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Analytical Methods

Determination of tetracyclines in multi-specie animal tissues by pressurized liquid extraction and liquid chromatography–tandem mass spectrometry

Cristina Blasco [a], Antonio Di Corcia [b], Yolanda Picó [a,*]

[a] Laboratori de Bromatologia i Toxicologia, Facultat de Farmàcia, Universitat de València, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, València, Spain
[b] Dipartimento di Chimica, Università "La Sapienza", Piazza Aldo Moro 5, 00185 Roma, Italy

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ABSTRACT

A specific, sensitive and robust pressurized liquid extraction (PLE) and liquid chromatography tandem mass spectrometry (LC–MS/MS) method for determining tetracycline, chlortetracycline, oxytetracycline and doxycycline in bovine, swine, poultry and lamb muscle tissues is presented. PLE was performed using an ASE® 200 from Dionex and water as extractant, followed by solid-phase extraction (SPE) using an Oasis HLB cartridge. The method was validated for beef, chicken, pork and lamb meat in compliance with the requirements set by Commission Decision, 2002/657/EC [Commission Decision 2002/657/EC (2002). Implementing Council Directive 96/23/EC concerning the performance of analytical methods and interpretation of results. *Official Journal of European Communities, L239*, 66–98. (Available at: <http://europe.eu.int>)]. The average recoveries of the different meat samples, spiked with the four tetracyclines at three levels (1, 100 and 200 μg kg^{-1} of each tetracycline), were always higher than 89% with intraday and interday precision lower than 15% and 17%, respectively. A good linearity was established for the four tetracyclines in the range from 5 to 10,000 μg kg^{-1} with $r > 0.995$. The limits of quantification (LOQs) were between 0.5 and 1 μg kg^{-1}, which are well below the tolerance levels set by the European Union. The decision limit (CCα) and the decision capability (CCβ) were in the range 101–116 and 112–130 μg kg^{-1}, respectively. Compared with previous methods, sample preparation time required for the analysis and LOQs, are reduced. The method demonstrated its successful application for the analysis of 100 meat samples. Two samples of beef and one sample of chicken out of 25 of each type tested positive while none of 25 samples of either, lamb or pork, tested positive.

1. Introduction

Tetracyclines are applied in veterinary medicine for treatment and prevention of microbial infections. Most widely used compounds within this group are tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and doxycycline (DC). They have a broad antibacterial spectrum against both, gram-positive and gram-negative bacteria, including the species *Spirochete*, *Actinomyces*, *Ricketsia* and *Mycoplasma* (Michalova, Novotna, & Schlegelova, 2004; Samanidou, Nikolaidou, & Papadoyannis, 2007). The benefits of antimicrobial treatments are numerous from the food safety point of view. However, meat from medicated animals may contain tetracycline residues, which can cause allergic reactions in some hypersensitive individuals and, consumed for long time, can also lead to problems regarding the spread of drug-resistant microorganisms (Mcevoy, 2002). A maximum residue limit (MRL)/tolerance of 100 μg kg^{-1} in muscle of all food-producing species has been officially established by different organisms (EU, FAO/WHO, FDA, and Japanese Ministry of Health, Welfare, and Labor) to ensure the safety of food to the consumers (Commission Regulation 508/1999/EC, 1999; FAO/WHO Food Standards Codex Alimentarius, 2007; Japanese Ministry of Health Welfare and Labor, 2008; US Food and Drug Administration, 1975).

Literature searches and published reviews reveal that many screening tests are used to detect tetracycline residues in meat and other animal products, but a confirmation and an accurate quantification by chemical methods is required to eliminate the risk of false non-compliant results (Blasco, Torres, & Pico, 2007; Di Corcia & Nazzari, 2002; Gentili, Perret, & Marchese, 2005; Masawat & Slater, 2007; Samanidou et al., 2007; Stolker & Brinkman, 2005; Stolker, Zuidema, & Nielen, 2007). A number of liquid chromatography–mass spectrometry methods for separating and determining the tetracyclines, isolated from foods, has been developed (Andersen et al., 2005; Cherlet, De Backer, & Croubels, 2006; De Ruyck & De Ridder, 2007; Goto, Ito, Yamada, Matsumoto, & Oka, 2005; Pena, Lino, Alonso, & Barcelo, 2007). Triple quadrupole (QqQ) instruments are the most frequently applied as they are nowadays suitable for routine use in many laboratories (Blasco et al., 2007; Gentili et al., 2005; Lee, Chung, Chung, & Lee, 2007; Samanidou

* Corresponding author. Tel.: +34 963543092; fax: +34 963544954.
E-mail address: yolanda.pico@uv.es (Y. Picó).

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et al., 2007; Stolker et al., 2007). Rigorous sample extraction and clean-up are required to remove most of the matrix and are also important to enrich the analyte of interest to obtain maximum measurement sensitivity. The commonly utilized techniques for extraction and clean-up of antibiotics in various matrices are: liquid extraction and solid-phase extraction (SPE) (Blasco et al., 2007; Di Corcia & Nazzari, 2002; Fritz & Zuo, 2007; Gentili et al., 2005; Samanidou et al., 2007; Stolker & Brinkman, 2005; Stolker et al., 2007). Aqueous based extractions are primarily employed because these systems provide greater solubility than most organic ones (excluding alcohols) for tetracyclines. Different acidic solutions (commonly at pH 4) have been successfully applied to food based extraction (Andersen et al., 2005; Cherlet et al., 2006; Cinquina, Longo, Anastasi, Giannetti, & Cozzani, 2003; Goto et al., 2005; Ishii, Horie, Murayama, & Maitani, 2006; Kaale, Chambuso, & Kitwala, 2008a; Pena et al., 2007). Unfortunately, liquid extraction has evident disadvantages: multiple extractions are required, automatization is clearly an utopia and too much consumption of time, sample and reactives are involved. Because of these disadvantages several methods are undoubtedly emerging as new alternatives for determining antibacterial residues. Among these alternatives a method based on the matrix solid-phase dispersion technique with heated water as the extractant, using a homemade sub-critical water extraction system was proposed (Bogialli, Coradazzi, Di Corcia, Lagana, & Sergi, 2007a; Bogialli, Coradazzi, Di Corcia, Lagana, & Sergi, 2007b; Bogialli, Curini, Di Corcia, Lagana, & Rizzuti, 2006). Heated water appeared to be an excellent extractant for tetracyclines in meat, since the absolute recovery data ranged between 70% and 78% (Bogialli et al., 2006).

In this paper, the development, optimization and validation of a multi-residue LC–ESI–MS/MS method, capable of quantifying four tetracyclines used in veterinary medicine, as well as their 4-epimers, in complex animal tissue matrices is presented. This work is an extension of a previous one (Bogialli et al., 2006) that uses hot water as extractant and contributes to provide clear and new advantages over the preceding one, such as the use of a commercial and automated system and the incorporation of a SPE clean-up step that allows to improve even more the obtained LOQs. This improvement is important because in addition to check regulatory compliance with the MRL values, the TC concentrations can provide information on their occurrence in a list of food groups representative of the diet in a population. To enhance the precision and accuracy of the method, the internal validation used was compliant with Commission Decision 2002/657/EC requirements (Commission Decision 2002/657/EC, 2002). The performance of the proposed extraction protocol has been compared to those obtained by the homemade pressurized system (Bogialli et al., 2006) and by liquid extraction with EDTA-McIlvaine buffer acidified at pH 4.0, followed by SPE clean-up using a polymeric sorbent (Cinquina et al., 2003; Pena et al., 2007). Finally, the definitive procedure was implemented as screening and confirmatory test to determine tetracycline residues in meat by a survey carried out over 100 samples taken in several markets of the Valencia city.

2. Experimental

2.1. Materials and reagents

TC, OTC, CTC, DC and demeclocycline (DMC) were purchased from Sigma (St. Louis, MO). DMC was used as internal standard (IS) because it is an obsolete antibiotic. The three epimers, 4-epitetracycline (e-TC), 4-epioxytetracycline (e-OTC), and 4-epichlortetracycline (e-CTC), were from Acros (Fisher Scientific, Schwerte, Germany). HPLC-grade methanol was purchased from Merck (Darmstadt, Germany). Formic acid, citric acid monohydrate, disodium hydrogenphosphate dehydrate, ethylene diaminetetraacetic disodium salt (Na_2EDTA) and trichloroacetic acid were analytical grade (Aldrich, Madrid, Spain). Deionised water (<8 MΩ cm resistivity) was obtained from the Milli-Q SP Reagent Water system (Millipore, Bedford, MA, USA). All the solvents and solutions were filtered through a 0.45 μm cellulose filter from Scharlau (Barcelona, Spain) before use. Acidic, neutral and basic alumina (Al_2O_3) were obtained from Merck, silica gel form scharlau, Florisil® from Aldrich, sea sand from Panreac and anhydrous sodium sulfate (analytical grade) from Scharlau. To block metal impurities 60 g of solid sorbent was placed in a Buchner funnel and 120 mL of 0.1 M EDTA was passed through the sorbent using vacuum. Oasis® HLB extraction cartridges (6 cc, 200 mg) were from Waters (Milford, MA, USA).

2.2. Liquid chromatography–mass spectrometry

A Quattro LC triple quadrupole mass spectrometer from Micromass (Manchester, UK), equipped with an LC Alliance 2690 system (waters) consisted of an autosampler and a quaternary pump, a pneumatically assisted electrospray probe, a Z-spray interface and a Mass Lynx NT software Ver. 4.1 were used. Analysis was performed in positive ion mode. The ESI source values were capillary voltage, 3.00 kV; extractor, 2 V; RF lens, 0.5 V; source temperature, 120 °C; desolvation temperature, 300 °C, and desolvation and cone gas (nitrogen 99.99% purity) flows, 600 L h^{-1} and 60 L h^{-1}, respectively. The analyzer settings were resolution, 12.0 (unit resolution) for the first and third quadrupoles; ion energy, 2.0; entrance and exit energies, −1 and 1; multiplier, 650; collision gas (argon, 99.995%) pressure 2.79×10^{-3} mbar; interchannel delay, 0.02 s; total scan time, 1.0 s. The mass spectrometer was operated in scan and product ion scan modes to optimize the conditions and select the transitions, and in selected reaction monitoring (SRM) mode to confirm the identity of analytes in the samples by selecting two transitions for each one and to quantify. Table 1 shows the particular conditions and transitions for each analyte. The analytical column was a Xterra C_{18} (100 × 2.1 mm I.D., 3.5 μm) from Waters. The mobile phase consisted of methanol and water, both with 10 mM formic acid at 0.2 mL min^{-1} in gradient that begins with 10% methanol, increases linearly in 9 min to 50% of methanol, maintaining this proportion for 2 min, then increases linearly in 9 min to 70% of methanol and return to the initial conditions in 10 min. The injected volume was 20 μL.

2.3. Meat samples

Meat samples (a portion from the leg of the slaughtered animals) were purchased in local markets. One hundred meat samples of beef, chicken, pork and lamb (25 of each) were analyzed. Samples of ca. 200 g were place in plastic boxes and preserved at 4 °C until arrive to the laboratory. In order to ensure that the selected portions were representative of the whole sample, each of them was homogenized to a semisolid/semiliquid consistency using a Bapitaurus food processor (Taurus, Berlin, Germany). After homogenization, the sample was transferred in 1 g portions to small plastic bags, labeled, and restored in the −80 °C freezer. Approximately 3–4 h after storage, the sample was prepared (in duplicate) by the method described below and analyzed by LC/MS/MS.

2.4. Analytical procedures

2.4.1. Preparation of standard solutions

Individual stock standard solutions of the analytes and the IS were prepared by accurately weighting 50 mg of standard powder and dissolving it in a 50 mL volumetric flask with methanol to obtain a 1 mg mL^{-1} concentration. Working standard solutions and

Table 1
LC–MS/MS conditions for confirming and quantifying the selected tetracyclines in meat[a].

Compound	t_r (min)	Quantifier SRM (m/z)	CV[b] (V)	CE[c] (V)	Qualifier SMR (m/z)	CV[b] (V)	CE[c] (V)	Ion ratios[d]
TC	8.23	445 → 410	18	12	445 → 427	18	18	0.536 ± 0.003
e-TC	6.23	445 → 410	18	12	445 → 427	18	18	0.502 ± 0.008
CTC	15.97	479 → 444	20	15	479 → 462	20	18	1.425 ± 0.009
e-CTC	10.72	479 → 444	20	15	479 → 462	20	18	0.729 ± 0.007
OTC	8.66	461 → 443	18	10	461 → 426	18	18	0.358 ± 0.003
e-OTC	7.53	461 → 443	18	10	461 → 426	18	18	0.504 ± 0.004
DMC (IS)	13.12	465 → 448	22	15	465 → 430	22	18	0.407 ± 0.010
DC	18.01	445 → 428	20	18	445 → 321	20	30	0.060 ± 0.001

[a] The criteria for residue identification were (i) four identification points through the measurement of two product ions plus the precursor ion; (ii) retention time of suspected analyte and reference standard within the tolerance interval of ±2.5%, and (iii) the ion ratio for each analyte in samples matches that of the standards within the maximum permitted tolerances (±20% for TC, e-TC, CTC. e-CTC and e-OTC; ±25 for OTC, and ±50 for DC).
[b] CV = cone voltage.
[c] CE = collision energy.
[d] Ion ratios = the ratio of the intensities of the two most abundant transitions of each tetracycline determined from the analysis of standards prepared in methanol–water (50:50 v/v) (n = 15).

mixtures of the required concentrations were prepared mixing (if required) aliquots of the standard solutions and diluted them with the appropriate volume of methanol–water (50:50). All solutions were stored at 4 °C in the dark and renewed after 1 month of use.

2.4.2. Pressurized liquid extraction (PLE) using ASE200® followed solid-phase extraction (SPE) concentration

The extraction of antibacterial agents from meat was performed by PLE, using an ASE 200 system from Dionex (Sunnyvale, CA, USA). The samples accurately weighted were placed in a mortar, added of the IS and gently blended with 11 g of EDTA washed sand using a pestle. This mixture was packed into a 22 ml pressure resistant stain steel extraction cell of the ASE. Any void space remaining after packing the solid material was filled with EDTA-treated sand. Circular glass microfiber filters of 1.98 cm diameter (Dionex Co.) were placed above and below the packing. Conditions used in the extraction were as follows: time heating cell 5 min, time of solvent in contact with the sample 10 min (static time), pressure 1500 psi, temperature 70 °C, time purging with nitrogen to expulse rest of solvent in the cell 60 s, water volume flushing respect the cell size in percentage 60%, and one times cycled. At the end of each extraction a total extract volume of 40 mL was obtained.

The PLE extract was then applied to a SPE HLB Oasis cartridge, previously activated with 5 mL of methanol and 5 mL of water. After passing the extract, the cartridge was washed with 2 mL of methanol 5% in water and, finally, tetracyclines were eluted with 2 mL of methanol acidified with 10 mM of formic acid. The solvent was removed using a multi-sample Turbovap LV Evaporator (Zymark, Hoptkinton, MA, USA), and the residue was redissolved in 1 mL methanol–water (50:50).

2.4.3. Heated water extraction using a homemade device

After a pretreatment similar to that described in the previous Section 2.4.2, the sample was analyzed in a homemade apparatus, previously described (Bogialli et al., 2006, 2007b). The mixture was filled in 15 cm × 8.3 mm i.d. stainless column that provided a similar volume (20.2 mL) to that of the cells of the ASE (22 mL). The column was then put into the oven and heated at 70 °C for 5 min. Ten milliliters of water at a 1 mL min^{-1} flow rate was then passed through the cell to extract the analytes. The extract was filtered and directly injected in the LC.

2.4.4. Water extraction followed by SPE

The water extraction was based on a previously reported procedure (Cinquina et al., 2003). A portion of 1 g of muscle, placed in a glass centrifuge was added of the IS and 2 mL of 20% trichloroacetic acid. The sample was shaken, 20 mL of McIlvaine buffer (11.8 g of citric acid monohydrate; 13.72 g of disodium hydrogenphosphate dehydrate; 36.62 g of Na_2EDTA diluted in 1 L of water 0.01 M) was added and the mixture centrifuged at 4000 rpm for 20 min. The supernatant was then applied to a SPE HLB Oasis cartridge and the tetracyclines extracted as reported in the Section 2.4.2.

2.4.5. Validation

Linearity, CCα, CCβ, LOD, LOQ, recoveries, precision, selectivity and stability were assessed according to the EU regulation 2002/657/EC [10]. The linearity of the analytical methods was proved by assessing the linearity of the calibration curves and determining the working range of the calibration for each compound, using meat extract without tetracyclines (n = 7) spiked in the 5–1000 μg kg^{-1}. Each level was prepared in triplicate. Linear regression analysis was performed. The goodness of fit of a statistical model describes how well it fits a set of observations. The correlation coefficient (r) and the quality coefficient (QC) (Laborda, Medrano, & Castillo, 2004; Van Loco, Elskens, Croux, & Beernaert, 2002). QC is a dimensionless parameter analogous to the well known standard deviation. The acceptance criteria were the correlation coefficient $\geqslant$ 0.995 and the QC < 10%.

The LOD was calculated at a signal-to-noise ratio of 3, while the LOQ value was calculated by using a signal-to-noise ratio of 10. LODs were obtained using the transition with higher signal/noise in SRM mode and LOQs were obtained using the lower one. For the LOD, the confirmatory transition should be visible in the chromatogram. Once estimated, LODs were checked by injecting three different extracts of samples spiked at the estimated concentration and LOQs by calculating recovery and precision. CCα and CCβ were calculated according to the Commission Decision 2002/657/EC for the case of substances an with established permitted limit (Commission Decision 2002/657/EC, 2002). CCα was established by analysing at least 20 blank materials per matrix fortified with the analyte(s) at the permitted limit. The concentration at the permitted limit plus 1.64 times the corresponding standard deviation equal the decision limit (α = 5%). CCβ was established in the same way but analysing the matrix fortified with the analyte(s) at the CCα.

The recovery experiments were carried out at three concentration levels (LOQ, MRLs and 2 MRL), each one in quintuplicate. For recovery studies, 1 g portion of meat was placed in the appropriate container according to the further extraction method and spiked with the antibacterial standard solution, taken care to uniformly spread them on the sample. The spiked sample was left for 10 min at room temperature to ensure the appropriate distribution in the matrix. Then, a constat concentration of IS (20 μL of a 5 μg mL^{-1} solution in methanol). The spiked samples were injected

with a seven points calibration curve using solution standards and ranging from 5 to 1000 μg kg^{-1}. Precision (within- and between-day) was calculated from the analysis of 16 blank samples of each beef, chicken, pork or lamb fortified with all analytes at each of the three specified fortification levels. Within-laboratory precision was obtained by following the same protocol, but analyses were performed in three different days. The specificity was assessed by analysing blank tissue samples. The stability was checked preserving standard and spiked samples at 4 °C and −20 °C at different time periods.

3. Results and discussion

3.1. Optimization of LC–MS

Fig. 1 displays typical total ion current (TIC) and mass chromatograms obtained for a lamb sample spiked with the four tetracyclines, the three 4-epimers and the IS. The TIC chromatogram is a summation of the ion signal generated by all the precursor → product ions transition showed in Table 1. Tetracyclines have a strong tendency to bind irreversibly to the silanol groups

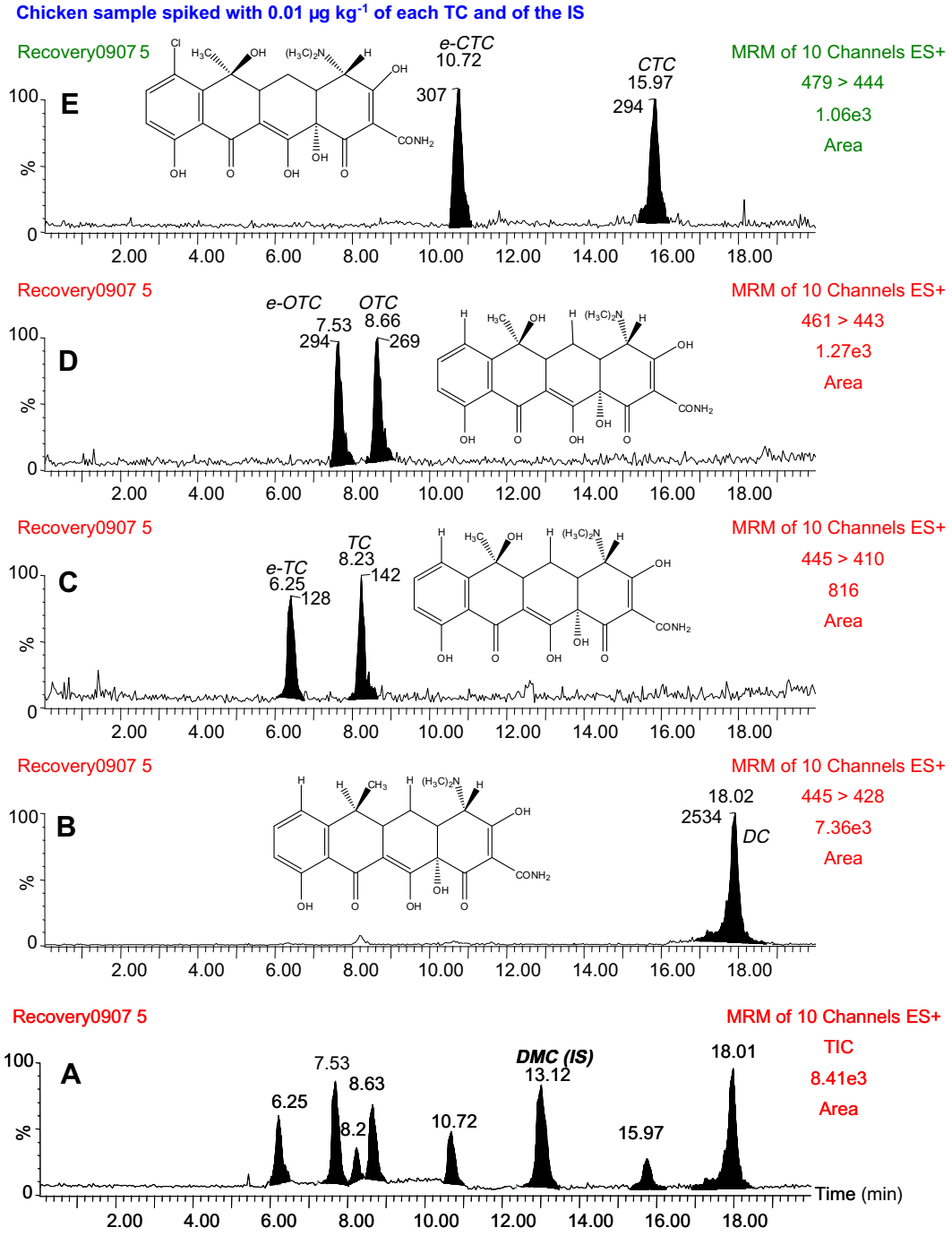

Fig. 1. LC–MS/MS chromatograms of a lamb sample spiked at 10 μg kg^{-1} of each tetracycline including the epimers and the internal standard (a tenth part of the tolerance level set by the European Union) (A) TIC of the ten precursor → product ion transitions reported in Table 1, (B) mass chromatogram for DC (445 → 428 transition), (C) mass chromatogram for TC and e-TC (445 → 410 transition), (D) mass chromatogram for OTC and e-OTC (461 → 443 transition) and (E) mass chromatogram for CTC and e-CTC (479 → 444 transition).

in silica based LC stationary phases, resulting in peak tailing (Kaale, Chambuso, & Kitwala, 2008b). The analytical column selected in this study is based on a fundamentally new organic/inorganic particle, which combines all the advantages of silica and polymeric chromatographic supports. This analytical column delivers sharp, symmetrical peaks for the tetracyclines and long column lifetimes using a mobile phase that has pH of 3.

Chemical transformation processes of tetracyclines, such as isomerization and epimerisation, have been reported giving rise to structurally related compounds. The EU authorities partially take into account these degradation products because the MRLs for tetracyclines, 100 μg kg^{-1}, are expressed as the sum of the parent drugs and their 4-epimers (Commission Regulation 508/1999/EC, 1999). For instance, CTC is converted to isochlortetracycline (iCTC) under alkaline conditions, while the epimerisation has been found to be catalyzed in acidic solutions in a pH range from 2 to 6 (Bogialli et al., 2006; Eichhorn & Aga, 2004). The possible interconversion between tetracyclines and their 4-epimeric forms during the extraction procedure was checked by spiking different meat samples with the tetracyclines or with the 4-epimers. No inter-conversion between the tetracyclines and its 4-epimers was observed (data not shown) However, the appearance of the small peaks of 4-epimers (6–7% of the parent compounds) when analysing muscle spiked with parent tetracyclines and extracted by PLE using water was reported in a previous study (Bogialli et al., 2006). Main difference observed between that study and this one is that the former redissolved the extract using acidified methanol, which can favor the formation of the 4-epimers. In the present study, the phenomenon of the inter-conversion between tetracyclines and their epimers was observed in both, standard and extract of the spiked samples, after preserving them more than 1 month in a freezer at −20 °C. In any case, the transformation of the tetracyclines in their 4-epimers is resolved because most regulations have defined tetracycline residues as the sum of the parent compound and the 4-epimers (Commission Regulation 508/1999/EC, 1999; FAO/WHO Food Standards Codex Alimentarius, 2007; Japanese Ministry of Health Welfare and Labor, 2008; US Food and Drug Administration, 1975).

Most problematic is the formation of other degradation analogues. In this sense, solutions, spiked extracts and/or extracts of CTC preserved for more than a month also displayed another peak, which also forms epi-analogues. This peak was also reported in a previous study (Bogialli et al., 2006) and was tentatively identified as iCTC based on the absence of the transition 679 → 444, which indicates the lack of hydroxyl group of the CTC. However, the iCTC formation is favoured under alkaline conditions, which are not used in the present study. Other studies explain the same phenomenon by the fact that CTC is subjected to the keto-enol tautomerisms resulting in its keto and enol forms (Cherlet et al., 2006). This isomerism also would explain the lack of hydroxyl group and the absence of the mentioned transition. The structural information obtained in this study was not conclusive on the identity of the degradation compound. These peak only appear in old solutions or extracts and was never detected in standard or extracts of spiked samples recently prepared.

3.2. Optimization of the extraction procedure

All parameters affecting the PLE extraction efficiency, such as temperature, pressure, treatment of sand, static time, cell size, number of extraction cycles and flush volume, were carefully evaluated by the absolute recovery obtained (without adding the IS). The optimum conditions were those reported in Section 2.4.2.

The sorbent used for the homogenization and dispersion of the meat and the optimum extractant were also optimized. Alumina (neutral and basic), Florisil®, silica and sea sand were tested. In addition, all these sorbents were tested both, previously washed with Na_2EDTA and without washing. The Na_2EDTA washed materials always provide better recoveries. An explanation is that Na_2EDTA deactivates metal impurities present in the sorbent surface and, probably, chelates also metals present in meat facilitating the decomplexation of tetracyclines bound to these metals. Silica and sea sand provided the better recoveries for all tetracyclines (up to 40%). Alumina, neutral or acid, does not recover these compounds and Florisil® provides good recoveries for oxytetracycline, chlortetracycline and their epimers but fails in recovering TC, e-TC and DC.

Water and methanol–water (50:50, v/v) were tested as extracts at different temperatures for both sorbents, silica and sea sand, to establish the better conditions. The best results were obtained using sea sand as dispersant ant the mixture of methanol water at 70 °C as extractant. However, the extraction with water, instead of with methanol–water, also provided very good recoveries (between a 4% and 10% lower than those obtained with the mixture of methanol–water). Water was finally selected because the coupling of the extraction with the clean-up by SPE is much easier using this extractant. With the mixture of methanol–water, the extract could not be directly applied to the SPE cartridge because the percentage of methanol in the extract solubilised the tetracyclines, which were only retained in the cartridge in a very small percentage. To clean-up these extract, an evaporation of the methanol present in the mobile phase was needed, involving another step that, if was not carefully performed, could constitute a source of losses of the analytes. Temperatures 110 °C provided the transformation of part of CTC and e-CTC to the iCTC or keto-CTC form. This can be used as an additional confirmation or as a way to quantify all the degradation products of CTC. However, it can be done only for CTC because the recovery and stability of the other TCs is poor.

3.3. Validation of the proposed method

The results of the linearity evaluation of the method for curves over three orders of magnitude, for standards prepared in methanol and in "blank" extracts of the different samples, are shown in Table 2. Every tetracycline showed good linearity with $r \geqslant 0.995$ and QC below 10%. Differences in the slopes of the calibration curves, prepared in methanol and in extracts of the different matrices, showed that matrix effect was negligible for all types samples, excepting lamb ones that showed a suppression effect in the response ca. 10% compared with that of the analytical standard.

Table 3 shows the LODs, LOQs, the MRLs, CCα and CCβ obtained for lamb spiked samples, which was the most problematic matrix as can be deduced from the previous results. The LODs and LOQs of the analytes of the method were estimated from a SRM mass chromatograms resulting from the analysis of lamb samples, spiked with the analytes at the 1 μg kg^{-1} level and confirmed by samples spiked at the appropriate concentrations (Table 3). The statistical risk of making a wrong decision is expressed by the CCα values with an error of 5% (probability of false non-compliance $\leqslant$5%) and the CCβ values with an error β = 5% (probability of falsely compliant samples $\leqslant$5%). These parameters were established for each tetracycline using both the quantifier and qualifier transitions. The values obtained are very similar. Table 3 also summarizes the good accuracy of the method for lamb samples. Repeatability and within-laboratory reproducibility of the method were lower than 15% and 17%, respectively. Recoveries varied between 89% and 99% with RSDs not higher than 18%.

Stability of standards and extracts was tested with a standard of 10 μg kg^{-1} prepared in methanol–water (50:50 v/v), and extracts of samples spiked at 10 μg kg^{-1}. After the preparation, they were stored at 4 and at −20 °C and measured immediately, 24 h, 48 h, one week and two weeks later. The results obtained point out that

Table 2
Results of linearity evaluation.

Compound	Concentration	Standard in	Slope	Intercept	r^a	QC[b]
TC	5–10,000	Methanol	28.83	−6.96	0.999	4.2
		Beef	28.19	−6.54	0.997	6.8
		Pork	27.25	−7.96	0.996	5.4
		Chicken	27.98	−7.66	0.996	8.1
		Lamb	22.35	−7.59	0.995	4.9
e-TC	5–10,000	Methanol	25.95	−10.22	0.998	4.6
		Beef	25.14	−10.41	0.997	5.9
		Pork	24.98	−10.05	0.996	5.3
		Chicken	25.73	−10.38	0.998	5.4
		Lamb	22.44	−6.88	0.996	8.8
CTC	5–10,000	Methanol	60.51	−14.49	0.999	4.9
		Beef	55.96	−13.66	0.998	5.6
		Pork	55.44	−12.35	0.998	3.5
		Chicken	56.54	−13.40	0.998	3.8
		Lamb	48.32	−8.63	0.997	7.8
e-CTC	5–10,000	Methanol	45.39	−12.13	0.998	4.4
		Beef	44.76	−11.54	0.997	4.8
		Pork	44.89	−10.22	0.997	4.6
		Chicken	45.01	−11.48	0.996	5.3
		Lamb	30.74	−8.25	0.996	6.9
OTC	5–10,000	Methanol	39.72	−11.30	0.998	6.3
		Beef	36.33	−10.19	0.996	5.2
		Pork	36.64	−10.36	0.996	6.4
		Chicken	37.98	−10.99	0.996	6.2
		Lamb	32.92	−7.85	0.996	7.0
e-OTC	5–10,000	Methanol	30.20	−6.22	0.999	4.9
		Beef	32.33	−6.30	0.997	4.2
		Pork	31.09	−610	0.998	3.7
		Chicken	30.99	−6.46	0.996	4.0
		Lamb	25.26	−5.26	0.997	8.2
DC	5–10,000	Methanol	321.64	−73.52	0.999	5.2
		Beef	310.23	−75.08	0.998	3.8
		Pork	312.23	−72.06	0.998	4.9
		Chicken	307.68	−73.54	0.998	5.0
		Lamb	272.68	−52.92	0.996	7.7

[a] r = Correlation coefficient (+criterion $\geqslant 0.995$).
[b] QC = The coefficient is based on the average relative deviation of the data points from the fitted equation: QC = 100 $x = \sqrt{\sum \frac{((y_i - y_i^2)/y_i)}{n-2}}$.

both, the standard and the extracts, are stable at least fifteen days stored at 4 °C and a month stored at −20 °C. Standards or extracts stored for longer periods showed the degradation phenomena described in the Section 3.1.

3.4. Comparison among different extraction procedure

The method was compared with other extraction procedures that present some common steps. One of the methods applies a similar hot water extraction but using a homemade pressurized liquid extractor and the other applies the clean-up step using HLB Oasis® cartridges but after conventional extraction with EDTA-McIlvaine buffer. Table 4 summarizes several parameters indicative of the analytical performance of the three methodologies. Recoveries, precision and linearity were satisfactory for the three methods. However, the proposed method is faster (only 45 min/per sample are required, including the chromatographic run), simpler (the ASE extracts automatically up to 26 samples) and more sensitive (more than five times) than the other two methods.

Although the use of hot water extraction without clean-up is more ecological and economical, since it avoid the use of polymeric cartridges or organic solvents, the method proposed here presents unquestionable advantages other than the improved sensitivity. The most important one is that the ASE system is much more robust and reliable than the homemade one. The latter periodically suffers of clogging of the extraction cell frits and connections. To avoid any clogging of the conductions, a very careful homogenization step with the EDTA-treated sand is required, which commonly takes much more time than the 10 min initially postulated by the authors. With some sample that contains small fat portion, homogenization can take more than half hour. Another disadvantage of the homemade system is that samples should be manually processed one after other, requiring longer time and a dedicated and qualified operator. The commercial system allows to process 24 samples automatically and it is much less sensitive to clogging, making effective the homogenization step of 10 min.

3.5. Monitoring residues in meat from the market

A total of 100 samples purchased from the local markets in Valencia (Spain) were analyzed, 25 of each (beef, pork, chicken and lamb). Three samples, two of beef and one of chicken, contained tetracycline residues. OTC and CTC were the tetracyclines involved in the incurred samples. OTC was found in one beef sample and in the chicken one at 160 μg kg^{-1} and 24 μg kg^{-1} with

Table 3
Limits of detection (LODs), limits of quantification (LOQs), detection capabilities (CCβs), decision limit (CCα), accuracy and precision obtained for spiked lamb samples.

Tetracycline	LODs (μg kg^{-1})	LOQs (μg kg^{-1})	CCβ (μg kg^{-1})	CCα (μg kg^{-1})	Concentration (μg kg^{-1})	Recovery x ± RSD, %	Repeatability RSD, %	Reproducibility RSD, %
TC	0.3[a]	0.5[a]	112[a]	105[a]	1	90 ± 18	14.3	16.5
		1.0[b]	114[b]	101[b]	100	90 ± 10	6.7	7.9
					200	91 ± 5	3.8	5.5
e-TC	0.2[a]	0.5[a]	115[a]	106[a]	1	90 ± 16	10.0	11.3
		1.0[b]	108[b]	109[b]	100	90 ± 12	11.2	13.5
					200	93 ± 8	7.4	10.2
CTC	0.3[a]	1.0[a]	125[a]	112[a]	1	89 ± 13	9.0	10.8
		0.5[b]	130[b]	115[b]	100	93 ± 9	7.6	8.7
					200	89 ± 11	6.5	7.5
e-CTC	0.3[a]	0.5[a]	120[a]	109[a]	1	92 ± 17	12.5	14.0
		1.0[b]	122[b]	103[b]	100	90 ± 14	10.8	11.4
					200	96 ± 10	8.8	7.6
OTC	0.1[a]	0.3[a]	122[a]	111[a]	1	92 ± 16	11.2	15.6
		0.8[b]	117[b]	116[b]	100	93 ± 14	8.2	13.6
					200	93 ± 11	6.5	10.2
e-OTC	0.1[a]	0.3[a]	119[a]	109[a]	1	94 ± 15	9.8	15.0
		0.6[b]	115[b]	101[b]	100	99 ± 10	8.6	13.4
					200	97 ± 7	4.5	9.9
DC	0.3	0.3[a]	114[a]	105[a]	1	95 ± 13	12.9	14.2
		1.0[b]	118[b]	110[b]	100	98 ± 11	9.6	12.6
					200	98 ± 6	7.6	10.2

[a] Calculated using the Quantifier SRM transition (see Table 1).
[b] Calculated using the Qualifier SRM transition (see Table 1).

Table 4
Methods' performance comparison.

Parameter	Proposed method	Hot water extraction	Conventional extraction
Spiking concentrations (μg kg^{-1})	1–200	10–100	50–100
Accuracy (% recovery)	>89	>88	80–84
Repeatability (RSD, %)	12–18	11–19	13–18
Linearity (r)	>0.995	>0.996	>0.998
Sensitivity (LOQ, μg kg^{-1})	⩽1	⩽10	⩽20
CCα (μg kg^{-1})	106–112	104–118	107–125
CCβ 8 μg kg^{-1})	113–125	112–124	108–130
Organic solvent consumption (mL sample^{-1})	7	–	9
SPE consumption (units sample^{-1})	1	–	1
Time required to process the sample (min)	<45	<120	<90 min
Characteristics	Fast, simple, sensitive	Ecological, simple cell frits and connections' clogging	Fast, simple less sensitive

RSDs (%) (n = 5) of 6% and 16%, respectively. The confirmatory ion ratios of OTC in incurred samples were <10% of the ion ratios determined in the analysis of standards (see Table 1). In addition, the t_r of the OTC in incurred samples are identical, within the instrumental variation. The corresponding LC–MS/MS chromatograms of the sample containing the lowest level are shown in Fig. 2A. It should be noted the nonappearance of e-OTC in the chromatogram. In this study, OTC was the most frequent identified tetracycline and one of the two contaminated samples contains a residue concentration higher than the MRL (100 μg kg^{-1}).

CTC was found in one beef sample at 6 μg kg^{-1} with RSDs (%) (n = 5) of 12% (corresponding to 2.6 μg kg^{-1} of e-CTC and 3.4 μg kg^{-1} of CTC). In contrast, to the OTC, the peak corresponding to e-CTC clearly show-up in the chromatogram. The confirmatory ion ratios and the t_r were also in agreement with those of the analytical standards. However, few observations should be made about this sample. The small peaks displayed by CTC at 9.42 min and by its epimer at 8.58, in the long term preserved standards and in the extracts of spiked samples, were predominant in this incurred sample. The most likely reason for this discrepancy was the higher rate of analyte degradation to iCTC or the different keto-enol equilibrium for incurred samples. The corresponding LC–MS/MS chromatograms are shown in Fig. 2B. This can be an embarrassing problem in the quantification of the CTC content in the sample,

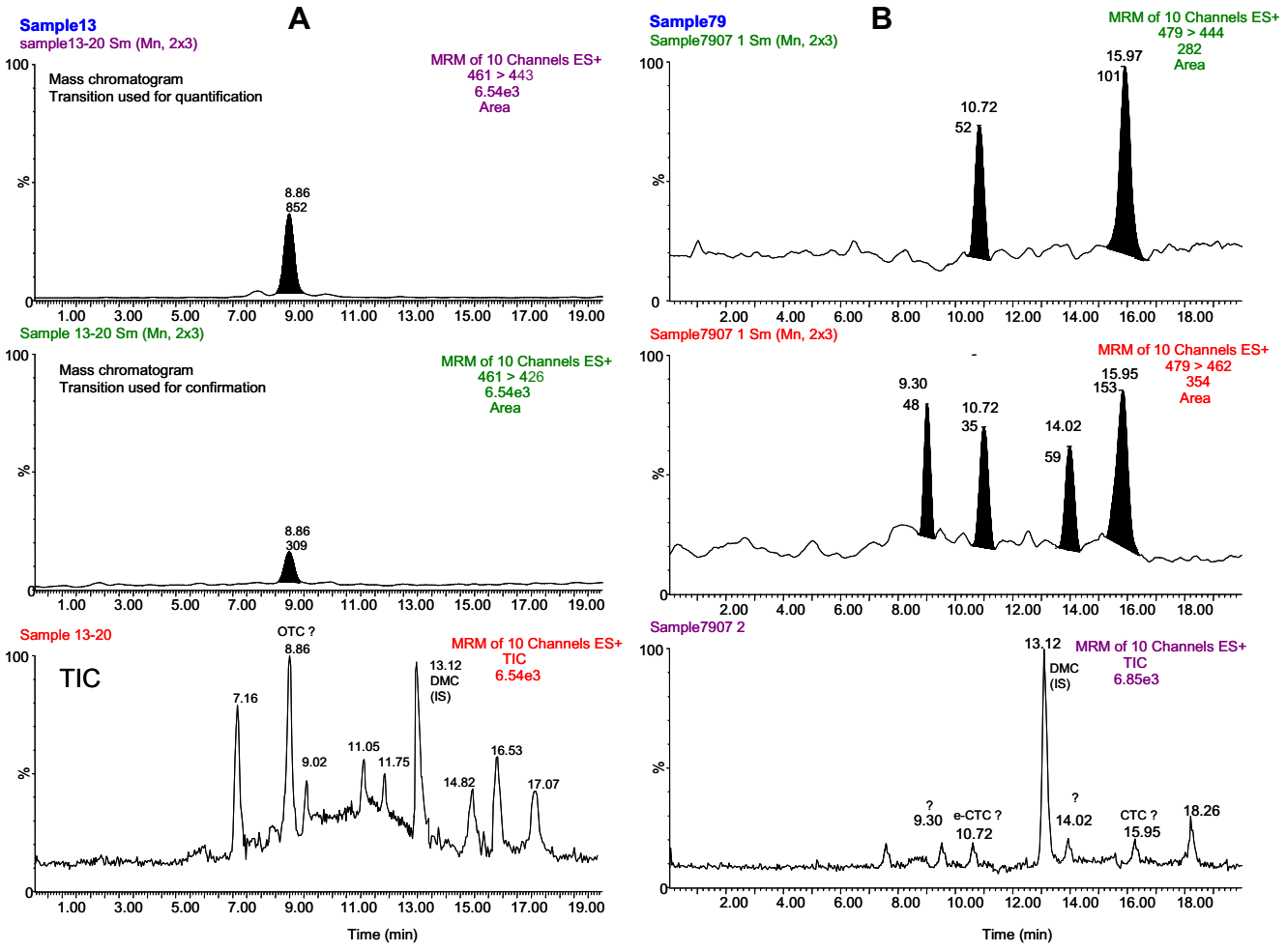

Fig. 2. LC–MS/MS chromatograms of (A) extract of the chicken sample in which oxytetracycline was found at 24 μg kg^{-1} and (B) extract of the beef sample that contains chlortetracycline at 6 μg kg^{-1}.

since there is not standard available. The extraction of this sample and an standard at 110 °C indicated an amount 15 μg kg^{-1} of CTC in its different forms or degradation products, which indicates that can be some underestimation of its concentration. In addition, this study can not contribute enough information to decide whether these peaks correspond to the keto-forms or to the iCTC and e-iCTC. The transition 479 → 444 achieves an accurate quantification of both species, CTC and e-CTC, without any interference of the degradation compounds.

4. Conclusion

The developed method, based on PLE extraction with hot water using an ASE® device, SPE clean-up with Oasis HLB® copolymer and LC–MS/MS determination, is suitable for the accurate determination of tetracycline residues in beef, chicken, pork and lamb samples. The procedure was validated in accordance with European Union regulation 2002/657/EC, proving unmistakably an appropriate compromise between the requirements of this regulation and the resources of an official laboratory with a high sample throughput. While accuracy and precision obtained by our method and by other extraction procedures was comparable, the proposed method was much simpler, faster and more sensitive. Quantification at 1 μg kg^{-1} for the different classes of meat samples by LC–MS/MS was readily achieved. Confirmation of the presence of one particular tetracycline in meat can be accomplished in <1 h upon sample receipt and extraction for multiple samples can be performed simultaneously. Summarizing, the developed method can be advantageously applied as routine procedure to identify and quantify tetracyclines in laboratories of food quality and safety control because its robustness and feasibility.

The effectiveness of this method has, actually, been further supported through a survey to determine tetracycline residues in meat samples taken randomly from local supermarkets. Data obtained in real samples indicate the importance of analytical control methods for food of animal origin and the need of further studies of contaminant sources in relation to food safety. The detection of tetracyclines in three samples, one of them with a violative level of OTC, confirms the usage of these antibacterials and established the need to implement monitoring programs.

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