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Analytical Methods

Development and validation of a method for the determination of low-ppb levels of macrocyclic lactones in butter, using HPLC-fluorescence

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ABSTRACT

An analytical method was developed and validated for the simultaneous determination of four macrocyclic lactones (ML) (abamectin, doramectin, ivermectin and moxidectin) in butter, using liquid chromatography with fluorescence detection. The method employed heated liquid–liquid extraction and a mixture of acetonitrile, ethyl acetate and water, with preconcentration and derivatization, to produce stable fluorescent derivatives. The chromatographic run time was <12.5 min, with excellent separation. The method validation followed international guidelines and employed fortified butter samples. The figures of merit obtained, *e.g.* recovery (72.4–106.5%), repeatability (8.8%), within-laboratory reproducibility (15.7%) and limits of quantification (0.09–0.16 μg kg^{-1}) were satisfactory for the desired application. The application of the method to real samples showed that ML residues were present in six of the ten samples evaluated. The method proved to be simple, easy and appropriate for simultaneous determination of ML residues in butter. To our knowledge, this is the first method described for the evaluation of ML in butter.

1. Introduction

The avermectins abamectin (ABA), doramectin (DOR) and ivermectin (IVM), and the milbemycin moxidectin (MOX) are macrocyclic lactones (ML) that are widely used to control parasitic infections in dairy cattle (Pereira, 2009). The occurrence of ML residues in milk and dairy products results from the topical application and oral or subcutaneous administration of these compounds to cattle (Gayard, Alvinerie, & Toutain, 1999). ML have a large, hydrophobic and complex ringed structure. The structure of avermectins consists of a 16-member macrocyclic ring containing a spiroketal group, a benzofuran ring and a disaccharide functionality. Milbemycins are structurally similar to avermectins, but they lack the disaccharide group (Danaher, Howells, Crooks, Cerkvenik-Flajs, & O'Keefe, 2006; Prichard, Ménez, & Lespine, 2012).

The avermectins are derived from fermentation products of the soil bacterium *Streptomyces avermitilis*. Avermectin B1, the predominant homologue, also known as abamectin (ABA), is the most important naturally produced compound. IVM and DOR result from synthetic modification of fermentation products. Milbemycins are derived from the fermentation of different soil bacteria, *Streptomyces hygroscopicus* or *Streptomyces cyaneogriseus*, and MOX results from a synthetic structural modification. Several aspects of the structural differences and the similarities of these compounds, biological activity and their mechanisms of action were recently reviewed (Prichard et al., 2012). The synthetic modifications result in different characteristics among each group of compounds, which are reflected in their Kow values and consequently in the distribution between plasma and lipids. IVM shows a log Kow of 3.2 while the other compounds show values between 4.4 and 4.8, indicating high affinity to lipids and adipose tissue.

After cattle are medicated, ML residues may be present in milk due to their partitioning between plasma and milk, which is a

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function of lipophilic properties and variables such as the drug formulation and the pharmacokinetics of each ML (Danaher et al., 2006; McManaman & Neville, 2003). Because mammals excrete large amounts of ML in their feces, only part the parent ML is transferred to milk, after they are mobilized from the adipose tissue. In terms of biotransformation, IVM was found to be 90% in the form of the parent drug in mammals (feces and tissues) (Danaher et al., 2006), while 85–99% of ABA residues were in the parent form in goats (muscle, milk and feces) (European Medicines Agency, 1999). A fraction of 67–92% of DOR was found as residues in sheep (fat, liver, kidney and muscle) (European Medicines Agency, 1997) and the parent moxidectin (MOX) was found to be the major residue in cattle (fat, liver, kidney and muscle) (Danaher et al., 2006).

ML are persistent in animal bodies and milks. IVM residues were detectable in the plasma of ruminants for 20–60 days after treatment (Anastasio et al., 2002; Cerkvenik et al., 2002) and sheep milk showed ABA residues for 23 days post-treatment (Cerkvenik et al., 2003). Residual levels of DOR were detectable 30–37 days after treatment (Cerkvenik-Flajs et al., 2005; Imperiale, Mottier, Sallovitz, Lifschitz, & Lanusse, 2003) and milk showed residues of MOX at 10–13 days post-treatment (European Medicines Agency, 2001).

The thermal stability of ML is also high. Residues of IVM were stable under cooking conditions (microwave heating, frying or boiling) (Rose, Farrington, & Shearer, 1998) or in milk samples frozen for one year at −20 °C (Cerkvenik, Doganoc, Skubic, Beek, & Keukens, 2001). IVM and MOX were stable under the conventional milk heating processes (65 °C for 30 min, 75 °C for 15 s and 100 °C for 10 s) that are widely used in the dairy industry (Cerkvenik et al., 2001; Imperiale et al., 2009).

High concentrations of IVM in milk are positively correlated with milk-fat content (Cerkvenik et al., 2004). The residual concentrations of IVM and MOX detected in cheese during the ripening period showed positive correlations with the percentages of water loss, total solids, and fat content (Cerkvenik et al., 2004; Imperiale, Busetti, Suárez, & Lanusse, 2004). The European Union (EU) prohibited the use of formulations containing ABA, DOR and IVM for dairy cows (EC, 2010) and established a withdrawal period of 49 days for medications containing IVM and administered to dairy cows by subcutaneous injection.

In order to assure that humans are not exposed to unsafe residue levels by consuming edible products obtained from treated animals, several governmental authorities have established maximum residue limits (MRL) or tolerance levels for veterinary drugs. The ML studied here, used for antiparasitic purposes, are included in Group B (veterinary drugs and contaminants) of Annex I to Council Directive 96/23/EC (EC, 1996). Only MOX has a MRL established in milk by Commission Regulation No. 37/2010 (EC, 2010). The marker residues are Avermectin B1a (ABA), Doramectin (DOR), 22,23-Dihydroavermectin B1a (IVM) and Moxidectin (MOX) (EC, 2010). The Codex Alimentarius (Codex, 2012) defined no MRL for ABA and MOX, indicating that these drugs should not be present in milk.

The analytical approaches used to detect residues of ML in food were reviewed by Danaher et al. (2006) and Danaher, Radeck, Kolar, Keegan, & Cerkvenik-Flajs (2012). Methods of HPLC-UV can be used for the determination of IVM in medications (da Costa & Pereira Netto, 2012), but not in foods because these methods are insufficiently sensitive. Most methods for ML determination in foods are based on high-performance liquid chromatography (HPLC) with fluorescence detection (HPLC-Fluo) after reaction with trifluoroacetic anhydride (TFAA) and 1-methylimidazole (MI) (Berendsen, Mulder, & Van Rhijn, 2007; Cerkvenik-Flajs et al., 2010; Gianetti et al., 2011; Rübensam et al., 2011) to produce a fluorescent group. Fluorescence detection is a suitable confirmatory method for the drugs listed in Group B of Annex I to Council Directive 96/23/EC (European Commission., 2002) and it is possibly the most commonly used method to detect ML (Galarini, Saluti, Moretti, Giusepponi, & Dusi, 2013; Sheridan & Desjardins, 2006; Turnipseed, Roybal, Andersen, & Kuck, 2005). Certain authors also consider that HPLC-Fluo is superior to HPLC–MS/MS to quantify ML, with respect to the achievable limits of detection (LOD) and quantification (LOQ) (Rübensam et al., 2011; Turnipseed et al., 2005), and the decision limit (CC_α) and detection capability (CC_β) (Rübensam et al., 2011).

To our knowledge, no publication has previously described the quantification of ML in butter or in high-fat milk products, despite the large number of studies that have quantified ML residues in milk and dairy products (Anastasio et al., 2002; Berendsen et al., 2007; Cerkvenik et al., 2001, 2004; Cerkvenik-Flajs et al., 2005, 2010; Galarini et al., 2013, 2011; Imperiale et al., 2003, 2009; Lobato, Rath, & Reyes, 2006; Rübensam et al., 2011).

Butter is a fatty product derived exclusively from milk and/or milk products, mainly in the form of a water–oil emulsion, with a minimum milk fat content of 80% for unsalted (Codex, 2010) and salted samples (Brasil, 1996). The occurrence of veterinary drug residues in butter is of particular concern because this is an inexpensive, favored and widely consumed food; moreover, butter is not usually included in monitoring programs. Therefore, the development and validation of methods for determination of ML residues in butter are essential to assure the quality of this food.

The initial hypothesis of this study assumed that because of the lipophilic characteristics of ML, they would probably become concentrated in fatty dairy matrices such as butter. Therefore, the aim of this study was to develop and validate a method for determination of ABA, DOR, IVM and MOX in butter. One difficulty for this study is the lack of MRL for ML in butters and certified materials for ML in butter. This is doubtless a result of the lack of previous data on ML residues in this food.

2. Material and methods

2.1. Chemicals and reagents

ABA, DOR, MOX (Fluka, Germany) and IVM (Sigma–Aldrich, MO, USA) were employed. Standard stock solutions of the individual ML (200 mg L^{-1}) were prepared by dissolving 2 mg of each standard in 10 mL of acetonitrile. A working standard solution of each compound (2 mg L^{-1}) was prepared by combining appropriate aliquots of each stock solution, and dilution using acetonitrile. The stock solutions were stored at −20 °C in amber glass flasks and discarded one month after preparation.

Acetonitrile and ethyl acetate (HPLC grade) were purchased from TediaBrazil (RJ, Brazil). The derivatization reagents triethylamine (TEA), trifluoroacetic anhydride (TFAA) and trifluoroacetic acid (TFA) were purchased from Sigma–Aldrich, and 1-methylimidazole (MI) was purchased from Fluka. Florisil (60–100 mesh), silica (230–400 mesh) and octadecylsilica were also obtained from Sigma–Aldrich (MO, USA). Ultrapure water was prepared in a Millipore Milli-Q system (Millipore, MA, USA), following reverse osmosis (Rios DI-3, Millipore).

Butter samples were purchased in supermarkets and represented the major brands available in the retail market of the cities of Niterói and Rio de Janeiro, Rio de Janeiro State, Brazil. In this state, the annual per capita consumption of butter is 0.303 kg (IBGE, 2010). Four imported butters from Argentina (2) and France (2), and six butters produced in Brazil were analyzed. All of them were purchased in July 2013, and according to the package labels were produced in May or June 2013. All butters were kept under refrigeration (4 °C) until analysis.

To obtain the analytical samples, the refrigerated butter packages were removed from the refrigerator and kept in the laboratory