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Preparation of Antibodies and Development of an Enzyme-Linked Immunosorbent Assay (ELISA) for the Determination of Doxycycline Antibiotic in Milk Samples

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ABSTRACT: This paper reports the development of an immunoassay for the specific analysis of doxycycline (DC), a congener of the tetracycline antibiotic family (TCs), in milk samples. This is the first time that DC antibody production is reported, based on a rationally designed and well-characterized immunizing hapten. The chemical structure of the immunizing hapten (13-[(2-carboxyethyl)thiol]-5-hydroxy-6-α-deoxytetracycline, TC1) was designed to maximize recognition of the tetracycline characteristic moiety defined as lower periphery of the TCs plus the region of the upper periphery composed by the hydroxyl group at position C_5 (B ring) and the dimethylamino group in ring A. Polyclonal antibodies raised against TC1 coupled to horseshoe crab hemocianyn (HCH) were used to develop a homologous indirect competitive enzyme-linked immunosorbent assay (ELISA). The microplate ELISA can detect DC in buffer down to 0.1 μg L^{-1}. The ELISA has been proven to tolerate a wide range of ionic strengths and pH values. The assay is very selective for DC with a minor recognition of methacycline (32% of cross-reactivity). Experiments performed with whole milk samples demonstrate that samples can be directly analyzed after a simple treatment method, reaching detectability values below 5 μg L^{-1}.

KEYWORDS: *doxycycline, immunoassay, ELISA, milk, tetracyclines*

INTRODUCTION

Tetracycline antibiotics (TCs) are nowadays extremely popular in human and veterinary medicine for the treatment of gastrointestinal, respiratory, and urinary, among others, infections.[1] This antibiotic family has a broad range of activity against a variety of Gram(+) and Gram(−) bacteria by oral administration via water and feed.[2] Only a small proportion of the TC dose administered is actually metabolized or absorbed in the body, a significant part being eliminated in feces and urine unaltered,[3] which has an important impact on the ecosystem, water, and soil-dwelling organisms. TCs are not usually found at high levels in the environment due to their chelating properties; they readily precipitate in the presence of divalent cations (e.g., Ca^{2+}, Mg^{2+}, or Zn^{2+}) accumulating in sewage, sludge, or sediments. On the other hand, TC residues have also been detected in many surface water resources that receive discharges from municipal wastewater treatment plants and agricultural runoff.[4] Besides the demonstrated persistence of these antibiotics in agricultural soils that have received manure containing antibiotics, the biodegradation may lead to even more toxic substances. Therefore, new strategies for improving tetracycline residue control and the efficiency of their removal in wastewater plants are necessary. Additionally, their widespread use together with the irresponsible and inappropriate administration of these antibiotics (and others) in animal husbandry, either for the prevention and treatment of diseases or as feed additives to promote growth,[5] favors the increase of bacterial resistance,[6] causing an important risk for human health due to diseases that can no longer be treated with the presently known antibiotics.[7] Moreover, consumption of food contaminated with antibiotic residues may cause allergic reactions and interferences with the intestinal flora while contributing to the appearance of resistant populations of bacteria. Furthermore, antibiotic residues cause important technological problems and economic losses in the diary industry related to the inhibition of the bacterial fermentation processes involved in the elaboration of cheese and cultured milk products.

There is also a social concern about all of these situations that is making consumers more exigent in terms of getting more natural and high-quality food products. As a result, recent trends in global food production, processing, and distribution are creating an increasing demand for food safety research to ensure a safer global food supply. An international approach to the management of antimicrobial resistance is essential for its surveillance, ensuring the safety of food for consumers. In Europe, the use of veterinary drugs is regulated through European Union (EU) Council Regulation 2377/90/EC and its annexes,[8] which describe the procedures for establishing maximum residue limits (MRLs)[9] for veterinary medicinal products in foodstuffs of animal origin (e.g., milk, eggs, or meat). In particular, Europe has established MRLs for tetracycline (TC), chlortetracycline (CTC), and oxytetracycline (OTC) compounds at 100 μg L^{-1} in milk samples. On the contrary, nowadays no MRL value has been settled for doxycycline (DC) or methacycline (MC) in these kinds of samples because they should not be used in farm animals for

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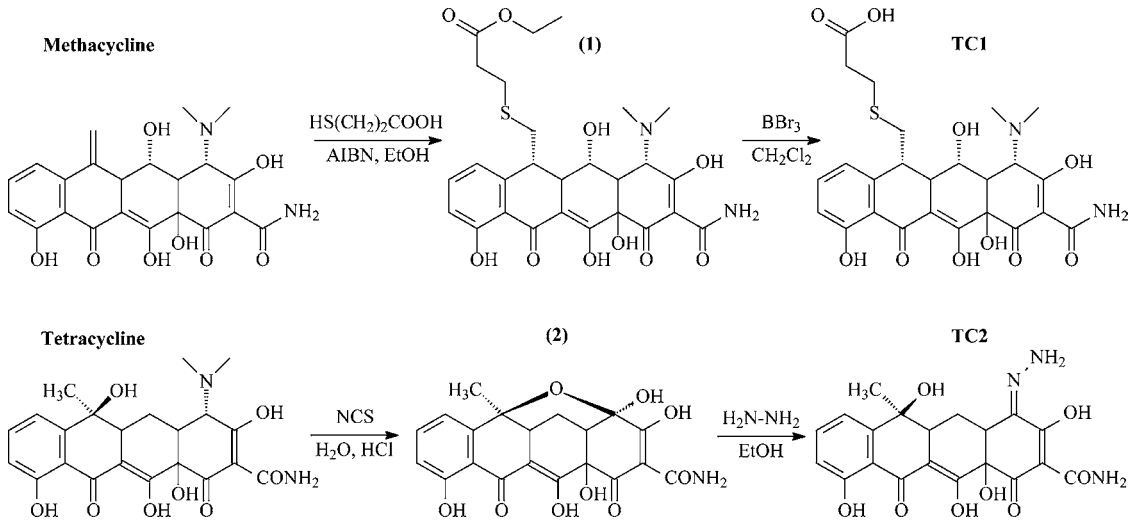

Figure 1. Synthetic pathways used to prepare TC1 and TC2 haptens.

milk production.[9] Thus, according to the Veterinary Medicine Evaluation Unit of the European Agency for the Evaluation of Medicinal Products, DC is indicated in cattle, pigs, poultry, turkeys, dogs, and cats for the treatment of infections, but not for use on lactating cattle and layers.[10] On the other hand, the U.S. Food and Drug Administration has restricted the use of DC for just pet dogs, and no use is permitted in pregnant, nursing, or growing animals.

Despite this, there exists a risk for illegal or inappropriate use of DC, which claims for the availability of analytical procedures to determine these residues in several tissues. Chromatographic techniques such as HPLC-UV[11] and/or HPLC-MS[12] provide high specificity and an excellent detectability but are not suitable for rapid residue analysis or screening of many samples. They usually require extensive sample preparation, sophisticated equipment, and skilled laboratory personnel. Alternatively, receptor-based assays such as dipsticks (i.e., lateral flow chromatography devices) are easy to use, being nowadays the most traditional on-site screening tests for TCs.[13,14] Current commercially available TC dipsticks are based on the use of DNA-regulatory proteins as receptors and show a quite broad recognition character for tetracycline congeners, including doxycycline. On the other hand, immunochemical analytical methods can also provide the necessary selectivity and detectability while offering the possibility to develop a wide variety of reliable, low-cost, and easy-to-use analytical configurations.[15] A few immunoassays for tetracyclines have been reported during recent years used to analyze preferentially honey[16,17] and milk[18,19] but also liver and muscle samples.[20] In particular, some of these immunoassays have proven to be suitable for the determination of single tetracycline congeners in milk samples. Thus, this paper presents the work performed addressed to produce polyclonal antibodies and the necessary immunoreagents to establish an immunochemical method for the specific analysis of DC residues. This is the first time that DC antibody production based on a rationally designed and well-characterized immunizing hapten is reported. Performance of the developed microplate-based ELISA has been demonstrated with milk samples.

MATERIALS AND METHODS

Chemistry. *General Methods and Instruments.* Thin-layer chromatography (TLC) was performed on 0.25 mm, precoated silica gel 60 F254 aluminum sheets (Merck, Darmstadt, Germany) predeveloped with saturated aqueous ethylenediaminetetraacetic acid solution (Na_2EDTA) followed by activation for 2 h at 130 °C. Unless otherwise indicated, purification of the reaction mixtures was accomplished by "flash" chromatography using silica gel as the stationary phase, being previously impregnated with saturated aqueous Na_2EDTA solution by dipping and further activated for 2 h at 130 °C. ^1H and ^{13}C NMR spectra were obtained with a Varian Inova-500 (Varian Inc., Palo Alto, CA) spectrometer (500 MHz ^1H and 125 MHz for ^{13}C). HPLC analyses were performed with a Merck Hitachi pump L-7100, a diode array detector L-7455, an autosampler L-7200, and an interface D7000 (Merck). The chromatograms were processed with HSM software (Merck). The column used was a C18 Purosphere column 15 cm × 4.6 mm, 5 μm, particle size (Merck), and the analyses were performed in isocratic mode using 0.01 M oxalic acid/acetonitrile (ACN)/MeOH 70:20:10 as mobile phase at a flow rate of 1.0 mL min^{-1}. The detection wavelengths to monitor the reactions were set at 250 and 365 nm. Liquid chromatography/electrospray ionization/mass spectrometry (LC/ESI/MS) was performed in a Waters (Milford, MA, USA) model composed by an Acquity UPLC system directly interfaced to a Micromass LCT Premier XE MS system equipped with an ESI LockSpray source for monitoring positive ions. Data were processed with MassLynx (V 4.1) software (Waters).

Molecular Modeling and Theoretical Calculations. Computational methodology was carried out by running Hyperchem 6.03 software package (Hypercube Inc., Gainsville, FL, USA) to compare physical−chemical features of the haptens with TC and DC. Minimum energy conformation, geometry, and molecular charge distribution were calculated using the semiempirical quantum model (PM3). Geometry was calculated for each acid−base form that could coexist at neutral (or close) pH. Conformational search was performed using a Monte Carlo method exploring all of the dihedral angles for the ring system and for the main substituents. Deprotonation enthalpies were calculated as a measure of acidity for comparison between haptens and analytes. The calculation results were also compared with the available bibliography data on the structures of tetracyclines and the immunoassay results.

Preparation of Tetracycline Haptens. Immunizing and competitor haptens TC1 and TC2 were synthesized following the scheme shown in Figure 1. Experimental details on the synthetic procedures and purification processes can be found below. Spectroscopic and spectrometric characterizations of the haptens are also provided.

Synthesis of Hapten TC1. (a) Ethyl 13-[(thio)propanoate]-5-hydroxy-6-α-deoxytetracycline (1). 3-Mercaptopropionic acid

(2.4 mL, 27.59 mmol) and azobis(isobutyronitrile) (AIBN, 25 mg) were added to a suspension of MC hydrochloride (0.5 g, 1.04 mmol) in EtOH (10 mL) placed in a round-bottom flask. The reaction mixture was kept on reflux with stirring for 12 h under N_2, until the complete disappearance of the starting material by TLC (9:1 CH_2Cl_2/MeOH as mobile phase) and HPLC-UV analysis and subsequently suspended in cold Et_2O (100 mL). The precipitate obtained was centrifuged to facilitate removal of 3-mercaptopropionic acid, and the solid collected was dissolved in water (3 mL) with the pH adjusted to 4.5. The aqueous solution was washed with $CHCl_3$ (5 mL) to remove the remaining mercaptan and finally extracted with CH_2Cl_2 (50 mL × 3). The organic layer was evaporated to dryness under vacuum to obtain a brownish-green solid (184.4 mg, 30.5%): ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.17 (3H, t, $-CH_2\mathbf{CH_3}$), 2.31 (2H, d, C_6-CH_2-S-), 2.43 (6H, s, $-N(CH_3)_2$), 2.55 (2H, m, $-CH_2-COOEt$), 2.69 (2H, m, $-S-\mathbf{CH_2}-CH_2-$), 3.04 (1H, m, $C_{4a}H$), 3.08−3.51 (3H, m (under water signal), C_5H, $C_{5a}H$, C_6H), 3.75 (1H, m, C_4H), 4.05 (2H, q, $-\mathbf{CH_2}CH_3$), 6.80 (1H, d, C_9H), 6.97 (1H, d, C_7H), 7.48 (1H, t, C_8H).

(b) 13-[(2-Carboxyethyl)thio]-5-hydroxy-6-α-deoxytetracycline (TC1). A solution of substrate **1** (308 mg, 0.54 mmol) in CH_2Cl_2 (15 mL) was cooled to −10 °C and then added dropwise to a stirred solution of BBr_3 (1 M in hexane, 3 mL, 3 mmol) followed by CH_2Cl_2 (15 mL). The reaction mixture was kept at −10 °C for 1 h under N_2 atmosphere and then at 25 °C until the complete disappearance of the starting material by TLC analysis (9:1 CH_2Cl_2/MeOH as mobile phase). The reaction was terminated by careful dropwise addition of water (30 mL), the organic phase was separated and washed with H_2O (3 × 20 mL), and the combined aqueous layers were evaporated to dryness. The residue was taken up in H_2O and chromatographed on Macro Prep, High Q Supgrat (ion-exchange resin) using a 5% acetic acid solution as mobile phase. Concentration in vacuum of the positive fractions led to the product as a brownish-yellow solid (214.1 mg, 73.0%): ^{1}H NMR (500 MHz, CD_3OD) δ 2.20 (2H, d, C_6-CH_2-S-), 2.51 (6H, s, $-N(CH_3)_2$), 2.63 (2H, m, $-CH_2-COOEt$), 2.69 (2H, m, $-S-\mathbf{CH_2}-CH_2-$), 3.13 (1H, m, $C_{4a}H$), 3.20−3.48 (3H, m (under water signal), C_5H, $C_{5a}H$, C_6H), 3.99 (1H, m, C_4H), 6.66 (1H, d, C_9H), 6.84 (1H, d, C_7H), 7.47 (1H, t, C_8H); HRMS (+EI) calcd for $C_{25}H_{29}N_2O_{10}S$ (M+) 549.1543, found 549.1523.

*Synthesis of Hapten TC2. (a) 4-Oxo-4-dedimethylaminotetracycline-4,6-hemiketal (**2**).* Powdered *N*-chlorosuccinimide (3.7 g, 26.2 mmol) was added to a solution of tetracycline hydrochloride (5 g, 10,4 mmol) in water (500 mL) containing concentrated HCl (1 mL) under vigorous stirring and at room temperature. The crude product that began to precipitate after a few minutes was recovered by filtration after 30 min of reaction and washed with water. Partial purification of the crude air-dried product (3.1 g) was achieved by adding water (75 mL) and Et_2O (200 mL) to the solid. The organic layer was washed with water (5 × 20 mL) and evaporated to dryness on a rotary vacuum evaporator. The residue was treated with water, and the product recovered by filtration was air-dried to obtain **2** partially purified (2.8 g, 65% yield) suitable for further transformations: ^{1}H NMR (500 MHz, DMSO-$d3_6$) δ 1.65 (s, 3H, CH_3), 2.29−2.34 (m, 1H, C_5H_β), 2.38−2.44 (m, 1H, C_5H_α), 2.80 (dd, 1H, C_4H), 2.92 (m, 1H, $C_{5a}H$), 6.96 (1H, d, C_9H), 7.19 (1H, d, C_7H), 7.66 (1H, t, C_8H).

(b) 4-Hydrazono-4-dedimethylaminotetracycline (TC2). A solution of **2** (2.7 g, 6.5 mmol) in 95% ethanol (19 mL) was added dropwise to a solution of hydrazine hydrate (0.4 mL, 12.6 mmol) in the same solvent (19 mL), and a precipitate began to appear immediately. The mixture was stirred for 1 h more and then left to stand overnight. The day after, the solid product was recovered by low-speed centrifugation and the crude was purified by a Na_2EDTA-impregnated silica gel column (after activation) with 9:1 CH_2Cl_2/MeOH as mobile phase to obtain the desired product (397.4 mg, 14% yield): ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.46 (s, 3H, $-CH_3$), 1.48 − 1.57 (m, 1H, $C_5H_ß$), 1.84−1.89 (m, 1H, C_5H_α), 2.96 (dd, 1H, $C_{4a}H$), 3.21−3.24 (m, 1H, $C_{5a}H$), 6.91 (1H, d, C_9H), 7.09 (1H, d, C_7H), 7.53 (1H, t, C_8H); HRMS (+EI) calcd for $C_{20}H_{20}N_3O_8$ (M+) 430.1250, found 430.1239.

Immunochemistry. *General Methods and Instruments.* The matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF-MS) used for analyzing the protein conjugates was a Perspective BioSpectrometry Workstation provided with Voyager-DE-RP software (version 4.03) developed by Perspective Biosystems Inc. (Framingham, MA, USA) and Grams/386 (for Microsoft Windows, version 3.04, level III) developed by Galactic Industries Corp. (Salem, NH, USA). The pH and the conductivity of all buffers and solutions were measured with a pH-meter pH 540 GLP and a conductimeter LF 340, respectively (WTW, Weilheim, Germany). Polystyrene microtiter plates were purchased from Nunc (Maxisorp, Roskilde, Denmark). Washing steps were performed on a SLY96 PW microplate washer (SLT Labinstruments GmbH, Salzburg, Austria). A Heidolph Titramax 1000 vibrating platform shaker (Brinkmann Instruments, Westbury, NY, USA) was used to shake the microplates at 900 rpm. Absorbances were read on a SpectramaxPlus (Molecular Devices, Sunnyvale, CA, USA). The competitive curves were analyzed with a four-parameter logistic equation using the software SoftmaxPro v4.7 (Molecular Devices) and GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA, USA). The chemical reagents used in the synthesis were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The preparation of the immunoreagents used is described below. Tetracyclines used for cross-reactivity studies were kindly supplied by Unisensor S.A. (Liege, Belgium).

Buffers. Unless otherwise indicated, phosphate-buffered saline (PBS) is 0.025 M phosphate buffer in a 2% saline solution (343 mmol L^{-1} NaCl, 6.8 mmol L^{-1} KCl) containing calcium divalent cation (1 mmol L^{-1} $CaCl_2$), and the pH is 5.5. PBST is PBS with 0.001% Tween 20. Borate buffer is 0.25 M boric acid−sodium borate, pH 8.7. Coating buffer is 0.05 M carbonate−bicarbonate buffer, pH 9.6. Citrate buffer is a 0.04 M solution of sodium citrate, pH 5.5. The substrate solution contains 0.01% 3,3′,5,5′-tetramethylbenzidine (TMB) and 0.004% H_2O_2 in citrate buffer. The McIlvaine buffer contains citric acid (0.1 M) and Na_2HPO_4 (0.2 M), pH 3.

Immunoreagents. (a) Immunogens. (1) TC1−HCH. Hapten TC1 was coupled to HCH following the mixed anhydride (MA) method as previously described.[21] Briefly, the carboxylic acid of TC1 (10 μmol) was activated with isobutyl chloroformate (14 μmol) in the presence of tributhylamine (12 μmol) in anhydrous dimethylformamide (DMF, 200 μL) and added to a solution of the protein (10 mg) in borate buffer (1.8 mL). The mixture was stirred for 3 h at room temperature overnight at 4 °C.

(2) TC2−HCH. Hapten TC2 (10 μmol in 100 μL of PBS) was added to a solution of HCH (10 mg in 0.9 mL of PBS) followed by a solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 50 μmol in 100 μL of PBS), and the mixture was reacted for 3 h at room temperature.

(b) Bioconjugates. (1) TC1−BSA, TC1−CONA, TC1−OVA, and TC1−HRP were prepared following the active ester (AE) procedure[22] by activating the hapten (10 μmol) with *N*-hydroxysuccinimide (NHS, 12.5 μmol) and dicyclohexylcarbodiimide (DCC, 25 μmol) in anhydrous DMF (200 μL) and adding the solution to horseradish peroxidase (HRP, 2 mg), bovine serum albumin (BSA, 10 mg), conalbumin (CONA, 10 mg), or ovalbumin (OVA, 10 mg) proteins in borate buffer (1.8 mL).

TC2−BSA, TC2−CONA, TC2−OVA, and TC2−HRP were prepared following different strategies. *Strategy A:* TC2 (10 μmol), in anhydrous DMF (100 μL) was coupled to HRP (2 mg) or to the proteins (10 mg each) in borate buffer (1.8 mL) by adding a freshly prepared solution of dimethyl pimelimidate dihydrochloride (DPM, 125 μL 10 μmol in borate buffer).[23] *Strategy B:* The proteins (BSA, CONA, OVA, 10 mg, each) or the enzyme (HRP, 2 mg) was coupled

to cyanuric chloride (CC, 10 μmol) in coating buffer as previously described[24,25] and in a second step a solution of TC2 (10 μmol) in anhydrous DMF (100 μL) was added to CC previously derivatized proteins (3 mg each) in coating buffer (675 μL).

All of the protein conjugates were purified by dialysis against 0.5 mM PBS (4 × 5 L) and Milli-Q water (1 × 5 L) and stored freeze-dried at −40 °C. Unless otherwise indicated, working aliquots were stored at 4 °C in 0.01 M PBS at 1 mg mL^{-1}. Hapten densities of the bioconjugates were estimated by measuring the molecular weight of the native proteins relative to that of the conjugates by MALDI-TOF-MS. Thus, MALDI spectra were obtained by mixing 2 μL of the freshly prepared matrix (*trans*-3,5-dimethoxy-4-hydroxycinnamic acid, 10 mg mL^{-1} in CH_3CN/H_2O 70:30, 0.1% TFA) with 2 μL of a solution of the conjugates or proteins in CH_3CN/H_2O 70:30, 0.1% TFA (10 mg mL^{-1}). The hapten density (δ hapten) was calculated according to the following equation: {MW(conjugate) − MW(protein)}/MW-(hapten)}. Coupling efficiency evaluated by MALDI-TOF-MS of the corresponding haptenized BSA immunoreagents is shown in Table 1.

Table 1. Hapten Densities of the BSA Conjugates[a]

immunoreagent	δ-hapten[b]	% conjugation[c]
TC1−BSA (MA)	3.3	9−11
TC2−BSA (EDC)	6.0	17−20
TC1−BSA (EA)	10.8	31−36
TC2−DMP−BSA	2.1	6−7
TC2−CC−BSA	1.0	2−3

[a]Analyses were performed by MALDI-TOF-MS. [b]Moles of hapten per mole of protein. [c]The conjugation is calculated on the basis of the assumption that the BSA has 30−35 free lysine groups.

Polyclonal Antisera. As180−As182 and As183−As185 were obtained by immunizing female white New Zealand rabbits weighing 1−2 kg with TC1−HCH and TC2−HCH, respectively, following a protocol already described.[22] The evolution of the antibody titer was assessed on a noncompetitive indirect ELISA, by measuring the binding of serial dilutions of each antiserum to microtiter plates coated with TC1−BSA or TC2−BSA. After an acceptable antibody titer was observed, the animals were exsanguinated, and the blood was collected on vacutainer tubes provided with a serum separation gel. Antisera were obtained by centrifugation and stored at −80 °C in the presence of 0.02% NaN_3.

Competitive Indirect TC1−OVA/As181 ELISA. Microtiter plates were coated with TC1−OVA (0.625 μg mL^{-1} in coating buffer, 100 μL/well) overnight at 4 °C and covered with adhesive plate sealers. The next day, the plates were washed four times with PBST (300 μL/well), and milk samples or standard (DC or other cross-reactants, from 50000 to 0.005 nM in PBST) were added to the wells (50 μL/well) followed by the antiserum As181 (1/500 diluted in PBST, 50 μL/well) and incubated for 30 min at room temperature, under shaking. The plates were washed as before, and a solution of anti-IgG−HRP (1/6000 in 10 mM PBST) was added to the wells (100 μL/well) and incubated for 30 min more at room temperature. The plates were washed again, and the substrate solution was added (100 μL/well). Color development was stopped after 30 min at room temperature with 4 N H_2SO_4 (50 μL/well), and the absorbances were read at 450 nm. The standard curves were fitted to a four-parameter equation according to the following formula: $y = (A - B/[1 - (x/C)^D] + B$, where A is the maximal absorbance, B is the minimum absorbance, C is the concentration producing 50% of the maximal absorbance, and D is the slope at the inflection point of the sigmoid curve. Unless otherwise indicated, data presented correspond to the average of at least two well replicates.

(a) Specificity Studies. DC, TC, MC, and OTC analytes used as standards were prepared from 10 mM stocks in dimethyl sulfoxide (DMSO) and CTC in 50 mM aqueous HCl and kept at 4 °C. Standard curves were prepared in PBST and run in the ELISA following the protocol described before. The cross-reactivity (CR) values were calculated according to the equation {IC_{50} [nM] (DC)/IC_{50} [nM] (cross-reactant)} × 100.

(b) Accuracy Studies. This parameter was assessed by preparing eight different blind spiked samples in PBST buffer and measuring them in duplicate in the ELISA.

Milk Experiments. (a) Samples. Skimmed milk samples free of antibiotics were supplied by the Agencia Española para la Seguridad Alimentaria y Nutricion (AESAN; Spanish Agency for Food Security).

(b) Sample Treatments. (i) Saturated ammonium sulfate (0.45 mL) or (ii) McIlvaine buffer (0.30 mL) solutions were added dropwise to whole milk samples (1 mL). The mixtures were shaken (0.5 min at room temperature), centrifuged (5000*g*, 10 min), and filtered (0.45 μm pore size) to separate the milk whey from the precipitate.

(c) Matrix Effect Studies. Nonspecific interferences produced by the milk have been assessed by preparing standard curves in milk whey at several dilution factors and running them in the ELISA to compare the parallelism with the standard curve prepared in buffer.

(d) Recovery Studies. The recovery of the analyte after sample treatment was assessed by spiking blank milk samples in triplicates at four concentration levels (25, 50, 100, and 200 μg kg^{-1}). After precipitation, the samples were analyzed using as reference standard curves prepared in milk whey.

(e) Accuracy Studies. This parameter was assessed by preparing again diverse blind spiked samples at different concentration levels and measuring them in the ELISA. In all cases data were corrected with the corresponding recovery factor.

RESULTS AND DISCUSSION

Immunoreagents for TCs have been developed with the objective to detect specifically doxycycline residues in food products. DC-specific polyclonal antibodies have been raised against two immunizing haptens. The chemical structures of the immunizing haptens were designed following chemical criteria with the aim to maximize exposure of the most important epitopes in the lower periphery of the tetracycline structure, an area with a great proportion of keto−enolic and hydroxyl groups and a primary amide. Moreover, attending to the chemical structure of DC, the presence of one hydroxyl group situated in the C_5 position (ring B) and that of the dimethylamino groups were considered to be relevant. Thus, hapten TC1 was designed to maximize recognition of these groups by introducing a spacer arm at the C_6 position in ring C. On the other hand, hapten TC2, although lacking the hydroxyl group at the C_5 position, allowed the preservation of the methyl group at C6, which could also be an important epitope for antibody recognition, by introducing the spacer arm through the amino group of ring A (see Figure 1 for chemical structures).

Hapten TC1 was synthesized following the procedures previously described by Blackwood et al.[26−29] to slightly modify 6-methylenetetracyclines. Reaction consisted on an anti-Markovnikov free radical addition of the 3-mercaptopropionic acid to the MC C_6 exocyclic double bond, using a catalytic amount of AIBN as radical initiator in ethanol. Contrary to the results reported by Nelson and co-workers,[30] the ethyl ester (MC−CH_2−S−$(CH_2)_2$−COOEt) instead of the carboxylic derivative was obtained in moderate yield. The ester was purified by flash column chromatography (silica gel pretreated with Na_2EDTA to avoid excessive retention of the product on the solid phase due to the formation of chelates with divalent cations) and hydrolyzed under strong basic conditions to obtain the desired TC1 hapten. On the other hand, synthesis of TC2 was accomplished by reacting TC with *N*-chlorosuccinimide, followed by the addition of the hydrazine.[29] The final mixture was also purified by EDTA-treated silica gel flash

column chromatography. Although the final yield of this reaction is very low, we suspect that a significant amount is lost during the purification procedure probably because of the lack of stability of the intermediate or final product.

TC1 was covalently coupled through its carboxylic groups to the lysine amino acid residues of the HCH using the mixed anhydride method, and the TC1−HCH bioconjugate was used to raise three antisera, As180, As181, and As182. On the contrary, TC2 was covalently coupled to the glutaric and aspartic residues of the same carrier protein but using 1-(3-dimethylaminopropyl)-3-ethylcabodiimide (EDC) in buffer solution, and the TC2−HCH bioconjugate was used to raise As183, As184, and As185. Similarly, both haptens were conjugated to HRP, BSA, CONA, and OVA proteins to use them as coating antigens or enzyme tracers to further develop the competitive ELISAs (see details under Immunochemistry/Immunoreagents). The screening of all possible antiserum/enzyme tracer (As/ET) and antiserum/coating antigen (As/CA) combinations was performed using DC as analyte. Previously, the concentrations of the immunoreagents were selected by two-dimensional checkerboard titration experiments in which the avidity of the antisera for the better-recognized coating antigens (CAs) was assessed.[15] Subsequently, the different competitor/antibody combinations were evaluated under competitive configuration to assess their capability to detect DC and to select the best coating antigen/antibody combination. In all cases, the antisera raised against TC1−HCH provided the best assays. In contrast, no usable assays (IC_{50} > 100 $\mu g\ L^{-1}$) were obtained with the As raised against TC2−HCH, indicating that the dimethylamino group played an important role in antibody recognition. Moreover, the indirect ELISA format was found to be more robust and reproducible.

Because of the simplicity we first tried to develop a direct competitive ELISA format; however, we did not succeed in establishing a good competitive immunochemical assay. On the contrary, Table 2 shows the features of the best competitive indirect ELISAs obtained, showing that DC could be detected under homologous conditions. Although several combinations rendered usable assays, As181/TC1−OVA was selected for further studies because of the excellent features and reproducibility observed on repetitive experiments. The TC1−OVA bioconjugate had a hapten density of 11 mol per mole of protein according to MALDI-TOF-MS analysis. The As181/TC1−OVA ELISA was further investigated with the aim to improve performance and to characterize their behavior in media with different physicochemical parameters (pH, ionic strength, etc.). No significant effects on the immunoassay detectability were observed after an overnight preincubation of the antisera with the analyte before the competitive step or by varying the length of the competitive step from 10 min to 1 h. In contrast, the concentration of Tween 20 in the assay buffer readily influenced the detectability of the assay. Thus, the best detectability was accomplished when the percentage of this detergent was reduced (see Figure 2A). With regard to the pH, the assay tolerated quite well pH values between 4 and 9, having the best performance at pH 5.5, which corresponds to the maximum concentration of the zwitterionic form according to the pK_a values reported for the TCs.[1] Above pH 9, the signal of the assay is almost inhibited, showing unexpectedly high IC_{50} values (see Figure 2B). With regard to ionic strength, in the absence of salts the signal of the detectability of the assay was significantly affected. On the other hand, when the ionic strength increased, an improvement of the detectability was observed. A conductivity of around 32 mS/cm was considered to be the most appropriate for this assay (see Figure 2C). Finally, the addition of Ca^{2+} seems to stabilize the TC analytes in terms of structure, owing to their tendency to form chelates, and improves the reproducibility of the assay (see Figure 2D). On the contrary, the addition of Mg^{2+} or Mg^{2+}/Ca^{2+} did not affect so much the assay and therefore did not improve the results obtained previously. The attempts to reduce the A_{min} and to improve signal-to-noise ratio with different blocking agents, such as BSA, casein, or milk powder, after coating the microplates with the antigen were unsuccessful.

According to the above studies, on further experiments a PBS, 0.025 M phosphate, 2% saline solution (343 mmol L^{-1} NaCl, 6.8 mmol L^{-1} KCl) containing calcium divalent cation (1 mmol L^{-1} $CaCl_2$) and 0.001% Tween with a pH of 5.5 was used. Figure 3 shows a standard calibration curve corresponding to the average of four assays performed on different days using two-well replicates using these conditions in the competitive step. The immunoassay features are shown in Table 3. As can be observed, the *detectability* accomplished is very good. The assay shows an IC_{50} of 1.26 ± 0.05 $\mu g\ L^{-1}$ and a limit of detection (LOD (90% of the zero dose) of 0.10 ± 0.03 $\mu g\ L^{-1}$). The working range was defined to be between 0.25 ± 0.06 and 6.70 ± 0.59 $\mu g\ L^{-1}$ (20−80% of the assay response at zero doses). Moreover, *specificity* studies performed measuring other tetracycline antibiotics (DC, TC, MC, OTC, CTC) showed that only DC and MC were significantly recognized within the concentrations evaluated (see Table 4). The recognition pattern demonstrates the importance of the hydroxyl group in the C_5 position at the B ring. Other TCs not containing this functional group, such as TC, CTC, or OTC, were not recognized.

This result is difficult to explain, although it is well-known that bioreceptors could be very sensitive to subtle modifications on particular functional groups (the so-called methyl group effect). Examination of the recognition profile of immunoassays for tetracyclines described in the literature[16−18,20,31,32] distinguishes two main groups: on the one hand, the immunoassays developed with antibodies raised against immunizing haptens keeping the 6-hydroxy group; on the other hand, those using antibodies generated against immunizing haptens lacking this group. The presence/absence of this group completely determined the recognition profile on each group of assays, recognizing for each of them only those congeners keeping or lacking this group, respectively. This observation deserves a more in-depth study to try to justify it. Computational chemistry could be a good tool for that because it could yield

Table 2. Immunoassay Features of the Best Competitive ELISA (TC1−HCH Immunogen)[a]

assay	A_{max}	A_{min}	IC_{50}[b]	slope	R^2
As182/TC1−BSA	0.533	0.158	4.22	−0.728	0.986
As181/TC1−BSA	0.619	0.189	2.00	−0.852	0.956
As181/TC1−OVA	0.808	0.129	5.27	−1.012	0.995
As182/TC1−CONA	0.701	0.234	3.82	−0.734	0.962
As180/TC1−OVA	1.061	0.243	5.13	−0.932	0.994
As182/TC1−OVA	1.214	0.340	8.26	−0.802	0.947
As181/TC1−CONA	1.251	0.556	2.42	−0.756	0.986

[a]Only some assays showing reasonable parameters and IC_{50} values are shown. [b]IC_{50} values are expressed in $\mu g\ L^{-1}$.

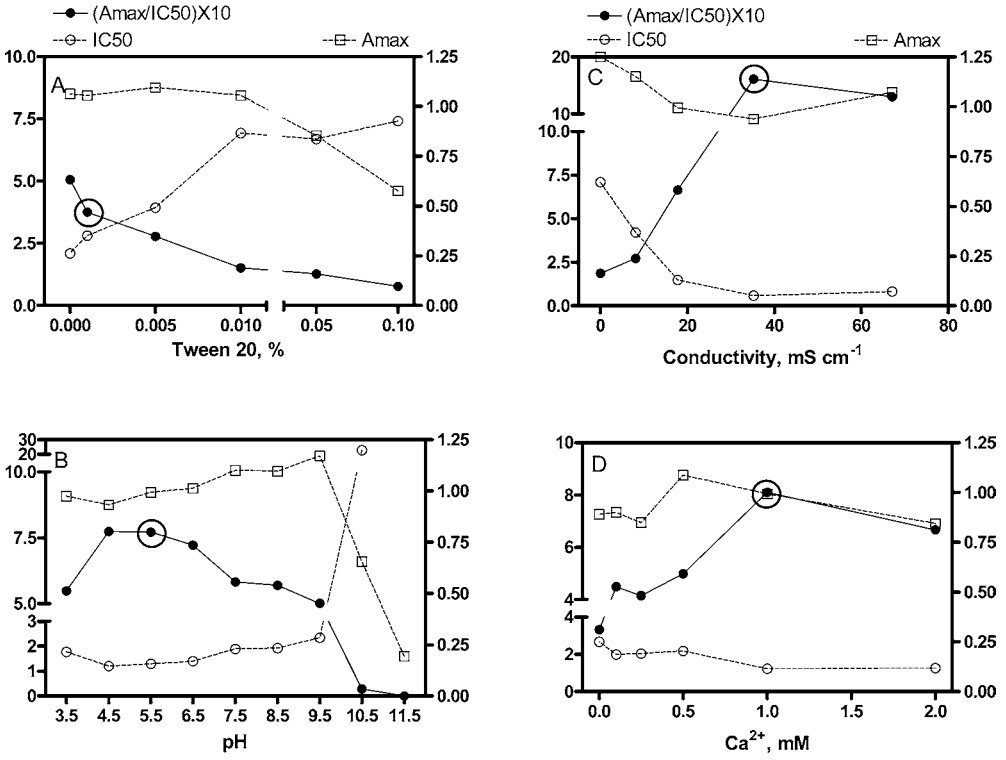

Figure 2. Effect of different physical−chemical parameters on the As181/TC1−OVA immunoassay: (A) concentration of Tween 20 in the assay buffer; (B) effect of pH; (C) effect of ionic strength; (D) effect of concentration of divalent cations (Ca^{2+}). The studies were performed by varying the composition of the buffer used in the competitive step. To evaluate the effect of the ionic strength, the concentration of NaCl was varied while the concentration of phosphate salts remained constant. To evaluate the effect of the concentration of divalent Ca^{2+}, Cl_2Ca was dissolved in PBS at different concentration values. All experiments were performed building calibration curves under different conditions. At least two-well replicates were employed for each standard concentration. Left axes indicate the value of the IC_{50} and the ratio $(A_{max}/IC_{50}) \times 10$. Right axes indicate the maximum absorbance. The IC_{50} values are expressed in nM.

information not only about the geometry of the haptens but also their electronic distribution, polarization, and, in some degree, comparative values for acidity. From the computational chemistry results it is evident that the conformation of the four-ring tetracycline system is highly conserved for tetracycline, doxycycline, and the immunizing hapten (it is taken as the reference structure), whereas the hydrazine derivative showed a large difference for rings A and B (see Figure 4 and Table 5). This fact is independent of the tetracycline protonation state. If we compare only the atoms in the ring system, the geometry differences between tetracycline, doxycycline, and the immunizing hapten are nonsignificant. If we add the common heavy atoms, the difference increases slightly but not enough to consider that the structures are different. In fact, the most important change is the 6-hydroxy group, which is perpendicular to the C/D ring system. If we compare the charge distribution between the four compounds another time, the hydrazine is clearly different for any protonation state. The other three compounds are very similar, with the obvious exception of the C-6 for tetracycline. Finally, if we compare the deprotonation enthalpies (an estimation of the acidity), the values are only appreciably different for the hydrazine compound. In this sense at pH 7 the populations of the different ionization states must be very different for this compound in relation to the other three.

The above data also explain the fact that TC2 did not provide usable assays. Thus, the hydrazine hapten shows large differences in geometry, electronic and charge distribution, and acidity. TC2 keeps the structure and properties of the target analytes with only one exception, the 6-hydroxy group, which is projected perpendicular to the ring system and highly exposed for interactions. This group is not trivial and could interact apparently at very large distance with respect to other parts in the molecule (in fact, the distance is not so large) as it is demonstrated in the oxidation reaction with NCS, in which this oxygen atom bridges with the C-4 (see Figure 1). Also, in the alkali-catalyzed TC isomerization this oxygen reacts with the carbonyl group at C-11, making a lactonization rearrangement that yields isotetracycline. This hypothesis of the highly interacting 6-hydroxy group is also coherent with the empirical observation from the published tetracycline immunoassays.

Finally, the assay *accuracy* was assessed by measuring several blind samples prepared in buffer. The results shown in Figure 5 correspond to the correlation found between the measured and the spiked concentration values. As can be observed, results obtained matched very well the spiked values. A slope near 1 was obtained (0.94) with a coefficient of correlation of R^2 = 0.989.

Due to the interest in controlling the presence of antibiotic residues in milk samples, studies were performed to assess potential nonspecific matrix effects. Results revealed that, at this point, milk samples could not be directly analyzed with the developed assay. Thus, the application of dilution factors of up to 500 times was needed to eliminate the nonspecific response observed (see Figure 3). For this reason, simple sample treatment methods were evaluated with the aim to implement this assay for high-throughput screening of milk samples. As can be observed in Figure 3, a simple precipitation procedure with

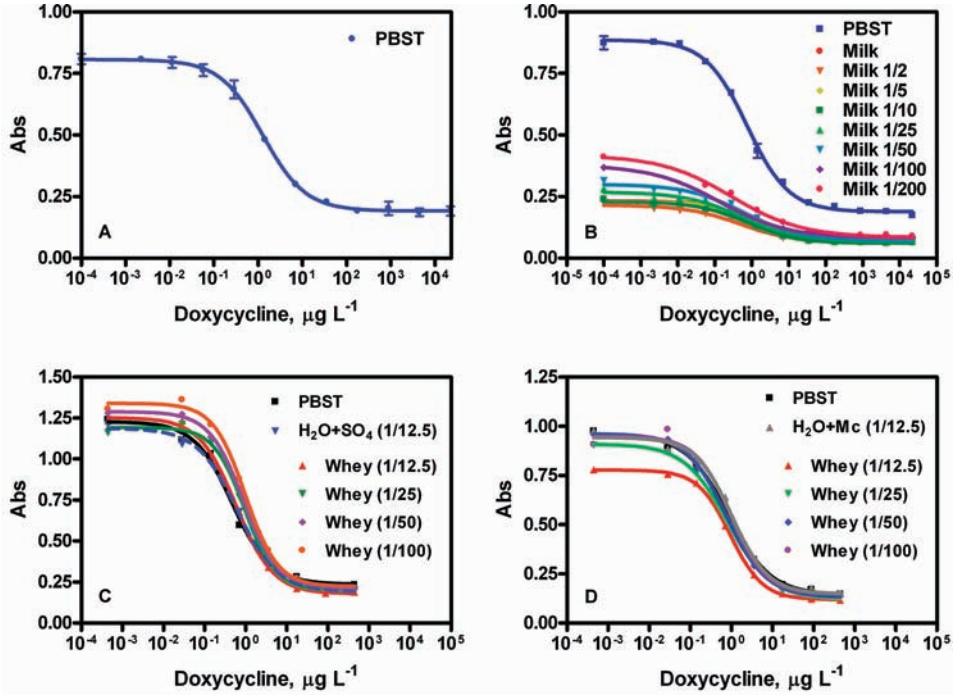

Figure 3. Graphs showing the calibration curve of the As181/TC1−OVA established ELISA for doxycycline under different conditions: (A) assay buffer; (B) untreated whole milk samples diluted to different proportions in the assay buffer; (C) whey milk prepared by treating whole milk with ammonium sulfate and further diluted with the assay buffer; (D) whey milk prepared by treating whole milk with the McIlvaine buffer and further diluted with the assay buffer. The data shown in graph A correspond to the averages of four assays performed on different days (see parameters in Table 3). The data shown in graphs B−D are the average and standard deviation of at least two-well replicates.

Table 3. Features of the Doxycycline ELISA

condition	value	As181/TC1−OVA assay parameter	value[a]
preincubation time	0 min	$signal_{min}$	0.19 ± 0.01
competition time	30 min	$signal_{max}$	0.81 ± 0.01
pH	5.5	slope	−0.90 ± 0.11
ionic strength	35.2 mS/cm (25 mM PBS)	R^2	0.998 ± 0.002
Tween 20	0.001%	IC_{50}, μg L^{-1}	1.26 ± 0.05
Ca^{2+} ($CaCl_2$)	1 mM	working range, μg L^{-1}	from 0.25 ± 0.06 to 6.70 ± 0.59
		LOD, μg L^{-1}	0.10 ± 0.03

[a]Values obtained correspond to the average and standard deviation of six calibration curves performed in four different days. Calibration curves were measured using three-well replicates.

Table 4. Cross-Reactivity of Related Compounds in the As181/TC1−OVA ELISA

	ELISA		
compound	IC_{50} (μg L^{-1})	LOD (μg L^{-1})	% CR[a]
doxycycline	1.26	0.10	100%
methacycline	3.89	0.28	32%
oxytetracycline	816.3	31.1	<1%
chlortetracycline	443.7	26.7	<1%
tetracycline	3605.0	127.8	<1%

[a]Cross-reactivity is expressed as a percent of the IC_{50} (nM) of the DC divided by the IC_{50} (nM) of the other compounds tested.

saturated ammonium sulfate or the McIlvaine buffer, followed by the corresponding centrifugation and filtration steps, was sufficient to minimize these undesired interferences. In both cases, the matrix effect, with respect to the PBS buffer, is almost negligible when milk whey is diluted 25 times. Moreover, as can be observed in the figure, milk could be directly measured with a lower dilution factor, because the effect observed after protein precipitation is very low. Thus, preparation of the standards in milk whey allowed the samples to be measured undiluted. Although both protein purification methods were found to perform very well, the use of the McIlvaine buffer was found to be slightly more reproducible. The DC recovery mean after the treatment was 42 ± 9%, being taken into account in subsequent LOD and accuracy studies. Moreover, shaking the microtiter plates during the competition step was found to be crucial because it minimized the formation of whey layers in the bottom of the microtiter wells, improving assay reproducibility and accuracy. Under these conditions, diluting the milk 25 times, the LOD achieved was close to 5 μg L^{-1}, which is still quite below the limits established for most of the tetracyclines allowed to be used on cattle producing milk for human consumption. Thus, preliminary experiments were performed by spiking milk samples at several concentrations around the MRL values of the rest of the tetracyclines, treated as described above and measured with the ELISA. Table 6 shows that the results obtained match very well the spiked concentration values.

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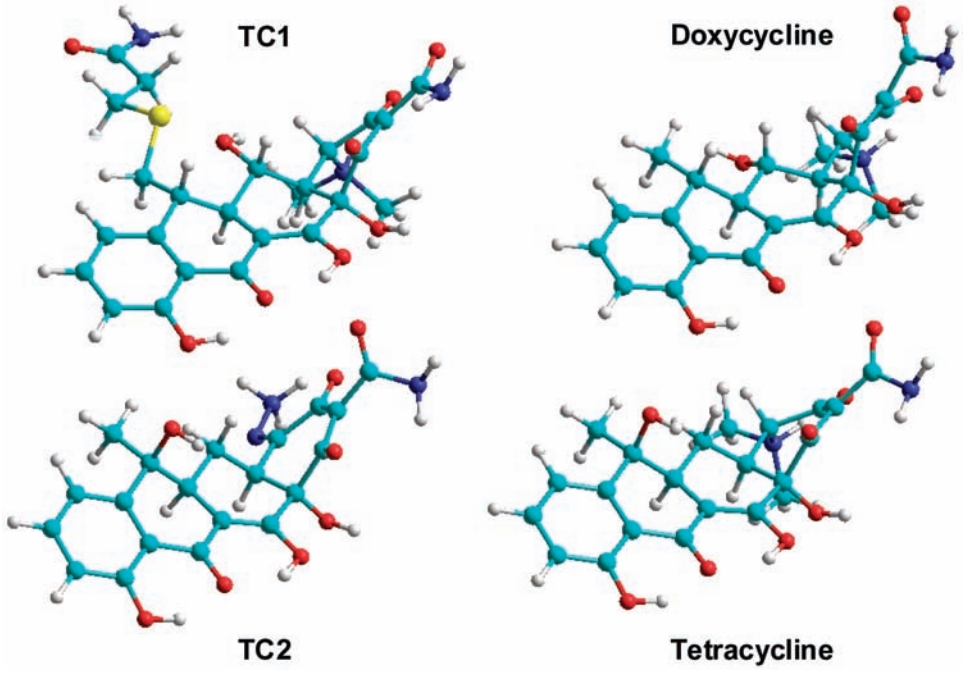

Figure 4. Calculated lower energy structures for the four compounds studied at the zwitterionic state.

Table 5. Geometry Fitting Error[a], Formation Enthalpy, and Deprotonation Enthalpy for TC1, TC, DC, and TC2

compound[b]	rms-rings (Å)	rms-common (Å)	ΔH_f (kJ/mol)	$\Delta\Delta H_{dp}$ (kJ/mol)
TC1+H			−545.5	
TC1+H−H			−1064.2	−518.7
TC1−H			−1195.4	−131.2
TC+H	0.153	0.218	−531.6	
TC+H−H	0.176	0.177	−1059.6	−528
TC−H	0.201	0.193	−1190.4	−130.8
DC+H	0.155	0.172	−523.0	
DC+H−H	0.184	0.178	−1056.7	−533.7
DC−H	0.169	0.156	−1190.7	−134.0
TC2+H	0.519	0.964	−334.6	
TC2+H−H	0.648	1.002	−922.4	−587.8
TC2−H	0.609	0.891	−1247.2	−324.8

[a]Expressed as rms for selected atoms referenced to the geometry of the corresponding hapten taken as reference. [b]+H or −H indicates the acid and base form with respect to the neutral tetracycline structures (+H is the form at high acidic pH, +H−H is the zwitterionic form, and −H is the form at moderate alkaline pH).

In summary, the immunochemical method reported here is a promising tool that could be used as an analytical screening method for monitoring the presence of DC residues in food samples. The antibodies raised, using TC1 immunizing hapten, have proven to be very specific for DC within the tetracycline antibiotic family. By combining these antibodies with a suitable competitor such as TC1−OVA, it has been possible to establish a competitive ELISA with excellent detectability. DC can be detected down to 0.1 μg L^{-1} in the assay buffer. Knowledge of the physicochemical properties of the TC antibiotic family has been critical to establishing a reproducible and robust method. Although the extent of nonspecific interferences caused by the milk samples is very high, a simple sample treatment method such as precipitating proteins using McIlvaine buffer has been sufficient to circumvent this drawback, yielding a reproducible

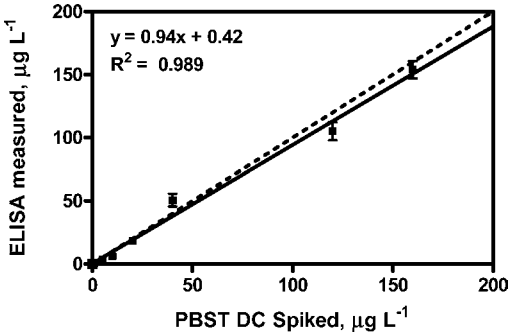

Figure 5. Results from the accuracy study performed in PBST. The graph shows the correlation between the spiked and measured concentration values using the DC ELISA. The dotted line corresponds to a perfect correlation (slope = 1). The data correspond to the average of at least three replicates.

Table 6. Accuracy of the DC Microplate-Based ELISA[a]

		measured	
spiked	*N*	PBST	whey (1/25)
200	3	220.4 ± 38.4	206.7 ± 29.3
100	3	118.7 ± 13.4	129.5 ± 32.3
50	3	81.43 ± 7.9	71.4 ± 5.7
25	3	31.15 ± 9.0	21.5 ± 7.0

[a]PBST and milk whey samples were spiked at distinct concentrations and measured with the DC ELISA. Spiked and measured DC concentration values are expressed in μg L^{-1}. Both types of samples were quantified using the calibration curve prepared in PBST buffer.

and accurate immunochemical analytical method. A LOD of 5 μg L^{-1} for DC in milk was achieved using the established immunochemical method and considering the recovery rate of the sample treatment step. Relative to the MRL values established for other TCs in milk, the detectability achieved by this ELISA is very good. Further research has to be performed for a complete validation in compliance with

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Commission Decision 2002/657/EC. In view of the excellent performance demonstrated by this assay to analyze DC in a complex biological sample, such as milk, there is great promise regarding the potential application of this immunoassay on new matrices such as water and soil samples in which antibiotics are considered to be emerging pollutants.

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) Riviere, J. E.; Spoo, J. W. Tetracycline antibiotics. In *Veterinary Pharmacology and Therapeutics*, 8th ed.; Adams, H. R., Ed.; Wiley-Blackwell: Ames, IA, 2001; pp 828−840.

(2) Kelly, M.; Tarbin, J. A.; Ashwin, H.; Sharman, M. Verification of compliance with organic meat production standards by detection of permitted and nonpermitted uses of veterinary medicines (tetracycline antibiotics). *J. Agric. Food Chem.* **2006**, *54* (4), 1523−1529.

(3) Kim, S.; Eichhorn, P.; Jensen, J. N.; Weber, A. S.; Aga, D. S. Removal of antibiotics in wastewater: Effect of hydraulic and solid retention times on the fate of tetracycline in the activated sludge process. *Environ. Sci. Technol.* **2005**, *39* (15), 5816−5823.

(4) Hirsch, R.; Ternes, T.; Haberer, K.; Kratz, K.-L. Occurrence of antibiotics in the aquatic environment. *Sci. Total Environ.* **1999**, *225* (1−2), 109−118.

(5) Michalova, E.; Novotna, P.; Schelegelova, J. Tetracyclines in veterinary medicine and bacterial resistance to them (review article). *Vet. Med. − Czech* **2004**, *49* (3), 79−100.

(6) Wegener, H. C. Antibiotics in animal feed and their role in resistance development. *Curr. Opin. Microbiol.* **2003**, *6* (5), 439−445.

(7) Hiramatsu, K.; Cui, L.; Kuroda, M.; Ito, T. The emergence and evolution of methicillin-resistant *Staphylococcus aureus*. *Trends Microbiol.* **2001**, *9* (10), 486−493.

(8) EC, Council Regulation 2377/90/EC of 26 June 1990 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. *Off. J. Eur. Union* **1990**, *L224* (Aug 18), 1−8.

(9) EC European Commission, Council Regulation 2377/90/EC (consolidated version of MRLs updated to 08.07.2008 obtained from EMEA); http://www.emea.europa.eu/htms/vet/mrls/a.htm.

(10) Unit, T. E. A. f. t. E. o. M. P. V. M. E. Committe for Veterinary Medicinal Products. *Doxycycline Summary Report*; EMA: London, U.K., 1997.

(11) Onal, A. Overview on liquid chromatographic analysis of tetracycline residues in food matrices. *Food Chem.* **2011**, *127* (1), 197−203.

(12) Chafer-Pericas, C.; Maquicira, A.; Puchades, R.; Company, B.; Miralles, J.; Moreno, A. Multiresidue determination of antibiotics in aquaculture fish samples by HPLC-MS/MS. *Aquac. Res. 41* (9), e217−e225.

(13) Link, N.; Weber, W.; Fussenegger, M. A novel generic dipstick-based technology for rapid and precise detection of tetracycline, streptogramin and macrolide antibiotics in food samples. *J. Biotechnol.* **2007**, *128* (3), 668−680.

(14) Alfredsson, G.; Branzell, C.; Granelli, K.; Lundstrom, A. Simple and rapid screening and confirmation of tetracyclines in honey and egg by a dipstick test and LC-MS/MS. *Anal. Chim. Acta* **2005**, *529* (1−2), 47−51.

(15) Estévez, M. C.; Kreuzer, M.; Sánchez-Baeza, F.; Marco, M. P. Analysis of nonylphenol: advances and improvements in the immunochemical determination using antibodies raised against the technical mixture and hydrophilic immunoreagents. *Environ. Sci. Technol.* **2005**, *40* (2), 559−568.

(16) Pastor-Navarro, N.; Morais, S.; Maquieira, Á.; Puchades, R. Synthesis of haptens and development of a sensitive immunoassay for tetracycline residues: application to honey samples. *Anal. Chim. Acta* **2007**, *594* (2), 211−218.

(17) Jeon, M.; Rhee Paeng, I. Quantitative detection of tetracycline residues in honey by a simple sensitive immunoassay. *Anal. Chim. Acta* **2008**, *626* (2), 180−185.

(18) Zhang, Y. L.; Lu, S. X.; Liu, W.; Zhao, C. B.; Xi, R. M. Preparation of anti-tetracycline antibodies and development of an indirect heterologous competitive enzyme-linked immunosorbent assay to detect residues of tetracycline in milk. *J. Agric. Food Chem.* **2007**, *55* (2), 211−218.

(19) Jeon, M.; Kim, J.; Paeng, K. J.; Park, S. W.; Paeng, I. R. Biotin−avidin mediated competitive enzyme-linked immunosorbent assay to detect residues of tetracyclines in milk. *Microchem. J.* **2008**, *88* (1), 26−31.

(20) Le, T.; Yu, H.; Guo, Y. C.; Ngom, B.; Shen, Y. A.; Bi, D. R. Development of an indirect competitive ELISA for the detection of doxycycline residue in animal edible tissues. *Food Agric. Immunol.* **2009**, *20* (2), 111−124.

(21) Galve, R.; Camps, F.; Sanchez-Baeza, F.; Marco, M. P. Development of an immunochemical technique for the analysis of trichlorophenols using theoretical models. *Anal. Chem.* **2000**, *72* (10), 2237−2246.

(22) Gascón, J.; Oubiña, A.; Ballesteros, B.; Barceló, D.; Camps, F.; Marco, M.-P.; González-Martínez, M. A.; Morais, S.; Puchades, R.; Maquieira, A. Development of a highly sensitive enzyme-linked immunosorbent assay for atrazine performance evaluation by flow injection immunoassay. *Anal. Chim. Acta* **1997**, *347* (1−2), 149−162.

(23) Marco, M. P.; Fabriàs, G.; Camps, F. Development of a highly sensitive ELISA for the determination of PBAN and its application to the analysis of hemolymph in *Spodoptera littoralis*. *Arch. Insect Biochem. Physiol.* **1995**, *30* (4), 369−381.

(24) Abuknesha, R. A.; Luk, C. Y.; Griffith, H. H. M.; Maragkou, A.; Iakovaki, D. Efficient labelling of antibodies with horseradish peroxidase using cyanuric chloride. *J. Immunol. Methods* **2005**, *306* (1−2), 211−217.

(25) Tsang, V. C. W.; Greene, R. M.; Pilcher, J. B. Optimization of the covalent conjugating procedure ($NaIO_4$) of horseradish peroxidase to antibodies for use in enzyme-linked immunosorbent assay. *J. Immunoassay Immunochem.* **1995**, *16* (4), 395−418.

(26) Blackwood, R. K.; Beereboom, J. J.; Rennhard, H. H.; von Wittenau, M. S.; Stephens, C. R. 6-Methylenetetracyclines. 1 I. A new class of tetracycline antibiotics. *J. Am. Chem. Soc.* **1961**, *83* (12), 2773−2775.

(27) Blackwood, R. K.; Stephens, C. R. 6-Methylenetetracyclines. II. Mercaptan adducts. *J. Am. Chem. Soc.* **1962**, *84* (21), 4157−4159.

(28) Blackwood, R. K.; Beereboom, J. J.; Rennhard, H. H.; von Wittenau, M. S.; Stephens, C. R. 6-Methylenetetracyclines. III. 1. Preparation and Properties. 2. *J. Am. Chem. Soc.* **1963**, *85* (24), 3943−3953.

(29) Blackwood, R. K.; Stephens, C. R. Some transformations of tetracycline at the 4-position. *Can. J. Chem.* **1965**, *43* (5), 1382−1388.

(30) Nelson, M. L.; Park, B. H.; Andrews, J. S.; Georgian, V. A.; Thomas, R. C.; Levy, S. B. Inhibition of the tetracycline efflux antiport

protein by 13-thio-substituted 5-hydroxy-6-deoxytetracyclines. *J. Med. Chem.* **1993**, *36* (3), 370−377.
(31) Faraj, B. A.; Ali, F. M. Development and application of a radioimmunoassay for tetracycline. *J. Pharmacol. Exp. Ther.* **1981**, *217* (1), 10−14.
(32) Burkin, M. A.; Galvidis, I. A. Improved group determination of tetracycline antibiotics in competitive enzyme-linked immunosorbent assay. *Food Agric. Immunol.* **2009**, *20* (3), 245−252.