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Occurrence of macrocyclic lactones in milk and yogurt from Brazilian market

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ABSTRACT

Avermectins and milbemycins belong to a family of compounds called macrocyclic lactones (ML) and are highly used as antiparasitic agents in the treatment of cattle for control of gastrointestinal nematodes, ticks and myiasis. In Brazil, there are five substances (ivermectin, abamectin, doramectin, eprinomectin and moxidectin) registered for bovines. The use of these compounds may bring benefits to the cows but indiscriminate use might result in the presence of residues in milk and dairy products. In this context, the objective of the study was to validate an analytical method for determination of five ML in dairy products and verify the occurrence of these compounds in milk and yogurt available in the Brazilian market. The method involved QuEChERS sample preparation, derivatization and determination by high performance liquid chromatography with fluorescence detection. The methodology was validated using organic samples of milk and yogurt for the following parameters: linearity, precision, accuracy, repeatability and limits of detection (LOD) and quantification (LOQ). The method showed good linearity. Average recovery, performed at three different levels varied from 83% to 112% (RSDs $< 14\%$). The method provides LOD ranging from 0.4 to 3.2 μg L^{-1} for milk and 0.6 to 0.9 μg kg^{-1} for yogurt. The LOQ was established according to the lower spike level (0.2–10 μg L^{-1} for milk and 2.5 μg kg^{-1} for yogurt). Repeatability and within-laboratory reproducibility were in satisfactory for both matrixes. In order to monitor milks and yogurts marketed in Campinas, SP, Brazil, 13 brands of UHT milk (135 samples), 8 brands of pasteurized milk (103 samples) and 13 brands of yogurt (104 samples) were acquired. A total of 342 samples were analyzed in duplicate for the presence of ivermectin, abamectin, doramectin, eprinomectin and moxidectin. Moxidectin was detected in one sample of pasteurized milk. No residue of the analyzed compounds was found in UHT milk or yogurt. Results indicate that the consumption of milk and yogurt does not represent a health risk for Brazilian consumers.

1. Introduction

Avermectins and milbemycins belong to a family of compounds called macrocyclic lactones (ML) and are highly used as antiparasitic agents in cattle and humans. Although the use of these substances bring benefits to the herd, the withdrawal time must be obeyed, as indiscriminate use of antiparasitic medicines, without the prescription instructions, may result in residues of these compounds in foods.

In Brazil, there are five compounds registered for use in cattle: ivermectin (IVE), abamectin (ABA), doramectin (DOR), eprinomectin (EPR) and moxidectin (MOX) totaling 135 commercial products. The most commercialized substances used in cattle are IVER, which is part of 75 products, and ABA, substance present in 56 products (SINDAN, 2013). In Brazil, only eprinomectin is indicated for use in cattle producing milk for human consumption, when other ML is being used the milk must be discarded (SINDAN, 2013).

In the European Union, compounds IVER, DOR and ABA are not allowed in lactating animals and maximum residue levels (MRLs) are established in bovine milk for EPR (20 μg/kg) and MOX (40 μg/kg) (Danaher, Howells, Crooks, Cerkvenik-Flajs, & O'Keeffe, 2006).

With the aim of protecting consumers and ensuring fair trade practices, these substances are monitored in food by regulatory

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agencies using established maximum residue limits (MRL). Codex Alimetarius does not establish an MRL for moxidectin in milk, while the MRL values for abamectin, ivermectin, doramectin and eprinomectin are set as 5 μg L^{-1}, 10 μg L^{-1}, 15 μg L^{-1} and 20 μg L^{-1}, respectively. In Brazil, veterinary drugs are monitored by the Brazilian Residues and Contaminants Control Plan (PNCRC) and the MRL established for cattle milk is 10 μg L^{-1} for abamectin, ivermectin and moxidectin. For doramectin and eprinomectin the MRL was set as 15 and 20 μg L^{-1}, respectively (Brasil, 2012).

In the last three decades, world milk production has increased by more than 50%, from 470 million tons in 1981 to 727 million tons in 2011. India is the world's largest milk producer, with 16% of global production, followed by the United States of America, China, Pakistan and Brazil, which produced 32 million tons of milk in 2011 (Embrapa, 2014; FAO, 2014). The consumption of milk and dairy products is associated with several benefits. They are considered a major source of nutrients and their daily consumption can contribute to the recommended daily intake of fat, protein, phosphorus, calcium, among other minerals, B vitamins, vitamins A and C. However, if it is not obtained within good veterinary practice, milk and other dairy products can be source of contaminants.

Most studies regarding contaminants in milk and dairy products present surveillance data indicating the presence and levels of occurrence of antibiotics, mycotoxins and product adulteration (Fonseca et al., 2009; Picinin et al., 2013; Souza et al., 2011). In products consumed on a daily basis, as is the case of milk and dairy products, it is vital that the levels of veterinary drugs used in livestock production are frequently monitored.

In 2003, Anastassiades et al., developed a sample preparation method for the determination of pesticide residues in fruits and vegetables that is known as QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe), which has been used for pesticide analysis in different matrix. Over the years this method has also been tested and successfully employed for the analysis of different compounds in foods, such as veterinary drugs, mycotoxins and polycyclic aromatic hydrocarbons (Aguilera-Luiz, Vidal, Romero-González, & Frenich, 2008; Cunha & Fernandes, 2010; Lesueur, Knittl, Gartner, Mentler, & Fuerhacker, 2008; Sadowska-Rociek, Surma, & Cieslik, 2014).

The objective of the present study was to validate an analytical methodology for determination of five ML in milk and yogurt using QuEChERS sample preparation and study the occurrence of these substances in samples commercially available in the Brazilian market.

2. Material and methods

2.1. Samples

Samples of milk and yogurt were collected monthly, throughout a year, in markets in the city of Campinas, State of São Paulo, Brazil. Milk samples were of two types: 13 brands of UHT milk (135 samples: 73 whole milk and 62 low fat) and 8 brands of pasteurized milk (103 samples: 67 whole and 36 low fat). Yogurts were obtained among 13 brands (104 samples), resulting in a total of 342 samples analyzed for the presence of ivermectin, abamectin, doramectin, eprinomectin and moxidectin. Organic samples of milk and yogurt were purchased to be used as blank samples.

2.2. Standards and reagents

Standards of doramectin, ivermectin, moxidectin, abamectin and eprinomectin were purchased from Sigma–Aldrich/Fluka, with purity >95%. Stock solutions of individual standards (10 mg L^{-1}) were prepared in acetonitrile and stored in the dark at −18 °C. The calibration standards solutions containing the 5 macrocyclic lactones were prepared in acetonitrile. These solutions were used for spiking organic milk and yogurt samples at different levels for recovery studies.

Reagent grade NaCl, acetic acid (Merck), anhydrous MgSO4, purity > 98% (Synth), N-methylimidazole (Sigma–Aldrich) and trifluoroacetic anhydride (Vetec) were used. The magnesium sulfate was heated in a muffle furnace for 5 h at 500 °C for phthalates and moisture removal. Primary secondary amine (PSA) sorbent (40 μm particle size) was obtained from Varian. Organic solvents used in the study were HPLC grade and purchased from Tedia.

2.3. Sample preparation

Samples were prepared by QuEChERS method followed by derivatization (Anastassiades, Lehotay, Štajnbaher, & Schenck, 2003; Kinsella et al., 2009). Ten mL of milk or 10 g of yogurt were transferred into a centrifuge tube, 10 mL of acetonitrile were added and the solution was mixed using a Vortex mixer for 1 min, then 4 g anhydrous MgSO4 and 1 g NaCl were added, the solution was mixed again for 1 min and the tube was centrifuged. Two milliliter aliquot of the upper layer was transferred to a centrifuge tube containing 100 mg PSA and 300 mg anhydrous MgSO4. The extract was mixed using a vortex for 30 s and then centrifuged. 550 mL of supernatant were transferred into a glass flask and the extract was evaporated in a water bath at 40 °C under nitrogen flow until total dryness. The extract was derivatized with 200 μL of 1-methylimidazole:acetonitrile (1:1), 200 μL of trifluoroacetic anhydride:acetonitrile (1:2) and 5 μL of acetic acid at 65 °C for 30 min. The extract was cooled, filtered through a Millex HV filter (0.45 μm, Millipore) and analyzed by HPLC with fluorescence detection.

2.4. HPLC-FLD analysis

The analyses were performed by HPLC-FLD on a Shimadzu system equipped with an LC-20AT pump, automatized injector SIL 20A (20 μL injection volume) and fluorescence detector RF-10A XL (excitation wavelength 365 nm and emission wavelength 470 nm). A C18 analytical column (Waters Nova-pak@, 150 × 3.9 mm, 4 μm particle size) at 25 °C and mobile phase of acetonitrile:methanol:acetic acid 2% (v/v) (55:40:5) at a flow rate of 1.2 mL min^{-1} were used for separation.

2.5. Method validation

The method validation was performed according to the Brazilian Institute of Metrology, Standardization and Industrial Quality (INMETRO) guidelines by the following parameters: linearity, accuracy, precision, limits of detection (LOD) and quantification (LOQ) (INMETRO, 2011). All the analyses were carried out using the same blank samples (organic milk and yogurt).

For milk, linearity was determined by the construction of calibration curves in acetonitrile in six concentrations in the range of 0–40 μg L^{-1} for eprinimectin; 0–30 μg L^{-1} for doramectin and 0–20 μg L^{-1} for ivermectin. These levels contemplate 0, 0.25, 0.5, 1, 1.5 and 2 times the MRL of the studied substances set by Codex Alimentarius. For moxidectin and abamectin, that do not have MRLs established by Codex, the concentrations studied were in the range of 0–20 μg L^{-1}. For yogurt, linearity was determined by the construction of calibration curves in acetonitrile at 7 concentration levels (0–25 μg L^{-1}).

Limits of detection (LODs) were estimated by seven independent analyses of organic milk and yogurt samples spiked with all ML at different levels. LODs were calculated as three times the standard deviation of the obtained responses. LOQs were

established as the lowest concentration level used in the recovery tests. Accuracy and precision (repeatability and within-laboratory reproducibility) were obtained through recovery studies carried out by spiking the blank samples with three different concentrations of ML standard solutions. Precision of the method was evaluated through the RSD associated with measurements during recovery tests.

3. Results and discussion

Fig. 1 presents representative chromatograms of a standard solution containing the five compounds analyzed, and a blank yogurt sample. As shown in the figure, an adequate separation of the 5 ML was achieved in a relatively short chromatographic run.

The ML showed linearity in the concentration studied, with correlation coefficients (r) higher than 0.9984. Table 1 presents the results obtained during method validation. As can be observed, recoveries for milk and yogurt varied between 96% to 109% and 83%–112%, respectively. The repeatability of the method was satisfactory for all ML, since the RSD were below 20% (European Commission, 2002; INMETRO, 2011). LODs varied from 0.1 μg L^{-1} (for ABA in milk) to 3.2 μg L^{-1} (for EPRI in milk). LOQs for milk were between 0.2 μg L^{-1}, for abamectin, to 10 μg L^{-1} for eprinomectin. As for yogurt, LOQ was 2.5 μg kg^{-1} for all compounds. Validation results are considered satisfactory as the method is shown to be able to detect and quantify the 5 ML studied in levels below the ones established by Codex.

Usually, ML residues are determined by HPLC-FLD or by LC–MS/MS and, although fluorescence detection requires derivatization, this is the most commonly used detector, due to its sensitivity, low cost and absence of matrix effect. Most of the procedures used for ML determination by HPLC-FLD involve extraction with acetonitrile and clean up by solid-phase extraction (Cerkvenik-Flajs et al., 2010; Danaher, O'Keeffe, & Glennon, 2000; Imperiale et al., 2009; Kolberg, Presta, Wickert, Adaime, & Zanella, 2009; Souza, Lima, Teodoro, & Junqueira, 2007; Turnipseed Roybal, Andersen & Kuck, 2005). In the latest years, QuEChERS sample preparation method has been applied for analysis of veterinary drugs, including ML, in different types of matrix (Aguilera-Luiz et al., 2008; Kinsella et al., 2009; Whelan et al., 2010). Nevertheless, the detection and quantification are usually performed by MS/MS. In the present study, a derivatization reaction was used in order to allow these compounds to be detected by fluorescence. Present results show that the use of QuEChERS sample preparation combined with derivatization also provides a satisfactory method for analysis of the five ML studied, being suitable for use as an alternative to the more expensive LC–MS/MS. As the present sample preparation method is less time consuming, less expensive and uses a smaller amount of solvent than extraction and clean up by SPE, it might be an option for ML analysis in quality control and official laboratories.

Among the 135 samples of UHT milk, none presented residues of the substances studied. As for the 103 samples of pasteurized milk analyzed, one sample presented MOX at a level of 2.2 μg L^{-1}, the other four ML were not detected in any sample (>LOD). This MOX level is below the MRL established by the European Union (40 μg L^{-1}), although no residue was expected to be found as the use of this compound is not indicated for lactating cows.

Most papers in this subject, published in the scientific literature, are focused on ML depletion studies (Danaher et al., 2006). There are only a few works on monitoring of these substances in milk. In

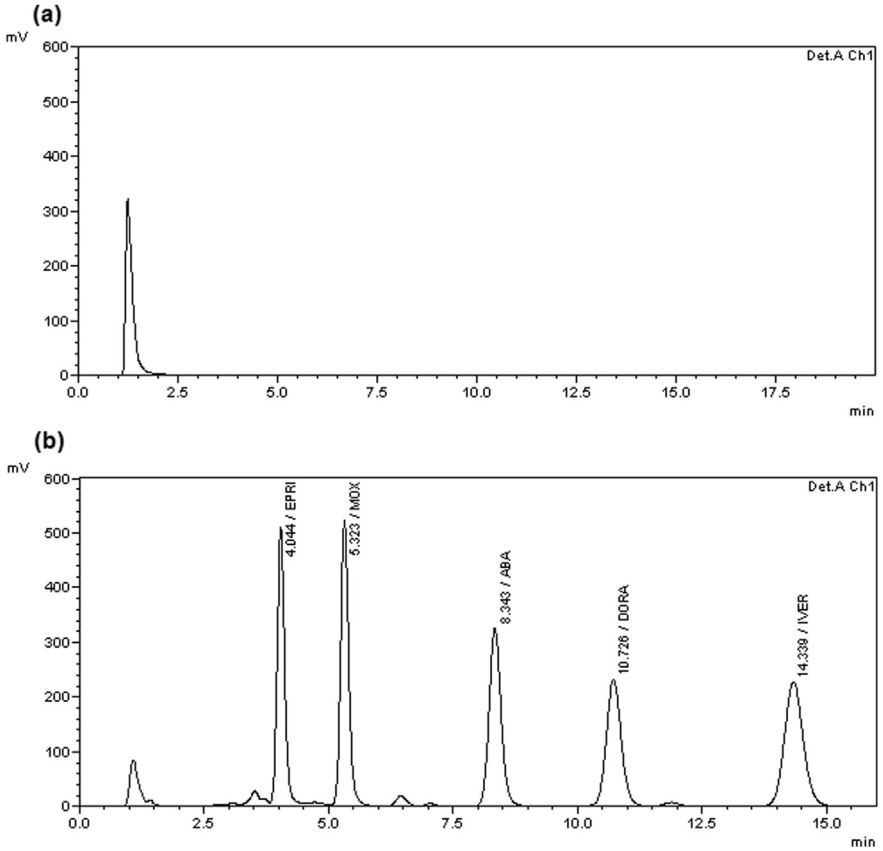

Fig. 1. HPLC-FLD chromatograms of (a) blank yogurt sample, (b) spiked yogurt sample. Conditions: Column C18 (Waters Nova-pak@, 150 × 3.9 mm, 4 μm particle size, at 25 °C). Detection by fluorescence: 365 nm (excitation) and 470 nm (emission). Mobile phase: acetonitrile:methanol:acetic acid 2% (v/v) (55:40:5). Flow rate: 1.2 ml min^{-1}.

Table 1
Validation parameters of the HPLC–FLD method.

Analite	Milk						Yogurt					
	Spiked level μg L^{-1}	*R* %	RSD_r ($n = 6$)	RSD_R ($n = 3$)	LOD μg L^{-1}	LOQ μg L^{-1}	Spiked level μg kg^{-1}	*R* %	RSD_r ($n = 6$)	RSD_R ($n = 3$)	LOD μg kg^{-1}	LOQ μg kg^{-1}
EPRI	10	108		7	3.2	10	2.5	109	10	4	0.9	2.5
	20	109	10	5			10	88		14		
	30	103		1			20	106		11		
MOX	1	104		5	0.4	1	2.5	111	7	4	0.6	2.5
	1.5	96	7	4			10	87		14		
	2	108		7			20	106		6		
ABA	0.2	103		5	0.1	0.2	2.5	109	5	3	0.6	2.5
	0.3	109	5	5			10	83		12		
	0.4	103		7			20	108		7		
DOR	7.5	105		8	1.0	7.5	2.5	112	6	3	0.6	2.5
	15	103	6	3			10	84		11		
	22.5	101		6			20	109		7		
IVER	5	98		6	1.2	5	2.5	109	4	5	0.6	2.5
	10	100	4	6			10	83		12		
	15	104		9			20	106		7		

R (%): Recovery was calculated using the average of all samples from recovery studies.
RSD_r Relative Standard Deviation under repeatability conditions (same day).
RSD_R – Relative Standard Deviation under within-laboratory reproducibility conditions (different days).
LOD: Limit of detection.
LOQ: Limit of Quantification.

Brazil, in a study conducted in the State of Paraná, 45% of the analyzed samples presented irregularity, a value far above of the one found in the present study (Jesus, 2007). Lobato, Rath, and Reyes (2006) monitored ivermectin residues in milk between 1999 and 2001 and detected residues in 18% of the samples, with levels below the MRLs. In China, Wang et al. (2011) analyzed the content of avermectins in several foods and no residue was detected in the 44 samples of milk studied.

As for the 104 yogurt samples analyzed, no residue of any of the 5 ML studied was detected (>LOD). In 2004, Cerkvenik et al., studied the fate of ivermectin applied in ewes and verified its stability during pasteurization and the production of yogurt. However, in the production of cheese, unlike results obtained by Anastasio et al. (2002), a loss around 35% has been verified. In a similar study, ivermectin and moxidectin were applied in sheep and the milk obtained was used in the production of cheese where the substances were detected (Imperiale, Busetti, Suárez, & Lanusse, 2004). The authors also concluded that the concentration of these substances tended to increase with the maturation of the cheese.

The low incidence of ML detected in this study may be explained by the fact that all the yogurt samples and most of the milk analyzed in this study originate from milk processing industries that use raw milk from several producers (farms) and therefore a possible contamination of a batch from one supplier can be masked during processing when raw material from different origins is mixed.

Brazil has an official monitoring program (PNCRC) established by the Ministry of Agriculture, Livestock and Food Supply (MAPA), where products of animal origin are monitored for the presence of residues of antibiotics, antiparasitics and other substances (Brasil, 1999) and, since 2009, there are no irregularities regarding ML residues in raw milk samples collected directly from the farm. There are no data available regarding industrialized milk and yogurt, since products from some establishments not monitored by MAPA may be in the market. The absence of irregularity indicates that the actions adopted by MAPA in Brazil have possibly led milk producers to adopt good practice for the use of these veterinary drugs.

The absence of residue in the samples analyzed indicate that the producers are complying with the good veterinary practice, and therefore, Brazilian consumers are apparently not exposed to risks regarding the presence of these substances in milk and yogurt.

4. Conclusion

The QuEChERS sample preparation combined with a derivatization reaction proved to be suitable for multiresidue analysis of eprinomectin, doramectin, moxidectin, abamectin and ivermectin in milk and yogurt. This reinforces the great versatility of QuEChERS technique, which has been widely used for analysis of different residues in various matrices. Residue of MOX was detected in one single milk sample, nevertheless level was below MRL established by European Union, therefore results indicate that the consumption of milk and yogurt does not represent risk to the population considering the five compounds studied.

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