A study of changes in the concentrations of tetracycline antibiotics in honey

Sandra Dluhošová, Lenka Kaniová, Ivana Borkovcová, Lenka Vorlová

Department of Milk Hygiene and Technology
Faculty of Veterinary Hygiene and Ecology
University of Veterinary and Pharmaceutical Sciences Brno
Brno, Czech Republic

Abstract

The work was focused on monitoring the stability of the tetracycline antibiotics oxytetracycline, tetracycline and chlortetracycline during sample processing and on optimising the analytical procedure for the determination of tetracycline antibiotics in honey. Stability was tested on standard solutions of tetracyclines in the most commonly used solvents – water, methanol and Na_2EDTA-McIlvaine buffer (citrate phosphate buffer). Subsequently, the preparation procedure was optimised on model honey samples spiked with tetracycline antibiotics at the concentration levels 1, 2 and 4 $mg \cdot l^{-1}$ using Na_2EDTA-McIlvaine buffer as a solvent. The stability of the tetracycline antibiotics was monitored for thirty days at an interval of 3 times a week. Antibiotics were determined by reversed-phased high-performance liquid chromatography with UV detection. Degradation of a multi component standard of tetracycline antibiotics was 69 – 85% in water, 28 – 61% in methanol and 46 – 53% in the Na_2EDTA-McIlvaine buffer. Degradation of a mixed standard of tetracyclines in samples of honey at the selected concentrations was 2 – 61%.

Chlortetracycline, HPLC, oxytetracycline, Solid - Phase Extraction, tetracycline

Introduction

Tetracyclines are broad-spectrum antibiotics used as veterinary drugs and supplementary additives. Their use is not permitted in the European Union for prevention and treatment in beekeeping. The presence of residues of these substances in honey is due to their use in countries outside the European Union where they serve to prevent and treat American foulbrood (*Paenibacillus larvae*) and European foulbrood (*Melissococcus plutonius*) (Peres et al. 2010 and Reybroeck et al. 2012). These residues may pose a risk to consumers in the form of allergies, toxic effects and bacterial resistance (Yang et al. 2014).

On the basis of knowledge of the poor stability of tetracyclines (degradation, formation of inactive complexes and epimerisation) (Oka et al. 2000; Pena et al. 2005 and Peres et al. 2010) we decided to compare their stability in mixed standards in various solvents and in honey. Na_2EDTA-McIlvaine buffer was used as a solvent for testing stability in honey. This buffer is the most suitable solvent for honey as it provides the best recovery of tetracyclines from the honey matrix by solid-phase extraction (SPE).

Materials and Methods

Chemicals

Chemicals of analytical grade purity were used. Methanol for HPLC and acetonitrile for HPLC (Merck, Germany); oxalic acid dihydrate, citric acid monohydrate, Complexone III, disodium hydrogen phosphate dihydrate (Penta, CR); standards of tetracycline hydrochloride, oxytetracycline hydrochloride and chlortetracycline hydrochloride (Sigma-Aldrich, Germany); SPE cartridge HLB Oasis 500 mg, 6 ml, chromatography column Nova-Pak C8, 150 x 3.9 mm, 4 μm (Waters, Ireland). Water of HPLC purity was prepared with the use of an Aqua Osmotic 03 device (Tišnov, CR).

A floral honey not containing tetracycline antibiotics was used as the model honey.

Working solutions of tetracyclines

A mixed solution of tetracycline antibiotics (TCs) was prepared, containing oxytetracycline (OTC), tetracycline (TC) and chlortetracycline (CTC) at a concentration 2 $mg \cdot l^{-1}$. Water of HPLC purity, methanol and McIlvaine buffer (pH = 4.0) with the addition of Na_2EDTA were used for dissolving. The solutions were filtered through a 0.22 μm membrane filter into dark (amber) vials and stored in the dark at 6 °C. The stability of the TCs was measured three times a week for a period of one month.

Address for correspondence:
MVDr. Sandra Dluhošová
Department of Milk Hygiene and Technology
Faculty of Veterinary Hygiene and Ecology
University of Veterinary and Pharmaceutical Sciences Brno
Palackého tř. 1946/1, 612 42 Brno, Czech Republic

E-mail: h14015@vfu.cz
Phone: +420 541 562 715
www.maso-international.cz

Sample preparation

Totally 10 g of honey was dissolved in 30 ml of Na_2EDTA-McIlvaine buffer. Standards of TCs dissolved in Na_2EDTA-McIlvaine buffer were added to honey mixture to produce solutions concentration levels 1, 2 and 4 $mg \cdot l^{-1}$. All samples were made up to a volume of 50 ml with Na_2EDTA-McIlvaine buffer. Samples of honey spiked with TCs were centrifuged at 5 000 rpm for 5 minutes and purified by means of SPE as in the previous study (Dluhošová et al. 2016). After elution, the samples were dried in a vacuum evaporator and reconstituted in 2 ml of mobile phase A (12 mM oxalic acid) and filtered through a 0.22 µm membrane filter into amber vials. The samples prepared in this way were stored in the dark at 6 °C and the stability of the TCs was monitored three times a week for a period of one month.

HPLC conditions

Tetracycline antibiotics were determined by reversed-phase high-performance liquid chromatography (RP-HPLC) with gradient elution. Analysis was performed using the Alliance 2695 separations module with a 2996 PDA detector. Separation was performed on Nova-Pak C8 column, 150 x 3.9 mm, 4 µm. The mobile phases were (A) 12 mM oxalic acid and (B) methanol : acetonitrile (50 : 50); the flow rate 0.8 ml per minute, column temperature 35 °C, injection volume 30 µl. Detection was performed in the UV area at a wavelength of 355 nm. The calibration curve method was used for evaluation using Empower 2 software.

Results and Discussion

The stability of tetracycline antibiotics in various solvents

Variable stability of tetracyclines was recorded in a mixed standard of TCs at the concentration 2 $mg \cdot l^{-1}$ depending on the solvent used, as shown in (Plate II, Fig. 1).

The stability of tetracycline antibiotics in honey

A mixed standard of TCs in honey, with the use of Na_2EDTA-McIlvaine buffer as a solvent, was assessed at concentrations 1, 2 and 4 $mg \cdot l^{-1}$. The OTC was the most stable at all the given concentrations. The stability of TCs in honey is demonstrated in (Plate II, Fig. 2).

We determined the high stability of OTC by comparing the stability of a mixed standard of TCs in Na_2EDTA-McIlvaine buffer and in a honey matrix with the use of Na_2EDTA-McIlvaine buffer and an SPE purification step. The stability of TC and CTC was lower in the honey matrix.

Chromatograms show TCs and their degradation products in various solvents on the first (Plate III, Fig. 3) and thirtieth (Plate III, Fig. 4) day of measurement. According to Oka et al. (2000) the degradation products of tetracycline antibiotics are epi-, iso-, anhydro-, apoxy-, epiiso- and epianhydroforms. A peak is eluted in (Plate III, Fig. 4) which, according to the available studies, is characterised as the degradation product subject to epimerisation C-4 4-epitetracycline (ETC). The peak eluted before CTC may be its degradation product 4-epi-chlortetracycline (ECTC), isochlortetracycline (ICTC) or 4-epiisochlortetracycline (EICTC). The precise identification of these degradation products is not possible due to the lack of standards of these compounds (Oka et al. 2000; Papadoyannis et al. 2000; Frizt and Zuo 2007 and Fletouris et al. 2008). These degradation products are also recorded during the analysis of a sample of honey in Na_2EDTA-McIlvaine buffer (Plate III, Fig. 5).

Tetracycline antibiotics in a solvent are generally considered very unstable, for which reason fresh working solutions are prepared before each analysis. The stability of tetracyclines is influenced by the process used in the preparation of samples and the use of laboratory glassware as they are subject to photodegradation (Viñas et al. 2004; Carrasco-Pancorbo et al. 2008 and Gajda et al. 2013). The falling stability of tetracyclines is evident from our results, for which reason the preparation of fresh working solutions before analysis is extremely important. Peres et al. (2010) evaluated the stability of TCs in honey and determined a stability under 5% for OTC, 24% for TC and 29% for CTC in a spiked sample of honey after sixty days of storage at 20 – 30 °C.

Conclusions

The stability of tetracycline antibiotics was determined in various solvents used. The influence of the solvents and of the type of matrix was proved on stability and recovery during experiments.

The highest recovery and stability were achieved with the use of Na_2EDTA-McIlvaine buffer. The fact that the stability of oxytetracycline in a honey matrix is extremely good may suggest further studies investigating the antimicrobial and antioxidant activity of honey in relation to residues of inhibitory substances.

Acknowledgements

This work was supported by the project IGA 214/2016/FVHE VFU Brno.

References

Carrasco-Pancorbo A, Casado-Terrones S, Segura-Carretero A, Fernández-Gutiérrez A 2008: Reversed-phase high-performance liquid chromatography coupled to ultraviolet and electrospray time-of-flight mass spectrometry on-line detection for the separation of eight tetracyclines in honey samples. J Chromatogr A **1195**: 107-116

Dluhošová S, Borkovcová I, Kaniová L, Vorlová L 2016: Determination of tetracycline antibiotics in honey using high-performance liquid chromatography. In: Proceeding of reports XVIII. Conference of Young Scientists with International Participation, May 25th 2016, Brno, Czech Republic, VFU Brno, 31-33 (In Czech)

Fletouris DJ, Papapanagiotou EP, Nakos DS 2008: Liquid chromatographic determination and depletion profile of oxytetracycline in milk after repeated intramuscular administration in sheep. J Chromatogr B Anal Technol Biomed Life Sci **876**: 148-152

Fritz JW, Zuo YG 2007: Simultaneous determination of tetracycline, oxytetracycline, and 4-epitetracycline in milk by high-performance liquid chromatography. Food Chem **105**: 1297-1301

Gajda A, Posyniak A, Bober A, Bladek T, Zmudziki J 2013: Oxytetracycline residues in honey analyzed by liquid chromatography with UV detection. J Apicultur Sci **57**: 25-32

Oka H, Ito Y, Matsumoto H 2000: Chromatographic analysis of tetracycline antibiotics in foods. J Chromatogr A **882**: 109-133

Papadoyannis IN, Samanidou VF, Kovatsi LA 2000: A rapid high performance liquid chromatographic (HPLC) assay for the determination of oxytetracycline in commercial pharmaceuticals. J Pharm Biomed Anal **23**: 275-280

Pena A, Pelantova N, Lino CM, Silveira MIN, Solich P 2005: Validation of an analytical methodology for determination of oxytetracycline and tetracycline residues in honey by HPLC with fluorescence detection. J Agr Food Chem **53**: 3784-3788

Peres GT, Rath S, Reyes FG 2010: A HPLC with fluorescence detection method for the determination of tetracyclines residues and evaluation of their stability in honey. Food Cont **21**: 620-625

Reybroeck W, Daeseleire E, De Brabander HF, Herman L 2012: Antimicrobials in beekeeping. Vet Microbiol **158**: 1-11

Viñas P, Balsalobre N, López-Erroz C, Hernández-Córdoba M 2004: Liquid chromatography with ultraviolet absorbance detection for the analysis of tetracycline residues in honey. J Chromatogr A **1022**: 125-129

Yang X, Zhang S, Yu W, Liu Z, Lei L, Li N, Zhang H, Yu Y 2014: Ionic liquid-anionic surfactant based aqueous two-phase extraction for determination of antibiotics in honey by high-performance liquid chromatography. Talanta **124**: 1-6

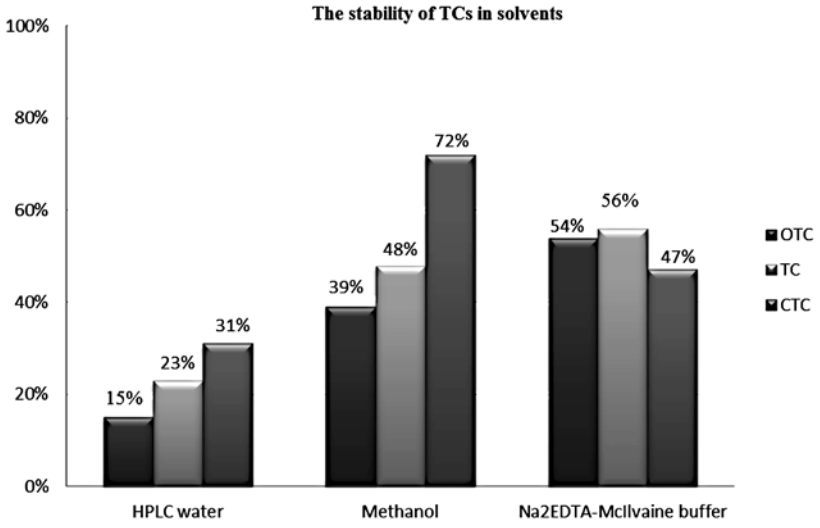

Fig. 1. The stability of a multi component standard of TCs (2 mg·l^{-1}) in solvents after thirty days of measurement; (OTC = oxytetracycline, TC = tetracycline, CTC = chlortetracycline)

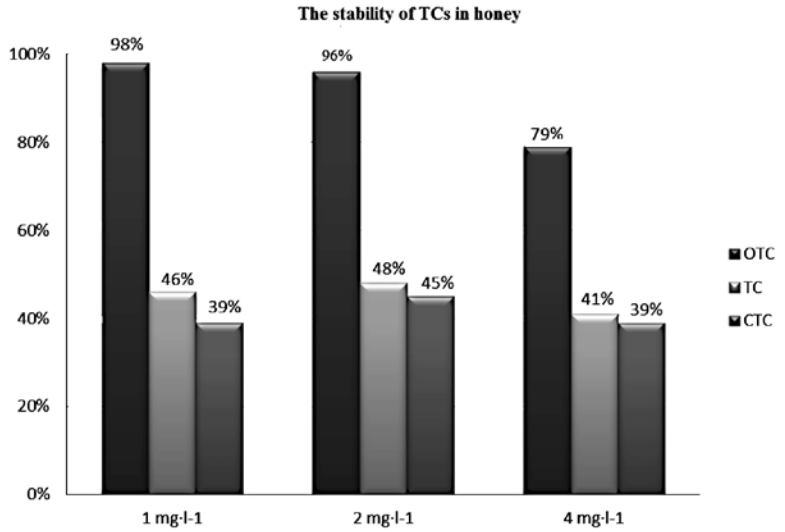

Fig. 2. The stability of a multi-parameter standard of TCs in honey (in Na2EDTA-McIlvaine buffer) after thirty days of measurement; (OTC = oxytetracycline, TC = tetracycline, CTC = chlortetracycline)

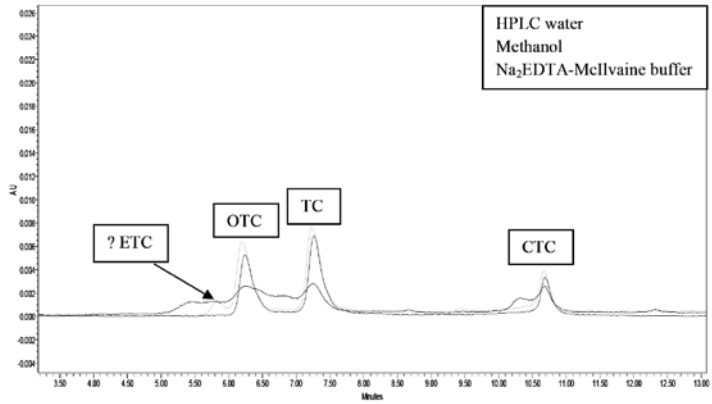

Fig. 3. Chromatogram of TCs content with the use of various solvents (Day 1 of measurement); (ETC = epitetracycline, OTC = oxytetracycline, TC = tetracycline, CTC = chlortetracycline)

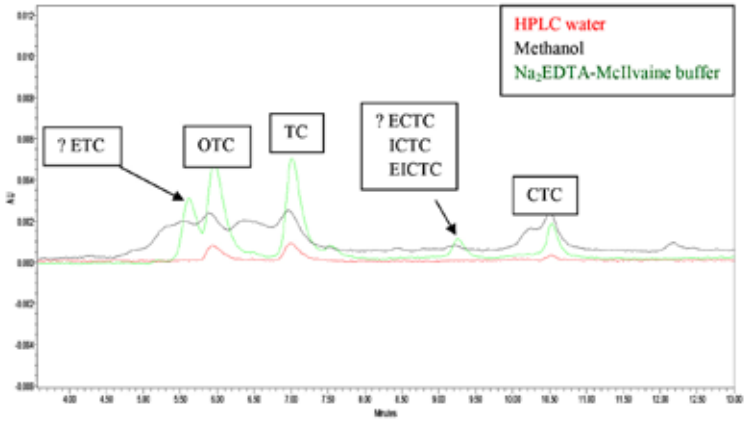

Fig. 4. Chromatogram of TCs content with the use of various solvents (Day 30 of measurement); (ETC = epitetracycline, OTC = oxytetracycline, TC = tetracycline, ECTC = epi-chlortetracycline, ICTC = isochlortetracycline, EICTC = epiisochlortetracycline, CTC = chlortetracycline)

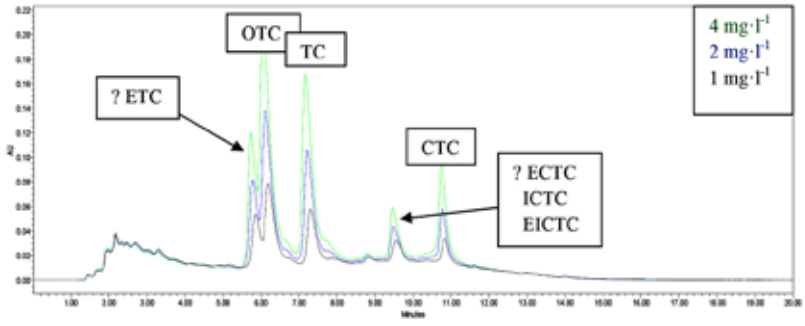

Fig. 5. Chromatogram of TCs content in a honey matrix with the use of the solvent Na_2EDTA-McIlvaine buffer (Day 30 of measurement);
(ETC = epitetracycline, OTC = oxytetracycline, TC = tetracycline, ECTC = epi-chlortetracycline, ICTC = isochlortetracycline, EICTC = epiisochlortetracycline, CTC = chlortetracycline)