄ acetylated derivative. Derivatives should be injected into the chromatographic system in less than 12 hours after derivatization.

### 2.7 Analysis methods

#### 2.7.1 HPLC-FLD

Derivatized eprinomectin B₁a residue analysis (limit of quantification of 2 µg kg⁻¹) was performed according to own home validated method. HPLC connected to a multi-wavelength FLD (fluorescence detector) system was used. The analytical system consisted in: automatic injector, Cₘ analytical column thermostatically controlled, ternary gradient pump with solvent degasser and auto sampler. An analytical column (ODS2 type: 250 × 4.6 mm di mm, 5 mm), maintained at 30°C, with a mobile phase of acetonitrile: water (97:3, v/v) and a flow rate of 1 mL min⁻¹ it was used. The fluorometric detector was set at an excitation wavelength of 355 nm and an emission wavelength of 455 nm.

In the range of: 0.005 – 1 µg (2.0 – 200 µg kg⁻¹), a linear calibration curve was obtained for eprinomectin B₁a, with the linear regression coefficient $r^2$: 0.9994. This obtained regression coefficient is greater than 0.9950 value wich denotes a good linearity for the selected range of concentration (Figs. B and C from the Additional material). To obtain the derivatized compound, the standard solution were treated under similar conditions to those used for the extract samples.

The limits of detection and quantification have been calculated by method based on the calibration curve of Miller and Miller, (2010) [29].

Quantification was done by using the calibration curve obtained by standard solutions containing 0.005 to 1.0 µg eprinomectin (2.0–200.0 µg kg⁻¹) under similar conditions to those used for the extract sample. The detection limit is given by the expression: $3.3 \frac{s_y}{s_x}/\text{slope}$; based on the assumptions:

a. the standard deviation of the signal of a solution with a concentration close to the blank solution is about standard deviation of $y$ (residual)

$$\frac{s_y}{s_x} = \sqrt{\frac{SS}{N-2}}$$

Where:

- $N$ is number of points on the curve;
- $SS$ is the sum of squared distances to curve points.

b. that there is a normal distribution at this concentration, probability of the α or β type errors that may occur is 5% and the curve intercepts zero value.

Arithmetic mean value of the different detection limits obtained with different calibration curves prepared fresh every day it was estimated. Consequently we obtained LOD = 1 µg kg⁻¹ and LOQ = 2 µg kg⁻¹.

The samples recovery percentage was calculated using the formula:

$$\% R = \frac{x_2 - x_1}{x_1} \times 100$$

Where:

- $x_1$ - is the measured blank
- $x_2$ - is the fortified sample measured
- $x_1$ - is the amount added

The residue concentration in the samples analyzed was calculated using calibration curves and recovery percentages given by the formula:
$\mu g \text{ kg}^{-1}$ eprinomectin $B_{1a} = c_{pr} \times 200 \times \frac{100}{R}$

Where:
$c_{pr}$ - is the eprinomectin $B_{1a}$ concentration in 5 grams of sample read in micrograms from calibration curve
$P$ - is the recovery percentage (where mean recovery must be between 70–110%).

### 2.7.2. LC-MS/MS

Eprinomectin residues in the purified non-derivatized sample extracts were confirmed by LC-MS/MS. For LC-MS/MS, technique (used in the aim of this method validation only) it was used an XTerra MS C$_{18}$ (Waters Alliance, USA) column (10 cm × 2.1 mm, 3.5 μm). The non-derivatized extract, was analyzed in ESI positive ionization mode with the following parameters: the drying gas (N$_2$) at 350 L h$^{-1}$, drying gas temperature 250°C, and ion spray voltage ~ 4500V.

The ions were monitored using Multiple Reaction Monitoring (MRM) mode for two transitions. MRM monitoring was accomplished for three characteristic ions: $m/z$ 896.1, $m/z$ 467.9 and $m/z$ 329.9, as tolerance permitted of ± 20%, resulting from the fragmentation of molecular ions [M-H]$^+$ ($m/z$ 914.6) of eprinomectin and the comparison of the abundance ratio of the fragmented ions was obtained in the booth, sample and standard at comparative concentrations.

A LC-MS/MS of a muscle sample (12.6 ppb), in working conditions (where a 3.31 minutes retention time it was established) is presented in Fig. 1 and the mass spectrum of eprinomectin it is shown in the figure A from the Additional material.

### 2.8 Statistics

The statistical software used to analyze the marker residues in tissues and milk was GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego, USA). The results were expressed as mean ± SEM. For the evaluation of differences between groups, two-way ANOVA with Bonferroni’s as post hoc test was used. Statistical differences were considered significant when $P < 0.05$ or lower.
3 Results and discussion

The method of extraction with acetonitrile and the dispersive purification proved to be effective, quick, simple and cheap. Since the classical derivatization method requires a long time, of about 2 hours, we agree with the authors [20,24] who tried to find the reaction conditions that would shorten this time. Based on observations of other authors such as the environmental acidity that influences speed reaction, we have used helpfully the addition of acetic acid in the mixture of derivatization [28].

The best results we achieved adding to the dry residue; obtained from the 1:1, (v/v) extraction of N-methylimidazole in acetonitrile solution (200 µL); trifluoroacetic anhydride in acetonitrile solution (1:2, v/v) (300 mL) and glacial acetic acid (50 µL), stirring in the vortex for 10 seconds between additions, and then maintained for 30 minutes at 65°C. The tube was cooled to 4°C (3 min) and left to stand in the dark, at room temperature for 12 minutes. An amount of 20 µL derivatized solution was then injected in the chromatographic system. In these conditions, we observed that the derivatization of eprinomectin B1a (with trifluoroacetic anhydride, N-methylimidazole in the presence of acetic acid), required a much shorter time for 30 minutes at 65°C, in comparison with not less than 90 minutes, in acetic acid absence case (Fig. 2).

While great majority of ML endectocide representatives (e.g. abamectin, doramectin, ivermectin, moxidectin, milbemectin), in the presence of 1-methylimidazole and trifluoroacetic anhydride, at the room temperature, almost instantly is forming fluorescent derivatives; eprinomectin different from these, requires much longer time (minimum 90 minutes) and a higher temperature (minimum 65°C). By glacial acetic acid adding to the derivatized mixture, the time needed for derivatization was sensibly reduced and the fluorescent derivative formed presented a remarkable stability confirming other authors’ observation [20,28].

Preliminary tests were performed to ascertain the best extraction method, derivatization conditions, the excitation wavelength and of fluorescent compound emission, and also the mobile phase composition. Following, it was established, that the excitation and emission maximum of the fluorescent derivative, were 355 nm and 455 nm (see figure D from Additional material).

For HPLC-FLD, mobile phase composition was optimized at the acetonitrile: water ratio (97:3 v/v) and to flow rate of 1 mL min⁻¹, in order to obtain a proper separation. In the working conditions, a retention time of about 12.0 minutes was achieved for the peak of eprinomectin B1a and asymmetry factor T < 2 (Figs. 3 and 4).

Methods’ precision was expressed in terms of recovery from bovine tissues and milk were studied by analyzing spiked samples at three different levels of concentration (0.5 × MRL; 1.0 × MRL and 1.5 × MRL) in replicate measurements. Method repeatability and precision it was ascertained on six replicates samples by analyzing samples fortified with known amounts of eprinomectin and recovery calculation expressed by the percentage of deviation of the concentration found from the theoretical concentration (Tables 3 and 4).

The obtained repetability Relative Standard Deviation (RSD) was comprised between 2.2 and 6.9% (Table 4). RSD values found for repeatability were under maximum value of 15% established by legislation [27]. Recovery from fortified samples, analyzed in different days is presented in Table 5.

![Figure 2: Comparative evolution of the eprinomectin derivatization process; with or, without acetic acid.](image)

Table 3: Repeatability on different fortified bovine samples.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Obtained concentration µg kg⁻¹</th>
<th>X µg kg⁻¹</th>
<th>SD µg kg⁻¹</th>
<th>RSD %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle</td>
<td>45.4 46.9 45.3 45.2</td>
<td>47.0 47.3</td>
<td>46.5 1.0</td>
<td>2.2</td>
</tr>
<tr>
<td>Fat</td>
<td>82.0 75.2 85.0 83.0</td>
<td>86.7 81.3</td>
<td>82.2 4.0</td>
<td>4.9</td>
</tr>
<tr>
<td>Liver</td>
<td>86.4 78.5 83.2 80.0</td>
<td>94.6 88.2</td>
<td>85.2 5.9</td>
<td>6.9</td>
</tr>
<tr>
<td>Kidney</td>
<td>88.0 93.8 87.5 85.5</td>
<td>86.2 84.7</td>
<td>87.6 3.3</td>
<td>3.7</td>
</tr>
<tr>
<td>Milk</td>
<td>17.8 19.1 18.8 16.7</td>
<td>17.5 18.2</td>
<td>18.0 0.9</td>
<td>4.9</td>
</tr>
</tbody>
</table>
Registered recoveries were higher than 80% by coefficients of variation (CV) less than 15%. Recovery was determined on fortified control samples 0.5 ×, 1 × and 1.5 × as follows: muscle: 25 μg kg⁻¹, 50 μg kg⁻¹ and respectively 75 μg kg⁻¹; fat: 50 μg kg⁻¹, 100 μg kg⁻¹ and respectively 150 μg kg⁻¹; liver: 750 μg kg⁻¹, 1500 μg kg⁻¹ and respectively 2250 μg kg⁻¹; kidney: 150 μg kg⁻¹, 300 μg kg⁻¹, 450 μg kg⁻¹ and milk: 10 μg kg⁻¹, 20 μg kg⁻¹ and respectively 30 μg kg⁻¹).

In our case, the average recovery coefficients were found in the range: 83.7‒97.3%, being placed between the accepted values (70‒110%).

Table 4: The precision of the analysis in bovine samples.

<table>
<thead>
<tr>
<th>Spiked eprinomectin B₁a content in fortified samples (μg kg⁻¹)</th>
<th>Analysed Eprinomectin B₁a content in fortified samples μg kg⁻¹ (average ± SD) (n = 6)</th>
<th>Repeatability variation %</th>
<th>Intermediate reproducibility variation %</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>8.04 ± 0.85</td>
<td>8.18</td>
<td>10.57</td>
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<tr>
<td>25</td>
<td>20.6 ± 1.8</td>
<td>7.39</td>
<td>9.22</td>
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<tr>
<td>50</td>
<td>42.6 ± 2.1</td>
<td>3.82</td>
<td>4.93</td>
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<tr>
<td>100</td>
<td>88.4 ± 3.2</td>
<td>2.92</td>
<td>3.62</td>
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<tr>
<td>500</td>
<td>436.2 ± 12.0</td>
<td>2.43</td>
<td>2.75</td>
</tr>
</tbody>
</table>

Table 5: Recovery from fortified samples (0.5 × MRL, 1 × MRL and 1.5 × MRL) analysed in different days.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Concentration level (μg kg⁻¹)</th>
<th>Average recovery % (n = 6)</th>
<th>Standard Deviation (SD)</th>
<th>CV %</th>
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<tbody>
<tr>
<td>Muscle</td>
<td>25</td>
<td>78.3</td>
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<td></td>
<td>50</td>
<td>93.0</td>
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<td></td>
<td>100</td>
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<td><strong>5.1</strong></td>
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<td>87.3</td>
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In Figs. 3 and 4 are presented the chromatograms of muscle samples with a content of eprinomectin B₁a, respectively, near the limit of quantification (Fig. 3) and a comparison between the chromatograms obtained for fortified liver to 10 μg kg⁻¹ and to 100 μg kg⁻¹, containing eprinomectin B₁a (Fig. 4).

Comparative analytical values obtained for the marker residues of B₁a eprinomectin in edible tissues and milk after treatment with the antiparasitic product, are presented in Figs. 5, 6 and 7.

The highest concentrations and persistent residues were found in liver followed by kidney, fat and muscle, but at any time of slaughter and in any tissues weren't found residues above the accepted MRL values [4,5]. Marker residue levels in samples from untreated control animals were below the detection limit of the analytical method (1 μg kg⁻¹) (Fig. E from Additional material).

Statistical results (by Anova) showed that the marker residue reached the maximum concentration approximately 3 days after the administration in all tissues.
At this time the marker residue in the liver was between 330–680 μg kg⁻¹ and dropped to 230–380 μg kg⁻¹ in 7 days. Depletion in other tissues followed the same pattern as that observed in the liver. The highest concentrations of marker residue found in the kidney and fat were of 98 μg kg⁻¹ and 24 μg kg⁻¹ respectively.

To all milk samples eprinomectin B₁a residues were found to be below the maximum limit allowed of 20.0 μg kg⁻¹, the maximum concentration found being 12.4 μg kg⁻¹ (Tables A and B from Additional material).

In Figs. 8 and 9, chromatograms of a milk sample fortified to 2 μg kg⁻¹ (LOQ) and a comparison between chromatograms obtained for fortified samples to 10 μg kg⁻¹ and 25 μg kg⁻¹ are presented.

The maximum peak ranged from 48–96 hours after the ant parasitic treatment to bovines. The average concentrations at 48, 72 and 96 hours were: 5.9 μg kg⁻¹; 7.8 μg kg⁻¹ and respectively 6.6 μg kg⁻¹. At 8 days after treatment, concentrations were within the limits of quantification (2 μg mL⁻¹). Similar values to ours were obtained in milk in a comprehensive comparative macrocyclic lactones study by Durden (2007) [30]. This method was validated in Romania according to the requirements of the Order ANSVSA 187/2007 for the
following parameters: identity confirmation, detection limits and quantification, linearity and range of linearity, interferences, storage stability and measurement uncertainty [31].

4 Conclusions

This method is a short and applicable one for the antiparasitic determination in edible tissues and milk residues. Our study revealed that the detection limit of the method used was 1 μg kg⁻¹, and the limit of quantification, based on acceptable precision was of 2 μg kg⁻¹, for all edible tissues. These values are fitting into the requirements for the residues analysis methods in products intended for human consumption, for which maximum residue limits are settled. The recoveries were greater than 80% with coefficients of variation below 15%. The amount of eprinomectin residues from this veterinary product and its biotransformation are comparable to those of standard medication used in similar purposes.

In our eyes the advantages of this specific QuEChERS – HPLC-FLD method provides a good: sensitivity and precision and so, this application, can contribute to enlarge the useful eco-friendly methods to researchers interested into perform various routine edible residues or other pharmacological or pollutant studies. The proposed methodology can be considered as simple and rapid, with linearity in usual residues range of concentration and very good precision.

Abbreviations

QuEChERS = Quick Easy Cheap Effective Rugged Safe
HPLC-FLD = High Performance Liquid Chromatography with Postcolumn Fluorescence Derivatization
SPE = Solid Phase Extraction
LC-MS/MS = Liquid chromatography-tandem mass spectrometry
MRM = Multiple Reaction Monitoring
GABA = Gamma Amino Butyric Acid
ML = Macrocyclic Lactones
μg kg⁻¹ BW = micrograms per kilogram bodyweight
μg L⁻¹ = micrograms per Litre
EMA-CVMP = European Medicines Agency – Committee for Medicinal Products for Veterinary Use
ADI = Acceptable Daily Intake
NOAEL = No Observed Adverse Effect Level
MRL = Maximum Residual Level
GLP = Good Laboratory Practice
a.u.v. = ad usum veterinarium = for veterinary use
CRS standard = Chemical Reference Substances
UPS = United States Pharmacopoeia
ESI = Electrospray Ionization
LOD = Limit of Detection
LOQ = Limit of Quantitation

References


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