

Development of a method for analysis of tetracycline residues in cow's milk by liquid chromatography/tandem mass spectrometry

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Abstract

An Ultra Performance Liquid Chromatography/tandem mass spectrometric (UPLC-MS/MS) multi-residue method for the simultaneous quantitative determination of tetracycline, chlortetracycline hydrochloride, oxytetracycline hydrochloride and doxycycline hydrochloride in milk has been developed in this work. Liquid-liquid extraction with trichloroacetic acid and Mcllvaine buffer was used for the isolation of the selected residues from milk. The sample was centrifuged and solid-phase extraction with an OASIS® HLB extraction column was used to clean up the sample extract. The samples were analysed with the instrument operated in the Multiple Reaction Monitoring acquisition mode. UPLC separation was performed on a reversed-phase C18 column using gradient elution with a mobile phase consisting of acetonitrile and 20 mM formic acid. The tetracycline analytes were detected with a quadrupole mass spectrometer using positive ion electrospray ionisation. The confirmatory method has an acceptable detection limit and was validated according to the European requirements for veterinary drug residues according to Commission Decision 2002/657/ES. The recovery of tetracyclines from milk ranged from 80.2 to 93.8% with relative standard deviation no larger than 7.3% for a concentration of 100 µg·l⁻¹. Furthermore, this analytical method is a useful tool for food safety monitoring and controlling veterinary drug use.

Food Safety, SPE, Tandem Mass Spectrometry, Tetracycline, Ultra Performance Liquid Chromatography, UPLC-MS/MS

Introduction

Antibiotics were first defined by Waksman as “chemical substances produced by microorganisms possessing the ability to kill or to inhibit the growth of bacteria and other microorganisms” (Sevilla-Santos 1982 and Zakeri and Wright 2008). Tetracycline antibiotics (TCAs) are broad-spectrum medicinal drug compounds active against a number of gram-positive and gram-negative bacteria. For this reason, they have been successfully used worldwide both in veterinary and human medicine. Because of their broad-spectrum activity and cost effectiveness, these antibiotics are nowadays used as veterinary drugs for food-producing animals. For this reason and for their low-cost efficiency they are used worldwide. The commonly used TCAs in veterinary medicine are tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and doxycycline (DC) (Bruno et al. 2002; De Ruyck and De Ridder 2007). The first three are metabolic products of tetracycline-producing *Streptomyces* bacteria such as *S. aureofaciens* and *S. rimosus*, the last is a modified semisynthetic derivative of TC (Betsy and Keogh 2012).

Over the last few years, the use of drugs in medicine for the preservation of human and animal health has had the undesirable effect of accumulation and persistence in the environment (Daghrir and Droguí 2013). Tetracyclines are commonly used in livestock animals for therapeutic treatment and for growth promotion (Arikan et al. 2006). After administration, they are absorbed and subjected to metabolic reactions. TCA residues subsequently get into muscle tissue and milk, though the largest proportion is excreted from the body. TCAs can, therefore, often be found in the environment.

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In order to protect the consumer from exposure to residues of TCAs in edible tissues of animal origin, maximum residue limit (MRL) values were laid down by the EU (Commission Regulation (EU) No. 37/2010). The tolerable MRL values for TC, CTC and OTC in milk are 100 mg·kg⁻¹. In this regulation, the marker residue is, however, the sum of the specific antibiotic and its 4-epimer. For DC, the MRL limits are not authorised for lactating dairy cows.

From the chemical perspective, TCAs consist of a 2-naphthacenecarboxamide framework containing four fused rings. Various TCAs differ by substitution at the C5, C6 and C7 positions on the main backbone (Fig. 1). Tetracyclines are susceptible to conformation degradation to 4-epimers at their two distinct sites of common structure. This conversion or metabolism can occur both in aqueous solutions and also in biological matrices during sample preparation and varies as a function of pH and temperature (Clive 1968).

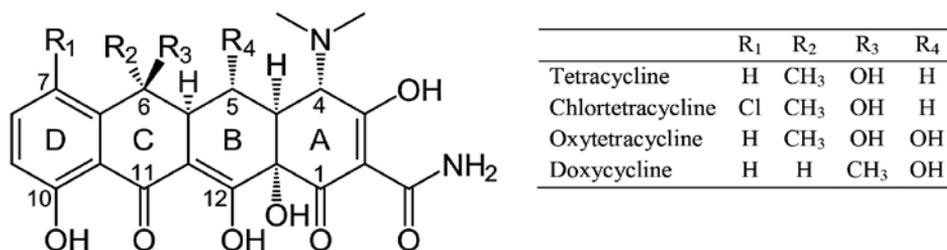


Fig. 1. Chemical structure of tetracyclines

Many liquid chromatography/mass spectrometry (LC/MS) methods for residue analysis of TCAs have been published, almost all of them using tandem mass spectrometry (MS/MS). For example, Bruno et al. (2002) and De Ruyck et al. (2007) have described a relatively fast and simple method for determination of TCAs in milk. Khong et al. (2005) also evaluated the epimerisation phenomenon in honey samples. Like all the authors who have closely studied this problem, they came to the conclusion that this effect has a large impact on sample preparation and storage. Many countries rely on detection by MS for the analysis of these compounds and other antibiotics in foodstuffs. European Commission Decision No. 96/23/EC states that “Methods based only on chromatographic analysis without the use of molecular spectrometric detection are not suitable for use as confirmatory methods” (Commission Decision (EC) No. 657/02), for which reason analysis by means of an Ultraviolet detector is no longer adequate. Furthermore, the previously described methods are time consuming and preparation of the sample is laborious. In addition, most of the methods use large analytical columns, 100 or 150 mm long, meaning that the performance of analysis is rather time consuming. As a result, the run time is about 30 minutes. The positives are that the peaks have better separation. On the other hand, longer time run requires a large volume of mobile phases.

The aim of this study was, therefore, to develop a simple quantitative method based on LC/MS/MS with an electrospray interface (ESI) ion source for analysis of four tetracyclines (TC, CTC, OTC and DC) in milk. Another goal was for the analysis time to be significantly shorter than in other studies, consequently providing more economically efficient analysis for food safety monitoring and controlling veterinary drug use.

Materials and Methods

Reagents and chemicals

Analytical standard materials of tetracycline (TC) and hydrochlorides of chlortetracycline (CTC), oxytetracycline (OTC) and doxycycline (DC) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

The solvent acetonitrile, used for the mobile phase, was of High Performance Liquid Chromatography (HPLC) grade and the additive for the mobile phase, formic acid, was of Liquid Chromatography-Mass Spectrometry (LC-MS) grade. These were also supplied by the same company. Distilled water was purified by the aquaMax™ Ultra 370 series (Young Lin Instruments, Korea) to HPLC grade.

Methanol of HPLC grade, used for extractions, was purchased from Fischer Scientific (Waltham, MA, USA). All other chemicals, trichloroacetic acid, citric acid monohydrate, disodium hydrogen phosphate dihydrate and disodium salt of ethylenediaminetetraacetic acid, were of analytical grade (Sigma-Aldrich).

Oasis® HLB 6cc (200 mg) SPE extraction cartridges were supplied by Waters (Milford, MA, USA).

Standard solutions

The analytical standards were dissolved in methanol at a concentration of 1 - 2 mg·ml⁻¹. The working standard solutions were made by dilution with water containing 0.1% formic acid. Standard solutions of 1 and 5 µg·ml⁻¹ water containing 0.1% formic acid were used to tune the mass spectrometer and to acquire the analyte identification spectra.

The stock solutions were kept at -20 °C, wrapped in aluminium foil, while the working solutions were stored in a fridge at +4 °C. The stabilities of the stored solutions were tested – the stock solutions were prepared fresh every 3 months and working solutions were replaced every 2 weeks.

Calibration curves

The linearity of the detector response was verified with TC, CTC, OTC and DC standard solutions over the range 25 - 500 µg·l⁻¹. Calibration curves were prepared monthly and estimates of the amount of the analytes in the samples were interpolated from these graphs.

Devices

A vortex mixer (Keison Products, UK), an EBA 21 centrifuge (Hettich, MA, USA), a vacuum SPE manifold (Phenomenex, CA, USA) and an N86KN.18/IP20 vacuum pump (KNF Neuberger GmbH, Germany) were used during sample preparation. The HPLC-MS/MS system consisted of an LC system (Dionex Ultimate 3000 RS, Germany) with a vacuum degasser, dual gradient analytical pump, autosampler and column compartment which were coupled with an API 2000 tandem quadrupole mass spectrometer (AB Sciex, USA) with electrospray ionisation interface. The LC-MS system was fully controlled by Analyst 6.0. The LC separations were carried out on a ZORBAX RRHD Eclipse Plus C18 reversed-phase column (Agilent Technologies), 1.8 µm, 50 x 3 mm i.d., protected with a ZORBAX Eclipse Plus C18 guard cartridge, 1.8 µm, 2.1 x 5 mm i.d.

Sample preparation

Sample preparation was adapted and modified from Cinquina et al. (2003). An aliquot of 5 mL of milk was transferred into a 50 mL plastic centrifugal tube. If necessary, tetracycline antibiotic (TCA) standards were added at this stage, following which the spiked sample was vortexed for 1 minute for the distribution of analytes. Afterwards, the sample was made acidic with 2 mL of 20% trichloroacetic acid in water and shaken for 5 min. After shaking, 20 mL of McIlvaine buffer (11.80 g of citric acid monohydrate; 13.72 g of disodium hydrogen phosphate dehydrate; 33.62 g ethylenediaminetetraacetic acid disodium salt diluted in 1 litre of water; 0.01 M; pH 3.5) was added and vortexed for 1 minute, after which the sample was centrifuged at 6000 rpm for 15 min. The supernatant was immediately applied to an SPE HLB Oasis cartridge, previously activated with 3 mL of methanol and 2 mL of water. After sample loading, the cartridge was washed with 4 mL of water and dried for 1 min. while drawing full vacuum. TCA residues were eluted with 3 mL of methanol, after which the solvent was removed under a stream of nitrogen in a thermoblock at 40 °C. The residue was dissolved in 1 mL of methanol and filtered on a 0.22 mm nylon filter. Finally, an aliquot (5 µL) was injected into the HPLC-MS/MS system.

Liquid chromatography

The LC separation of the tetracycline analytes was performed at 15 °C on a reversed-phase column with an organic mobile phase. Mobile phase A consisted of water containing 0.1% (v/v) formic acid to increase the ionisation intensity and mobile phase B was acetonitrile. Gradient elution was applied to increase the separation capacity and to decrease the retention times. For this reason, the gradient programme consisted of 80A:20B (0 - 0.1 min), 80A:20B to 25A:75:B (0.1 - 2 min.), 25A:75B to 20A:80B (2 - 3 min.), 20A:80B (3 - 5.5 min.), 20A:80B to 80A:20B (5.5 - 7 min.), 20A:80B (7 - 10 min.). The flow rate of the mobile phase was 0.35 mL/min. and a volume of 5 µL of cleaned sample extract was injected into the LC-MS/MS system, while the temperature of the autosampler was maintained at 7 °C.

Mass spectrometry

A tandem quadrupole mass spectrometer was used with positive ion electrospray ionisation (ESI⁺) for the detection, identification and quantification of tetracycline analytes. The eluent was sprayed into the mass

spectrometer without splitting. Nitrogen gas flows of 60 and 600 L·h⁻¹ for nebulising the LC eluent and drying the solvent was used. The probe temperature was 450 °C and the capillary voltage 5.25 kV. Fragmentation of the precursor ions into product ions was carried out in the collision cell using nitrogen. For each analyte, diagnostic fragment ions were obtained by in-source collision-induced dissociation (CID) of the protonated molecule [M+H]⁺. The analytes were detected by MS/MS using two Multiple Reaction Monitoring (MRM) mode.

Results and Discussion

Mass spectrometry

Optimisation of the mass spectrometer was performed by direct infusion of separate standard solutions with a concentration of 5 mg·l⁻¹ using a syringe pump. Table 1 summarises the transition of [M+H]⁺ precursor ions into product ions and the optimised Mass Spectrometry (MS) operating parameters of the examined tetracyclines.

Table 1. Summary of diagnostic product ions and MS operating parameters

Analyte	Precursor ion [m/z]	Product ions [m/z]	Cone voltage [V]	Collision energy [eV]
Tetracycline	445.0 ⁺	427.1 ⁺ / 410.1 ⁺ ^a / 154.1 ⁺	16	24 / 25 / 37
Chlortetracycline	479.0 ⁺	462.1 ⁺ / 444.1 ⁺ ^a / 154.1 ⁺	21	26 / 27 / 39
Oxytetracycline	461.1 ⁺	443.1 ⁺ / 426.2 ⁺ ^a / 201.1 ⁺	16	24 / 25 / 51
Doxycycline	445.1 ⁺	428.2 ⁺ ^a / 410.1 ⁺ ⁺ / 267.1 ⁺	16	24 / 27 / 51

^aMost abundant product ion

Fig. 2 shows the mass spectra of the examined analytes obtained during the optimisation of the mass spectrometer. All the analytes showed the maximum intensity in the positive ionisation mode, for which reason the protonated molecular ion [M+H]⁺ was chosen as the precursor ion. TC and OTC exhibited neutral losses of 18 Da, corresponding to the H₂O molecule ([M-H₂O+H]⁺) and then generated the ([M-H₂O-NH₃+H]⁺) ion with the loss of the H₂O and NH₃ molecule. Other analytes, CTC and DC, exhibited neutral losses of 17 and/or 35 Da, corresponding to the ammonium molecule ([M-NH₃+H]⁺), with the subsequent loss of a water molecule ([M-NH₃-H₂O+H]⁺).

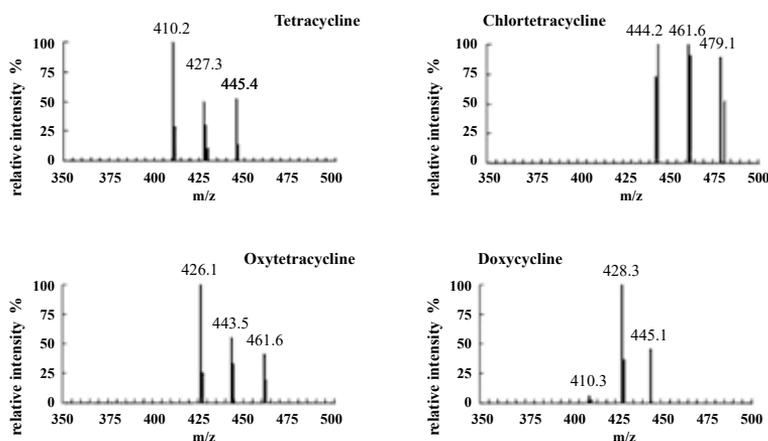


Fig. 2. Mass spectra of the examined analytes obtained after direct infusion with a concentration of 10 mg·l⁻¹

The most abundant product ions for TC, CTC and OTC at m/z 410, 444 and 426, respectively, correspond to $[M-NH_3-H_2O+H]^+$ and for DC at m/z 428, which correspond to $[M-NH_3+H]^+$. These fragmentation patterns agreed with those of TC, CTC, OTC and DC in previous papers (Bruno et al. 2002; De Ruyck and De Ridder 2007). The loss of ammonia occurs from the carboxyl amide group in position A-2. Whilst, the cleaving of a water molecule occurs in the presence of a hydroxyl group in position C-6 of the TCAs' general structure. However, DC does not contain a hydroxyl group in position C-6, and for this reason shows the most abundant ion intensity at m/z 428.

The drug compounds involved in this study belong among the drug substances of group B, veterinary drugs and contaminants in Annex I of Council Directive 96/23/EC (Commission Decision (EC) No. 657/02). For this reason, at least three Identification Points (IPs) have to be obtained for confirmation of drug compounds using mass spectrometry. In this method, the detection of the analytes in standard solutions and in spiked milk samples was performed by the MRM transition mode. The two most abundant product ions were detected from the precursor ion. The precursor ion accounts for 1 IP and each product ion for 1.5 IPs. This method therefore represents 4 IPs.

LC/MS analysis

Once the MS/MS parameters had been optimised, the separation of all TCAs, standard solutions, was performed using a ZORBAX RRHD Eclipse Plus C18 reversed-phase column (Agilent Technologies), 1.8 μm , 50 x 3 mm i.d. Gradient elution was applied for fast analysis and good resolution. To increase the ionisation intensity, 0.1% (v/v) formic acid was used as mobile phase A and pure acetonitrile as B, starting at 20% of B and increasing to 80% in 3 min. The composition was then held for 2.5 min. Afterwards, the column was equilibrated with the initial mobile phase composition for 3 min., with a total run time of 10 min. 350 μL flow rate was used to decrease the run time. Moreover, the higher content of mobile phase B and fast flow rate generated sharper peaks of TCAs. As previously mentioned, the temperature of the analytical column greatly influenced the shape, retention time and intensity of TCAs during separation, for which reason a lower temperature, 15°C, was applied. The retention times of OTC, TC, CTC and DC were 1.29 min., 1.70 min., 2.22 min. and 2.28 min., respectively. An example of a chromatogram of a blank milk sample fortified with TCAs is shown in Fig. 3.

Optimisation of the extraction process

Although multiple extraction methods had already been tested (Bruno et al. 2002; De Ruyck and De Ridder 2007), sample preparation was adapted and slightly modified from Cinquina et al. (2003). Removal of proteins was performed with a 20% solution (w/w) of trichloroacetic acid in water (which was stored at 4 °C). The centrifuge was set at 6000 rpm for 15 min. to obtain a clearer supernatant. As a precaution, the samples were stored in a refrigerator during preparation, for which reason epimerisation reactions were minimised. The supernatant was then applied on the SPE HLB Oasis extraction cartridge and a clean extract was obtained. Removal of the solvent was performed under a stream of nitrogen in a thermoblock at 40°C. Taking into account the researchers' experience with the limited stability of spiked samples, the prepared samples were stored frozen for a maximum of 2 weeks before analyses.

Linearity

Under the instrumental conditions reported in the experimental section, the linearity was demonstrated by five-point standard calibration in a concentration range of 0 - 500 $\mu g \cdot l^{-1}$ obtained by a six-point standard calibration. The slope and intercept values for the standard regression lines of TC, OTC, CTC and DC were 16.989 and 439.33, 18.045 and 326.11, 18.551 and 15.90, and 64.161 and 158.21, respectively.

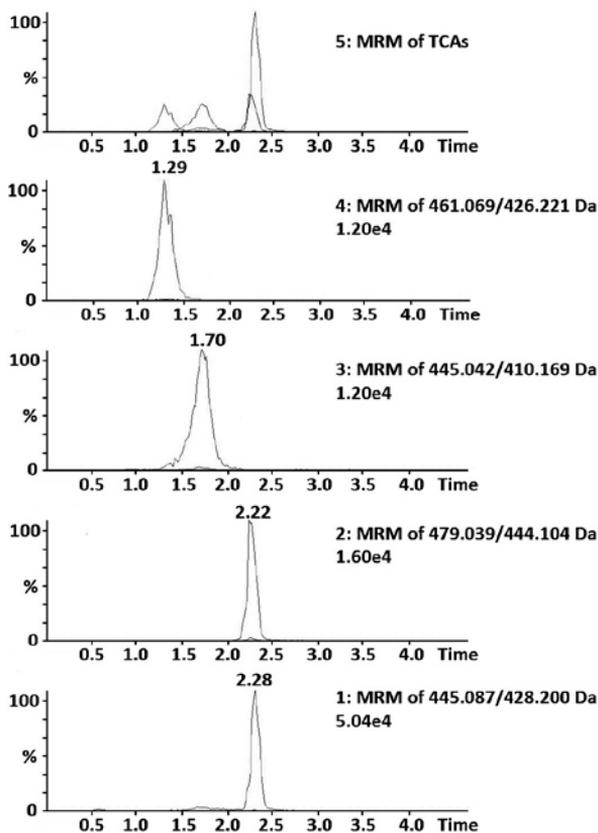


Fig. 3. Example of a chromatogram of a blank milk sample fortified at $2 \text{ mg} \cdot \text{l}^{-1}$ for each compound

Extraction recovery

The ability of TCAs to form complexes with Ca^{2+} and Mg^{2+} , which are abundantly present in milk, is well known. The first attempts to perform extraction on SPE Oasis[®] HLB (hydrophilic-lipophilic balance) using the extraction procedure described by De Ruyck et al. (2007) gave unsatisfactory recoveries (TC 20.8%, OTC 27.5%, CTC 69.2% and DC 13.3%). This problem might have been caused by the absence of the chelating agent in the described method. Cinquina et al. (2003), in contrast, used McIlvaine buffer (pH 3.5) with Na_2EDTA . With this agent, our recoveries were much higher, and the overall recovery values ranged from 78.9 to 92.2% as can be seen in Table 2.

Analytical limits

The limit of detection (LOD) and limit of quantification (LOQ) were estimated from LC-MS/MS chromatograms resulting from the analysis of $50 \text{ } \mu\text{g} \cdot \text{l}^{-1}$ of each tetracycline in the milk sample. Subsequently, the peak to averaged background noise ratio was calculated. A summary of all obtained analytical limits is presented in Table 3. As can be seen, for TC, CTC and OTC, the values of LOD ranged from 7.86 to $11.5 \text{ } \mu\text{g} \cdot \text{l}^{-1}$ and the values of LOQ from 26.22 to $38.22 \text{ } \mu\text{g} \cdot \text{l}^{-1}$. DC has an LOD of 3.40 and an LOQ of 11.32.

Table 2. Analyte recovery values for the determination of tetracyclines in fortified milk samples (mean (%), s_r (%), $n = 6$ for each concentration)

Spiked level [$\mu\text{g}\cdot\text{l}^{-1}$]	Tetracycline		Chlortetracycline		Oxytetracycline		Doxycycline	
	Mean	s_r	Mean	s_r	Mean	s_r	Mean	s_r
50	87.9	2.98	68.9	3.87	73.1	3.27	70.7	3.61
100	93.6	2.06	93.8	2.84	92.7	3.60	80.2	3.62
150	95.2	2.35	96.5	3.45	94.9	3.15	85.9	3.47
Overall recovery	92.2	2.46	86.4	3.39	86.9	3.34	78.9	3.57

Table 3. Summary of analytical limit values

[$\mu\text{g}\cdot\text{l}^{-1}$]	Tetracycline	Chlortetracycline	Oxytetracycline	Doxycycline
LOD	11.50	7.86	9.96	3.40
LOQ	38.22	26.22	33.20	11.32

These LOD values are acceptable when compared with the values of other authors such as Bruno et al. (2002) (TC: 2; CTC: 7; OTC 0.5; DC: 4 $\mu\text{g}\cdot\text{l}^{-1}$) and De Ruyck and De Ridder (2007) (TC: 7.5; CTC: 25; OTC 7.5; DC: 5 $\mu\text{g}\cdot\text{l}^{-1}$).

Conclusions

In this study, a simple, fast and specific LC-MS/MS confirmatory multi-residue method has been developed for the simultaneous determination of tetracycline, chlortetracycline, oxytetracycline and doxycycline in milk at residue levels between 3.40 and 11.50 $\mu\text{g}\cdot\text{l}^{-1}$. The presented technique is also suitable for the quantification of residues of TCAs at the maximum residue limit according to Commission Decision 2002/657/ES. In addition, an economic saving was also achieved by decreasing the run time to just 10 minutes. This approach, with a slight modification in the extraction procedure, can also be applied to other matrices. Therefore, this tool can be helpful in determining the MRL of TCAs in milk or meat by local farmers during and after the treatment of bovine mastitis.

In conclusion, this analytical method is useful for food safety monitoring and controlling veterinary drug use.

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