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Advances in Chromatographic Analysis of Tetracyclines in Foodstuffs of Animal Origin—A Review

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Abstract: Tetracyclines (TCs) are broad-spectrum antibiotics in human and veterinary medicine, characterized by their exceptional chemotherapeutic efficacy against a wide range of Gram-positive and Gram-negative bacteria, rickettsiae, spirochetes, large viruses, chlamydia, mycoplasmas and protozoan parasites. The use of tetracyclines is increasing, as they are used not only in treatment but also prevention of illnesses. Moreover TCs are given to animals destined for human consumption to promote growth and may potentially result in the presence of residues in edible animal tissues, which can be toxic and dangerous for human health and potentially cause allergic reactions. An extended and comprehensive review on the analytical methodologies concerning tetracyclines in foodstuffs of animal origin reported in literature is provided in the present article. Emphasis is given on sample preparation regarding isolation and purification, chromatographic conditions and method validation according to the legislation. Results of published assays are comparatively presented and criticized.

Keywords: Tetracyclines, antibiotics, foodstuff, chromatography, analysis

INTRODUCTION

Tetracycline antibiotics (TCs) were first discovered in 1945. They derived from a species of *Streptomyces* bacteria. They are broad-spectrum antibiotic

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agents extensively used to control bacterial infections in humans and animals. They exhibit activity against infections caused by both Gram-positive and Gram-negative bacteria, chlamydia, mycoplasmas, rickettsiae and protozoan parasites. Additionally TCs are given to animals destined for human consumption to promote growth. However the presence of residues in edible animal tissues may potentially cause allergic reactions or may lead to toxic and dangerous effects on human health. In order to ensure human safety and health the regulatory agencies in the European Union (EU), the United States and in individual countries have enacted permitted limits (PL) or Maximum Residue Levels (MRLs) for the presence of TCs in animal products (1–5).

The determination of residual TCs in animal products is consecutively very important. It has been the subject of several analytical studies using different techniques. Microbiological assays and immunoassays are routinely used as screening methods to detect tetracycline residues in food, despite their important disadvantages, like poor sensitivity and selectivity, semi-quantitative measurements of residues detected and false positive results. However the confirmation and quantification of TCs residues certainly require chemical methods. At present chromatographic techniques are applied at most as confirmatory for TCs residues analysis in food of animal origin. Despite the simplicity and the low cost of TLC, HPLC coupled with UV, fluorescence and mass spectrometry remains the technique of choice for multiresidue analysis.

Prior to the determination step, TCs have to be isolated from tissues usually by an extraction step with a suitable solvent system like McIlvaine buffer (citric acid with disodium hydrogen phosphate), succinate, phosphate and oxalate buffers followed by a cleanup procedure. More details on the analytical methodologies for TCs'extraction conditions, analysis, detection published within the last two decades are described in the following paragraphs.

The great interest on these compounds is reflected by the significant number of review articles that can be found in literature after 1990. The first review is written by Barker and Walker in 1992. The authors present an overview of chromatographic methods for the separation and determination of tetracyclines isolated from foods. The methods reviewed include microbial inhibition, immunoassay and receptor technologies for detection, techniques for isolation from food matrices, and thin-layer chromatographic, high-performance liquid chromatographic, gas chromatographic and mass spectrometric procedures for determination of these compounds. A discussion of the variables involved in methodologies and a review of method criteria are offered from 81 reviewed articles (6).

One year later, Shaikh and Moats presented a review of developments in the liquid chromatographic (LC) methods of analysis for the residues of antibiotics (aminoglycosides, chloramphenicol, sulfonamides, tetracyclines macrolides, β-lactams, etc.) in food products of animal origin. This review

covers cleanup procedures, such as ultrafiltration, liquid-liquid partition, solid-phase extraction, immunoaffinity, and matrix solid-phase dispersion, for use as extraction, deproteination, and concentration steps (7).

Five review papers were found in 1998. Niessen presented an overview of the analysis of antibiotics by liquid chromatography–mass spectrometry. Currently available data on different compound classes including tetracyclines were reviewed with special emphasis on aspects relevant to LC–MS and on the mass spectral information obtained (8).

Oka et al. in their review summarized the recent developments in mass spectrometric analysis of tetracycline antibiotics (TCs) in foods. The mass spectrometric techniques discussed are: the collision-activated decomposition mass-analysed ion kinetic energy spectrometry (CAD MIKES), thin-layer chromatography (TLC)–fast atom bombardment (FAB) mass spectrometry (MS), particle beam (PB) liquid chromatography (LC)–MS, LC-frit FAB-MS, thermospray (TSP) LC–MS, atmospheric chemical ionization (APCI) LC–MS and tandem electrospray (ESI) LC–MS. The advantages and limitations of the above-mentioned techniques are described in the confirmation of TCs in foods (9).

Schenck and Callery presented an overview of the recent developments in the chromatographic determination of antibiotic residues, including tetracyclines, in milk that may result from the widespread use of antibiotics in dairy cattle management. They conclude that rapid screening tests are commonly used to detect the presence of antibiotics in milk. However more accurate chromatographic methods are required by government regulatory agencies to identify and confirm the identity and quantity of antibiotic present (10).

Fedeniuk and Shand published a short review summarizing the basic theory and practices of the extraction and cleanup of agricultural antibiotics from biomatrices, providing information for the analysis of residues of ionophores, β-lactams, macrolides, chloramphenicol, aminoglycosides, tetracyclines and peptide antibiotics (11).

Marzo and Dal Bo described the analytical methods employed in the pharmaco-kinetics of antibiotics (cephalosporins, aminoglycosides, tetracyclines, rifamycin and quinolones). The pharmacokinetic characteristics of each class of drugs are briefly described, and some historical and chemical notes on the various classes are given (12).

Two years later, Oka et al. in their review on the chromatographic analysis of tetracycline antibiotics in foods presented an overview of the recent developments in chromatographic analysis methods for TCs in foods. This review covers separation techniques like thin layer chromatography, capillary electrophoresis, high-performance liquid chromatography, and sample preparation including extraction and cleanup procedures (13).

Three review articles are found for 2002. Di Corcia and Nazzari present an overview of liquid chromatographic–mass spectrometric methods for analyzing antibiotic and antibacterial agents in animal food products focusing on sample treatment (14).

Joshi reviewed the HPLC separation of antibiotics present in formulated and unformulated samples. Chromatographic conditions such as column and mobile phase for the various classes of antibiotics viz. penicillins, cephalosporins, macrolides, tetracyclines, aminoglycosides, quinolones, rifamycins, etc. published from April 1998 to November 2000 are presented providing a brief discussion on chemical structure, spectrum of activity and action mechanism of each class (15).

McEvoy wrote on the contamination of animal feeding stuffs as a cause of residues in food: a review of regulatory aspects, incidence and control. This paper reviews the legislative framework controlling the use of veterinary medicines and zootechnical food additives such as sulphonamides, tetracyclines, nitroimidazoles, nitrofurans, ionophore coccidiostats and nicarbazin, in the EU. The literature on each of these is reviewed and examples of interventions to minimize contamination are given (16).

Hernández et al. in 2003 presented an overview on the analysis of antibiotics in biological samples by capillary electrophoresis. Antibiotic groups studied include β-lactam antibiotics (penicillins and cephalosporins); aminoglycosides; quinolones; and tetracyclines (17).

One year later, Michalova et al. in their article: "Tetracyclines in veterinary medicine and bacterial resistance to them" present the history and classification of tetracyclines, their application in veterinary medicine, their mode of action and information on the mechanisms of bacterial resistance (18).

Smyth and Brooks presented a critical evaluation of HPLC-ESI-MS and CE-ESI-MS for the detection and determination of small molecules of significance in clinical and forensic science. Tetracyclines are studied among several drugs. Analytical information on sample preparation and analytical techniques are provided (19).

In 2005, Anderson et al. wrote on the complexities in tetracycline analysis, chemistry, matrix extraction, cleanup and liquid chromatography. Their review article compiles, compares, and discusses the results and observations found in published methods, focusing on chemical principles in order to aid chemists to develop useful analyses. Seventy-four articles are reviewed (20).

From all works stated above it is obvious that there is a constantly increasing interest for this class of antibiotics especially after 1995. This trend is definitely shown in Figure 1. Herein an extended and comprehensive overview of the analytical methodologies for the determination of tetracycline antibiotics in foodstuffs of animal origin recently reported in literature is provided. The chemistry and antimicrobial activity of tetracyclines in human and veterinary medicine is studied. Legislation aspects for veterinary antibiotics are taken into consideration. Emphasis is given on the determination of these compounds in edible animal tissues such as bovine, porcine and chicken muscle, kidney, liver, eggs, milk, cheese, shrimps, fish and honey. Results of various published assays are presented comparatively and are criticized regarding extraction methods during sample preparation, analytical conditions and method validation.

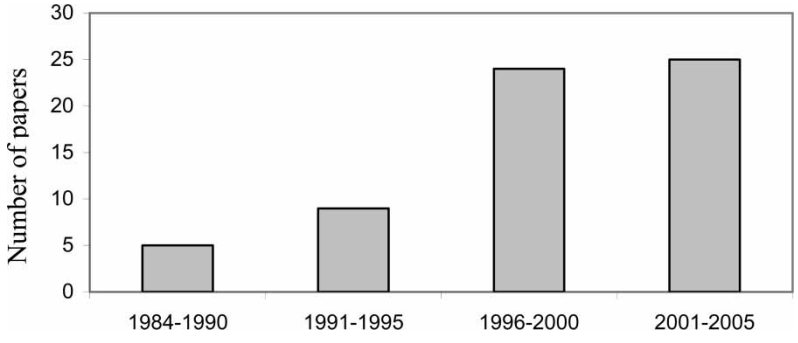


Figure 1. Number of published papers on the determination of tetracyclines by various analytical techniques over the period 1987–2005.

CHEMISTRY AND ANTIMICROBIAL ACTIVITY OF TETRACYCLINES

Structure, Chemical Characteristics and Stability

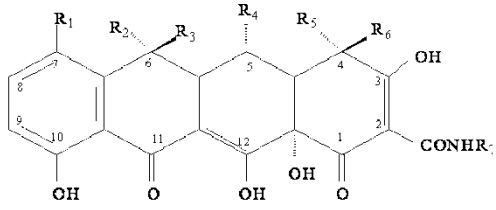
Tetracyclines belong chemically to the polyketides. Under the name "tetracycline" (1,4,4a,5,5a,6,11,12a-octahydronaphthacene) a number of antibiotics of either natural, or semi-synthetic origin, derived from a system of four linearly fused six-membered rings (A, B, C and D) as shown in Figure 2, to which a variety of functional groups are connected. Five asymmetric centers exist on the tetracycline molecules: C-4, C-4a, C-5a, C-6, C-12a. (1, 5).

The antimicrobial activity of tetracyclines is strongly connected with their chemical structure and stereochemistry and more specifically with the maintenance of the linear fused tetracycle, the stereochemical configurations at the 4a, 12a (A-B ring junction) and 4, 6 positions and the keto-enol system (11, 12 positions). If functional groups at positions 1, 2, 3, 10, 11 and 12 are changed the activity of the compounds is completely lost or significantly decreased. However, the addition of substituents to the amide nitrogen at 2 position increases the water solubility and still maintain activity. The 4-position can be altered slightly, having a monosubstituted amine. A variety of functional groups placed at positions 5, 6, 7 and 9 improve antimicrobial activity or stability. R_4 at position 5 can be either a hydroxyl, and a keto group, or hydrogen. The 7-position is substituted with chlorine, fluorine, bromine and nitro groups. Substituents at 6 position can be hydrogen, hydroxyl and methylene groups while on 8-position any electron donating group is accepted. At the 9-position, the introduction of an acylamido group has led to the developemed of new tetracyclines, named glycycyclines, which exhibit activity against tetracycline-resistant bacteria (2).

Eight tetracyclines are commercially available: tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC), doxycycline (DC), minocycline (MNC), methacycline (MTC), demeclocycline (DMC), and rolitetracycline (RTC) and can be used in human and veterinary medicine.

Tetracyclines are solid, crystalline, odorless compounds with yellow or pale yellow color, usually in the form of hydrates or hydrochloride salts. They are soluble in dilute acids and in solutions of alkali hydroxides, slightly soluble in water and methanol and practically insoluble in ethers and chloroform. Their ultraviolet spectra show strong absorption at 270 and 350 nm. They are amphoteric compounds with dissociation constants corresponding to the acidic hydroxyl group at 3-position at pK_a 2.8–3.3, the dimethylamino group at 4-position at the pK_a 7.3–8.3, and the hydroxyl group at 12-position at pK_a 9.3–10.2 (3).

Tetracyclines are relatively unstable compounds. Although they are stable in air, exposure to strong sunlight or high temperatures (over 90°C) for chlortetracycline, causes them to darken. Sunlight produces various degradation products under extreme acidic, basic or temperature conditions. When exposed to dilute acid (pH < 3) tetracyclines undergo epimerization at C-4 as well as dehydration at C-6, to yield 4-epi-TCs



Compound		Chemical name	R_1	R_2	R_3	R_4	R_5	R_6	R_7
TC	Tetracycline	2-naphthacenecarboxamide, 4-(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-monohydrochloride	H	CH_3	OH	H	$N(CH_3)_2$	H	H
OTC	Oxytetracycline	5-hydroxy-tetracycline	H	CH_3	OH	OH	$N(CH_3)_2$	H	H
CTC	Chlortetracycline	7-chloro-tetracycline	Cl	CH_3	OH	H	$N(CH_3)_2$	H	H
DC	Doxycycline	6-deoxy-5-hydroxy-tetracycline	H	CH_3	H	OH	$N(CH_3)_2$	H	H
MNC	Minocycline	7-dimethylamino-6-demethyl-6-deoxy-tetracycline	$N(CH_3)_2$	H	H	H	$N(CH_3)_2$	H	H
MTC	Methacycline	6-methylene-5-hydroxy-tetracycline	H	$=CH_2$		OH	$N(CH_3)_2$	H	H
DMC	Demeclocycline	6-demethyl-7chloro-tetracycline	Cl	H	OH	H	$N(CH_3)_2$	H	H

Figure 2. Chemical structures of examined tetracyclines.

(*continued*)

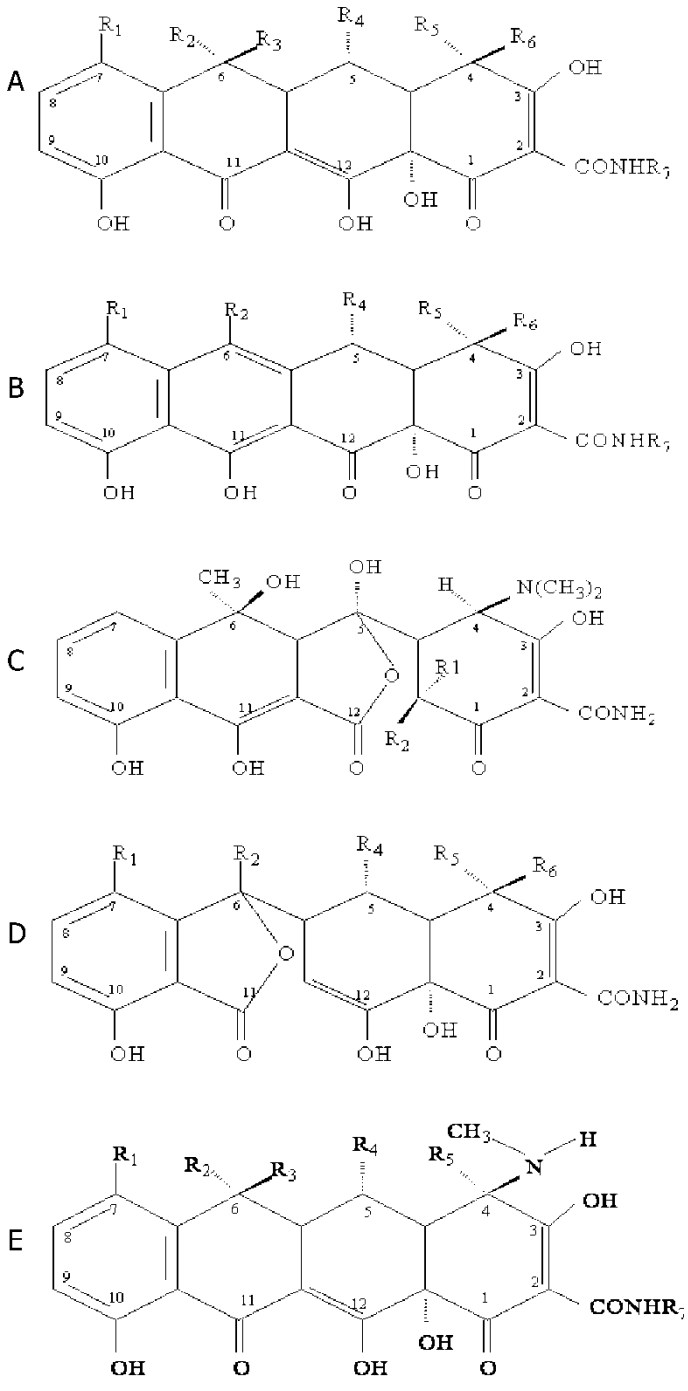


Figure 2. Continued.

Compound		Structure	R_1	R_2	R_3	R_4	R_5	R_6	R_7
4-ETC	Epi-tetracycline	A	H	CH_3	OH	H	H	$N(CH_3)_2$	H
4-EOTC	Epi-oxytetracycline	A	H	CH_3	OH	OH	H	$N(CH_3)_2$	H
4-ECTC	Epi-chlortetracycline	A	Cl	CH_3	OH	H	H	$N(CH_3)_2$	H
4-EDC	Epi-doxycycline	A	H	CH_3	H	OH	H	$N(CH_3)_2$	H
ATC	Anhydro-tetracycline	B	H	CH_3	-	H	$N(CH_3)_2$	H	H
AOTC	Anhydro-oxytetracycline	B	H	CH_3	-	OH	$N(CH_3)_2$	H	H
ACTC	Anhydro-chlortetracycline	B	Cl	CH_3	-	H	$N(CH_3)_2$	H	H
EATC	Epi-anhydro-tetracycline	B	H	CH_3	-	H	H	$N(CH_3)_2$	H
EAOTC	Epi-anhydro-oxytetracycline	B	H	CH_3	-	OH	H	$N(CH_3)_2$	H
EACTC	Epi-anhydro-chlortetracycline	B	Cl	CH_3	-	H	H	$N(CH_3)_2$	H
ADTC	2-acetyl-2-decarboxamido TC	A	H	CH_3	OH	H	$N(CH_3)_2$	H	CH_3
ADOTC	2-acetyl-2-decarboxamido OTC	A	H	CH_3	OH	OH	$N(CH_3)_2$	H	CH_3
ADCTC	2-acetyl-2-decarboxamido CTC	A	Cl	CH_3	OH	H	$N(CH_3)_2$	H	CH_3
α-APOTC	α-apo-ocytetracycline	C	H	OH					
β-APOTC	β-apo-ocytetracycline	C	OH	H					
IOTC	Iso-oxytetracycline	D	H	CH_3	-	OH	$N(CH_3)_2$	H	-
ICTC	Iso-chlortetracycline	D	Cl	CH_3	-	H	H	$N(CH_3)_2$	-
N-DMOTC	N-desmethyl-oxytetracycline	E	H	CH_3	OH	OH	-	H	H
N-DMCTC	N-desmethyl-chlortetracycline	E	Cl	CH_3	OH	H	-	H	H

Figure 2. Continued.

and anhydro-TCs respectively. Under strong acidic conditions (pH $<$ 2) anhydro form of OTC, whose substituent R_4 is a hydroxyl group, suffers cleavage and lactonization to apo-derivatives. Mild alkali attacks C-11a to form a carbonate group. In TC, OTC and CTC where R_3 is a hydroxyl group, a C-ring opening occurs as the hydroxyl group attacks the freshly formed carboxylic acid at 11-position to form iso-derivatives. These degradation products are inactive or even toxic (anhydro-TCs) for humans and animals (1, 2).

Thermostability experiments in liquid media have shown that OTC is unstable in water at 100°C with a half-life of 2 min, but more stable in oil

at 180°C with a half-life of 8 min. Thermal stability of TCs in various food matrices differs a lot. OTC is more stable in salmon tissues than in buffer systems and also more stable in milk than in chicken or beef tissues. A heat treatment at 120°C for 60 min reduces OTC residues in meat tissues about 35–75% and CTC residues about 50%, depending on the cooking process. Certain food additives also influence significantly the rate of TCs degradation with pyrophosphates, hexametaphosphates and nitrites decreasing this rate (4).

Tetracyclines are also chemically characterized by their strong tendency to form fluorescent complexes with various chemical species, like Fe^{3+}, Fe^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+}, Be^{2+}, Al^{3+}, phosphates, citrates, salicylates, *p*-hydroxybenzoates, saccharin anion, caffeine, urea, thiourea, polyvinylpyrrolidone, because of its B- and C-ring oxygen atoms. Due to their polar character, TCs bind with lipoproteins, albumins and globulins (1).

History and Classification of TCs

Systematic research on antibiotics after World War II led, in the 1940s, to the discovery of the first member of TCs class, chlortetracycline. In 1948, Benjamin M. Duggar, Professor of Plant Physiology at the University of Wisconsin first isolated a tetracycline member. The substance (chlortetracycline) extracted from a type of soil that contained a unique fungi (*S. aureofaciens*), found in the vicinity of cemeteries, was named "aureomycin" due to its yellow color. Terramycin (oxytetracycline) was isolated 2 years later by Finley from cultivation of *Streptomyces rimosus.* The parent compound, tetracycline, was first reported in 1953 after catalytic hydrogenolysis of aureomycin, but its production is also possible by cultivation of some Streptomyces strains. In 1957, McCormick discovered 6-demethylchlortetracycline (demeclocycline), a metabolite of a *S. aureofaciens* mutant. Other important tetracyclines like doxycycline, minocycline, methacycline, and rolitetracycline were produced semi synthetically. In the early 1990s a new generation of TCs was discovered, named glycycyclines, with addition of a 9-glycylamido group (1).

All these tetracyclines mentioned above belong to the "typical tetracyclines,", characterized by the same antimicrobial activity and the same mechanism of action (inhibition of proteinosynthesis) due to a very specific chemical structure. A second class of TC antibiotics, "atypical tetracyclines" includes nortetracyclines (the B-ring comprises a five-member carbocycle), thiatetracyclines (posses a sulfur atom at C-ring) and some of typical TCs degradation products like anhydro- and epianhydro- derivatives. These molecules are characterized by a bacteridical activity by attacking cytoplasmic membranes and lack of any therapeutic interest because of their low-level bacteriostatic effect and their adverse side effects in

humans due to their ability to interact either with prokaryotic or with eukaryotic cells (18).

Antimicrobial Activity

Tetracyclines are antibiotics having a very broad spectrum of antimicrobial activity against a wide range of gram-positive (*staphylococcus, streptococcus, pneumonococcus, enterococcus*) and gram-negative bacteria (*gonococcus, cholera, dysentery bacillis, brucella*), including some anaerobes. They are also active against chlamydia, *mycoplasmas, rickettsiae, spirochetes* and some large viruses, such as members of the lymphogranuloma group.

It should be noticed that due to the development of resistant bacteria strains, use of TCs for treatment of staphylococcal, streptococcal or pneumonococcal infections is significantly limited. The broad-spectrum of antimicrobial activity of TCs in connection with other characteristics, like their good absorbance, low toxicity and relative low cost, explains the wide use of this class of antibiotics in human and veterinary medicine for therapy of various bacterial and non-bacterial infections. Their use is also widespread in animal husbandry as promoting growth agents and for the prophylaxis of food-producing animals from infections (2).

Veterinary Medicine

Tetracyclines are provided in veterinary medicine for treatment of gastrointestinal (gastritis, hepatitis), respiratory (pneumonia, reunites), genito-urinary (mastitis, uterine) and skin bacterial infections, infectious diseases of locomotive organs as well as for systemic infections, cholera and sepsis. TCs that can be used in veterinary medicine according to EU and FDA legislation are tetracycline, oxytetracycline, chlortetracycline and doxycycline, while animal species in which these drugs can be provided are beef, cattle, pig, sheep, goats, horses, dogs, cats, poultries, rabbits and fishes (18).

Tetracyclines are administered either orally or parenterally, in doses from 10 to 50 mg/per kg of body, depending on drug preparation and animal species. Oral preparations of tetracyclines cannot be administered to ruminants because of the destruction of the ruminal microflora. Rapid intravenous administration of tetracyclines can result in cardiovascular dysfunction and collapse in any species. The electrocardiographic abnormalities may be due to chelation of free calcium ions. Tetracyclines ideally should be diluted in fluids and administered slowly if given by the intravenous route.

Administration of TCs is forbidden during last-half of pregnancy and in small animals until 1 month of age, as these drugs were found to decolorize teeth and cause significant problems to bone formation and development due to formation of complexes with calcium ions. However, any combinations

of TCs with antacids, calcium, iron, sodium and magnesium supplements, phenobarbital, terefthalic acid or microsomal enzyme inducers should be avoided as these medications changes blood concentrations of TCs (3).

As already mentioned, TCs are also used in veterinary medicine as feed additives to promote growth in animal husbandry. Growth-promoting properties of tetracyclines were first discovered in 1949 after chickens being fed with chlortetracycline supplemented feed. Subtherapeutic doses of OTC, TC and CTC were widely used for many years as growth-promoting agents in calves, chickens, turkeys, sheep and pigs. That practice contributes significantly to the development of resistant bacteria strains, a fact which threats the future use of TCs as antibiotics either in human or in veterinary medicine. Moreover, TC residues in animal food products may lead to health problems to sensitive target groups of population. For all these reasons administration of TCs as feed additives is not allowed in the EU by legislation since 1975 (18).

Human Medicine

The main indications for the administration of tetracyclines to humans are infections of *E. coli* and *H. influenzae*, infections of the bile duct, bacterial respiratory disorders including bronchitis prophylaxis, mixed infections arising from the mouth, pharynx, or intestinal tract, brucellosis, tularemia, plague and other pasteurelloses, leptospirosis, lymphogranuloma inguinale, cholera, and rickettsiosis. Eight TCs are available to human medicine: TC, OTC, CTC, DC, MNC, DMC, MTC, and RTC. They are usually administered orally, although some are available as parenteral products e.g., RTC, which is provided only parenterally.

Administration of TCs in humans is not indicated in cases of pregnancy, hepatic or renal deficiency and for children below 8 years old. Most important adverse effects are gastrointestinal disorders, colorization of skin and teeth, hepatic insufficiency, Fanconi syndrome. TCs interactions with penicillins, theophylline, antacids, magnesium, iron or calcium supplements, carbamazepine and vitamin A should be avoided (21, 22).

Mechanism of Action and Resistance to Tetracyclines

Tetracyclines prevent bacterial growth by inhibiting protein synthesis. These drugs bind directly and specifically to the 30 S ribosomal subunit of protein S7 and block attachment of bacterial aminoacyl t-RNA to the acceptor sites on m-RNA, preventing the function of translation (addition of aminoacids to the growing peptide chain). Association of TCs with the ribosomes is a reversible process supported by various ribosomal proteins and certain genes of 16S-rRNA.

To interact with these molecule-targets, TCs need to penetrate through the bacterial cell and traverse cell membranes. In case of gram-negative bacteria, tetracyclines pass through the outer membrane with an energy-dependent process in the form of positively charged magnesium-antibiotic complexes and through the cytoplasmic membrane with passive diffusion as uncharged lipophilic molecules. Traverse of cytoplasmic membrane of gram-positive bacteria is also energy-dependent. Within the cytoplasm, TCs are likely to form complexes since the internal pH and divalent metal ion concentrations are higher than those outside the cell. Consequently the active form of these drugs that binds to the ribosome is a magnesium-tetracycline complex.

Weak inhibition of protein synthesis by 80 S ribosomes and poor accumulation of TCs by mammalian cells explains the absence of antieukaryotic activity. However, TCs inhibit protein synthesis by 70 S ribosomes in mitochondria and therefore some side effects can be caused in humans, but only in high doses (21).

During the last two decades, usage of tetracyclines in many infections has been limited due to the widespread growth of tetracycline resistant bacteria. Mainly there are two different tetracycline resistant mechanisms: (a) ribosomal protection, where certain bacterial genes attach and protect the ribosome the tetracyclines bind with and (b) efflux of the drug outside the cell through protein efflux-pumps. The ribosomal protection is developed above all from gram-positive bacteria and from the efflux system from gram-negative.

To overcome problems arising from the spreading of resistant bacteria, research started during the early 1990s to discover new TC members that exhibit activity against organisms resistant to older ones. Scientists confirmed that when the acyl group of 9-acylamido derivatives of minocycline was modified to include an N,N-dialkyamine group, the molecule not only contained antibacterial activities of other TCs but also exhibited activity against bacteria that have developed protection mechanisms. Glycycyclines (9-glycilamido derivatives of TCs) are steel in clinical trials, but many scientists believe they represent the future of TCs as antibiotics (2).

Pharmacokinetic Profile

Tetracyclines are rapidly absorbed from the gastrointestinal tract. The degree of absorption after oral administration, as well as bioavailability, is directly in proportion with the lipophilicity of the drugs. The least lipophilic and worst absorbed is OTC, while DC is the best-absorbed tetracycline, as it is characterized by high lipophilicity, insignificant interaction with food components and less tendency to chelate with divalent ions. In cases of parenteral administration, absorbance and bioavailability are strongly dependent on the site of administration. Intramuscular administration into the shoulder of animals is

preferred. Bioavailability of TCs to chickens varies from 1% for CTC, 9.4% and 23% for OTC and TC to 41% for DC, while on pigs is 4.8% for OTC, 19% for OTC, 25% for TC and 60% for DC. Bioavailability of OTC after parenteral administration is about 99.5%.

Tetracyclines are widely distributed in the body as far as they are lipid soluble, with higher levels in kidney and liver cells. Important part of the administered dosages are concentrated in liver, excreted in bile and reabsorbed from the intestines. Due to this enterohepatic circulation, small amounts of TC antibiotics are present in the blood for a long period of time. DC, which is the most lipophilic, shows the greatest degree of penetration. Due to TCs tendencies to form chelate complexes with divalent ions, significant amounts are accumulated in the bones and egg cells. TCs also bind strongly with lipoproteins, with this tendency decreasing from DC to CTC, TC and OTC. They undergo minimal or no metabolism before elimination, and they are excreted in urine and feces, either unchanged or in a microbiological inactive form (1–3).

Main Tetracycline Members

Chlortetracycline

Chlortetracycline was the first member of the tetracyclines class to be isolated in 1948 from *S. aureofaciens*. Its chemical name is 2-naphthacenecarboxamide, 7-chloro-4-(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a pentahydroxy-6-methyl-1,11-dioxo-onohydrochloride with molecular formula $C_{22}H_{23}N_2O_8Cl \cdot HCl$ and molecular weight 515.34. It is a yellow, crystalline, odorless powder, stable in air but slowly affected by light. CTC is sparingly soluble in water, soluble in solutions of alkali hydroxides and carbonates, slightly soluble in alcohol, practically insoluble in acetone, in chloroform, in dioxane and in ether. Most important degradation products are epi-, anhydro, epianhydro and iso-derivatives.

CTCs antimicrobial activity spectrum and the mechanism of action are common with that of other TCs as described before. Most important preparations used in veterinary medicine in the USA are aureomycin uterine tablets for acute uterine infections in cattle, pigs and sheep, aueromycin soluble powder USP for treatment of enteritis and pneumonia in calves and pigs and for cholera, synovitis and chronic respiratory disease in poultries, and chlortetracycline for medicated feed for enteritis, pneumonia, *E. coli* infections in calves, cattle, poultries, pigs and sheep (1–5).

Oxytetracycline

Oxytetracycline with chemical name 2-naphthacenecarboxamide, 4-(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,5,6,10,12,12a hexahydroxy-6-methyl-1,11-dioxo-dihydrate, molecular formula $C_{22}H_{24}N_2O_9 \cdot 2\,H_2O$ and

molecular weight 496.46, was isolated from strains of *Streptomyces rimosus* and named terramycin in 1950. It is a pale yellow to tan, odorless, crystalline compound. It is stable in air, but exposure to strong sunlight causes it to darken. It loses potency in solutions of pH below 2, and is rapidly destroyed by alkali hydroxide solutions. OTC is very slightly soluble in water, freely soluble in 3 M hydrochloric acid and in alkaline solutions, and sparingly soluble in alcohols. Epi-, anhydro, epianhydro, apo-, N-desmethyl- and iso-derivatives are OTC's main degradation products.

Most frequently administered preparations of OTC in veterinary medicine are: oxytetracycline hydrochloride soluble powder for enteritis and pneumonia in calves, cattle, pig and sheep as well as for synovitis, hexamitiasis and chronic respiratory disease in poultry, terramycin tablets USP for treatment of enteritis and pneumonia in calves, oxytetracycline injection USP (parenteral administration) for treatment of antinobacillosis, anaplasmosis, uterine infections in cattles and for Ehrlichiosis and arthritis in horses (1–5).

Tetracycline

Under the chemical name, tetracycline, (2-naphthacenecarboxamide, 4-(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-monohydro-chloride), the parent drug, was the third member to be produced after catalytic hydrogenolysis of aureomycin, but it can also be isolated from cultivations of *Streptomyces viridofaciens.* The molecular formula is $C_{22}H_{24}N_2O_8$ and the molecular weight 443.43. It is a yellow, odorless, crystalline powder; moderately hygroscopic, stable in air, but exposure to strong sunlight in moist air causes it to darken. It loses potency in solution at pH below 2, and is rapidly destroyed by alkali hydroxide solutions. It is soluble in water and in solutions of alkali hydroxides and carbonates, slightly soluble in alcohol and practically insoluble in chloroform and in ether. Most important degradation products are epi-, anhydro, epi-anhydro-derivatives.

Some important preparations are: TC uterine tablets for treatment of uterine infections in cows, TC boluses USP for enteritis and pneumonia in calves, TC hydrochloride capsules USP for Rocky Mountain fever and other susceptible bacterial infections in dogs, TC · HCl soluble powder USP for enteritis and pneumonia in calves and pigs and for cholera, synovitis and chronic respiratory disease in poultry and TC oral suspension USP for bacterial gastroenteritis in cats and dogs (1–5).

Doxycycline

Doxycycline is semisynthetically produced and not isolated from *Streptomyces*. Its chemical name is 2-naphthacenecarboxamide, 4-(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,5,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-monohydrate with molecular formula $C_{22}H_{24}N_2O_8 \cdot H_2O$ and

molecular weight 462.45. Doxycycline differs a lot from other TCs. It's a yellow, crystalline powder, stable in air but less sensitive to light exposure, soluble in water and in solutions of alkali hydroxides and carbonates, slightly soluble in alcohol and practically insoluble in chloroform and in ether. DC is the most stable tetracycline due to absence of hydroxyl group at R_3 substituent. 4-epi-DC is the only degradation derivative that is formed.

Important differences can be noticed in pharmacology and pharmacokinetics as DC is more completely absorbed from the gastrointestinal tract than the tetracyclines developed earlier and absorption is less likely to be affected by food or calcium or other divalent or trivalent metals. Doxycycline is also more lipid-soluble than other TCs. In dogs, it is eliminated primarily through intestinal excretion (1–5).

Future of Tetracyclines

One of the biggest problems with all antibiotic classes is that they are used much more frequently and sometimes in inappropriate ways, so that they have no effect on diseases and only increase the chance of causing resistant bacterial strains. Antibiotics like TCs used widely in livestock (TCs represented 66% of the total volume of antibiotics used for therapy in animals in the European Union) have led to an explosion of resistant strains that even effect the human population. Between approximately 50–90% of cattle, pig and poultry strains are found resistant to TCs. Glycylcyclines are the next generation of tetracyclines that will hopefully surpass resistance problems, representing the future of TCs as antibiotics (23).

Except from their bacteriostatic activity, in the early 1990s, tetracyclines have surprised researchers with unexpected properties that are entirely independent of their antimicrobial characteristics. It was found that TCs, especially DC, enhances natural inhibitors of matrix metalloproteinases. This family of enzymes are very important as they mediate the breakdown of various proteins (collagens, glycoproteins, proteoglycans), a procedure that is involved in many pathological processes responsible for a variety of disorders, like atherosclerosis, aortic aneurysms, corneal ulcers of the eye, inflammation, cancer development. The possibility of administration of TCs in some of these disorders as inflammatory or anticancer drugs is in a very early stage of study (2).

LEGISLATION FOR VETERINARY ANTIBIOTICS

As already mentioned, the use of tetracycline antibiotics has become a serious problem with regards to infectious diseases, as they are substances that leave residues e.g., in milk or meat, which when consumed can be directly toxic or

cause allergic reactions in some hypersensitive individuals. Even low-level doses of antibiotic in foodstuffs consumed for long periods can lead to problems regarding the spread of drug-resistant micro-organisms.

Food safety and consumer health protection is a very important target for the EU. Thus, a number of regulations and directives are occupied with food safety matters, some of which regulate the use of antibiotics in veterinary medicine and livestock, as well as the control of their residues in foods of animal origin. To ensure human food safety, the European Union has set tolerance levels for many drugs in animal products. Sales of animal health antibiotics (excluding coccodiostatics) in 1997 within the EU were estimated at a total of 5,093 tons, with therapeutics being 69% of the total amount and growth promoters 31%. Approximately two-thirds of the therapeutic antibiotics were tetracyclines (66%) (23).

The use of veterinary antibiotics, including tetracyclines, as feed-additives and growth promoting agents in husbandry is not allowed in EU countries with 70/524/EEC directive (24), whose application started in 1975. Furthermore, the EU, in order to assure consumer protection, has established maximum residue limits (MRLs) for veterinary medicinal products in foodstuffs, through council regulation 2377/90/EC (25). According to this regulation, all kinds of residues are classified in four categories. The first includes substances for which final MRLs have been set; the second one includes substances for which it was not considered necessary to establish MRL values; and the third and fourth categories include substances with provisional MRLs and with no tolerance levels, respectively.

Tetracyclines belong to the first category. The MRL of 100 μg/kg has been set for meat tissues and milk for all TC members, while for eggs, liver and kidney, MRLs established are 200, 300 and 600 μg/kg, respectively. These values include not only the parent TC but also the 4-epimer derivatives. Table 1 presents the MRL values set by the EU.

Another important regulation is council directive 93/23/EC (26), which defines the conditions and methods for the residue control of food producing animals and their primary products, such as meat, milk, eggs and honey. The directive divides all residues into two groups. Group A comprises substances with no tolerance level, and group B substances with a certain MRL set, including veterinary drugs. Through this directive, a network of laboratories, responsible for monitoring of residues, is organized in various levels (field, national and community laboratories).

In the EU, there are no defined standardized techniques and methods, which are used in obligatory fashion from laboratories in the residue control. Directive 96/23/EC sets forth performance characteristics, limits and criteria that should be fulfilled from techniques and methods chosen to be used.

The council decision 657/2002/EC (27), which supplements the directive 96/23/EC, defines with exactness the performance characteristics of analytical techniques and methods that can be used as confirmatory

Table 1. Allowed residue limits of TCs in different target tissues in the EU and USA

Pharmacologically active substances	Marker residue	Animal species	MRLs (μg/kg or ppb)	Target issues
European Union				
Tetracycline	Sum of parent drug and its epimer	All food-producing species	600	Kidney
Oxytetracycline			300	Liver
Chlortetracycline			100	Muscle
			100	Milk
			200	Eggs
Pharmacologically active substances	**Marker residue**	**Animal species**	**Tolerances (mg/kg or ppm)**	**Target issues**
USA				
Tetracycline	Sum of the three TCs	Beef cattle, dairy cattle, calves, swine, sheep, chickens, turkeys, catfish, lobster, and salmonids	12	Kidney
Oxytetracycline			6	Liver
Chlortetracycline			2	Muscle
			3	Milk
			12	Fat

methods in residue control of foodstuffs of animal origin. These techniques are liquid chromatography (LC) and gas chromatography (GC) coupled with mass or infrared spectroscopy (MS or IR) for substances included in group A, while for substances in group B, LC with ultraviolet, diode array and fluorescence detectors can also be used. Specificity, precision, robustness, stability, recovery, linearity, reproducibility, limit of decision and limit of detection are some of the performance criteria that should be examined (28).

Unlike in the European Union, veterinary drugs are regulated in the United States as two distinct assemblies, one including drugs for therapeutic purposes and one for growth-promotion purposes. So, certain antimicrobials, including tetracyclines, are approved by FDA (Food and Drug Administration) for growth promotion in "subtherapeutic" doses and infections therapy (29). The Union of Concerned Scientists (UCS) estimates that approximately 24 million pounds, or 70% of antibiotics in the United States are routinely placed in the feed and water of healthy livestock. More than half of these drugs are identical or nearly identical to antibiotics routinely

used for humans. The Institute of Medicine (IOM) estimates that about 20 million pounds of antimicrobials are given to farm animals each year and about 80% of these are used for nontherapeutic reasons. Tetracyclines possess about 30% of the total use of antibiotics in animal husbandry (30).

Concerns about antibiotic overuse in veterinary medicine and animal livestock are widespread and well founded in the United States the last decade, as the problem of resistant bacterial strains increases. In recent years, Federal Legislation was introduced to deal with this problem. "The Preservation of Antibiotics for Human Treatment Act of 2002," has been introduced in the U.S. Senate and House, in order to restrict antibiotics use as additives to animal feed. This is also supported by the American Medical Association, the American Public Health Association, the American College of Preventive Medicine and numerous other health organizations. This decision would withdraw federal approval for nontherapeutic agricultural use of eight named antimicrobials or classes of antimicrobials: penicillins, tetracyclines, macrolides, lincomycin, bacitracin, virginiamycin, aminoglycosides, and sulfonamides (29).

To protect the public, the Food and Drug Administration (FDA) and the U.S. Department of Agriculture (USDA), through its Food Safety and Inspection Service (FSIS), cooperate in a program to monitor the use of these animal drugs, identify improper use and take action to prevent future illegal use by a producer. FDA establishes tolerances to include a safety factor to assure that the drug will have no harmful effects on consumers of the food product. The Agency first determines the level at which the drug does not produce any measurable effect in laboratory animals. From this, an acceptable daily intake (ADI), the drug tolerance and withdrawal times are determined so that the concentrations of drug residues in edible tissues are below the ADI. Depending on the drug, "safety factors" of between 100-fold to 2,000-fold are included in the calculations used to set the tolerances (31).

According to the valid legislation, which was last revised at 1998, tolerances established for TCs are: (a) acceptable daily intake (ADI). The ADI for total residues of tetracyclines including chlortetracycline, oxytetracycline, and tetracycline is 25 μg per kg of body weight per day; (b) for beef cattle, dairy cattle, calves, swine, sheep, chickens, turkeys, catfish, lobster, and salmonids, tolerances are established for the sum of residues of the tetracyclines including chlortetracycline, oxytetracycline, and tetracycline, in tissues and milk as follows: 2 ppm in muscle, 6 ppm in liver, 12 ppm in fat and kidney, 0.3 ppm in milk (32, 33).

The USDA regularly monitors tissue samples from slaughtered animals for many individual chemical compounds including: antimicrobials (sulfonamides, penicillins, streptomycin, tetracyclines, erythomycin, neomycin, gentamicin, and tylosin), other drugs (coccidiostats, growth promoters, antiparasitics) and pesticides and industrial chemicals (chlorinated hydrocarbon pesticides, organophoshates). The main goal of the USDA's National Residue Program is to protect consumers from adulterated meat and poultry

products. The program is divided into three major activities: Monitoring, Surveillance, and Exploratory phases (31). Maximum residue limits (MRLs) in Japan have been established for OTC, TC, and CTC of 0.2–1.2 μg/g in edible animal tissues being their sum (34).

ANALYTICAL METHODOLOGY

Figure 3a shows the frequency at which the different analytical techniques are applied t o the TC's analysis. HPLC is the technique that is used at most (~80%) for the determination of tetracyclines antibiotics in foodstuff of animal origin. In the following paragraphs, information on analytical methodology as well as sample preparation procedures is cited.

HPLC Methods-Chromatographic Conditions

A common problem in the chromatographic determination of tetracyclines is that due to the presence of two ketone groups they can readily chelate to metal ions. Thus they have a tendency to bind irreversibly to the silanol groups in silica-based LC stationary phases, resulting in peak tailing. This problem has been overcome either by adding oxalic acid to the mobile phase or by using polystyrene–divinylbenzene LC columns.

As shown in Figure 3b, from the analytical columns used in applications reported in the literature, 19% are polymeric, 58% are C_{18}, 21% are C_8 and only in one paper is a C_{16} column used. Within the 48 reviewed papers that apply HPLC, gradient elution is applied to 12 papers, while isocratic elution is used in 36. By taking into consideration only the papers that separate more than 2 analytes which are 39, only 10 involve a gradient program, and the rest apply isocratic elution.

Almost half of the proposed methods involve the use of oxalic acid in the mobile phase, 18% use phosphate buffer, 10% formic acid and 10% EDTA (as illustrated in Figure 3c). An internal standard is used only in the 15% of the reviewed papers. The chromatographic conditions found in the literature for the determination of tetracyclines are cited in brief in the next paragraphs. Table 2 provides all information extracted from the reviewed papers. An end-capped LiChrospher RP-8, 5 μm analytical column was used for the determination of TC, CTC, OTC and DC, with 7% acetic acid-ACN (35:65 v/v) as the mobile phase (35).

A LiChrospher RP-18, 5 μm, analytical column was used with 0.02 M orthophosphoric acid (pH 2.3) and ACN (76:24 v/v) for OTC determination (36). The same column has been used with 0.01 M oxalic acid–ACN (50:50 v/v) for the determination of TC and ETC, with 1% H_3PO_4/ACN (65:35 v/v) for ATC-EATC (37) and with 1% H_3PO_4/ACN (75:25 v/v) for OTC (38). The use of 0.01 M oxalic acid–ACN (60:40 v/v) was applied for

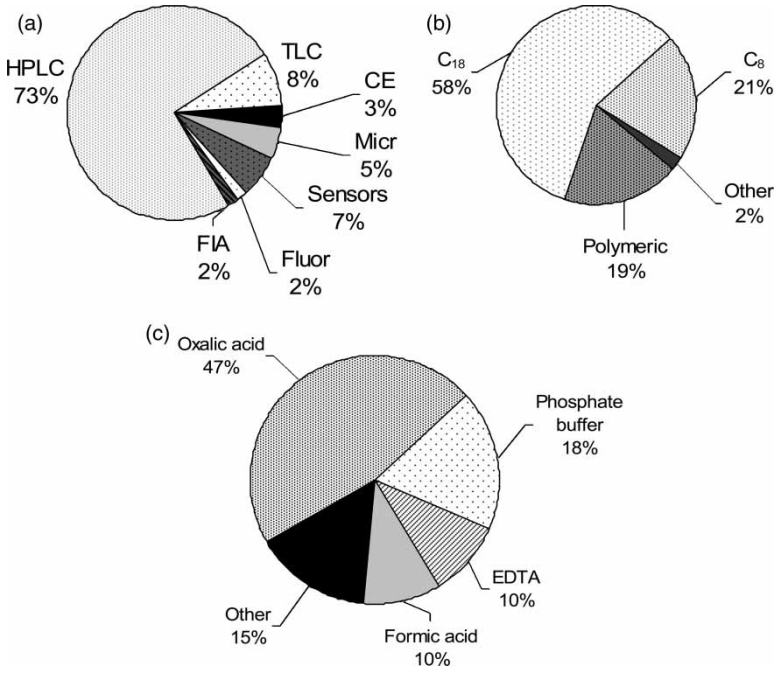


Figure 3. (a) Percentage distribution of analytical techniques used for tetracyclines determination. (b) Analytical columns used in TCs analysis. (c) Mobile phases used in TCs analysis.

the separation of TC, CTC, ETC and ECTC (38). A mixture of H_2O/ACN/70% $HClO_4$, 0.6 mM Na_2EDTA, 5 mM oxalic acid (699:298.5:2.5 v/v/v) (pH 2.5) was used for DC determination using DMC as internal standard (39). The separation of OTC, TC, CTC, epimers (40) as well as TC, CTC and epimers (41) was achieved on this column with 0.01 M oxalic acid–ACN (50:50 v/v) as the mobile phase.

A Hypersil ODS, 5 μm, analytical column operated at 22°C, was used with 0.05 M sodium dodecyl sulphate/5% 1-butanol (pH 3) for the separation of OTC, TC, CTC, DC, MNC, and epimers (42), as well as with 0.05 M NaH_2PO_4 (pH 2.2)/ACN (78:22 v/v) for the determination of TC, OTC,CTC, DC and MTC (43). A mixture of ACN/THF/0.01 M oxalic acid (pH 3) (15:3:82 v/v/v) was used for the determination of OTC using TC as internal standard (44). TC was analysed on the same column with: ACN/1 M imidazol buffer with 10 mM Na_2EDTA and 50 mM magnesium acetate (10:90 v/v) (45).

An end-capped LiChrosorb RP-18, 10 μm, analytical column was used with 0.05 M diethanolamine (pH 7.3 with phosphoric acid) with a mobile phase consisted of 0.001 M Na_2EDTA/isopropanol (86:14 v/v) for the separation of TC, OTC and CTC (46) and with 0.01 M oxalic acid/CH_3OH /ACN

Table 2. Overview of HPLC methods for the determination of tetracyclines in foodstuffs of animal origin

Analytes	Sample	Sample preparation	Chromatography	Detection	Linear range	Recovery (%)	Ref
TC, OTC, CTC, DC	Liver	Extr. with 0.1 M Na_2-EDTA/McIlvaine buff. (pH 4), homog., centr., filt., residue extr. $\times 2$ with Na_2EDTA/McIlvaine buff. SPE of combined extracts on C_{18}Baker cartridges	LiChrosorb RP-18, 10 μm El: 0.01 M oxalic acid/CH_3OH/ACN (2.5:1:1.5 v/v/v), pH 2 FR: 2 mL/min	UV 350 nm	LOD: 0.05 ng/μL for OTC, TC 0.1 ng/μL for CTC, DC	Chicken: OTC: 86, TC: 81, CTC: 81, DC: 72; Bovine kidney: OTC: 91, TC: 86, CTC: 92, DC: 85; Pork kidney: OTC: 89, TC: 86, CTC: 86, DC: 72; Bovine muscle: OTC: 92, TC: 87, CTC: 93, DC: 72; Pork muscle: OTC: 93, TC: 90, CTC: 90, DC: 72; Milk: OTC: 87, TC: 83, CTC: 85, DC: 72; Egg: OTC: 82, TC: 80, CTC: 88, DC: 72; Yellow tail: OTC: 91, TC: 88, CTC: 93, DC: 72	(47)
TC, OTC, CTC	Tissues	Extr. with 0.02 N HCl-0.1 M Na_2EDTA, heat 98°C, homog., centr., cool in ice, filt., add. of 0.002 N HCl, heat 98°C, cool, centr., add. of CH_2Cl_2, homog., filt. evap., recon. with CH_3OH	LiChrosorb RP-18, 10 μm, endc.El: 0.05 M dethanolamine (pH 7.3 with phosphoric acid) with 0.001 M Na_2EDTA/IPA (86: 14 v/v) FR: 1.5 mL/min	UV 254 nm		OTC: Beef liver-muscle: 50–70; Beef kidney: 75–85; CTC: Turkey liver: 13–21; Turkey muscle: 23–32; TC: Beef liver: 41–51; Muscle: 27–34; Kidney: 34–41; Turkey liver-muscle: 70–95	(46)

(*continued*)

Table 2. Continued

Analytes	Sample	Sample preparation	Chromatography	Detection	Linear range	Recovery (%)	Ref
TC, OTC, CTC, DC, MTC, DMC, MNC	Honey	Extr. with 0.1 M Na_2-EDTA/McIlvaine buff. (pH 4), homog., centr., filt., SPE C_{18}, SPE, -COOH cartridges	Chemosorb C_8, 3 μm; El: 0.01 M oxalic acid/CH_3OH/ACN (16:3:2 v/v/v), pH 3 FR: 1 mL/min	UV 350 nm		OTC: 92.8, TC: 91.7, CTC: 99.6, MTC: 83.7, DC: 97.0, DMC : 87.8, MNC : 85.7	(71)
CTC	Poultry/ swine feeds	Extr. with 4 N HCl-acetone-H_2O (1: 8: 6v/v/v), homog., centr., filt., SPE C_{18}Clean-up for CTC: add. of water saturated with CH_2Cl_2, homog., add. of CH_2-Cl_2saturated with water, homog., centr., filt. of aqueous layer	Nova-Pack C_{18}El: 0.01 M oxalic acid/CH_3OH/ ACN (3.5:1.5:1 v/v/v), pH 2 with HCl (60:40)FR: 1 mL/min	UV 370 nm		Premix: 94.6; Ruminant feed: 74.8–83.2; Poultry/swine feed: 70.5–82.9	(57)
TC, OTC, CTC,	Milk	Milk samples centr. Using M.W. cutoff system, SPE, C_{18} varian	Novapack C_{18}, 5 μm; El: 0.05 M oxalic acid/ ACN/CH_3OH (30:50:20 v/v/v) and 0.05 M oxalic acid/ ACN (40:60 v/v). FR: 0.5 mL/min.	PB-MS/MS	LOD: Standards 20 ng, Milk samples: 100 ng/ mL		(59)

OTC	3 Species of marine shellfish	Extr. with 0.1 M Na_2-EDTA/McIlvaine buff. (pH 4), vortex, centr., filt., SPE C_{18}, recon. 0.1 M perchloric acid, centr., filt.	LiChrospher RP-18, 5 μm; El: 0.02 M orthophosphoric acid (pH 2.3)–ACN (76:24 v/v) FR: 1.2 mL/min	UV 355 nm	LR: 0.1–1.5 μg/g	79.8	(36)
TC, OTC, CTC	Bovine, porcine muscle	Extr. with 0.1 M Na_2-EDTA/McIlvaine buff. (pH 4) (4°C), homog., centr. (4°C), add. Of TCA (1 g/mL), homog., filt., SPE, Sep-Pack C_{18}, endc.	Nova-Pack C_{18}, 4 μm; El: 0.01 M citric acid-0.01 MK_2HPO_4/ACN (72:28 v/v) with 0.005 M TMA chloride, 0.1 g/LEDTA. FR: 2.4 mL/min	UV 365 nm	LOD: 10 ng/g	Calf: OTC: 70, TC: 58, CTC: 50	(56)
TC, OTC, CTC, DC, MTC, DMC, MNC	Milk	Centr. (10°C), lower layer Extr. with succinate buff. (pH 4), homog., centr., filt., MCAC, ultrafilt.,	PLRP-S 5 μm; El: 0.01 M oxalic acid/CH_3OH/ACN. Grad.el.: 0–1 min 100:0:0 v/v/v, from 1–5 min to 70:8:22 v/v/v, from 5–11 min 70:8:22 v/v/v. FR: 1 mL/min	UV 355 nm	LOD: 0.42–1.27 ng/mL	OTC: 82–91, TC: 70–79, CTC: 66–80, MTC: 65–75, DC: 72–79, DMC: 69–81, MNC: 63–37	(79)
TC, OTC, CTC, DC, MTC, DMC	Kidney	Extr. with 0.05 M succinate buff./3.7% Na_2-EDTA (pH 4), vortex, centr., filt., MCAC, SPE (C_{18})	Chromspher C_8, 5 μm; El: 0.01 M oxalic acid–ACN (80:20 v/v)FR: 0.8 mL/min	UV 365 nm	LR: 0.03–3 μg/mL; LOD: 4 μg/mL for OTC and TC 12 μg/mL for CTC, DC, MTC, DMC	OTC: 59–73, TC: 44–53, CTC: 56–67, MTC: 50–71, DC: 48–77, DMC: 49–65	(69)

(*continued*)

Table 2. Continued

Analytes	Sample	Sample preparation	Chromatography	Detection	Linear range	Recovery (%)	Ref
TC, OTC	Tissue samples	Extr. with 10 mM, Na_2EDTA/McIlvaine (pH 4)/CH_3OH (3:7), centr., residue re-Extr. and extr. combined and evap. to 50 mL. SPE with C_{18} and SCX cartridges, Eluate adj. pH to 13 with 10 M NaOH.	ODP-50, 5 μm, 40°C; El: Sorensen buff. (pH 12)/ACN (90:10v/v).FR: 1 mL/min	FL (from λ_{exc} 350 nm, λ_{em} 420 nm to λ_{exc} 374 nm, λ_{em} 506 nm in 7 min)	2–40 ng for OTC1–40 ng for TC	Bovine: OTC: 80–82, TC: 79–87; Swine: OTC: 74–86, TC: 72–74; Chicken: OTC: 63–75, TC: 62–70	(81)
OTC	Fish Feed	Extr. with ethyl acetate, addit. of 0.01 M EDTA, evap. of organic layer, centr., SPE C_{18}	Hypersil ODS 5 μm ACN/THF/0.01 M oxalic acid (pH 3) (15:3:82 v/v/v). FR: 1 mL/min I.S.: TC	UV 365 nm	0.1–6.4 μg/g LOD: 10 ng/g	93.6–98.1	(44)
TC, OTC, CTC, DMC	Animal tissues	Extr. with 0.1 M succinate buff. (pH 4), homog., centr., filt., dilut. With SEPSA sol. SPE C_8 or XAD-2 resins and on-line MCAC	PLRP-S 5 μm; El: A: 0.1 M NaH_2PO_4–0.01 M Citric acid–0.01 M EDTA. B: ACN-CH_3OH-A (25:10:65 v/v/v) Grad. El. from 100% A to 100% B in 10 min and then is. el.100% B for 10 min FR: 1 mL/min	UV 350 nm	LOD: OTC, TC: 10 μg/kg CTC, DMC: 20 μg/kg	SPE C_{18}: Sheep liver: OTC: 58–83, TC: 50–86, CTC: 36–65, DMC : 40–60XAD-2: Cattle kidney: OTC: 64–69, TC: 61–89, CTC: 59–88, DMC: 44–58	(77)

CD	Turkey plasma	Extr. with 6% w/v ascorbic acid and phosphate sulphite buff. (pH 6) and ethyl acetate, vortex, centr. Addit. in org. layer 0.2% w/v ascorbic acid, evap., recons. in MP, add. of hexane, centr., aqueous layer	LiChrospher RP-18, 5 μm; El: H_2O/ACN/70% $HClO_4$, (699:298.5:2.5 v/v/v), 0.6 mM Na_2EDTA, 5 mM oxalic acid (pH 2.5). FR: 1 mL/minI.S.: DMC	UV 350 nm	0.2–600 μg/mL; LOD: 0.2 μg/mL	66.1–72.3	(39)
OTC, EOTC, α-apoOTC, β-apoOTC	Porcine muscle, water	Extr. with 0.1 M Na_2-EDTA/McIlvaine buff. (pH 4), homog., centr., filt., SPE C_{18} Bond Elut.	Inertsil C_8, 5 μm; El: 0.1 M ammonium acetate (pH 3)/ACN/THF (72.5:12.5:15 v/v/v)FR: 1 mL/min	DAD 350 nm for OTC, EOTC 250 nm for α- and β-apoOTC	OTC: 40 ng/mL, EOTC; 20 ng/mL, α-apoOTC:50 ng/mL, β-apoOTC: 140 ng/mL	Water: OTC: 90–99, EOTC: 66–83, α-apoOTC: 66–83, β-apoOTC: 58–84; Muscle: OTC: 70–84, EOTC: 49–62, α-apoOTC: 26–40, β-apoOTC: <20	(68)
TC	Swine tissues	Extr. with ACN/1 M imidazol buff. with 10 mM Na_2EDTA and 50 mM magnesium acetate (15: 85v/v) and hexane, vortex, centr., evap., ultrafilt	Hypersil ODS 5 μm; El: ACN/1 M imidazol buff. with 10 mM Na_2EDTA and 50 mM magnesium acetate (10: 90v/v). FR: 1 mL/min	FL (λ_{exc} 380 nm, λ_{em} 520 nm)	1.25–20 ngLOD: 0.04 μg/gLOQ: 0.05 μg/g	Muscles: 58–67; Kidney ; 57–66	(45)

(*continued*)

Table 2. Continued

Analytes	Sample	Sample preparation	Chromatography	Detection	Linear range	Recovery (%)	Ref
TC, OTC, CTC, DC	Eggs, animal tissues	Extr. with 0.1 M sodium succinate buff. (pH 4), homog., add. of 20 mL CH_3OH, votr., centr., filt.MCAC and extr. with SDB-RPS cation exchange membranes	PLRP-S 8 μm; El: 0.01 μ oxalic acid–ACN. Grad. El. from 85:15 v/v to 60:40 v/v. FR: 1 mL/min	FL (λ_{exc} 406 nm, λ_{em} 515 nm). Post-column addition of 5% (m/v) zirconyl chloride octahydrate	OTC: 2–1000 μg/kg, TC: 3–1000 μg/kg, CTC: 4–1000 μg/kg, DC: 5–1000 μg/kg	Eggs: OTC: 50 TC: 45, CTC: 37, DC: 53; Pork kidney: OTC: 60, TC: 45, CTC: 46, DC: 58; Pork muscles: OTC: 69, TC: 54, CTC: 53, DC: 66 Bovine liver: OTC: 58, TC: 39, CTC: 42, DC: 53	(75)
TC, OTC, CTC	Animal tissues, Milk, cheese	Extr. with 0.1 M Na_2-EDTA/McIlvaine buff. (pH 4), vortex, centr., filt., residues twice re-extr. combined super. Extr. with SPE, C_{18} cartridges or for milk MSPD, C_{18} sorbent	LiChrosorb RP-18El: 0.01 M oxalic acid–CH_3OH–ACN (65:17.5:17.5 v/v/v) FR: 1.4 mL/min	UV-DAD	SPE: 15–22 ng/μL. MSPD: 30 ng/μL	Tissues: OTC : 86–90, TC: 62–67, CTC: 52–66. Cheese: OTC : 52–65, TC: 45–47, CTC: 59–66. Milk (SPE)OTC : 53, TC: 52, CTC: 51(MSPD), OTC : 79–93, TC: 74–89, CTC: 56–68	(48)
OTC, TC CTC	Tissues	Extr. with Glycine-HCl buff., homog., add ammonium sulphate, homog., centr., filt., residue re-Extr., SPE, cyclohexyl endc.	Prodigy C_{18}, 2 μm. El: A: CH_3CN/0,01 μ oxalic acid, 0,01 mM EDTA, 0.04% HFBA (10:90) B: CH_3CN/0.01 μ oxalic acid, 0.01 mM EDTA, 0,04% HFBA (90:10). Grad. El.: 0 min 10%B, 10 min 50%B, 11 min 90%B, 14 min 90%B, 16 min, 10%B. FR: 1 mL/min	APCI-MS/MS	Muscles: 10 ng/g; Kidney: 20 ng/g	Muscle: OTC: 58–96, TC: 52–87, CTC: 60–99; Kidney: OTC: 57–112, TC: 61–101, CTC: 61–83	(62)

OTC, TC CTC	Bovine tissues	Extr. with 0.1 M Na_2-EDTA/McIlvaine buff. (pH 4) × 3, homog., centr., filt., SPE C_{18} Bond Elut.	TSK Gel Super C_8, 2 μm.El: ACN/0.05% TFA (1:4 v/v). FR: 0.5 mL/min	ESI-MS/MS		Liver: OTC: 73.8, TC: 70.3, CTC: 61.7, DC: 55.5; Kidney: OTC: 75.5, TC: 71.3, CTC: 64.9, DC: 58.1; Muscle: OTC: 79.0, TC: 79.1, CTC: 73.0, DC: 69.9	(73)
TC, OTC, CTC, DMC	Eggs, animal tissues	Extr. with 1 M citrate buff. pH 4 (for chicken liver only) or 5 and ethyl acetate, homog.,vortex, centr., residue re-extr. × 2 with ethyl acetate (2 × 12 mL) combined extr.: filt., evap. to dryness (40°C) and recon. with CH_3OH. Direct inj. and on-line MCAC on a Anagel-TSK precolumn. Column switching.	PLRP-S 5 μm; El: A: 0.1 M NaH_2PO_4– 0.01 M Citric acid– 0.01 M EDTA.B: ACN- CH_3OH-A (25:10:65 v/v/v) Grad. El.from 100% A to 100% B in 10 min. Is. El. 100% B for 10 min FR: 1 mL/min	UV 350 nm	OTC: 10–100 μg/kg; TC, DMC: 16–100 μg/kg. CTC: 20–10 μg/kg.	Eggs: OTC: 91–104, TC: 64–59, CTC: 59–77, DMC: 66–105	(74)
MNC	Bovine tissues and serum	Tissues: Extr. with H_2O, homog., centr. SPE C_{18}Serum add. of ACN, centr., filt., SPE C_{18}	μ-Bondapak C_{18} 5 μm; El: 100% CH_3OH. FR: 1 mL/min	UV 350 nm	LOD: 100 ng/μL	Tissues: 61; Serum: 54	(52)

(*continued*)

Table 2. Continued

Analytes	Sample	Sample preparation	Chromatography	Detection	Linear range	Recovery (%)	Ref
OTC, TC, CTC	Animal tissues	Extr. with 1 M oxalic acid and ACN, homog., addit. of oxalate buff., homog., centr., filt., SPE, SDB cartridges	PLRP-S 5 μm; El: 0.01 M oxalic acid–ACN (50:50 v/v)FR: 0.9 mL/min	UV 360 nm	20–1000 ng/g; LOD: Tissues: OTC, TC: 10 ng/g, CTC: 15 ng/g. Kidney, liver: OTC, TC: 20 ng/g, CTC: 25 ng/g.	Bovine: Tissues: OTC: 83, TC: 82; CTC: 82; Liver: OTC: 85, TC: 83CTC: 82; Kidney: OTC: 84, TC: 82, CTC: 81; Pork:Tissues: OTC: 83, TC: 83, CTC: 84; Liver: OTC: 84, TC: 82, CTC: 81; Kidney: OTC: 84, TC: 85, CTC: 83	(78)
TC, OTC, CTC, DC	Pork and chicken meat	Extr. with 0.1 M sodium succinate buff. (pH 4), homog., add. of 20 mL CH_3OH, votr., centr., filt. MCAC and Extr. with SDB-RPS cation exchange membranes	PLRP-S 8 μm; El: 0.01 M oxalic acid–ACN. Grad. El. from 85:15 to 60:40 v/v in 16 min. FR: 1 mL/min, IS : DMC; For MS: Symmetry C_{18} 5 μm, El: A: CH_3OH/0.069% TFA (30:70) Grad. El. from 100% A to 100% CH_3OH. FR: 0.3 mL/min	FL (λ_{exc} 350 nm, λ_{em} 420 nm). Post-column addition of 5% m/v zyrconyl chloride octahydrate; ESI-MS/MS	LOD: 0.42–1.38 μg/kg		(49)
TC, OTC, CTC, DC, DMC, MNC	Milk, shrimps	Milk samples Extr. with acetic acid, homog., centr. Shrimp samples Extr. with 0.1 M succinic acid (pH 4), homog., centr., MCAC, SPE, supelclean ENVI	PLRP-S, 5 μm; El: 0.01 M oxalic acid/CH_3OH (58: 42 v/v)FR: 0.5 mL/min	PB-MS		~50	(80)

TC, OTC, CTC, DC	Tissues	Extr. with 0.1 M Na_2-EDTA/McIlvaine buff. (pH 4) × 3, homog., centr., filt., SPE C_{18} Bond Elut.	Bakerbond C_8, 5 μm (30°C). El: 0.01 M oxalic acid/CH_3OH/ACN (55: 27:18 v/v/v), pH 2FR: 1 mL/min	APCI-MS/MS	LOD: 0.001 ppm OTC, TC 0.004 ppm CTC, 0.002 ppm DC	Bovine liver: OTC: 61–67, TC: 72–78, CTC: 65–72, DC: 60–65; Kidney: OTC: 68–77, TC: 67–78, CTC: 66–70, DC: 64–72; Muscle: OTC: 67–83, TC: 74–81, CTC: 63–73, DC: 69–72; Swine liver: OTC: 67–73, TC: 68–78, CTC: 63–79, DC: 62–70; Kidney: OTC: 72–80, TC: 77–80, CTC: 70–78, DC: 68–70; Muscle: OTC: 80–82, TC: 74–80, CTC: 67–84, DC: 70–72	(72)
TC, OTC, DMC, MNC, ATC, EATC, MTC	Milk	Stirring and heating samples (65°C), sat.with KCl (only for water samples), SPME, 50 μm Carbowax/templated resins	Purosphere RP-18, 3 μm; El: (A) ACN with 0.2% HCOOH and (B) H_2O with 0.2% HCOOH. Grad. El.: 0 min 16: 84 v/v, 1.5 min 24: 76 v/v, 2 min 30:70 v/v, 7 min 50:50 v/v. FR: 1 mL/min	ESI-MS/MS	LOD: TC: 14, OTC: 30, DMC: 9, MNC: 40, ATC: 4, EATC: 4, MTC: 6 ng/mL; Milk: 100 ppb	TC: 25, OTC: 20, DMC: 57, MNC: 17, ATC: 92, EATC: 31, MTC: 60	(58)

(*continued*)

Table 2. Continued

Analytes	Sample	Sample preparation	Chromatography	Detection	Linear range	Recovery (%)	Ref
TC, CTC, ETC, ECTC	Animal bones	Extr. with 1 M HCl for 10 h, homog., centr., residue re-extr. with 1 M HCl. Super. filt., XAD-2 resins	LiChrospher RP-18, 5 μm; El: 0.01 M oxalic acid–ACN (60:40 v/v) FR: 0.4 mL/min	UV-DAD, 360 nm.			(38)
TC, OTC, CTC, ICTC, N-DMCTC, N-DMOTC, 4-N-DMICTC	Eggs	Extr. with 0.3 M sodium citrate buff., vortex, centr., on-line dialysis, on-line SPE, PLRP – S 70 μm	Supershere RP-8, 4 μm; El: 0.01 M or 0.005 M oxalic acid–ACN (50:50 v/v). FR: 1 mL/min. For LC-MS/MS El: ACN/0.5% HCOOH (40:60 v/v). FR: 0.4 mL/min.	UV (310 and 360 nm) FL (λ_{exc} 350 nm, λ_{em} 420 nm). Post-column additionof 0.4 M glycine buff. pH 12. ESI-MS/MS	Whole egg: 50–600 μg/kg, Egg white and yolk: 100–600 μg/kg, Serum: 200–60 μg/kg.	Whole egg: OTC: 97, TC: 96, CTC: 91, ICTC: 98; Egg white: OTC: 96, TC: 96, CTC: 95, ICTC: 99; Egg yolk: OTC: 94, TC: 94, CTC: 90, ICTC: 95; Serum: OTC: 95, TC: 96, CTC: 93, ICTC: 97	(70)
TC, OTC, CTC.	Animal tissues	Extr. with H_2O, homog., addit. of ACN, vortex, addit. of 0.1 M H_3PO_4, homog., centr., filt., L/L Extr. with hexane and CH_2Cl_2, evap. to 2 mL, filt	Phenomenex ODS 3; El: 4 mM oxalic acid, 4 mM sodium oxalate, 4 mM sodium dodecyl-sulfonate/ACN (70:30 v/v).FR: 1 mL/min	UV 370 nm		Pork tissues: OTC: 93, TC: 109, CTC: 98; Pork kidney: OTC: 94, TC: 93, CTC: 110; Pork liver: OTC: 99, TC: 91, CTC: 95; Bovine tissues: OTC: 91, TC: 101, CTC: 92; Bovine kidney: OTC: 98, TC: 105, CTC: 92	(50)

OTC	Animal bones	Extr. with 1 M HCl for 10 h, homog., centr., residue re-Extr. with 1 M HCl. Super. filt., XAD-2 resins	LiChrospher RP-18, 5 μm; El:1% H_3PO_4/ACN (75:25 v/v)FR: 0.7 mL/min	UV-DAD 360 nm	0.05 mg/kg		(38)
TC, ETCATC, EATC	Animal feed	High temperature treatment after homog. in 133 and 100°C for 20, 30 and 45 min. Extr. with 1 M HCl, homog., centr., filt., adj. pH to 4 with NaOH, centr., MCAC, SPE, C_{18}	LiChrospher RP-18, 5 μm; El: 0.01 M oxalic acid–ACN (50:50 v/v) for TC-ETC and 1%H_3PO_4/ACN (65: 35 v/v) for ATC-EATC FR: 0.9 mL/min. For LC-MS/MSPuresil C_{18} 5 μm; El: 0.5% HCOOH/ACN (23: 77)	UV-DAD 360 nm; ESI-MS/MS		TC: 65, ETC: 63; ATC: 71, EATC: 65	(37)
OTC, TC, CTC, epimers	Animal feed and bones	Extr. with succinate buff. (pH 4) or 1 M HCl, homog., centr. filt., MCAC, SPE C_{18}.	LiChrospher RP-18, 5 μm; El: 0.01 M oxalic acid–ACN (50:50 v/v) FR: 0.9 mL/min	UV-DAD 360 nm	LOD: OTC, TC: 0.5 and CTC: 1 μg/kg	OTC, TC: 65; CTC: 50	(40)
TC, CTC, epimers	Animal bones	High temperature treatment after homog. in 133 and 100°C for 20,30 and 45 min. Extr. with succinate buff. (pH 4) or 1 M HCl, homog., centr., filt., MCAC, SPE, C_{18}	LiChrospher RP-18, 5 μm; El: 0.01 M oxalic acid–ACN (50:50 v/v) FR: 0.9 mL/min	UV-DAD 360 nm	LOD: TC: 1.5 and CTC: 5 μg/kg	TC: 67; CTC: 52	(41)

(*continued*)

Table 2. Continued

Analytes	Sample	Sample preparation	Chromatography	Detection	Linear range	Recovery (%)	Ref
OTC, TC, CTC, DC, MNC, epimers	Animal feeds	Extr. with 1: 1 ACN/H_2O sol. (pH 3 with 0.01 M citric acid), vortex, centr., filt.	Hypersil ODS 5 μm, 22°C; El: 0.05 M sodium dodecyl sulphate/5% 1-butanol (pH 3)FR: 1 mL/min	DAD 364 nm	5–100 μg/g; LOD: 0.1–0.4 μg/g	OTC: 52–80, TC: 64–88, CTC: 66–89, MNC: 84–95, DC: 74–89	(42)
TC, OTC, CTC, DC	Bovine milkand muscle	Two step chemical extr.: 1. with 20% TCA, vortex, 2. with McIlvaine buff., vortex, centr., SPE, HLB Oasis	Hypersil C_8 5 μm; El: 0.01 M oxalic acid–CH_3OH–ACN(60:25:15, v/v/v)FR: 1 mL/min	UV 365 nm	0.1–5 ng/mL	Milk: OTC: 91.9–93.3, TC: 80.3–84.1, CTC: 84.0–84.9, DC: 85.3–86.9; Muscles: OTC: 84.3–84.9, TC: 81.8–84.6, CTC: 83.1–89.5, DC: 84.2–84.9	(65)
TC, OTC, CTC, DC	Milk	Dil. with water 1:5 v/v; SPE syringe, ISOLUTE-C8	LiChrospher RP-8 (endc.) 5 μm El: 7% acetic acid-ACN(35:65 v/v) FR: 1 mL/min	UV 267 nm	0.1–1 μg/mL	Milk: OTC: 91.2–93.5, TC: 90.8–95.5, CTC: 80.8–87.3, DC: 81.2–88.1	(35)
TC, OTC, CTC and their 4-epimers	Eggs, environmental samples	Extr. with 1 M citrate buff. (pH 5), homog., addit. of ACN, centr. Residue re-Extr. with H_2O and ACN. Combined super. evap., recon. with CH_3OH.	Puresil C_{18}El: 0.5% HCOOH, 1 mM ammonium oxalate/ACN (80:20 v/v).FR: 1 mL/min	Microb. assay: *S. aureus* in brain –heart broth middle (pH 5.9), photometrical det. 595 nm ESI-MS/MS	LOD: MS: 20 μg/kg; Micr: 150 μg/kg	MS: OTC: 71–98, TC: 85–109, CTC: 88–90	(51)

TC, OTC, CTC, DC, 4-epimers	Pig tissues	Extr. with 0.1 M succinate buff. (pH 4), homog., centr., add. 20% (w/v) TCA, vortex, centr., filt., SPE C_8, Oasis.	PLRP-S, 8 μm (60°C); El: 0.001 M oxalic acid, 0.5% (v/v) HCOOH, 3% (v/v) THF (A)/ THF(B). Grad. El.: 0–20 min 100% A, 20.1–27 min 95%A, 27.1–34 min 90%A, 34.1–40 min 100%AFR: 1 mL/min, IS: DMC	ESI-MS/MS	LOD : 0.5–4.5 ng/g	Muscle: EOTC: 57, OTC: 47, ETC: 50, TC: 53, ECTC: 34, CTC: 45, EDC: 38, DC: 28; Skin-fat: EOTC: 40, OTC: 42, ETC: 22, TC: 18, ECTC: 42, CTC: 45, EDC: 44, DC: 37; Liver: EOTC: 27, OTC: 26, ETC: 24, TC: 25, ECTC: 23, CTC: 18, EDC: 17, DC: 12; Kidney: EOTC: 61, OTC: 58, ETC: 54, TC: 57, ECTC: 53, CTC: 58, EDC: 57, DC: 44	(76)
TC, OTC, CTC, DC, MNC, MTC	Honey	Extr. with 0.1 M Na_2-EDTA/McIlvaine buff. pH 4, vortex, centr., filt., SPE, Discovery phenyl-cartridges	Discovery RP-Amide C_{16} 5 μm. El: 0.01 M oxalic acid–ACN (88:12 v/v). Is. el. for 9 min, change into 82:18 v/v in 0.01 min, is .el. for 16 min. FR: 1 mL/min	UV (270 and 350 nm)	MNC, OTC, TC: 50–650 ng/mL, CTC, MTC, DC: 100–850 ng/mL	MNC: 97; OTC: 91; TC: 83; CTC: 81; MTC: 72; DC: 91	(82)
TC, OTC, CTC, DC, MTC	Milk	Extr. with 1 M HCl, addit. of ACN, sonic., centr., addit. of hexane, vortex, evap. Until 10 mL, Dil. with CH_3OH, filt.	Hypersil ODS 5 μm; El: 0.05 M NaH_2PO_4 (pH 2.2)/ACN (78:22 v/v). FR: 1 mL/min	ESA electr. Det. (400, 600, 680, 700 mV)	LOD: OTC: 12.5, TC: 20, CTC: 25, MTC: 10, DC: 25 ng/mL	OTC: 93–96, TC: 90–94, CTC: 89–93, MTC: 93–94, DC: 91–94	(43)

(*continued*)

Table 2. Continued

Analytes	Sample	Sample preparation	Chromatography	Detection	Linear range	Recovery (%)	Ref
TC, OTC, CTC, DC	Shrimps	Extr. with 0.1 M H_3PO_4 and ACN, centr., filt., liq-liq. extr. with hexane and CH_2Cl_2. Aqueous layer centr., filt.	C_{18}, 5 μm; El: 0.1 M HH_2PO_4 (pH 2.5 with H_3PO_4)/ACN (80:20 v/v)FR: 1.5 mL/min	Electr. Det., mixed valent Ru oxide-Ru cyanide glassy carbon electrode (1.1 V)	LOD: OTC: 0.1 ng/μL, TC, CTC: 0.5 ng/μL	OTC: 72.5–80.0, TC: 80.0–91.5, CTC: 75.2–111.0	(55)
CTC	Swine plasma and water	Prec. with ACN, vortex, centr.	Hypersil C_8, 5 μm; EI: ACN/0.5% HCOOH (70:30 v/v) FR: 0.5 mL/min IS: OTC	ESI-MS/MS	Linear range: 20–2000 ng/mL	Swine plasma: 97.7–100.3; Water: 93.0–101.1	(66)
TC, OTC, CTC	Shrimps milk	Extr. with 0.1 M succinate buff., vortex, centr., filt., residues twice re-extr. combined super. extr. with SPE, Oasis	Hydrobond C_8, 5 μm (endc), 35°C; El: A: 0.01 M oxalic acid for milk or 0.1% HCOOH for shrimps; A/ACN/CH_3OH (75:18: 7 v/v/v). FR: 1 mL/min	UV, 370 nm ESI-MS/MS	UV : 50–400 μg/kg; MS: Shrimp: 25–400 μg/kg; Milk: 50–400 μg/kg	Shrimps: OTC : 81–91, TC: 92–97, CTC: 72–82 Milk: OTC : 75–95, TC: 86–88, CTC: 75–85	(67)
TC, OTC, CTC,	Eggs and honey	Extr. with Tetrasensor® kit buff., vortex, centr., dilut. with CH_3OH 1:9 and 1:3 for honey and eggs.	Genesis C_{18} 4 μm; El: 0.2% HCOOH and 0.1 mM oxalic acid/CH_3OH. Grad. El.: 90:10 (0 min), 25:75 (1 min), 25:75 (2.5 min), 90: 10 (2.6 min). FR: 0.3 mL/min	ESI-MS/MS	Micr. Method; Honey: 25 μg/kg; Eggs: 125 μg/kg	Eggs: OTC: 67.5; TC: 71.4; CTC: 60.4	(53)

OTC, TC, MTC	Honey	Extr. with 2% citric acid sol. (pH 4.5 with 40% NaOH). Add. of 0.1 M phosphate buff. (pH 4) XAD-2 resins.	XDB-C_{18}, 5 μm; EI: 0.001 M phosphoric acid/ACN (84:16 v/v). FR: 0.5 mL/min	DAD (274 nm for OTC and TC, 350 nm for MTC) and CL det. With potassium permanganate sodium sulfite-β-cyclodextrin	LR: 8–800 ng/mL. LOD: with DAD: OTC: 2 ng/mL, TC: 2, MTC: 9 with CL: OTC: 3 ng/mL, TC: 5, MTC: 0.9	DAD: OTC: 96–118, TC: 94–116, MTC: 97.2–103.2; CL: OTC: 104.118.0, TC: 92.6–122.0, MTC: 99.6–105.8	(61)
TC, OTC, CTC, DC	Shrimps	Extr. with 0.1 M Na_2-EDTA/McIlvaine buff. (pH 4), homog., centr., filt., SPE C_{18} Phenomenex.	Inertsil C_{18}, 3 μm; El: 0.01 M phosphate buff. (pH 2.5)/ACN (80:20 v/v)FR: 1 mL/min	Electr. det. with a boron-doped diamond thin film electrode (1.4 V)	LOD: OTC, TC: 0.01 ng/μL CTC, DC: 0.05 ng/μL	OTC: 83.3–96.5, TC: 88.4–96.9, CTC: 86.6–93.3, DC: 90.6–102.0	(54)
TC, OTC, CTC, DC4-epimers	Honey	Extr. with oxalate buff. (pH 4), vortex, centr., SPE, oasis HLB	Atlantis dC_{18}, 3 μm; EI: (A) water with 0.1% HCOOH (B) CH_3OH/ACN (50:50 v/v). Grad. El.: 0–5 min 15%B, 5–10 min 40%, 10–13 min 90%B, 13–23 min 15%B. fr: 0.3 mL/min. I.S.: DMC	ESI-MS/MS	LR: 0–100 μg/kg		(60)

(*continued*)

Table 2. Continued

Analytes	Sample	Sample preparation	Chromatography	Detection	Linear range	Recovery (%)	Ref
OTC, TC, CTC, DC, MNC	Bovine muscle	Extr. with 0.3 M citrate buff. Clean-up on NEXUS SPE	Inertsil ODS-3 5 μm. Multi-step grad. El. with 0.05 M oxalic acid and ACN. FR: 1.65 mL/min.	DAD 351 nm	LOQ 40 μg/kg for MNC, CTC and DC and 25 μg/kg for OTC and TC CCα 104.7–109.8 μg/kg, CCβ 108.4–116.7 μg/kg	98.7–103.3	(64)
OTC, TC, CTC	Animal (bovine and swine) muscle, kidney, and liver	Ultrafiltr. Ultrafree MC/PL membranes	TSK-Guardgel ODS-80 Ts column (5 μm, 15 mm × 3.2 mm I.D.; at 30°C Grad. El. El: A: 0.05% HCOOH. B: MeOH with 0.05% HCOOH. FR: 0.2 mL/min. Grad. El.: $t = 0.00–0.50$ min, %B = 0; $t = 0.51–6.00$, %B = 100. IS: DMC	ESI-MS-MS	0.002 ppm	70–115	(63)

TC, OTC	Pig kidney	Extr.with EDTA-McIlvaine's buff., MI-SPE	A 25 cm × 0.4 cm i.d. Tracer Extrasil ODS2, 5 μm, ec, Teknokroma. El: Milli-Q water with 3% of MeOH and 1% of acetic acid (A), and ACN (B). FR: 1 ml min^{-1}. Grad. El. 10–15% B from 0 to 15 min, 100% B at 35 min and then is. el. for 2 min. 35°C	UV 350 nm	66–69	(34)

(2.5:1:1.5 v/v/v), pH 2 for the determination of TC, OTC, CTC and DC (47). A mixture of 0.01 M oxalic acid–CH_3OH–ACN (65:17.5:17.5 v/v/v) was used for the determination of TC, OTC and CTC (48).

The separation of TC, OTC, CTC, and DC was also achieved on a Symmetry C_{18}, 5 μm, column with gradient elution starting with 100% CH_3OH/0.069% TFA (30:70) and ending at 100% CH_3OH (49). A Phenomenex ODS-3 analytical column was used with 4 mM oxalic acid, 4 mM sodium oxalate, 4 mM sodium dodecylsulfonate/ACN (70:30 v/v) for the separation of TC, OTC and CTC (50).

A Puresil C_{18} analytical column with 0.5% formic acid and 1 mM ammonium oxalate/ACN (80:20 v/v) was used for the separation of TC, OTC, CTC and their 4-epimers (51). Prior LC-MS/MS analysis a Puresil C_{18}, 5 μm, was used with 0.5% formic acid/ACN (23:77) as mobile phase for the determination of TC, ETC, ATC and EATC (37).

A μ-Bondapak C_{18}, 5 μm, column was used with 100% CH_3OH as eluent for the determination of MNC (52). A Genesis C_{18} (4 μm) analytical column was used with 0.2% formic acid and 0.1 mM oxalic acid/CH_3OH as eluent for the separation of TC, OTC and CTC. Gradient elution was applied, starting with 90:10 (v/v), changing to 25:75 (v/v) in 1 min, remaining for 2.5 min and going back to the initial conditions at 2.6 min (53).

An Inertsil C_{18}, 3 μm, analytical column was used with 0.01 M phosphate buffer (pH 2.5)/ACN (80:20 v/v) for the separation of TC, OTC, CTC and DC (54). A C_{18}, 5 μm, analytical column with no more information with regards to the sorbent material was used with 0.1 M KH_2PO_4 (pH 2.5 with H_3PO_4)/ACN (80:20 v/v) for the separation of TC, OTC, CTC, and DC (55). A Nova-Pack C_{18}, 4 μm, column was used with 0.01 M citric acid-0.01 M K_2HPO_4/ACN (72:28 v/v) with 0.005 M tetramethylammonium chloride, 0.1 g/L EDTA for the determination of TC, OTC and CTC (56), as well as with 0.01 M oxalic acid/CH_3OH /ACN (3.5:1.5:1 v/v/v), pH 2 with HCl (60:40 v/v) for the determination of CTC (57).

The separation of TC, OTC, DMC, MNC, ATC, EATC and MTC was achieved on a Purosphere RP-18, 3 μm column with gradient elution starting at A: B 16:84 v/v, changing to 24:76 v/v after 1.5 min, to 30:70 v/v after 2 min and ending to 50:50 v/v after 7 min, where A is ACN with 0.2% formic acid and B is H_2O with 0.2% formic acid (58).

A Novapack C_{18}, 5 μm, column was used for TC, OTC and CTC determination, with 0.05 M oxalic acid/ACN/CH_3OH (30:50:20 v/v/v) and 0.05 M oxalic acid/ACN (40:60 v/v) as mobile phase (59).

An Atlantis dC_{18}, 3 μm, analytical column was used with A: water with 0.1% formic acid and B: CH_3OH /ACN (50:50 v/v) for the separation of TC, OTC, CTC, DC, and their 4-epimers. DMC was used as internal standard. A linear multi-step gradient elution program was applied starting with 15%B, changing to 40% with the next 5 min, and to 90%B after 3 min. An equilibrium step of 10 min was applied to bring the initial conditions (60).

An XDB-C_{18}, 5 μm, column was used with 0.001 M phosphoric acid/ACN (84:16 v/v) for the determination of OTC, TC, and MTC (61). A Prodigy C_{18}, 2 μm, analytical column was used for the determination of OTC, TC and CTC using A: CH_3CN/0.01 M oxalic acid, 0.01 M EDTA, 0.04% HFBA (10:90 v/v) and B: CH_3CN/0.01 M oxalic acid, 0.01 M EDTA, 0.04% HFBA (90:10 v/v). Gradient elution was applied starting with 10%B, changing to 50%B after 10 min, to 90%B after 1 min, and remaining isocratic for 3 min. An equilibration step was applied to bring back the system to the initial conditions for 2 min (62).

A TSK-Guardgel ODS-80 Ts column (5 μm, 15 mm × 3.2 mm I.D) was used for the sample enrichment of TC, OTC and CTC at 30°C. A stepwise gradient was applied. Mobile phase A was distilled water containing 0.05% formic acid. Mobile phase B was methanol containing 0.05% formic acid. The gradient conditions were as follows, base on time (t) set at the pump: $t = 0.00–0.50$ min, hold %B = 0; $t = 0.51–6.00$, hold %B = 100. DMC was used as internal standard (63).

An Inertsil ODS-3, (5 μm, 250 × 4 mm, analytical column) was used for the determination of OTC, TC, CTC, DC and MNC in bovine muscle, at ambient temperature. A multi-step linear gradient elution was applied using A: 0.05 M oxalic acid and B: CH_3CN. Initial conditions were 92:8 v/v, changing to 80:20 after 2 min, 75:25 after 3 min, 70:30 after 2 min, and remaining isocratic for 2 min more and reaching 65:35 after 2 min. Anthranilic acid was used as internal standard (64). A typical chromatogram is shown in Figure 4.

A Hypersil C_8, 5 μm, analytical column was used with 0.01 M oxalic acid–CH_3OH–ACN (60:25:15, v/v/v) as eluent for the separation of TC, OTC, CTC and DC (65), while CTC was determined with ACN/0.5% formic acid (70:30 v/v) with OTC as internal standard (66).

An end-capped Hydrobond C_8, 5 μm, analytical column was used at 35°C with A: 0.01 M oxalic acid for milk or 0.1% formic acid for shrimps A/ACN/CH_3OH (75:18:7 v/v/v) as mobile phase for the separation of TC, OTC and CTC (67). An Inertsil C_8, 5 μm column was used with 0.1 M ammonium acetate (pH 3)/ACN/THF (72.5:12.5:15 v/v/v) for the separation of OTC, EOTC, α-apoOTC and β-apoOTC (68). A Chromspher C_8, 5 μm, column was used with 0.01 M oxalic acid–ACN (80:20 v/v) for the determination of TC, OTC, CTC, DC, MTC and DMC (69).

A Supershere RP-8, 4 μm, analytical column was used for the separation of TC, OTC, CTC, ICTC, N-DMCTC, N-DMOTC, and 4-N-DMICTC with 0.01 M or 0.005 M oxalic acid–ACN (50:50 v/v) as eluent. For LC-MS/MS the mobile phase consisted of ACN/0.5% formic acid (40:60 v/v) (70). A Chemosorb C_8, 3 μm column was used with a mobile phase consisted of 0.01 M oxalic acid/CH_3OH /ACN (16:3:2 v/v/v), pH 3 for the separation of TC, OTC, CTC, DC, MTC, DMC and MNC (71).

A Bakerbond C_8, 5 μm analytical column operated at 30°C was used with 0.01 M oxalic acid/CH_3OH /ACN (55:27:18 v/v/v) at pH 2, for the

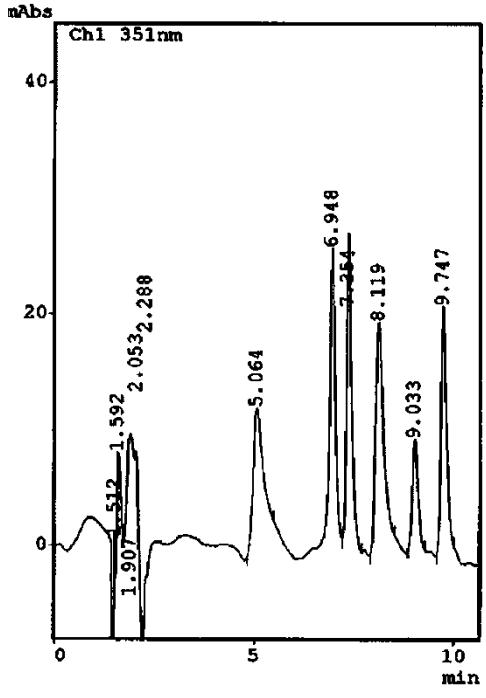


Figure 4. High performance liquid chromatogram of tetracyclines in spiked bovine muscle tissue at 8 ng/μL, after SPE using the conditions described in text. Peaks: (1) MNC 5.064 min (2) OTC 6.948 min (3) TC 7.354 min (IS) anthranilic acid 8.119 min (4) CTC 9.033 min and (5) DC 9.747 min.

determination of TC, OTC, CTC and DC (72). A TSK Gel Super C_8, 2 μm, column was used with ACN/0.05% TFA (1:4 v/v) as mobile phase for the determination of OTC, TC and CTC (73).

A polystyrene–divinylbenzene PLRP-S, 5 μm, analytical column was used for the determination of TC, OTC, CTC and DMC. A linear gradient elution from 100% A to 100% B in 10 min and then isocratic 100% B for 10 min was applied with A: 0.1 M NaH_2PO_4–0.01 M Citric acid–0.01 M EDTA and B: ACN-CH_3OH-A (25:10:65 v/v/v) (74). A similar analytical column PLRP-S, 8 μm, was used for the separation of TC, OTC, CTC and DC with 0.01 M oxalic acid–ACN. Linear gradient elution was applied changing from 85:15 v/v to 60:40 v/v (75). The same column was used for the determination of TC, OTC, CTC, and DC with DMC as internal standard. Gradient elution was applied starting from 85:15 v/v and changing to 60:40 v/v in 16 min. with 0.01 M oxalic acid–ACN (50). TC, OTC, CTC, DC and 4-epimers with DMC as internal standard were separated on the same column, operated at 60°C, with 0.001 M oxalic acid, 0.5% (v/v) formic acid, 3% (v/v) THF (A)/THF(B) as mobile phase.

Gradient elution was applied starting with 100% A for 20 min, changing to 95% A, in 7 min, 90% A for 7 min and brought back to the initial conditions for 6 min (76). The same column was used with A: 0.1 M NaH_2PO_4–0.01 M citric acid–0.01 M EDTA and B: ACN-CH_3OH-A (25:10:65 v/v/v). Linear gradient elution changing from 100% A to 100% B in 10 min and then isocratic 100% B for 10 min was used for the separation of TC, OTC, CTC, and DMC (77), while an isocratic method with 0.01 M oxalic acid–ACN (50:50 v/v) achieved the separation of OTC, TC and CTC (78). A PLRP-S, 5 μm, column was used for the separation of TC, OTC, CTC, DC, MTC, DMC and MNC with 0.01 M oxalic acid/CH_3OH/ACN as eluent. Gradient elution was applied under the following timetable: 0–1 min 100:0:0 v/v/v, linear change from 1–5 min to 70:8:22 v/v/v, from 5–11 min 70:8:22 v/v/v (79), while the separation of TC, OTC, CTC, DC, DMC and MNC was achieved on the same column with 0.01 M oxalic acid/CH_3OH (58:42 v/v) (80).

Other columns used involve an ODP-50, 5 μm column operated at 40°C with Sorensen buffer (pH 12)/ACN (90:10 v/v) for the determination of TC and OTC (81) and a Discovery RP-Amide C_{16} 5 μm analytical column for the separation of TC, OTC, CTC, DC, MNC and MTC. In the latter case 0.01 M oxalic acid–ACN (88:12 v/v) was used as the mobile phase under a gradient program of isocratic elution for 9 min, changing to 82:18 v/v in 0.01 min, and isocratic elution for 16 min (82).

Thin Layer Chromatography

Thin layer chromatography (TLC) was also applied to the tetracyclines determination. An overview of the thin-layer chromatographic method used in TC's determination in food samples is presented in Table 3. A Merck Silica gel HPTLC plate, pre-developed with Na_2EDTA, activated at 130°C, was used with CH_3Cl/CH_3OH/5% Na_2EDTA (60:20:5 v/v/v) as mobile phase for the determination of TC, OTC, CTC and DC in tissues, milk and egg (83), as well as for the separation of MNC, CTC, DC, MTC, OTC (71). RP-8 TLC plates with 0.5 M oxalic acid/ACN/CH_3OH (4:1:1 v/v/v), pH 2 as mobile phase were used for the determination of and MNC, OTC, TC, DMC and CTC (71).

A Merck Silica gel plate, pre-developed with 10% sodium acetate pH 8, activated at 110°C, wsa used with CH_2Cl_2/CH_3OH/H_2O (59:35:6 v/v/v) for TC, 60:35:5 v/v/v for CTC, 58:35:7 v/v/v) as well as for the determination of OTC, TC, ETC, ATC, EATC, ADTC, OTC, EOTC, ADOTC, α-apoOTC, β-apoOTC, CTC, CTC, ADCTC, MTC and DMC in animal feeds and premixes. Fluorescence densitometry was applied after plates been dipped in liquid paraffin in hexane (30:70) with LOQ values 0.2 ng for TC, CTC and their impurities and 0.1 ng for OTC and its impurities (84).

A silica gel plate Si60 F_{254} was used for the determination of DC with 0.05 M citric acid/CH_3OH/isopropanol (1:3:1 v/v/v) in milk using UV densitometry at 366 nm. A linear range of 0.1–0.5 μg was obtained (85).

Table 3. Overview of TLC methods for the determination of tetracyclines in foodstuffs of animal origin

Analytes	Sample	Sample preparation	Chromatography	Detection	LOD/Linear range	Recovery (%)	Ref
TC, OTC, CTC, DC	Tissues, milk, egg	Extr. with 0.1 M Na_2-EDTA/McIlvaine buff. (pH 4), homog., centr., filt., SPE C_{18} Sep-Pack.	Merck Silica gel HPTLC plate (pre-dev. with Na_2EDTA, activ. at 130°C); El: CH_3Cl/CH_3OH/5% Na_2EDTA (60:20:5 v/v/v)	UV Dens. and with det. reagents (1% Fast violet B, 120°C)	LOD: 0.1 ng/μL	UV detection: Milk: OTC: 85.6, TC: 73.0, CTC: 79.0, DC: 80.6; Egg: OTC: 76.6, TC: 72.3, CTC: 75.2, DC: 61.0; Chicken: OTC: 65.7, TC: 70.5, CTC: 73.0, DC: 72.2; Bovine: OTC: 75.7, TC: 62.4, CTC: 65.5, DC: 61.0 Pork: OTC: 78.0, TC: 70.3, CTC: 66.7, DC: 68.2; Spray reagent: Milk: OTC: 75.4, TC: 66.6, CTC: 73.3, DC: 73.3; Egg: OTC: 65.8, TC: 73.3, CTC: 83.3, DC: 59.2; Chicken: OTC: 75.0, TC: 65.8, CTC: 65.8, DC: 65.0; Bovine: OTC: 61.7, TC: 65.8, CTC: 66.7, DC: 65.0; Pork: OTC: 70.0, TC: 83.3, CTC: 81.1, DC: 56.6	(83)
(A):MNC, CTC, DC, MTC, OTC (B): MNC, OTC, TC, DMC, CTC	Honey	Extr. with 0.1 M Na_2EDTA/McIlvaine buff. (pH 4), homog. centr., filt., SPE C_{18} Baker cartridges.	Merck Silica gel HPTLC plate (pre-dev. with Na_2EDTA, activ. at 130°C); El: CH_3Cl/CH_3OH/5% Na_2EDTA (60:20:5 v/v/v)RP-8 TLC plates; El: 0.5 M oxalic acid ACN/CH_3OH (4:1:1 v/v/v), pH 2.	Det. reagents for HPTLC: 0.2 M $MgCl_2$ for RP-TLC: 0.2 M $MgCl_2$ and 10% TEA in methanol UV (360 nm)	HPTLC: MNC: 0.03 μg; Others: 0.01 μg; RP-TLC: 0.01 μg		(71)

TC, OTC, CTC, DC	Bovine tissues	Extr. with 0.1 M Na_2-EDTA/McIlvaine buff. (pH 4) × 3, homog., centr., filt., SPE C_{18} Baker cartridges	RP-8 TLC plates; El: 0.5 M oxalic acid/ACN/CH_3OH (4:1:1 v/v/v), pH 2	FAB-MS/MS	LOD: 0.1 ng/μL	No quantitative results	(86)
DC, FL	Milk	Milk samples inj. on the TLC plate pre-dev. with hexane to remove lipid fraction	Silica gel plate Si60 F_{254}. El: 0.05 M citric acid/CH_3OH/IPA (1:3:1 v/v/v)	UV Dens. (254 nm for FL, 366 nm for DC)	LR: 0.1–0.5 μg	DC: 98.2–101.2; FL: 96.4–129.3	(85)
TC, ETC, ATC, EATC, ADTC, OTC, EOTC, ADOTC, α-apoOTC, β-apoOTC, CTC, ECTC, ADCTC, MTC, DMC	Animal feeds, premixes	Premixes: extr. with CH_3OH/1 M HCl (99:1 v/v), ultrasonic, filt., dil. with CH_3OHFeeds: add. of Kieselgur, homog. Add. of CH_3OH/1 M HCl (99:1 v/v), dil. with CH_3OH, filt.	Merck Silica gel plate (pre-dev. with 10% sodium acetate pH 8, activ. at 110°C); El:CH_2Cl_2/CH_3OH/H_2O (59:35:6 v/v/v for TC, 60:35:5 v/v/v for CTC, 58:35:7 v/v/v for OTC.	FL Dens. (400 nm) after plates been dipped in liquid paraffin in hexane (30:70)	LOQ: 0.2 ng for TC, CTC and their impurities; 0.1 ng for OTC and its impurities.	Animal feeds: TC: 72.6–80.1%; Premixes: TC: 79.2; CTC: 87.2–99.2; OTC : 7.3	(84)

TC, OTC, CTC and DC were determined in bovine tissues using RP-8 TLC plates with 0.5 M oxalic acid/ACN/CH_3OH (4:1:1 v/v/v), pH 2, as mobile phase and detection FAB-MS/MS with LOD: 0.1 ng/μL (86).

Other Techniques

As already mentioned, the vast majority of the reviewed papers apply HPLC for the separation of tetracyclines antibiotics. However other techniques can be found in literature concerning the determination of tetracycline antibiotics. Among them capillary electrophoresis (87, 88), fluorimetry (89), microbiological assays (90–92), electrochemical biosensors (93–96), FIA (97) are also used for tetracycline analysis. These methods are summarized in Table 4.

Capillary electrophoresis using a Fused silica capillary of 27 cm × 75 μm i.d. (20°C) and 500 mM magnesium acetate tetrahydrate in N-methylformamide as medium, 15 kV and fluorescence detector set at λ_{exc} 325 nm, λ_{em} 515 nm provided a linear range: 50–1000 ng/mL for TC, OTC, CTC, DMC, ETC and EATC determination in milk and plasma samples (88).

OTC in catfish was determined by Capillary Electrophoresis using a coated capillary cartridge of 24 cm × 25 μm i.d. (25°C), Medium: 0.2 M phosphate buffer (pH 2), 8 kV and UV detection at 265 nm. A linear range: 0.1–25 ng/μL and LOD: 0.05 ng/μL were obtained (87).

Fluorimetry was applied for the determination of TC, OTC and CTC in chicken tissues at λ_{exc} 375 nm, λ_{em} 515 nm LOD: TC: 0.15 μg/g, OTC:0.327 μg/g, CTC: 0.25 μg/g (89). Microbiological assays used for the determination of TC, OTC, CTC and DC in milk provided LOD values of 1500 μg/kg for CTC, 260 μg/kg for DC, 560 μg/kg for OTC and 480 μg/kg for TC (91).

OTC and CTC were determined in tissue samples by microbiological test yielding an LOD value of 600 μg/kg (92). Microbiological tests for the determination of TC, OTC, CTC and DC in tissues provided an LOD of 3 ng for OTC, 4 ng for TC, 0.3 for CTC and 0.6 ng for DC (93).

TC and OTC were determined in honey by FIA. As TCs inhibit electrochemiluminescence (ECL) from $Ru(bpy)_3^{+2}$–tripropylamine system, detection was performed by inhibited ECL with a Pt working electrode at 1.05 V (vs Ag/AgCl). LOD values achieved were 4×10^{-9} g/mL for TC and 3.8×10^{-9} g/mL for OTC (97).

Two luminescent biosensors were used for the determination of TC, OTC, CTC, DC, DMC, MNC MTC and DMC in pork serum. Luminescence was measured after the addition of D-luciferin. LOQ values achieved were 50 ng/mL (96). A Whole-cell biosensor was also used for CTC determination. Measurements of the produced β-galactosidase fluorescent protein provided an LOD of 0.03 mg/kg (95).

Table 4. Overview of other analytical methods for the determination of tetracyclines in foodstuffs of animal origin

Analytes	Sample type	Sample preparation	Analytical technique	Linear range/LOD	Recovery (%)	Ref
OTC	Catfish	Extr. with 50% TCA, 1 M HCl and Na_2EDTA(s), vortex, centr., filt., SPE, Sep-Pack C_{18} cartridges.	CE: Coated capillary cartridge of 24 cm × 25 μm i.d. (25°C), Medium: 0.2 M phosphate buff. (pH 2), 8 kV, UV 265 nm	LR: 0.1–25 ng/μL LOD: 0.05 ng/μL	92.9	(87)
TC, OTC, CTC, MC, ETC, ATC	Milk, plasma	Precip. of proteins with 10% TCA, centr., SPE MP1 microcolumns.	CE: Fused silica capillary of 27 cm × 75 μm i.d. (20°C), Medium: 500 mM magnesium acetate tetrahydrate in N-methylformamide, 15 kV, FL λ_{exc} 325 nm, λ_{em} 515 nm	LR: 50–1000 ng/mL	OTC: 63.3–97.2	(88)
OTC, CTC	Tissues	Homog.	Microb. tests: test strain: *B. sereus*, growth medium: agar sol. pH 6.5,supplement: $MgSO_4 \cdot 7H_2O$, incub. at 30°C for 18–24 h. test strain: *B. subtilis*, growth medium: agar sol. pH 6, supplement: no incub. at 30°C for 18–24 h.	LOD: 600 μg/kg		(92)

(*continued*)

Table 4. Continued

Analytes	Sample type	Sample preparation	Analytical technique	Linear range/LOD	Recovery (%)	Ref
CTC	Milk	Milk samples centr., filt., add. to Luria broth with bacterial culture *E. coli MC4100* (3 h, 37°C)	Three cell biosensors: containing TC promoter (P_{tet}) and reporter gene (tet R, three different gene systems of *E. coli*, lacZYA, luxCDABE or gfp). Meas. of the produced β-galactosidase, green fluorescent protein	LOD: 0.01 μg/mL		(94)
TC, OTC, CTC, DC, DMC, MNC MTC, MC	Pork serum	Dil. with 1:1 with bacterial culture	Two luminescent biosensors: media: Luria-Bertani medium, *E.coli* K12 pTetLuc1 and *E. coli* K12 pTetLux1. (37°C for 90 min). Meas.: luminescence after addit. of D-luciferin	LOQ: 50 ng/mL		(96)
CTC	Pig feces	Extr. with double distilled H_2O, vortex, centr.	Whole-cell biosensor: medium: Luria broth, bacterial culture: *E. coli GM10.1* (3 h, 37°C). Meas. of the produced β-galactosidase, fluorescent protein	LOD: 0.03 mg/kg		(95)
TC, OTC, CTC + Fluoroquinolones	Chicken tissues	Extr. with 1% acetic acid, homog., centr., add. of NH_4OH, CH_3OH, $MgCl_2$, mix., centr., filt.	FL λ_{exc} 375 nm, λ_{em} 515 nm	LOD: TC: 0.15 μg/g, OTC: 0.327 μg/g, CTC: 0.25 μg/g	22–36	(89)

TC, OTC, CTC, DC	Tissues	For the Tetrasensor test, dil. with a buff., centr., filt.	Microb. tests: Two agar diffusion methods in pH 6 medium with *B. subtilis* and *B. sereus* (incub. at 30°C for a night) in thick plates for meat fluid and in thin plates for intact or minced meat.Two methods: Premi test (inhibitor test with *B. strarothermophilus*, incub.at 64°C for 2 h). Tetrasensor (receptor test)	LOD: with *B. subtilis* OTC: 8, TC: 5, CTC: 0.5, DC: 1 ng; With *B. sereus*OTC: 3, TC: 4, CTC: 0.3, DC: 0.6 ng		(90)
TC, OTC, CTC	Milk	Add. of milk to cultural medium (Mueller-Hinton agar) with bacterial culture *E. coli ATCC 11303* (2 h, 37°C).	Electr. biosensor: measurement of CO_2 production rate in relation to inhibition of microbial growth (*E. coli ATCC 11303*). Sensor: galvanic cell with a RE, and an IE. WS: KCl in water with 10^{-4} M Na_2CO_3	LOD: 25 μg/L		(93)
TC, OTC, CTC, DC	Milk	Add. of 4 μL/mL acidiol solution	Microb. test: Eclipse 100. test strain: *B. stearothermophilus*, growth medium: sol. commercially available, incub. at 64°C for 2 h, 30 min	LOD: CTC: 1500, DC: 260, OTC: 560, TC: 480 μg/kg		(91)
TC, OTC	Honey	Extr. with citric acid (pH 4), homg., adj. pH at 4.5 with 2% citric acid, phosphate buff., XAD-2 resins	FIA: TCs inhibit ECL from $Ru(bpy)_3^{+2}$ – tripropylamine system. Det.: inhibited ECL with a Pt working electrode at 1.05 V (vs. Ag/AgCl)	LOD: 4×10^{-9} g/mL for TC and 3.8×10^{-9} g/mL for OTC	TC: 82–108	(97)

Three cell biosensors were used for CTC determination in milk. Quantifications based on measurements of the produced β-galactosidase green fluorescent protein. LOD: 0.01 μg/mL (94). An electrochenical biosensor was applied for TC, OTC and CTC in milk samples with LOD: 25 μg/L (93).

Extraction Conditions—Cleanup Sample Preparation

It is widely accepted that the most important step and the most time-consuming one in any analytical method applied to food analysis is, beyond any doubt, the extraction and isolation step.

The isolation of TCs from tissues combines an extraction step with a suitable solvent system and a cleanup procedure. A common problem in the analysis of TCs is that they have the tendency to bind irreversibly to the silanol groups on silica-based materials (C_8, C_{18}) due to the presence of the two ketone groups. Moreover, as they readily form chelate complexes with divalent metal ions and combine with sample matrix proteins, an acidic deproteinizing agent is demanded as extracting solvent.

Half of the reviewed methods for the determination of tetracyclines found in literature as they are presented in Tables 2–4, are applied to tissue samples of various animals, while milk, honey and animal feed samples possess a quite important percentage, about 10% each one. In most of these methods three or four tetracyclines are separated, TC, OTC, CTC and DC above all, as these drugs are used in veterinary medicine.

Tetracyclines are usually extracted from sample matrix using an acidic buffer solution, sometimes in combination with a chelating agent. Figure 5a shows the frequency of usage of the different extraction media. McIlvaine buffer solution (a mixture of citric acid and disodium hydrogen phosphate) with Na_2EDTA is the prevailing medium for TC's extraction from liver, honey, shellfish, shrimps, bovine, porcine muscle, milk, shrimps, cheese, eggs. The next medium of choice for TC's extraction from milk, shrimp, eggs, animal bones, kidney, bovine, porcine muscle, and chicken meat is succinate buffer. Citrate buffer in different concentrations is also used.

For further cleanup, solid phase extraction (SPE) on C_{18}-solid phase extraction (SPE) cartridges or hydrophilic-lipophilic balanced-SPE (HLB-SPE) cartridges is the extraction technique most frequently used (47%) prior to the chromatographic analysis as shown in Figure 5b. Other techniques used include liquid-liquid extraction, solid-phase microextraction, matrix solid phase dispersion and metal chelate affinity chromatography. However liquid-liquid extraction is rarely used as it is time consuming and needs large amounts of organic solvents. Metal chelate affinity chromatography (MCAC) off- and on-line, based on the chelating ability of TCs, as well as exchange extraction membranes and XAD-2 resins have been employed as purification methods. Disadvantages of these procedures are mainly focused

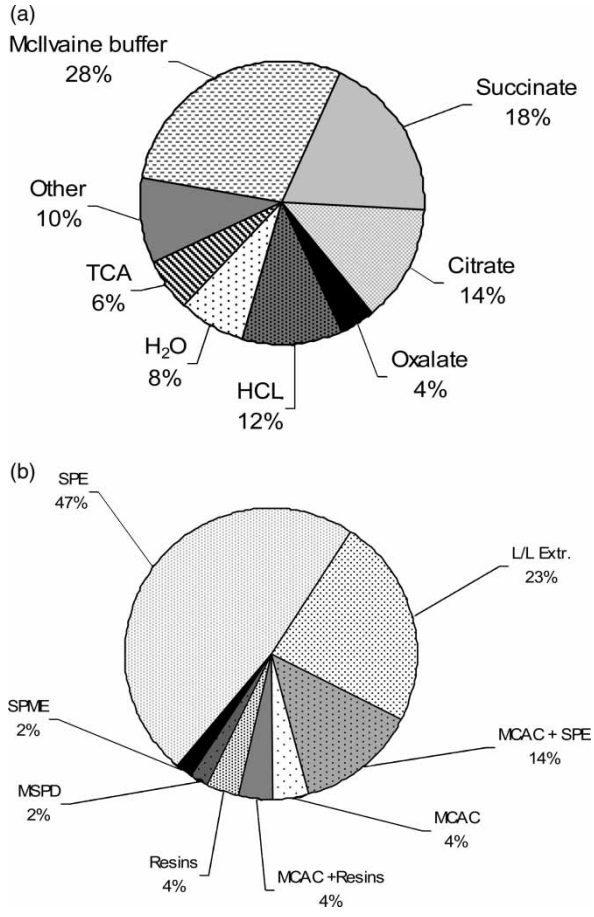


Figure 5. (a) Solutions used for the extraction of TCs from food samples. (b) Percentage distribution of sample preparation used for tetracyclines determination in food samples.

on their complication and their poor recoveries, so that in most cases an extra solid phase extraction step is demanded.

A common feature encountered when developing extraction methodologies for tetracyclines analysis besides their tendency to form strong complexes with multivalent cations is the fact that they are amphoteric compounds soluble in polar and moderately polar organic solvents. Binding to silanols has resulted in low recoveries when C_{18} SPE columns are used for cleanup extracts. To avoid this problem, C_{18} SPE columns have been silylated or pre-treated with EDTA.

As stated above to decrease the tendency for tetracyclines to bind to cations in the matrix the majority of the researchers utilize aqueous

solutions containing chelating agents e.g., EDTA, oxalic and citric acids. McIlvaine's buffer, used in many extraction procedures contains citric acid. The use of EDTA–McIlvaine's buffer, combined with SPE using alkyl-bonded silica cartridges for cleanup, was established by Oka et al. (71) appearing to be the current standard for the extraction of tetracyclines from tissue matrices (68). However in a recent paper of Samanidou et al. (64) 0.3 M citrate buffer provided better recovery rates as shown in Table 2. Nexus polymeric cartridges were used with CH_3OH/CH_3CN/0.05 M oxalic acid (30:30:40 v/v) providing a >90% recovery of the examined TCs. To optimize extraction of TCs namely OTC, TC, CTC, MNC and DC from bovine muscle tissue, various buffer solutions were tested. As shown from the results summarized in Table 5, citrate buffer 0.3 M at pH = 4 presents higher extraction efficiency (86.4–107.6%). It is noteworthy that citrate buffer provided better recovery than McIlvaine buffer, which by now prevails in most extraction schemes reported in the literature. The overall extraction scheme is presented in Figure 6 (64).

An increased interest is noticed in using the chelating abilities of tetracyclines when developing extraction methodologies. The use of metal chelating affinity columns was used to cleanup succinate buffer extracts of porcine kidney and muscle, and bovine liver for tetracycline, oxytetracycline and chlortetracycline analysis. Recoveries varied from 40 to 70%, with no apparent effects due to the tetracycline type or tissue. This may be of some benefit, as Blanchflower et al. (62) showed that chlortetracycline is especially prone to epimerization in aqueous solutions, which may account for its low recoveries.

Extraction with 0.1 M Na_2EDTA/McIlvaine buffer (pH 4) was applied for the isolation of TC, OTC, CTC, DC, MNC and MTC from honey. Further purification was achieved on Discovery phenyl-cartridges. High recovery rates were obtained that is 97% for MNC, 91% for OTC and DC, 83% for TC, 81% for CTC and 72% for MTC (82).

The same extraction medium was used for the isolation of TC, OTC and CTC from animal tissues, milk and cheese (48), for TC, OTC, CTC and DC from shrimps (54), for OTC from three species of marine shellfish (36), for

Table 5. Extraction recoveries of the examined Tcs from spiked bovine muscle tissue (1 g) using different buffer solutions prior to SPE (64)

Analytes	Acetate buffer 0.1 M, pH = 4	Acetate buffer 0.1 M, pH = 5	McIlvaine buffer, pH = 4	0.1 M Na_2EDTA/ McIlvaine buffer pH = 4	Citrate buffer 0.3 M, pH = 4
MNC	12.4	18.0	54.7	55.3	86.4
OTC	37.8	19.0	72.3	50.8	104.7
TC	19.3	11.7	71.0	61.7	94.4
CTC	32.0	43.6	65.9	57.0	92.2
DC	28.6	18.8	70.9	62.6	107.6

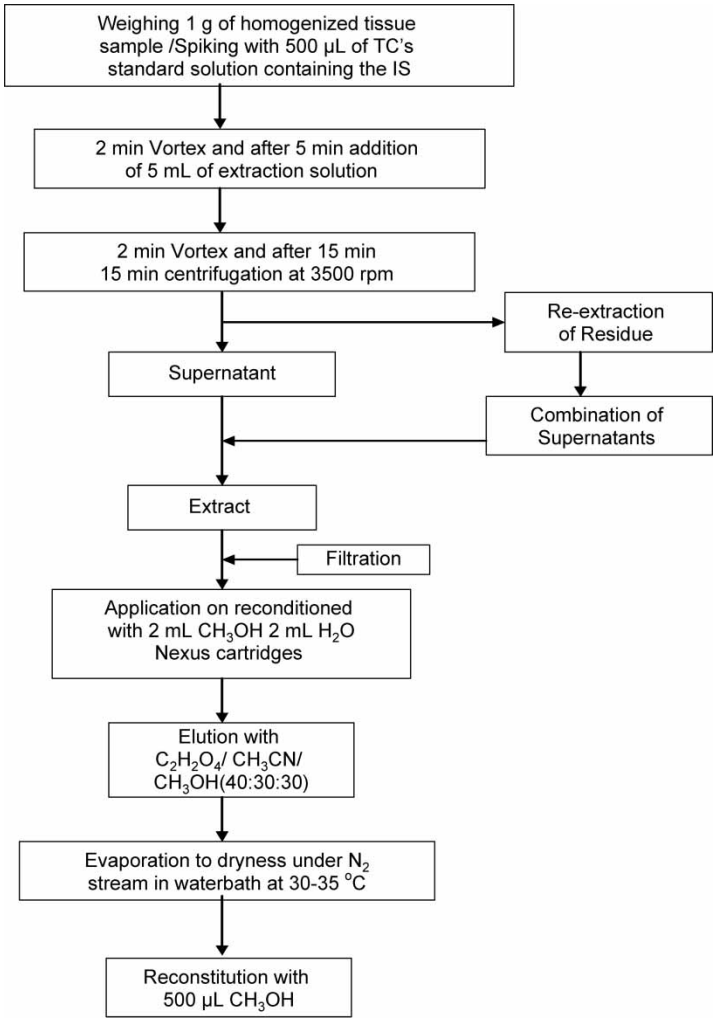


Figure 6. Extraction procedure for the isolation and cleanup of TCs from animal tissues using SPE.

TC, OTC and CTC from bovine and porcine muscle (56), for TC, OTC, CTC, DC, MTC, DMC and MNC from honey (71), for TC, OTC,CTC and DC from liver (47) for OTC, TC and CTC from bovine tissues (73), for TC, OTC, CTC and DC from tissues, milk and egg (83), for TC, OTC, CTC and DC from bovine tissues (86), for OTC, EOTC, α-apoOTC, β-apoOTC from porcine muscle and water (68), as well as for TC, OTC, CTC and DC from tissues (72). Relevant recovery results are shown in Tables 2–4.

TC and OTC were extracted from different tissue samples (bovine, swine and chicken) with 10 mM Na_2EDTA/McIlvaine (pH 4)/CH_3OH (3:7). SPE

was performed on C_{18} and SCX cartridges. Recovery ranging from 62 to 87% was dependent on matrix type as shown in Table 2 (81).

Dilution with water 1:5 v/v and SPE on ISOLUTE-C_8 was used for the extraction of TC, OTC, CTC and DC from milk, providing high recovery rates (80.8–95.5%) (35). A two-step chemical extraction with 20% TCA, and McIlvaine buffer and SPE on HLB Oasis cartridges was applied for the extraction of TC, OTC, CTC and DC from bovine milk and muscle. Recovery rates were 84.3–84.9% for OTC, 81.8–84.6 for TC, 83.1–89.5% for CTC and 84.2–84.9% for DC (65). Citrate buffer 1 M, pH 4, was used for chicken liver and ethyl acetate for the extraction of TC, OTC, CTC and DMC from eggs and animal tissues. Column switching was used (74).

The combination of extraction with 0.1 M sodium succinate buffer (pH 4), MCAC and extraction with SDB-RPS cation exchange membranes was applied for the isolation of TC, OTC, CTC and DC from eggs and animal tissues. Percentage recovery was in the range 37–50 for egg samples, 45–60 for pork kidney, 53–69 for pork muscles and 39–66 for bovine liver (75). Liquid-liquid extraction was applied to lipid fraction removal with hexane for the isolation of DC from milk samples prior to TLC analysis. Recovery achieved was 98.2–101.2% (85).

TC, OTC, CTC, ICTC, N-DMCTC, N-DMOTC and 4-N-DMICTC were extracted from eggs using 0.3 M sodium citrate buffer and on-line dialysis using a modified ASTED system, on-line SPE on PLRP–S 70 μm, (70). TC, OTC, CTC and their 4-epimers were isolated from eggs and enviromental samples extracted with 1 M citrate buffer (pH 5) (51). Succinate buffer 0.1 M was applied to the extraction of TC, OTC and CTC from shrimps and milk and further cleanup by SPE using Oasis cartridges (67).

TC, OTC, CTC and DMC were extracted from animal tissues with 0.1 M succinate buffer (pH = 4) and with succinate buffer pH = 4.0 containing 3.72% EDTA and 0.3% pentanesulphonic acid. SPE C_8 or XAD-2 resins and on-line MCAC were used (77). TC, OTC, CTC, and DC were extracted from pork and chicken meat with 0.1 M sodium succinate buffer (pH = 4). Further cleanup was performed by MCAC and extraction with SDB-RPS cation exchange membranes (49). A similar procedure was applied to the extraction of TC, OTC, CTC, DC, DMC and MNC from milk and shrimps with cleanup, desalting and concentration using MCAC and SPE on Supelclean ENVI-Chrom P cartridges (80).

TC, OTC, CTC, DC, MTC and DMC were extracted from kidney with 0.05 M succinate buffer/3.7% Na_2EDTA (pH = 4). Further purification was achieved using MCAC and SPE on C_{18} (70). TC, OTC, CTC, DC and 4-epimers were extracted from pig tissues with 0.1 M succinate buffer (pH = 4), 20% (w/v) TCA, and further cleanup on SPE C_8 and Oasis (76).

OTC, TC, CTC and epimers as well as TC, CTC and epimers were extracted from animal feed and bones with succinate buffer (pH = 4) or 1 M HCl. MCAC and SPE C_{18} are further applied respectively (40, 41). OTC, TC and CTC were extracted from animal tissues with 1 M oxalic acid and ACN and SPE on SDB cartridges (78).

TC, OTC, CTC, DC, MTC, DMC and MNC were extracted from milk with succinate buffer (pH = 4). For further cleanup MCAC and ultrafiltration were applied. Extraction recoveries ranged from 63 to 91% (79). The same buffer was used for the extraction of TC, OTC, CTC, DC and 4-epimers from honey. SPE with oasis HLB was further applied (60). OTC from fish feed was extracted with ethyl acetate, 0.01 M EDTA and SPE on C_{18} (44). OTC and TC, CTC, ETC, ECTC were extracted from animal bones with 1 M HCl. XAD-2 resins were used for further purification (38).

TC, OTC and CTC were extracted from animal tissues with H_2O, ACN, 0.1 M H_3PO_4. Liquid-liquid extraction with hexane and CH_2Cl_2 was applied to the extraction of OTC, TC and CTC from pork tissues, kidney and liver. High recovery rates were noticed 92–110% (50). MNC was extracted from bovine tissues and serum with H_2O and SPE C_{18} (52).

TC, ETC, ATC and EATC were extracted from animal feed with 1 M HCl (at 133 and 100°C). Further cleanup was achieved by MCAC on a chelating sepharose column and SPE on C_{18} cartridges (37). TC, OTC and CTC were extracted from eggs and honey with Tetrasensor kit buffer (53). TC, OTC, CTC, DC, MTC were extracted from milk with 1 M HCl 89–96% (43). DC was extracted from turkey plasma with 6% w/v ascorbic acid and phosphate sulphite buffer (pH 6) and ethyl acetate (39). OTC, TC, CTC, DC, MNC, and epimers were extracted from animal feeds with 1:1 ACN/H_2O at pH = 3 with 0.01 M citric acid (42).

OTC, TC and MTC were extracted from honey with 2% citric acid solution (pH = 4.5 with 40% NaOH). Further purification was performed using XAD-2 resins (61). TC, OTC, CTC and DC were extracted from shrimps with 0.1 M H_3PO_4 and ACN, and LLE with hexane and dichloromethane (55). TC was extracted from swine tissues (muscles and kidney) with ACN/1 M imidazol buffer with 10 mM Na_2EDTA and 50 mM magnesium acetate (15:85 v/v) and ultrafiltration (45). OTC was extracted from fish feed with ethyl acetate, 0.01 M EDTA. SPE on C_{18} lead to a high recovery in the range 93.6–98.1 (44).

TC, OTC, CTC and DMC were extracted from animal tissues with 0.1 M succinate buffer (pH = 4), containing 3.72% EDTA and 0.3% pentanesulphonic acid. SPE C_8 or XAD-2 resins and on-line MCAC with SPE C_{18} were used for further sample preparation (77).

TC, OTC, CTC, ICTC, N-DMCTC, N-DMOTC, and 4-N-DMICTC were extracted from eggs with 0.3 M sodium citrate buffer. On-line dialysis was subsequently performed by on-line SPE, PLRP–S 70 μm (70).

OTC, TC, CTC and epimers were extracted from animal feed and bones with succinate buffer (pH = 4) or 1 M HCl. Further cleanup was performed by MCAC and SPE on C_{18} (40). Similar conditions were applied for TC, CTC and epimers extraction from animal bones (41).

TC, OTC and CTC were extracted from shrimps and milk with 0.1 M succinate buffer and isolated by SPE using Oasis cartidges (67). TC, OTC, and CTC from tissues were extracted with 0.02 M HCl–0.1 M Na_2EDTA,

at 98°C. LLE with methylene chloride was further applied (46). OTC, TC and CTC were extracted from bovine tissues (liver, kidney and muscle) with 0.1 M Na_2EDTA/McIlvaine buffer (pH 4). Further cleanup was achieved by SPE C_{18} Bond Elut (73).

CTC from poultry/swine feeds was extracted with 4 M HCl-acetone-H_2O (1:8:6 v/v/v), SPE C_{18}. Additional cleanup for CTC was performed by LLE with methylene chloride (57). OTC, TC and CTC from tissues were extracted with glycine-HCl buffer and SPE, on cyclohexyl end-capped cartridges (62).

Two molecularly imprinted polymers (MIPs) were synthesized using TC and OTC antibiotics as template molecules in non-covalent molecular imprinting procedures. After a chromatographic evaluation, the performance of the MIPs as selective SPE sorbents was evaluated. Extraction recovery from pig kidney tissue extract was 66–69%. The polymers were prepared by the non-covalent approach using TC and OTC as the template molecules, respectively. The same functional monomer (MAA) was used in both polymerisations but the porogenic solvents were different. The pre-polymerisation mixture for the tetracycline MIP comprised TC, MAA, the cross-linking monomer EGDMA and the initiator AIBN dissolved in the porogen, acetonitrile in a 25 mL thick-walled glass tube. Prior to MISPE pig kidney tisuue was extracted with EDTA-McIlvaine's buffer (34).

TC, OTC, DMC, MNC, ATC, EATC and MTC were extracted from milk by SPME using 50 μm Carbowax/templated resins (58). TC, OTC, CTC were extracted from milk samples after centrifugation using M.W. cutoff system. Further purification was achieved by SPE on C_{18} Varian cartridges (59). TC, CTC, ETC and ECTC were extracted from animal bones with 1 M HCl. For further purification XAD-2 resins were used (38). TC, OTC, CTC, DC, MTC and DMC were extracted from kidney with 0.05 M succinate buffer/3.7% Na_2EDTA (pH = 4). Further cleanup was achieved by MCAC and SPE on C_{18}(69). OTC from catfish was extracted with 50° TCA, 1 M HCl and Na_2EDTA. SPE on Sep-Pack C_{18} was subsequently applied with high recovery 92.9% (87).

TC, OTC, CTC, DMC, ETC and EATC were extracted from milk and plasma after precipitation of proteins with 10% TCA. SPE was performed using MP1 microcolumns (88). TC, OTC, CTC and Fluoroquinolones were extracted from chicken tissues with 1% acetic acid (89). TC and OTC were extracted from honey with citric acid (pH 4). XAD-2 resins were further used (97). Ultrafiltration was used for the extraction of TC, OTC and CTC from animal (bovine and swine) muscle, kidney, and liver using ultrafree MC/PL membranes (63).

Detection Techniques

UV detection in various wavelengths is the dominant detection technique applied in the 57% of the reviewed papers as shown in Figure 7. However,

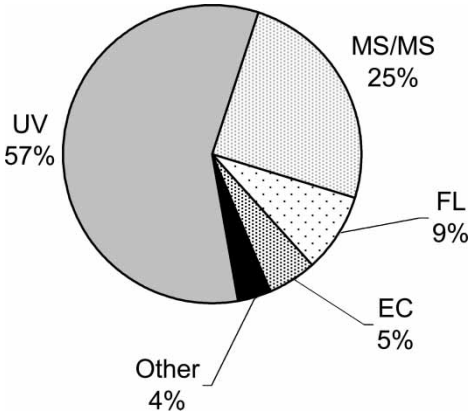


Figure 7. Percentage distribution of detection techniques used in TC determination in food samples.

the last 8 years the use of mass spectrometry is significantly increased (25%). Fluorescence and electrochemical detection are used to a lesser extent.

UV detection is employed mainly at wavelengths within the range 350–370 nm. More specifically TC, OTC and CTC are determined in animal tissues at 370 nm (50) as well as CTC in poultry/swine feeds (57) and TC, OTC, CTC in shrimps and milk with LOD values 50–400 μg/kg (67).

TC, OTC, CTC and DC are determined in bovine milk and muscle at 365 nm with an LOD of 0.1–5 ng/mL (65). At the same wavelength OTC is determined in fish feed with LOD of 10 ng/g (44), TC, OTC, CTC, DC, MTC and DMC in kidney with LOD values of 4 μg/mL for OTC and TC and 12 μg/mL for CTC, DC, MTC, DMC (69), as well as TC, OTC and CTC in bovine and porcine muscle with 10 ng/g as LOD (56).

OTC, TC, CTC, DC, MNC and epimers isolated from animal feeds were monitored at DAD 364 nm yielding LOD values of 0.1–0.4 μg/g (42).

A significant number of papers determine TCs at 360 nm. OTC, TC, CTC, epimers in animal feed and bones were determined with LOD values 0.5 μg/kg for OTC and TC and 1 μg/kg for CTC (40), 0.05 mg/kg for OTC in animal bones (38). TC, CTC, ETC, ECTC in animal bones (50) and TC, ETC, ATC, EATC in animal feed were monitored at the same wavelength as well (37). TC, CTC, epimers in animal bones were measured with LOD values 1.5 μg/kg for TC and 5 μg/kg for CTC (41), OTC, TC and CTC in animal tissues 20–1000 ng/g with LOD values 10 ng/g for OTC, TC in tissues and 15 ng/g for CTC. Respective values for kidney and liver are 25 ng/g for CTC and 20 ng/g for OTC and TC (78).

OTC was determined in three species of marine shellfish at 355 nm with linear range: 0.1–1.5 μg/g (36) while TC, OTC, CTC, DC, MTC, DMC and MNC in milk with LOD within the range 0.42–1.27 ng/mL (79).

OTC, TC, CTC, DC and MNC were determined in bovine muscle at 351 nm. Achieved method's LOQ values were 40 μg/kg for MNC, CTC and DC and 25 μg/kg for OTC and TC. The decision limits (CCα) were in the range 104.7–109.8 μg/kg, detection capability (CCβ) was in the range 108.4–116.7 μg/kg for all compounds (64).

The majority of developed methods determined TCs at 350 nm. TC, OTC, CTC, DMC in eggs and animal tissues were determined with LOD values 10–100 μg/kg for OTC, 16–100 μg/kg for TC and DMC and 20–10 μg/kg for CTC (74) as well as MNC in bovine tissues and serum with LOD: 100 ng/μL (52), TC, OTC, CTC and DMC in animal tissues with LOD values 10 μg/kg for OTC and TC and 20 μg/kg for CTC and DMC (77), DC in turkey plasma with LOD: 0.2 μg/mL (39), TC, OTC, CTC, DC, MTC, DMC and MNC in honey (71), TC, OTC,CTC, DC in liver with LOD values of 0.05 ng/μL (47), as well as OTC, EOTC, α-apoOTC, β-apoOTC in porcine muscle and water. Monitoring of α- and β-apoOTC and EOTC at 250 nm yielded LOD values 40 ng/mL for α- and β-apoOTC and OTC, 20 ng/mL for EOTC, 50 ng/mL for α-apoOTC, 140 ng/mL for β-apoOTC: (68).

TC, OTC, CTC, DC, MNC and MTC in honey were determined at 270 and 350 nm with LOD values of 50–650 ng/mL for MNC, OTC, TC and 100–850 ng/mL for CTC, MTC and DC (82).

OTC, TC and MTC in honey using DAD were monitored at 274 nm for OTC and TC and 350 nm for MTC leading to a linear range: 8–800 ng/mL (61). TC, OTC, CTC and DC detection in milk at 267 nm gave an LOQ of 0.1 μg/mL (35). TC, OTC and CTC in tissues were also determined at 254 nm (46).

Electrospray-MS/MS detection technique has been used for TC, OTC and CTC in shrimps, and milk with LOD values 25–400 μg/kg and 50–400 μg/kg respectively, (67). Same technique was applied for TC, ETC, ATC and EATC in animal feed (37), for TC, OTC, CTC, DC 4-epimers in honey with linear range: 0–100 μg/kg (60). For TC, OTC, DMC, MNC, ATC, EATC and MTC in milk LOD values obtained were 14 ng/mL for TC, 30 ng/mL for OTC, 9 ng/mL for DMC, 40 ng/mL for MNC, 4 ng/mL for ATC, and EATC and 6 ng/mL for MTC. In milk samples the LOD was 100 ppb (58). For CTC determination in swine plasma and water linear range was 20–2000 ng/mL (66). OTC, TC and CTC in bovine tissues (73) and TC, OTC, CTC, DC, 4-epimers in pig tissues were also determined by ESI-MS/MS (positive ion mode) with LOD values: 0.5–4.5 ng/g (76) TC, OTC and CTC in honey and eggs with respective LOD values 25 μg/kg and 125 μg/kg (53). TC, OTC, CTC and their 4-epimers in eggs and environmental samples by microbiology assay using *S. aureus* in brain–heart broth middle (pH = 5.9) and photometrical detection at 595 nm and ESI-MS/MS provided LOD values 150 μg/kg and 20 μg/kg respectively (51). TC, CTC, OTC were determined in animal (bovine and swine) muscle, kidney, and liver by ESI-MS-MS with LOD 0.002 ppm (63).

Particle beam PB-MS/MS detection technique is applied for TC, OTC, CTC, DC, DMC and MNC in milk and shrimp (80) as well as for TC, OTC and CTC in milk with LOD value 100 ng/mL (59). APCI-MS/MS detection is applied for OTC, TC and CTC in tissues with LOD value 10 ng/g in muscles and 20 ng/g in kidney (62), for TC, OTC, CTC and DC in tissues with LOD values 0.001 ppm for OTC, TC, 0.004 ppm for CTC and 0.002 ppm for DC (72).

Electrochemical detection is used for the determination of TC, OTC, CTC and DC in shrimps by mixed valent Ru oxide-Ru cyanide glassy carbon electrode (1.1 V) with LOD 0.1 ng/μL for OTC and 0.5 ng/μL for TC and CTC (55). TC, OTC, CTC and DC in shrimps by electrochemical detection with a boron-doped diamond thin film electrode (1.4 V) yielded LOD values 0.01 ng/μL for OTC and TC and 0.05 ng/μL for CTC and DC (54). Electrochemical detection is also used for the determination of TC, OTC, CTC, DC, and MTC in milk with LOD values: 12.5 ng/mL for OTC, 20 ng/mL for TC, 25 ng/mL for DC and CTC and 10 ng/mL for MTC (43).

Fluorescence detection is used for TC determination in swine tissues with $\lambda_{exc} = 380$ nm, and $\lambda_{em} = 520$ nm with LOD and LOQ values 0.04 μg/g and 0.05 μg/g respectively (45), TC and OTC in tissue samples (from $\lambda_{exc} = 350$ nm, $\lambda_{em} = 420$ nm to $\lambda_{exc} = 374$ nm, $\lambda_{em} = 506$ nm in 7 min), 2–40 ng for OTC 1–40 ng for TC (81) as well as for TC, OTC, CTC and DC in pork and chicken meat. In the latter method, fluorescence was induced by complexation of TCs with 5% m/v zirconyl chloride octahydrate. Detection was achieved at $\lambda_{exc} = 350$ nm and $\lambda_{em} = 420$ nm yielding an LOD: 0.42–1.38 μg/kg. Results were also confirmed by ESI-MS/MS (49). TC, OTC, CTC and DC in eggs and animal tissues were determined at $\lambda_{exc} = 406$ nm, $\lambda_{em} = 515$ nm after post-column addition of 5% (m/v) zirconyl chloride octahydrate. LOD values obtained are 2–1000 μg/kg for OTC, 3–1000 μg/kg for TC, 4–1000 μg/kg for CTC and 5–1000 μg/kg for DC (75).

Chemiluminescence is applied for the determination of OTC, TC and MTC in honey. Chemiluminescence detection was applied with potassium permangate-sodium sulfite-β-cyclodextrin. LOD values achieved are 3 ng/mL for OTC, 5 ng/mL for TC and 0.9 ng/mL for MTC: (61). TC, OTC, CTC, ICTC, N-DMCTC, N-DMOTC, 4-N-DMICTC were determined in eggs using different detection modes: UV (310 and 360 nm), FL ($\lambda_{exc} = 350$ nm, $\lambda_{em} = 420$ nm) after post-column addition of 0.4 M glycine buffer pH 12 and ESI-MS/MS. (70).

Method Validation-Validation Criteria

In the majority of the published methods the analytical procedure is validated in terms of various parameters such as linearity, accuracy, precision, specificity, selectivity, sensitivity, limit of quantification (LOQ), limit of detection (LOD), applicability and robustness or ruggedness.

Linearity of the method is usually checked using spiked blank tissue samples. For each calibration curve, a number of different concentration levels are used usually six, including a zero level and/or not the MRL level. Peak areas of TCs or peak area ratios between TCs and IS are plotted against concentrations and linear regression analysis is performed. The accuracy defined as the closeness of agreement between the true value and the mean results of a series of experiments ($n = 6$) is usually determined by analyzing a number, usually six, blank tissue samples spiked at the same concentration level and by comparing the measured concentration to the spiked concentration.

The precision defined as the "within laboratory repeatability" can be divided into repeatability and intermediate precision since it includes the use of the same method on identical test material, in the same laboratory by the same operator using the same equipment within short intervals of time. It is expressed as the relative standard deviation (RSD, in %), being the ratio between the standard deviation (SD) and the mean found concentration (in %). The "within-run or within-day" precision was determined on the same samples as for the accuracy, while the "between-day" precision was determined on blank tissue samples spiked at the MRL level and analyzed on different days. Both accuracy and precision are evaluated at three different concentrations. The precision of the method was determined by calculating the relative standard deviation (RSD, %) for the repeated measurements. The accuracy of the method (expressed as the relative error RE, %) is determined by assessing the agreement between the measured and nominal concentrations of analysed samples. The recoveries are obtained by spiking food samples at three different concentrations and then by analysing the samples six times on three different days ($n = 18$).

Linearity is studied by matrix samples fortified with a series of mixed standards of the five TCs, covering the entire working range. Calibration curves are constructed with these samples, injected at least three times, using analyte/internal standard peak area ratio. Precision and accuracy expressed in terms of TCs recovery from food sample. Recovery is calculated as the percentage of the found mass of the analyte on the spiked sample towards the mass that was initially spiked and was tested after replicate analysis of spiked samples in various concentrations.

The limit of quantification (LOQ) is defined as the lowest concentration for which the method is validated with accuracy and precision within the specified ranges. Moreover, as recommended by the EU, the LOQ has to be at least equal to half the MRL. The limit of detection (LOD) is defined as the lowest measured content from which it is possible to deduce the presence of the analyte with reasonable statistical certainty. The specificity was evaluated by the analysis of blank tissue samples. The absence of the possible interference of endogenous compounds with the same retention time, as well as the absence of the possible interference of other compounds belonging to the same class of compounds as the analyte, was demonstrated.

In compliance with the draft SANCO/1805/2000 revision (99), the validation procedure includes the determination of detection limit (CCα), detection capability (CCβ), and specificity for the quantitative confirmation method. CCα is defined as: "the concentration above which it can be determined with a statistical certainty of $1 - \alpha$ that the identified analyte content is truly above MRL." CCα is calculated by spiking 20 blank samples at MRL (100 μg/kg in the case of TCs). CCβ is "the concentration at which the method is able to detect MRL concentrations with a statistical certainty of $1 - \beta$. The β error should be less than or equal to 5%. CCβ was calculated by analysing 20 blank spiked samples at corresponding CCα level for each analyte in both matrices.

The decision limit, CC*a*, is calculated as the mean measured concentration at the MRL (100 μg/kg tissue) plus 1.64 times the standard deviation of within-day precision at this concentration. The detection capability, CCβ, was calculated as CC*a* plus 1.64 times the standard deviation of within-day repeatability at CC*a*. Statistical analysis was performed at the 95% confidential level and the number of replicate analyses was 20.

From the published methods only a limited number of papers on the quantitative determination of the tetracyclines propose methods validated by a set of parameters which are in compliance with the recommendations as defined by a regulatory agency. More specifically Cherlet et al. in their article (76) on the quantitative multi-residue analysis of tetracyclines and their 4-epimers in pig tissues by HPLC-ESI-MS, validated the proposed method for the quantitative determination of the tetracyclines by a set of parameters which are in compliance with the recommendations with the requirements as defined in the Rules Governing Medicinal Products in the European Community as defined by the EC (98, 99).

Cinquina et al. in their article "Validation of a high-performance liquid chromatography method for the determination of oxytetracycline, tetracycline, chlortetracycline and doxycycline in bovine milk and muscle," validated the method in compliance with requirements set by draft SANCO/1805/2000 European Decision (65).

Blanchflower et al. in their article "Confirmatory assay for the determination of tetracyline, oxytetracycline, chlortetracycline and its isomers in muscle and kidney using liquid chromatography-mass spectrometry," validated their method at the maximum residue limit, the maximum residue limit as well as at two times the maximum residue limit (62).

Andersen et al. in their article (67) on the determination of tetracycline residues in shrimp and whole milk using liquid chromatography with ultraviolet detection and residue confirmation by mass spectrometry proposed two methods designed to simplify sample extraction and cleanup steps and to be fast and convenient for routine testing in a regulatory environment. The objective of this study was to develop a method that would be simple and fast for routine regulatory analysis of the three tetracycline residues

OTC, TC, and CTC in shrimp and whole milk. Regulatory aspects that were taken into consideration include the U.S. Code of Federal Regulations, Title 21, Part 556, Sections 150, 500, and 720. U.S. Government Printing Office, Washington, DC, 2003 (Chapter 1), as well as The European Agency for the Evaluation of Medicinal Products, EMEA/MRL/023/95 (99).

Alfredsson et al. in their article "Simple and rapid screening and confirmation of tetracyclines in honey and egg by a dipstick test and LC–MS/MS" did not evaluated the method as a quantitative one, since the use of tetracyclines is illegal in honey production. Therefore, the confirmation method has just to fulfil the confirmation criteria according to Commission Decision 2002/657/EC (53).

Samanidou et al. in their article "Development and validation of an HPLC confirmatory method for the determination of tetracycline antibiotics residues in bovine muscle according to the European Union regulation 2002/657/EC," performed the validation of the method they propose according to the European Union regulation 2002/657/EC determining selectivity, stability, decision limit, detection capability, accuracy and precision using spiked samples at various concentrations (64).

Trends in the TCs Analysis in Food Samples of Animal Origin

After a thorough study of the literature, from the up to now published methods for the determination of TCs in foodstuffs of animal origin, an increase can be noticed by time, especially after 1995. The majority of the analytical techniques used for that purpose are separation techniques (80%), among which HPLC is the most frequently used.

From the various HPLC methods developed so far about 60% use columns with C_{18} cartridges and 21% C_8. The use of ACN as an organic modifier in mobile phase and addition of oxalic acid for better separation are usual practices, with very few exceptions using other buffers, like citric acid, formic acid or THF. As regards with the detection techniques, UV detectors are used in percentage larger than 50%, while the last 8 years the use of mass spectrometry is significantly increased (20%).

About 50% of the developed methods presented in Tables 2–4 are applied to tissue samples of various animals, while milk, honey and animal feed samples possess a quite important percentage, about 10% each one. In most of these methods, three or four tetracyclines are separated, TC, OTC, CTC and DC above all, as these drugs are used in veterinary medicine. Many different acid buffers are used for the extraction of TCs from the matrix of samples, like McIlvaine buffer (25%), succinate buffer (18%), citrate buffer (10%), HCl (14%) and H_3PO_4 (5%). Solid phase extraction (SPE) is the most frequently used (50%) technique for cleaning up these extracts. Other techniques used are liquid/liquid extraction (20%) and metal chelate affinity chromatography (15%).

CONCLUDING REMARKS

The use of tetracyclines has become a serious problem as regards with infectious diseases, since they leave residues in food products of animal origin such as milk, eggs or meat which can be directly toxic or else cause allergic reactions in some hypersensitive individuals. Even low-level doses of antibiotic in foodstuffs consumed for long periods can lead to problems regarding the spread of drug-resistant micro-organisms. In the present paper the methods for the determination of tetracyclines in foodproducts of animal origin are reviewed and comparatively presented. HPLC is the predominant technique used for this purpose. Method development however must be followed by method validation according to regulatory agencies in order to obtain methods that can be readily applied by authorised laboratories that execute analyses of food samples to ensure the protection of human health. Anyway concerning the Europoean Union all methods currently applied for the analysis of official samples of the substances in group B of Annex I of Council Directive 96/23/EC will have to comply with the decision 2002/657/EC by the 1st of September 2007.

ABBREVIATIONS

4-ECTC	Epi-chlortetracycline
4-EDC	Epi-doxycycline
4-EOTC	Epi-oxytetracycline
4-ETC	Epi-tetracycline
ACN	Acetonitrile
ACTC	Anhydro-chlortetracycline
Activ.	Activated
ADCTC	2-acetyl-2decarboxamido CTC
Add.	Addition
ADI	Acceptable daily intake
Adj.	Adjusted
ADOTC	2-acetyl-2decarboxamido OTC
ADTC	2-acetyl-2decarboxamido TC
AIBN	2,2′-azobisisobutyronitrile
AOTC	Anhydro-oxytetracycline
ATC	Anhydro-tetracycline
buff.	Buffer solution
CE	Capillary electrophoresis
Centr.	Centrifugation
CL	Chemiluminescence
Conc.	Concentrated
CTC	Chlortetracycline
DC	Doxycycline

Dens.	Densitometry
Det.	Detection
Dil.	Dilution
DMC	Demeclocycline
EACTC	Epi-anhydro-chlortetracycline
EAOTC	Epi-anhydro-oxytetracycline
EATC	Epi-anhydro-tetracycline
ECL	Electrochemiluminescence
EGDMA	Ethylene glycol dimethacrylate
El.	Eluent/elution
Electr.	Electrochemical
endc.	End-capped
ESI	Electrospray ionization
EU	European Union
Evap.	Evaporation
Extr.	Extraction
FDA	Food and Drug Administration
Filt.	Filtration
FL	Fluorescence
Fluor.	Fluorimetry
FR	Flow rate
FSIS	Food Safety and Inspection Service
Grad. El.	Gradient elution
HFBA	Heptafluorobutyric acid
Homog.	Homogenization
ICTC	Iso-chlortetracycline
Incub.	Incubation
Inj.	Injection
IOM	Institute of Medicine
IOTC	Iso-oxytetracycline
IPA	Isopropanol
IS	Internal Standard
Is. El.	Isocratic elution
LLE	Liquid liquid extraction
LR	Linear Range
MAA	Methacrylic acid
MCAC	Metal chelate affinity chromatography
Meas.	Measurement
Microb.	Microbiological
MIP	Molecularly imprinted polymers
MISPE	Molecularly imprinted polymers-solid phase extraction
Mix.	Mixing
MNC	Minocycline
MP	Mobile phase

MTC	Methacycline
N-DMCTC	N-desmethyl-chlortetracycline
N-DMOTC	N-desmethyl-oxytetracycline
OTC	Oxytetracycline
Precip.	Precipitation
Pre-dev.	Pre-developed
PS-DVB	Polystyrene-divinylbenzene
Recons.	Reconstitution
Sol.	Solution
Sonic.	Sonication
SPE	Solid phase extraction
SPME	Solid phase microextraction
TC	Tetracycline
TCA	Trichloroacetic acid
TEA	Triethanolamine
TFA	Trifluoroacetic acid
THF	Tetrahydrofurane
TMA	Tetramethylammonium
UCS	Union of Concerned Scientists
Ultrafilt.	Ultrafiltration
USDA	United States Department of Agriculture
USP	United States Pharmacopeia
α-APOTC	α-apo-ocytetracycline
β-APOTC	β-apo-ocytetracycline

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