Food Control 21 (2010) 620–625

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

A HPLC with fluorescence detection method for the determination of tetracyclines residues and evaluation of their stability in honey

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ARTICLE INFO

Article history:
Received 19 May 2009
Received in revised form 6 September 2009
Accepted 23 September 2009

Keywords:
Honey
Tetracyclines
Antibiotic residue
Antimicrobials

ABSTRACT

A high-performance liquid chromatography with fluorescence detection method for the simultaneous determination of oxytetracycline (OTC), tetracycline (TC) and chlortetracycline (CTC) residues in honey was developed and validated. Sample preparation was done in a single solid phase extraction step. The chromatographic separation was performed on a C_8 column. Matrix matched calibration curves showed linearity higher than 0.99. The accuracy values lay between 86% and 111% and the precision was lower than 11%. Limits of detection and quantitation were 8 μg kg^{-1} and 25 μg kg^{-1}, respectively. The method was employed to evaluate the stability of the tetracyclines in honey during 60 days of storage. The residue levels of OTC, TC and CTC were reduced 97%, 76% and 71%, respectively. Honey samples ($n = 22$) collected from the retail market were analyzed. No tetracyclines residues were detected.

1. Introduction

Tetracyclines (TCs) are antimicrobial substances that have been used worldwide in apiculture as prophylactic or therapeutic agents in the prevention and treatment of bacterial diseases such the American Foulbrood (AFB) and the European Foulbrood (EFB). Residues of these veterinary drugs can be found in honey after beehive treatment (Martel, Zeggane, Drajnudel, Faucon, & Aubert, 2006; Thompson et al., 2005), exposing consumers to the risks associated with ingestion of these contaminants, like allergic reactions. Non-human usage of antimicrobials has also an impact on the occurrence of resistant bacteria in animals and foods (WHO, 2003). The presence of antimicrobial residues in honey is a hurdle to the international trade of the product. Since Brazil became an important honey exporter, producers, authorities and researches are more concerned about the problem and have taken efforts to avoid honey contamination and, consequently, improve apiculture in the country.

Some countries have established maximum residue limits (MRL) for TCs in honey, while others do not tolerate any residue level. For instance, Codex Alimentarius and the European Union (EU) have not established MRL for veterinary drugs in honey, and in member countries of the EU it is prohibited the use of antimicrobials in beekeeping. However, the United States allows the use of OTC for treatment of AFB (USFDA, 2008), and in Brazil the National Program for Honey Residues Control established by the Ministry of Agriculture has set a group MRL for OTC, TC and CTC as 200 μg kg^{-1}, as well as established the limit of quantitation (LOQ) required for the method of analysis as 25 μg kg^{-1} (Brasil, Ministério da Agricultura, Pecuária e Abastecimento, 2006). Furthermore, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established a group Acceptable Daily Intake (ADI) for OTC, TC and CTC as 0–0.03 mg kg^{-1} bw (WHO, 2003).

Chromatographic analysis of TCs in foods involves problems of analytes recovery from the matrix and peak resolution. The main causes are the interaction of the silanol groups in the silica-based columns with highly polar groups distributed on the analyte molecules and the formation of metal chelates in the matrix and in the chromatographic system. These problems have been surpassed by the use of end-capped modified silica columns, synthesized from 99.99% pure silica, the development of new columns with polymeric materials, free of silanols, the use of oxalic acid in the mobile phase and the use of ethylenediaminetetraacetic acid (EDTA) in the extraction buffer and in the SPE conditioning step (Anderson, Rupp, & Wu, 2005; Fedeniuk & Shand, 1998; Huq, Garriques, & Kallury, 2006; Oka, Ito, & Matsumoto, 2000).

In the last 20 years, some methods using HPLC have been reported as the most suitable technique for the determination of TCs in honey, including HPLC with mass spectrometry (Alfredsson, Branzell, Granelli, & Lundström, 2005; Carrasco-Pancorbo, Terrones, Segura-Carretero, & Fernández-Gutiérrez, 2008; Debayle, Dessalces, & Grenier-Loustalot, 2008; Hammel, Mohamed, Gremaud, LeBreton, & Guy, 2008; Kaufmann, Roth, Ryser, & Widmer, 2002; Khong, Hammel, & Guy, 2005; Lopez, Pettis, Smith, & Chu, 2008; Nakazawa et al., 1999; Oka et al., 1994), HPLC with

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doi:10.1016/j.foodcont.2009.09.006

chemiluminescence detection (Wan et al., 2005) and HPLC with ultra-violet (UV) detection (Diaz, Cabanillas, & Salinas, 1990; Diegez, Soraci, Bedascarrasbure, & Libonatti, 2004; Li et al., 2008; Martel et al., 2006; Oka, Ikai, Kawamura, Uno, & Yamada, 1987; Pagliuca, Gazzoti, Serra, & Sabatini, 2002; Viñas, Balsalobre, López-Erroz, & Hernandez-Córdoba, 2004). Although TCs exhibit fluorescence (Anderson et al., 2005), this property has not been frequently used in the development of HPLC methods for the determination of TCs in honey. Pena, Pelantova, Lino, Silveira, and Solich (2005) reported a method using fluorescence detection for the determination of OTC and TC in honey. The post-column effluent was mixed with magnesium acetate solution to promote complex formation between magnesium ions and TCs, increasing the fluorescence of the analytes. Recently, Fujita et al. (2008) reported a method with fluorescence detection for OTC, TC and CTC in honey using an isocratic mobile phase and no post-column reaction for fluorescence enhancement. Both methods reported an extensive sample preparation procedure with at least two sequential clean-up steps using SPE polymeric cartridges and metal chelate affinity chromatography to obtain a suitable final extract.

The aim of this work was to develop and validate a method for simultaneous determination of OTC, TC and CTC residues in honey using HPLC with fluorescence detection and a simple sample preparation technique with no post-column reaction. The method was applied to evaluate tetracycline residue stabilities during honey storage and to analyze different honey types collected from the retail market.

2. Experimental

2.1. Chemicals and solvents

Otherwise specified all the chemicals were analytical grade. Chemicals employed were: sodium hydroxide (Merck, Darmstadt, Germany), citric acid (Merck, Rio de Janeiro, Brazil), sodium phosphate dibasic hepthydrate (Ecibra, São Paulo, Brazil). Ethylenediaminetetraacetic acid dissodium salt dihydrate ($Na_2EDTA{\cdot}2H_2O$) (Titriplex® III), sodium acetate dihydrate and HPLC grade acetonitrile (J.T. Baker, Xalostoc, Mexico), HPLC grade methanol (Tedia, Fairfield, USA), ethyl acetate suitable for pesticide residue analysis (Merck, Darmstadt, Germany). Octadecyl (C_{18}) solid phase extraction cartridges (500 mg, 3 mL) were from Varian (Lake Forest, USA). Water was purified using a Milli-Q system (Millipore, USA).

2.2. Standard and extraction buffer solutions

CTC (purity 99%), DC (purity 98.5%) and TC hydrochloride (purity 95%) analytical standards were purchased from Sigma–Aldrich (St. Louis, USA) and OTC dihydrate analytical standard (purity 98.5%) was obtained from ICN Biomedicals (Aurora, USA).

Stock solutions containing 1 mg mL^{-1} of each compound were prepared in methanol and stored in tightly closed amber vessels at −18 °C for a maximum period of 1 month.

Working solutions of OTC, TC and CTC were prepared daily from the stock solutions in water:methanol (1:1 v/v). These solutions were used to construct calibration curves for all the analytes, after spiking blank honey samples at six concentration levels (25, 50, 100, 200, 300 and 500 μg kg^{-1}). The internal standard (DC) was also spiked in the blank honey samples at a concentration of 200 μg kg^{-1}.

McIlvaine buffer (pH 4.0) was prepared using 0.10 mol L^{-1} citric acid solution and 0.20 mol L^{-1} sodium phosphate dibasic hepthydrate. The extraction buffer was prepared by dissolving 37.22 g of Na_2EDTA in one liter McIlvaine buffer (pH 4.0).

2.3. HPLC–FD equipment and chromatographic conditions

The chromatographic system was constituted of a Waters 600E Multisolvent Delivery System operated by the Millennium Software (Waters, Milford, USA), equipped with a Rheodyne 7725i manual injector (Rheodyne, Rohnert Park, USA) with a 50 μL loop. Detection used a Shimadzu RF-10A spectrofluorometric detector (Shimadzu Corporation, Kyoto, Japan) operating at an excitation wavelength of 390 nm and an emission wavelength of 512 nm. The results were recorded and processed by a Waters 746 Computing Integrator (Thermo Separation Products, San Jose, USA). Analytical (4.6 × 250 mm, 5 μm) and guard (4.6 × 12.5 mm, 5 μm) columns were Zorbax Eclipse XDB-C8 (Agilent Technologies, Wilmington, USA). Mobile phase flow rate was 1.0 mL min^{-1} and was constituted of an aqueous (A) and an organic phase (B). Mobile phase A was an aqueous solution containing 0.075 mol L^{-1} sodium acetate dihydrate, 0.035 mol L^{-1} calcium chloride dihydrate and 0.025 mol L^{-1} Na_2EDTA with the pH corrected to 6.5 with 5 mol L^{-1} sodium hydroxide solution, using a Digimed DM 20 pHmeter (Digicrom Analítica, Brazil). Mobile phase B was HPLC grade methanol:water (95:5 v/v). Both mobile phases were filtered through 0.45 μm nylon membranes (Sartorius, Goettingen, Germany) and degassed for 5 min in an ultrasonic bath (Thornton, Vinhedo, Brazil) under vacuum. The separation was performed under gradient elution with mobile phase B increasing linearly from 30% to 50% in 13 min, keeping these proportions until 15 min and then returning to the original conditions in 5 min. A 3 min pause was then observed before the next injection to equilibrate the column.

2.4. Sample preparation procedure

Three grams of honey were exactly weighted into 50 mL polyethylene centrifuge tube and 60 μL of a 10 μg mL^{-1} DC solution were added as internal standard. Then, 15 mL of McIlvaine buffer (pH 4.0) with 0.10 mol L^{-1} Na_2EDTA were added and the mixture was vortexed (Phoenix, Araraquara, Brazil) until the honey dissolved completely. By using a manifold Visiprep™ (Supelco, USA), the SPE cartridge was conditioned with 5 mL of methanol and 5 mL of McIlvaine buffer (pH 4.0) containing 0.10 mol L^{-1} Na_2EDTA. After condition, 5 mL of sample were allowed to pass through the cartridge followed by 2.5 mL of McIlvaine buffer (pH 4.0): methanol (85:15 v/v) and 2.5 mL of water. The cartridge was dried for 2 min by aspiration using a negative pressure in the manifold chamber (40 kPa) and another washing step with 2.5 mL acetonitrile was done. Cartridge was dried again for one minute and analytes were eluted with 3.0 mL of ethyl acetate:methanol (75:25 v/v). The elution mixture was evaporated until dryness under a gentle nitrogen flow in a water bath (30–35 °C) and the residue was dissolved in 1 mL methanol: water (15:85 v/v). Final extracts were filtered through 0.45 mm syringe filters (Millipore, Brazil) and injected onto the HPLC.

2.5. Method validation

The method was *in-house* validated by the evaluation of the following performance parameters: linear range, linearity, selectivity, precision, accuracy, limits of detection and quantitation.

2.6. Samples

Five kilograms of multi-flower honey, free of antimicrobials, were supplied by a producer located in Campos do Jordão, São Paulo, Brazil and it was used to develop the method and in the TCs stability study. Twenty samples from different floral origins (orange, multi-flower and eucalyptus) and regions of Brazil (south, southeast, north-east) and two samples without declared origin in the