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# A Rapid Confirmatory Method for Analyzing Tetracycline Antibiotics in Bovine, Swine, and Poultry Muscle Tissues: Matrix Solid-Phase Dispersion with Heated Water as Extractant followed by Liquid Chromatography-Tandem Mass Spectrometry

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A rapid, specific, and sensitive procedure for determining four widely used tetracycline antibiotics and three related epimers in bovine, swine, and poultry muscle tissues is presented. The method is based on the matrix solid-phase dispersion technique with heated water as the extractant followed by liquid chromatography (LC)-tandem mass spectrometry (MS) equipped with an electrospray ion source. Target compounds were extracted from tissues with 5 mL of water heated at 70 °C. After acidification and filtration, 100 $\mu$L of the aqueous extract was injected in the LC column. MS data acquisition was performed in the multireaction monitoring mode, selecting two precursor ion to product ion transitions for each target compound. Heated water appeared to be an excellent extractant, since the absolute recovery data ranged between 70 and 78%. The accuracy of the method was determined at three spike levels, using minocycline as a surrogate analyte, in any different kind of muscle tissues considered and varied between 88 and 109% with relative standard deviations ranging between 3 and 11%. Limits of quantification were estimated to range between 1 (chlortetracycline) and 9 ng/g (4-epioxytetracycline), based on a signal-to-noise ratio of 10, and are well below the tolerance levels set by the European Union. The effects of the extraction temperature, volume of the extractant, and washing of the material supporting the biological matrix with ethylenediamine tetraacetic disodium salt on the analyte recovery were studied.

**KEYWORDS: Tetracycline antibiotics; muscle tissues; bovine; swine; poultry; matrix solid-phase dispersion; heated water as extractant; LC-MS/MS**

## INTRODUCTION

Relatively high levels of antibiotic residues in foodstuffs can provoke allergic reactions in some hypersensitive individuals and transfer drug-resistant bacteria from food to humans (*1*). In addition to immediate adverse effects, there are also long-term effects to the exposure of low levels of residues that are still unknown.

For the rapid detection of antibiotics in food, microbiological assays are routinely used because they are easy to perform and inexpensive. The main disadvantage of these tests is their lack of specificity; that is, they often detect growth inhibition of a sensitive test strain, whether a generic substance with antimicrobial activity is present or not (*2*). More sophisticated test systems allow the detection of at least a structural or functional group, but there is still no possibility for identification or quantification of the specific drug. Nevertheless, these methods continue to be used as screening assays because of their simplicity and low cost. However, before samples are condemned for containing levels of antibiotics exceeding the tolerance levels, it is well-recognized that these methods need to be supported by highly selective and sufficiently sensitive chemical methods. Public Health Agencies in many countries rely on detection by mass spectrometry (MS) for unambiguous confirmation of antibiotics in foodstuffs. The European Commission Decision 2002/657/EC states that, "Methods based only on chromatographic analysis without the use of molecular spectrometric detection are not suitable for use as confirmatory methods" (*3*).

Tetracycline antibiotics (TCAs) have a broad range of activity against Gram-positive and Gram-negative bacteria and are inexpensive. For these reasons, TCAs are widely used antibiotics in animal husbandry for the prevention and treatment of diseases and as feed additives for growth promotion. TCAs are licensed for use in a variety of food-producing animals including cattle, pig, sheep, poultry, and fish (*4*). In 1998, the European

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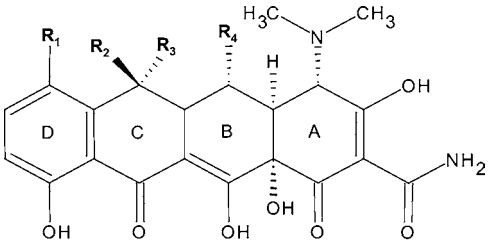


| | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| Minocycline | $N(CH_3)_2$ | H | H | H |
| Tetracycline | H | $CH_3$ | OH | H |
| Oxytetracycline | H | $CH_3$ | OH | OH |
| Demeclocycline | Cl | H | OH | H |
| Chlortetracycline | Cl | $CH_3$ | OH | H |
| Doxycycline | H | H | $CH_3$ | OH |

**Figure 1.** Molecular structures of selected TCs.

Federation of Animal Heath conducted a survey on the veterinary use of antibiotics in the 15 member States of the European Union (EU) and Switzerland. In this survey, it was estimated that TCAs represented almost 65% of all antibiotics and antibacterials consumed for therapeutic and prophylactic use and ranked of the sum of antibiotics used for growth promotion with 1599 t. The basic structure of TCAs consists of a hydronaphthacene framework containing four fused rings. The various TCA components differ chiefly by substitution at the C5, C6, and C7 positions on the backbone (**Figure 1**). Of the eight commercially available TCAs, chlortetracycline (CTC), oxytetracycline (OTC), tetracycline (TC), and, to a lesser extent, doxycycline (DC) are the most commonly applied to food-producing animals.

To ensure the safety of food for consumers, the EU has laid down maximum residue limits (MRLs) for TC, OTC, CTC, and DC in various food products. In particular, the tolerance levels set for cattle, poultry, and swine muscle tissues are 100 ng/g and this limit should comprise the sum of the parent drug and its main metabolite (except for the DC), which is the 4-epimer (*6*). Withdrawal periods of 5−20 days before slaughter are recommended for food-producing animals, depending on the species and nature of the food product.

In general, the analysis of TCA residues in food can present a number of problems. TCAs rapidly chelate to metal ions. Consequently, materials containing no traces of metals have to be used in any step of the analytical procedure (*7*, *8*). Alternatively, a metal-blocking agent, such as oxalic acid or ethylenediamine tetraacetic disodium salt ($Na_2EDTA$), can be used in both the sample treatment step and the analysis of the final extract (*9*).

A further complication in the determination of TCAs, in particular of CTC, is the fact that they can rapidly isomerize even under mild conditions to form 4-epimers (*10*) (**Figure 2**). Besides to 4-epichlortetracycline (epiCTC), some authors (*5*, *11*) demonstrated the presence of abundant amounts of isochlortetracycline (ICTC) in incurred egg samples (see again **Figure 2**). Some researchers (*8*) suggested that the actual EU legislation (*6*) relating to MRLs for TCAs may have to be further modified to take account of all isomers and not only epimers.

Following the introduction of the liquid chromatography−mass spectrometry (LC-MS) technique, several analytical procedures based on this technique have been developed for TCA residue determination in food (*9*). However, some of these methods involve the use of LC-MS interfaces that are no longer commercially available and/or have limits of quantification (LOQs) higher than tolerance levels cited above. In addition, despite the use of the specific MS detector, most of the methods proposed still make use of time-consuming, labor-intensive multistep sample preparation procedures. This topic has been reviewed in two articles (*12*, *13*).

After the pioneering work of Barker and his colleagues (*14*), many researchers have successfully adopted the so-called matrix solid-phase dispersion (MSPD) technique for extracting contaminants from solid biological matrices (*15*). A fine dispersion of the biological matrix onto a suitable solid support is easily obtained by blending the sample and a suitable support with a mortar and pestle. After blending, this material is packed into a column and analytes are eluted by a suitable organic solvent. The abrasive action of the sorbent during blending has been demonstrated to disrupt the gross architecture of the matrix (*15*), so that a tight and quasi-homogeneous layer of the matrix components is formed on the support surface. Over classical sample treatment procedures, MSPD offers distinct advantages in that (i) the analytical protocol is drastically simplified and shortened; (ii) the possibility of emulsion formation is eliminated; (iii) the consumption of toxic, flammable, and expensive solvents is substantially reduced; (iv) and last but not least, the extraction efficiency of the analytes is enhanced as the entire sample is exposed to the extractant. On the basis of this technique, two methods have been developed for analyzing TCA residues in fish (*16*), milk, cheese, and meat (*17*). Although, to a reduced extent, methods based on MSPD still use nonselective organic solvents as extractants. Consequently, the sample treatment protocol generally includes a cleanup step before injection of the extract into the LC column.

Recently, several simple and rapid confirmatory methods have been developed for determining sulfonamide antibacterials in milk, eggs (*18*) and bovine tissues (*19*, *20*), carbamate insecticides in milk (*21*) and vegetables (*22*), amoxicillin and ampicillin in bovine tissues and milk (*23*), aminoglycoside antibiotics in milk (*24*), and algal toxins in fish (*25*). These procedures are based on extraction by the MSPD technique and LC-MS equipped with a single or triple quadrupole for final extract analysis. The uniqueness of these methods is that the analytes are extracted with heated water. Over conventional extraction with organic solvents, the use of heated water as extractant offers distinct advantages in that no use of toxic and expensive solvents is made and selective extraction can be achieved by suitably controlling the extraction temperature. Selective extraction reflects in that many endogenous compounds are not coextracted with the analytes. In this way, relatively large volumes of the extract can be injected into a reversed-phase LC column after little and simple manipulation of the extract itself.

The aim of this work has been that of developing a LC-tandem MS confirmatory method able to determine four TCAs and three related epimers in bovine, swine, and poultry muscle tissues at the EU regulatory levels by suitably modifying the sample treatment procedure mentioned above.

## EXPERIMENTAL PROCEDURES

**Reagents and Chemicals.** TC, OTC, CTC, DC, minocycline (MC), and demeclocycline (DMCTC) were purchased from Sigma (St. Louis, MO). MC and DMCTC were used, respectively, as surrogate analyte (SA) and internal standard (IS), as the former substance is not used in Europe for veterinary use while the latter one is an obsolete antibiotic. Three TCA epimers, e.g., 4-epitetracycline (4-epiTC), 4-epioxytetracycline (4-epiOTC), and 4-epiCTC, were from Acros (Fisher Scientific, Schwerte, Germany). Individual stock solutions of the analytes, the SA, and the IS were prepared by dissolving each compound in methanol

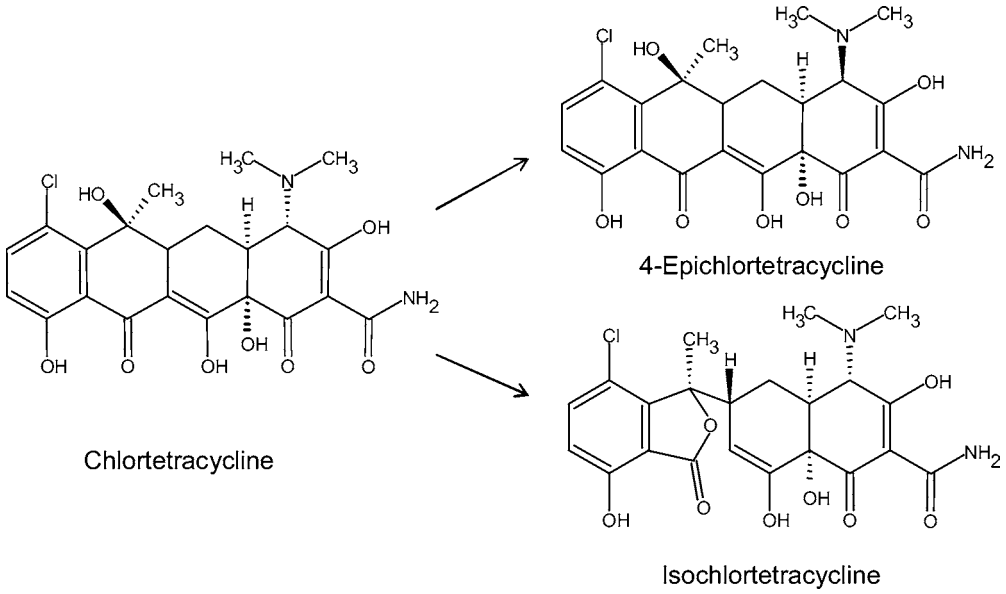


**Figure 2.** Structures of the isomers of CTC.

to obtain a 1 mg/mL concentration. After preparation, these solutions were stored at 4 °C in the dark and freshly prepared every 2 months. A composite working standard solution of the target compounds and the SA was prepared by mixing the above solutions and diluting with methanol to obtain analyte concentrations of 3 $\mu$g/mL. A 6 $\mu$g/mL solution of the IS was prepared by diluting the stock solution with methanol. When unused, all solutions were stored at 4 °C in the dark and renewed after 1 month of use.

For LC, distilled water was further purified by passing it through a Milli-Q Plus apparatus (Millipore, Bedford, MA). Methanol "Plus" of gradient grade was obtained from Carlo Erba (Milan, Italy). Formic acid was from Sigma. $Na_2EDTA$ dehydrate and sand (crystobalite, 40−200 mesh size), a material obtained by heating silica at about 1500 °C, were from Fluka AG (Buchs, Switzerland). Before use, metal impurities on the crystobalite surface were blocked by treatment with $Na_2EDTA$. In particular, 60 g of sand was packed in a plastic tube (2.6 mm i.d.) and 120 mL of 0.1 mol/L EDTA was passed through the tube at flow-gravity. Partial drying of the sand was carried out by vacuum with a water pump. Thereafter, crystobalite was completely dried in an oven at 100 °C.

**Samples.** Bovine, swine, and poultry muscle tissue samples used for this study were collected from local markets. Before use, any sample was determined to be free of the antibiotics considered.

**Extraction Apparatus.** The design of the homemade extraction apparatus used in this work was very similar to that shown in a previous paper (*26*), with the exception that nitrogen was bubbled in water to eliminate any trace of dissolved oxygen and the analyte-containing water leaving the extraction cell was collected in a calibrated glass tube instead of a sorbent cartridge. A 7.5 cm × 8.3 mm i.d. stainless steel column was used as an extraction cell. Stainless steel frits (2 $\mu$m pore size) were located above and below the matrix/sand material.

**Sample Preparation and Extraction.** Prior to blending with $Na_2EDTA$-treated crystobalite, muscle samples were minced with a meat grinder. A 1.5 g portion of tissue was put in a porcelain mortar and spiked with variable volumes of the working standard solution and that of the SA, taking care of uniformly spreading it on the sample. Intimate contact between the analytes and the sample was obtained by pounding with the pestle for about 1 min. The mixture was allowed to equilibrate for 1 h by storing the mortar at 4 °C. A 4.5 g amount of crystobalite was then added to the mortar, and the mixture was blended with the pestle for about 10 min, until an apparently dry material was obtained. This material was then packed into the extraction cell. To ensure homogeneous packing of the cell, close attention was paid to pour the material into the tube in 3−4 aliquots, tapping the tube firmly for 10−15 s after the addition of each aliquot. Any void space remaining after packing the solid material was filled with crystobalite. The tube was then put into the oven and heated at 70 °C for 5 min. Five milliliters of water at a 1 mL/min flow rate was then passed through the cell to extract the analytes. Extracts of any type of tissue considered appeared slightly opalescent and pale yellow colored with a pH of 5.6−5.9, depending on the kind of the muscle tissue. After 300 ng of the IS was added, extracts were acidified to pH 3−3.1 with 10 mol/L formic acid (11−15 $\mu$L). The precipitate was eliminated by filtration through a glass fiber filter (1.2 $\mu$m pore size, 2.5 cm diameter, Alltech, Sedriano, Italy), and 100 $\mu$L of the final extract was injected into the LC column. By following the procedure described above, the guard column was replaced with a new one after more than 120 injections of extracts.

**Instrumental Analysis.** The liquid chromatograph consisted of a Waters pump (model 600 E, Milford, United States), a 100 $\mu$L injection loop, and Alltima 5 $\mu$m C-18 guard (7.5 mm × 4.6 mm i.d.) and analytical (250 mm × 4.6 mm i.d.) columns (Alltech) thermostated at 20 °C and was interfaced by an electrospray ion (ESI) source to a benchtop triple-quadrupole mass spectrometer (model Micromass Quattro Micro API, Waters). Before use, any peak tailing for TCAs caused by the presence of metal impurities on the stationary phase was permanently eliminated by washing the LC column with an $Na_2EDTA$ solution, following a procedure previously reported (*27*). Mobile phase component A was methanol, and component B was water, both acidified with 10 mmol/L formic acid. At 1.0 mL/min, the mobile phase gradient profile was as follows ($t$ in min): $t_0$, A = 0%; $t_8$, A = 0%; $t_9$, A = 45%; $t_{14}$, A = 61%; $t_{15}$, A = 100%; $t_{18}$, A = 100%; $t_{19}$, A = 0%; and $t_{28}$, A = 0%. Analytes retention times varied ≤1% over 2 weeks. A diverter valve led the effluent into the ion source, operating in the positive ion mode, with a flow of 400 $\mu$L/min only between 9 and 23 min of the chromatographic run. High-purity nitrogen was used as drying and curtain gases; high-purity argon was used as the collision gas. The nebulizer gas was set at 650 L/h while the cone gas was set at 50 L/h; the probe and desolvation temperatures were maintained at 120 and 350 °C, respectively. The gas pressure in the collision cell was 3 mTorr. The capillary voltage was 3000 V, and the extractor voltage was 2 V. Declustering potential, collision energy, and other transmission parameters were optimized for each analyte and are reported in **Table 1**. Mass axis calibration of each mass-resolving quadrupole $Q_1$ and $Q_3$ was performed by infusion of a sodium and cesium iodide solution at 10 $\mu$L/min. The unit mass resolution was established and maintained in each mass-resolving quadrupole by keeping a full width at half-maximum of approximately 0.7 amu. All of the source and instrument parameters for monitoring TCAs were optimized by standard solutions of 5 $\mu$g/mL infused at 10 $\mu$L/min by a syringe pump. The multireaction monitoring (MRM) mode was used for quantitation by selecting at least two molecular ion decomposition reactions for each analyte (see again **Table 1**).

**Quantitation.** Absolute recovery of each analyte and the SA added to any muscle tissue sample was assessed by summing the ion current

**Table 1.** MRM Conditions for Detecting TCAs by Tandem MS

| compound | transition (*m/z*) | product ion | cone voltage (V) | collision energy (eV) |
|---|---|---|---|---|
| TC | 445 > 410 | $[M + H - NH_3 - H_2O]^+$ | 18 | 12 |
| | 445 > 427 | $[M + H - H_2O]^+$ | | 18 |
| 4-epiTC | 445 > 410 | $[M + H - NH_3 - H_2O]^+$ | 18 | 12 |
| | 445 > 427 | $[M + H - H_2O]^+$ | | 18 |
| MC[a] | 458 > 441 | $[M + H - H_2O]^+$ | 30 | 20 |
| OTC | 461 > 426 | $[M + H - NH_3 - H_2O]^+$ | 18 | 17 |
| | 461 > 443 | $[M + H - H_2O]^+$ | | 10 |
| epiOTC | 461 > 426 | $[M + H - NH_3 - H_2O]^+$ | 18 | 17 |
| | 461 > 443 | $[M + H - H_2O]^+$ | | 10 |
| DMCTC[b] | 465 > 430 | $[M + H - NH_3 - H_2O]^+$ | 22 | 18 |
| | 465 > 448 | $[M + H - NH_3]^+$ | | 15 |
| CTC | 479 > 444 | $[M + H - NH_3 - H_2O]^+$ | 20 | 18 |
| | 479 > 462 | $[M + H - H_2O]^+$ | | 15 |
| epiCTC | 479 > 444 | $[M + H - NH_3 - H_2O]^+$ | 20 | 18 |
| | 479 > 462 | $[M + H - H_2O]^+$ | | 15 |
| DC | 445 > 321[c] | | 20 | 30 |
| | 445 > 428 | $[M + H - NH_3]^+$ | | 18 |

[a] SA. [b] IS. [c] As postulated by us, the ion at *m/z* 321 formed by opening of the C ring and consequent neutral loss of $HOC_6H_5CH_2OH$.

profiles relative to the transitions considered, normalizing them to the peak area of the IS, and comparing these ratios to those obtained by injecting a related blank sample extract to which the analytes were added postextraction. We followed this procedure to obviate a "positive" matrix effect that strengthened the analyte ion signal intensities, as compared to signals observed by injecting TCAs from a standard solution. The validation of the method at any given concentration and for any muscle tissue considered was performed in an analogous way, with the difference that signals of targeted compounds were normalized to those of the SA.

## RESULTS AND DISCUSSION

**General Remarks.** When analyzing muscle extracts spiked with only authentic TCAs, we noted the appearance of small peaks for 4-epimers of TC, OTC, and CTC in the mass chromatogram. The peak area for any epimer accounted for 6−9% of that of the related parent compound. It has to be pointed out that this is not a drawback of the method as the EU regulation states that TCAs have to be quantified as sum of the parent compounds and their 4-epimers. Also, the ion signal profile of CTC displayed a small peak that was not present when analyzing extracts spiked with the analytes postextraction. On the basis of mass spectrometric considerations, this species was tentatively identified as ICTC, considering that no ion signal was evident at the retention time of the postulated ICTC for the $[M + H]^+ > [M + H - NH_3 - H_2O]^+$ transition selected for MS/MS detection of CTC (**Table 1**). It is known that loss of water provoked by the CID process of TCA ions derives from the presence of the hydroxyl group in the C ring and ICTC lacks this group (see again **Figure 2**). Interestingly, the picture described above did not significantly change from a quantitative point of view by varying the extraction temperature. On this basis, we supposed that some enzymatic isomerization of TCAs occurred before extraction, while dispersing spiked tissues on crystobalite. In the recent past, we had experimental evidence for the fact that a sulfonamide antibacterial, i.e., sulfoquinoxaline (SQX), was rapidly degraded to an oxygenated metabolite during deposition of a SQX-spiked tissue on the solid support (*20*). At our best knowledge, the EU legislation relating to MRL for CTC has not yet been modified to take into account all CTC isomers, apart from epiCTC. Thus, we estimated that partial conversion of CTC to ICTC occurring during sample treatment, if not considered for CTC quantification, should produce an underestimation of CTC in incurred samples no larger than 5−6%.

**Table 2.** Effect of the Treatment of the Matrix Supporting Material (Crystobalite) with 0.1 mol/L $Na_2EDTA$ on the Recovery of Some Selected TCs in a Bovine Muscle Tissue Sample[a]

| | recovery[b] (%) (RSD, %) | |
|---|---|---|
| compound | untreated support | $Na_2EDTA$-treated support |
| TC | 50 (23) | 76 (6) |
| MC (SA) | 32 (28) | 75 (11) |
| OTC | 57 (24) | 75 (7) |
| CTC | 20 (25) | 70 (12) |
| DC | 29 (30) | 78 (7) |

[a] Spike level, 100 ng/g; extractant, water heated at 70 °C. [b] Mean values from triplicate experiments.

Initial extraction experiments gave poor recovery of all TCAs (**Table 2**). We supposed this negative result could be due to the presence on the surface of the siliceous material (crystobalite) supporting the biological matrix of metal impurities able to strongly bind TCAs by complex formation. Treatment of crystobalite with 0.1 mol/L $Na_2EDTA$ reached the goal of achieving good recovery of all of the TCAs considered (see again **Table 2**). The action of $Na_2EDTA$ should be that of deactivating metal impurities present on the crystobalite surface by formation of stable complexes. It has to be noted that, when eliminating any trace of unreacted $Na_2EDTA$ by washing crystobalite exhaustively with water, the benefit obtained by the $Na_2EDTA$ treatment partially disappeared. Probably, the excess of $Na_2EDTA$ on the crystobalite surface plays the role of also chelating metals present in the biological matrix so liberating that fraction of TCAs, which binds to cations during preparation of the support/matrix blend.

**Effect of the Temperature on Analyte Recoveries.** As its temperature is increased, water becomes more and more effective in extracting organic compounds from matrices. On the other hand, a risk inherent to the use of hot water as an extractant is that it could decompose those compounds that are thermolabile and/or prone to hydrolytic attack. Therefore, we evaluated the temperature effect on recoveries of the selected TCAs by performing extractions at various temperatures. The aim of this study was also that of finding the minimum extraction temperature able to give good recovery of the analytes and the lowest amount of matrix components that could contaminate the ion source and/or interfere with the rest of the analysis. For this study, a sample of bovine tissue was spiked with the analytes and the SA at 100 ng/g and 5 mL of water was passed through the extraction cell at a 1 mL/min flow rate. At each temperature, three extractions were carried out and results for some selected analytes are reported in **Figure 3**.

Raising the temperature of the extractant from 60 to 70 °C had the effect of improving the extraction yields of all of the analytes. A further increase of the water temperature to 80 °C resulted in some increase of the extraction yield of DC. This increase, however, was accompanied by a decrease of the recovery of the SA (MC) and OTC, maybe due to some decomposition of the two TCAs. Thus, the best compromise for achieving good recovery of all TCAs considered was that of using an extraction temperature of 70 °C. An analogous recovery against temperature behavior was observed when extracting TCAs from both swine and chicken muscles tissues (data not reported here).

**Effect of the Extractant Volume on Analyte Recoveries.** Besides affecting the extraction yield of the target compounds,

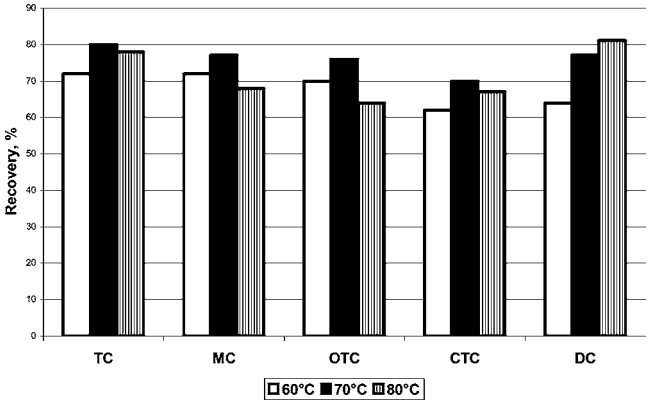


**Figure 3.** Effect of the water temperature on recovery of five TCAs added to a bovine muscle sample at the 100 ng/g level. Recovery data resulted from triplicate measurements.

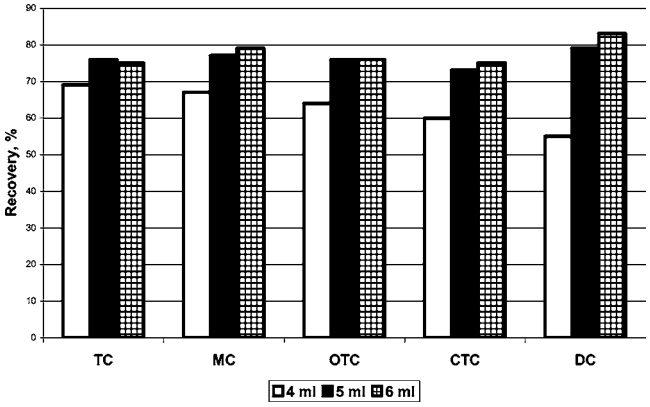


**Figure 4.** Effect of the extractant volume on recovery of five TCAs added to a bovine muscle sample at the 100 ng/g level. Recovery data resulted from triplicate measurements.

the water volume passing through the extraction cell can influence the sensitivity of the method, as this method does not include any concentration step of the extract. For the purpose of finding the minimum volume of water able to efficiently extract the analytes, experiments were performed by spiking a bovine tissue sample with the analytes and the surrogate IS at 100 ng/g level and extracting with increasing water volumes. Experiments were made in triplicate, and results for some selected analytes are shown in **Figure 4**. As can be seen, extracting with more than 5 mL of water did not significantly increase the analyte recovery. Thus, the best compromise between method sensitivity and extraction yield was that of passing through the extraction cell 5 mL of water heated at 70 °C. An analogous recovery against extractant volume behavior was observed when extracting TCAs from both swine and chicken muscles tissues (data not reported here).

**Matrix Effect.** We observed that ion signals of TCAs added postextraction to tissue extracts were more intense than those obtained when injecting analytes from a reference standard solution. In addition, this effect was remarkably dependent on the kind of the muscle tissue extract to which target compounds were added. This anomalous "positive" matrix effect was already observed by Heller et al. (*28*) on injecting aminoglycoside antibiotics from a milk extract. When analyzing contaminants in biological matrices with an ESI source, a "negative" matrix effect, or less commonly, a "positive" matrix effect is the rule more than the exception. To obviate this drawback, many authors have proposed the adoption of analyte-fortified control tissue extracts as reference standards (*28*−*35*). However, it is possible that the extent of the matrix effect can vary by varying the source of a specific biological matrix. In this case, using a generic analyte-fortified control tissue extract as a reference standard will affect analyte quantitation in incurred samples. For all of the TCAs considered here, including MC (SA) and DMCTC (IS), we conducted a study aimed at assessing if remarkable variations of the matrix effect occurred by varying the source of the different muscle tissues considered. For this purpose, six muscle samples, respectively, of cattle, swine, and poultry coming from six different breedings were extracted as reported in the Experimental Procedures. Prior to LC-MS analysis, the extracts were spiked with the analytes and the SA at 100 ng/g level. Quantification of the TCAs in final extracts was performed by comparing their absolute peak areas to those of the same compounds injected from a standard solution. For each muscle sample considered, results of these experiments for some selected analytes are presented in **Table 3**. As can be read, except in a few cases, the matrix effect affected significantly quantitation of all of the TCAs considered. Moreover, this "positive" matrix effect was dependent on the particular kind of animal from which the muscle tissue derived. Vice versa, the extent of the matrix effect for protonated TCAs appeared to be not remarkably dependent on the source of any particular muscle tissue, as RSDs were in all cases not higher than 14%. It has to be pointed out that EU guidelines (*3*) state that a method can be considered precise when relative standard deviations are not higher than 20%. Therefore, when analyzing bovine, swine, and poultry muscle tissues, a corresponding analyte-fortified control tissue extract could be used as a reference standard to improve the accuracy of the analysis of TCAs in incurred samples.

**Table 3.** Accuracy[a] and Precision[b] Data for Some Selected TCs Directly Added to Muscle Tissue Extracts of Cattle, Swine, and Poultry with Each Tissue Coming from Six Different Sources[c]

| compound | cattle | swine | poultry |
|---|---|---|---|
| TC | +6 (13) | +2 (10) | +31 (12) |
| MC (SA) | +21 (10) | +17 (8) | +48 (10) |
| OTC | +2 (12) | +4 (6) | +21 (9) |
| DMCTC (IS) | +34 (13) | +33 (8) | +37 (11) |
| CTC | +45 (12) | +38 (13) | +47 (12) |
| DC | +43 (12) | +28 (13) | +42 (12) |

[a] Calculated as [(mean calcd concn − spiked concn)/spiked concn] x 100. The concentration of each TC (included MC and DMCTC) in every tissue extract was calculated by comparing its absolute peak area to that of the same compound injected from a standard solution. [b] Expressed as relative standard deviation (RSD, %). [c] Spike level, 100 ng/g.

**Accuracy and Precision.** For any animal muscle tissue considered, that is, swine, chicken, and cow, this method was validated at three different concentrations corresponding to one-half of the MRL, the MRL, and 1.5 times the MRL, following criteria reported in the EU guidelines (*3*). At each analyte concentration, five measurements were performed with the criterion of adding the SA (MC) before analyte extraction. Quantification of the various concentrations of TCAs in muscle tissue was done by using negative tissue extracts spiked with the analytes and the SA as reference standards. Results are reported in **Table 4**. The accuracy data varied between 88 and 109% with standard deviations not higher than 11%. Thus, this method meets requirements reported in the EU guidelines (*3*) indicating that a method can be considered accurate and precise when accuracy data are comprised between 80 and 110% with relative standard deviations not higher than 20%.

**Table 4.** Accuracy (Recovery, %) and Precision (RSD, %) Data Obtained by Five Replicate Analyses of Bovine, Swine, and Poultry Muscle Tissue Samples Spiked with TCs at Concentrations Equal to or Close to Tolerance Levels (100 ng/g) Set by the EU

| muscle tissue | concn | TC | 4-epiTC | OTC + 4-epiOTC[a] | CTC | 4-epiCTC | DC |
|---|---|---|---|---|---|---|---|
| bovine | MRL/2 | 96 (7) | 106 (7) | 98 (5) | 90 (5) | 98 (8) | 96 (6) |
| | MRL | 98 (4) | 105 (5) | 102 (5) | 95 (5) | 102 (7) | 103 (10) |
| | 1.5 MRL | 101 (8) | 108 (6) | 98 (4) | 91 (9) | 100 (4) | 107 (11) |
| swine | MRL/2 | 95 (9) | 107 (10) | 102 (9) | 89 (7) | 103 (6) | 103 (6) |
| | MRL | 98 (6) | 104 (7) | 96 (11) | 95 (8) | 100 (8) | 108 (6) |
| | 1.5 MRL | 94 (3) | 102 (5) | 97 (7) | 92 (5) | 107 (5) | 105 (4) |
| poultry | MRL/2 | 103 (5) | 109 (6) | 95 (4) | 92 (6) | 103 (9) | 93 (4) |
| | MRL | 101 (4) | 109 (5) | 99 (3) | 95 (3) | 106 (8) | 99 (5) |
| | 1.5 MRL | 98 (8) | 106 (6) | 101 (7) | 88 (5) | 102 (6) | 96 (7) |

[a] Recovery of OTC and its epimer was measured as a total as these two compounds could not be differentiated either chromatographically or by mass spectrometry.

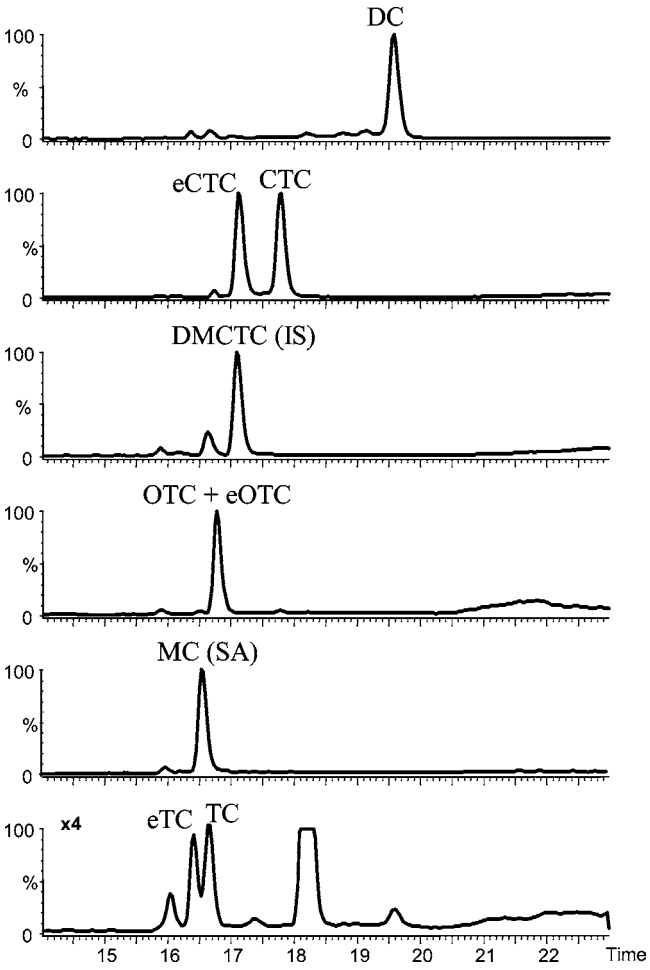


**Figure 5.** MRM LC-MS/MS chromatogram resulting from analysis of a swine muscle tissue spiked with eight TC (including the SA) antibiotics at the 50 ng/g level. 4-epiTC, 4-epitetracycline; MC (SA); epiOTC, 4-epioxytetracycline; DMCTC (IS); and epiCTC, 4-epichlortetracycline.

**Limits of Detection (LODs) and LOQs of the Method.** LOQs of the method were estimated from the MRM LC-MS/MS chromatogram shown in **Figure 5** and resulting from analysis of a bovine muscle tissue extract spiked with TCAs at 50 ng/g level. After the sum of the ion currents of the transitions selected for each analyte was extracted, the resulting trace was smoothed twice by applying the mean smoothing method (MassLynx 4.0 software, Waters). Thereafter, the peak height-to-averaged background noise ratio was measured. The background noise estimate was based on the peak-to-peak baseline near the analyte peak. LOQs were then calculated on the basis of a minimal accepted value of the signal-to-noise ratio (S/N) of 10. These data are listed in **Table 5**. In the same table, LODs of the method are also presented. When performing detection with a MS/MS arrangement, the most important condition to be satisfied for ascertaining the presence of a targeted compound is that at least two parent ion to daughter ion transitions give signals distinguishable from the background ion current. Accordingly, a definition of LOD (S/N = 3) of each analyte was adopted, considering in each case the transition giving the worst S/N. It can be read that TCAs can be detected in muscle tissues of food-producing animals at concentrations far below tolerance levels set by the EU. Tolerance levels of TC, OTC, and CTC residues in muscle tissues set by the U.S. Food and Drug Administration are 2000 ng/g (*36*). Thus, this method could also be advantageously adopted as a confirmatory method by U.S. regulatory laboratories.

**Table 5.** Limits of Detection (LOD) and LOQs of the Method for Determining TCs in Various Muscle Tissues

| compound | LOD (ng/g) | LOQ (ng/g) |
|---|---|---|
| 4-epitetracycline | 6 (445 > 427)[a] | 7 |
| tetracycline | 5 (445 > 427) | 6 |
| epioxytetracycline | 5 (461 > 443) | 9 |
| oxytetracycline | 4 (461 > 443) | 8 |
| epichlortetracycline | 1 (479 > 462) | 1 |
| chlortetracycline | 1 (479 > 462) | 1 |
| doxycycline | 6 (445 > 321) | 6 |

[a] *m/z* values of the transitions giving the worst signal-to-noise ratios are reported in parentheses.

**Linear Dynamic Range.** Under the instrumental conditions reported in the Experimental Procedures, the linear dynamic range of the ESI/MS/MS detector was estimated for all of the analytes. Amounts of each analyte varying from 0.3 to 600 ng and a constant amount of 30 ng of the IS were injected from suitably prepared standard solutions into the LC column. At each analyte amount, three replicate measurements were made. Signals against amount-injected curves were then constructed by averaging the peak area resulting from the sum of the signals for parent and fragment ions of each analyte and relating this area to that of the IS. Results showed that ion signals of the four target compounds and the surrogate IS were linearly correlated with injected amounts up to 400 ng, with $R^2$ values ranging between 0.9969 and 0.9999.

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