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Review

Chromatographic methods for tetracycline analysis in foods

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ABSTRACT

The tetracyclines have served for decades as an important class of antibiotics in food animal health and production. As such, they have also been a source of concern for residue monitoring authorities around the world. In response to this concern a number of microbial inhibition, immunoassay and bacterial receptor methods have evolved for the detection of this class of compounds in various foods of animal origin. However, these methods often lack specificity and are subject to false positive and false negative results. For these reasons a number of chromatographic methods for the separation and determination of the tetracyclines isolated from foods have been developed that are capable of identifying and quantifying individual tetracycline drugs. We present here an overview of tetracycline analytical methods, including 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 this class of compounds. A discussion of the variables involved in such methodology and a review of method criteria are offered.

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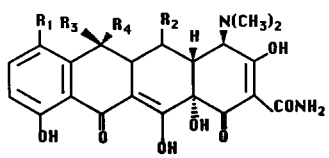
1. INTRODUCTION

Over the last two decades there has been an increased use and availability of veterinary therapeutic agents to maintain the health of and increase the production from food animals. This increased usage has been paralleled by a rising concern regarding the presence of residues from such agents in the food supply. The presence of drug residues is often due to the improper observance of drug withdrawal times and is most commonly seen, in the USA, for the various classes of antibiotic drugs [1]. The tetracycline antibiotics are one of these classes and have long been a source of concern to residue monitoring programs.

The Food Safety and Inspection Service (FSIS) branch of the United States Department of Agriculture (USDA) has included the antibiotics tetracycline (TC), chlortetracycline (CTC) and oxytetracycline (OTC; Fig. 1, a–c, respectively) in their "Compound Evaluation and Analytical Capability National Residue Program Plan" [2] for many years and routinely monitor for the presence of these compounds in tissues from food animals. Internationally, these compounds, as well as the compounds doxycycline (DC; 1d, Fig. 1) and minocycline (MC; 1e, Fig. 1) and others, are also of concern as residues in animal derived foods. The governmental tolerances and/or action levels for these compounds vary among countries and among animal species, based on differing predicted consumption rates and safety factors. The FSIS, for example, utilizes levels ranging from zero tolerance to 4 μg/g for monitoring the various species and tissues examined [2]. Monitoring for these compounds in the various species is conducted, in most cases, by a Swab test or other microbiological assay [2] utilizing transudate or homogenate from kidney, liver or muscle tissues. Preliminary identification of the particular residue involved can be based on thin-layer chromatographic analysis of the tissue extract after the conversion of the component tetracycline to its anhydro derivative [2,3]. More exacting determinative and confirmatory methodology is then applied to those samples suspected of being in violation.



a. Tetracycline, R_1=R_2= H, R_3= OH, R_4= CH_3
b. Chlortetracycline, R_1=Cl, R_2= H, R_3= OH, R_4= CH_3
c. Oxytetracycline, R_1= H, R_2=R_3= OH, R_4= CH_3
d. Doxycycline, R_1=R_3= H, R_2= OH, R_4= CH_3
e. Minocycline, R_1= $N(CH_3)_2$, R_2=R_3=R_4= H

Fig. 1. Structures of the various tetracyclines.

In this review we will focus on the latter aspect of tetracycline analyses, examining the chromatographic techniques that have been successfully applied to the isolation and determination of these compounds as they occur as residues in food animal matrices. A number of reviews concerning the analysis of tetracyclines in general or as residues in foods of animal origin appeared in the mid-1980s [4–8] and we will refer to these reviews *in toto* for some aspects of the matters addressed here. Further, given the importance of utilizing rapid screening technologies in food monitoring programs to determine to which samples these more elaborate determinative methods should be applied, we also provide an overview of the microbiological/immunoassay approaches presently available. From this information conclusions regarding the state of the art of tetracycline analysis and the directions for future investigation are drawn and offered for consideration.

2. MICROBIAL AND IMMUNOASSAY DETECTION OF TETRACYLINES

Tetracyclines have traditionally been detected in animal tissues and fluids by microbial inhibition tests (MIT). These MITs are in wide use today but a number of other detection methods have been de-

veloped in the last decade and are also available. These include the competitive bacterial receptor binding assay, enzyme immunoassay, and bioautography. At present such residue screening methods are not validated by any federal agency [9]. There are seven methods listed as AOAC Official Methods of Analysis for the detection of antibacterials in milk, but only one official method for the detection of tetracyclines [10]. No official methods are listed for the detection of tetracycline residues in animal tissues, however.

2.1. Microbial inhibition tests

All MITs are based on the inhibition of bacterial growth by residues of antibacterial compound(s) present in milk or tissue. Early assays for chlortetracycline and oxytetracycline residues in milk utilized the reduction of methylene blue as an indicator of bacterial growth [11,12]. Numerous MIT methods for the detection and quantitation of tetracycline have since been described [13–17]. MITs are non-specific and interferences from other antibacterials can occur. Selective sensitivity for tetracyclines or other antibiotics can be obtained by changes in the culture medium, indicator bacteria, or pH [18,19]. Microbial methods measure only the parent drug and microbiologically active metabolites [20]. Imprecision occurs as a result of zone size differences between plates. Zone size may vary as a result of differences in agar layer thickness, agar quality, uneven seeding of bacterial spores on the agar surface, or incubator temperature variation [14]. Plate assay MITs are performed by streaking a uniform suspension of indicator bacterial spores over an agar medium. Swabs or disks soaked in a body fluid are placed on the plate and incubated. A positive control is provided by a neomycin sensitivity disk. The observed end point can be a zone of inhibition surrounding a sample or a color change resulting from pH changes. MITs currently in use for screening tissues for tetracyclines by the USDA–FSIS are the Swab test on premises (STOP), live animal Swab test (LAST), and the calf antibiotic and sulfa test (CAST) [21]. The USDA–FSIS is currently evaluating the fast antibiotic screen test (FAST) as a replacement for the STOP and CAST methods [17].

2.2. Swab test on premises

The STOP is used to detect antibiotic residues in kidney and other tissues of slaughter animals [16]. The STOP method is relatively simple and requires only a few minutes [22]. A cotton swab is inserted directly into the meat sample, left in place for 30 min, and the cotton tip is placed on a test plate containing Difco antibiotic medium No. 5 previously streaked with a spore suspension of *Bacillus subtilis*. The plate is incubated at 29°C overnight (16–20 h) and observed for inhibition of bacterial growth surrounding the swab. Johnston *et al.* [16] reported 94% agreement with results of STOP and standard microbial assays. Korsrud and MacNeil [18] reported varying sensitivity with different media using standard solutions of tetracyclines. With the standard antibiotic medium No. 5, limits of detection (LODs, μg/ml) were 6.2 (CTC), 3.1 (OTC) and 1.6 (TC). With antibiotic medium No. 2, LODs were 0.06 (CTC) and 1.6 (OTC and TC). Minimal detectable levels using antibiotic medium No. 5 as reported by Johnston *et al.* [16], were 0.01 μg/ml (CTC) and 0.08 μg/ml for OTC and TC. In a comparison of STOP, HPLC, MIT and thin-layer chromatography–bioautography (TLC-B) by MacNeil *et al.* [23], STOP lacked the sensitivity of HPLC but had greater or lesser sensitivity for OTC than TLCB or MIT depending on the growth medium used.

2.3. Calf antibiotic and sulfa test

The CAST procedure was introduced by the USDA to increase sulfonamide detection sensitivity in bob veal calves but is also sensitive to a variety of other antimicrobials including tetracyclines. The degree of inhibition varies with the compound tested [24]. The CAST procedure is similiar to the STOP but uses Mueller–Hinton medium and *Bacillus megaterium* ATCC 9885 as the indicator bacteria, and is incubated at 44°C. Plates are read as for the STOP procedure and kidney is used as the sample tissue. Korsrud and MacNeil [18] reported the CAST procedure was more sensitive than the STOP procedure for standard solutions of 22 antibiotics tested including CTC, OTC and TC. Minimum detectable levels (μg/ml) were 0.2 (CTC), 0.8 (OTC) and 0.4 (TC).

2.4. Live animal swab test

The LAST procedure is a modification of the STOP procedure differing only in the amount of *B. subtilis* used [25]. It is used for preslaughter field screening of residues in urine and for prediction of residues in edible tissue. It was the first on-farm test available for screening live cattle for possible residues and is based on the correlation between urine and tissue residue levels. Urine or blood samples may be used [25,26]. Two sterile swabs are dipped in a urine sample and placed on the LAST plate containing antibiotic medium No. 5 streaked with *B. subtilis* ATCC 6633 spores . A neomycin disc is used as a positive control, and incubation and test interpretation are as for the STOP procedure. Several reports indicate a high incidence of false positive results using the LAST assay. In one study 75% (15 or 20) of untreated cows showed a positive result [27]. Tritschler *et al.* [25] reported 5.4% positive results and 19.9% questionable results from 221 untreated dairy cows and heifers. TerHune and Upson [28] had varying results for LAST detection of OTC when compared to standard quantitative OTC MIT procedures. LAST was 100% accurate when urine OTC concentration was >4.3 μg/ml and 60% accurate when urine OTC concentration was <4.3 μg/ml. Some 20% of LAST results were false positive and 20% were false negative. False positive results were associated with high urine osmolarity and high urine pH, apparently resulting in inhibition of bacterial growth. False negative samples were associated with dilute urine. In this study LAST was 100% accurate in detecting OTC in the urine and predicting tissue OTC residues when OTC concentration was at therapeutic levels. However, LAST did not detect OTC in the urine or predict OTC concentrations of 0.1–0.4 μg/g in tissue.

2.5. Fast antibiotic screen test

The FAST is a new procedure under evaluation by USDA–FSIS which provides results within 6 h. It has undergone field trials involving 10 000 samples for comparison with STOP and CAST for sensitivity. The FAST assay is similiar to the CAST procedure but the FAST growth medium contains sugar and a purple dye. Bacterial metabolism of the sugar results in acid production causing a color change from purple to yellow for the pH sensitive dye used. A sterile cotton swab is saturated with fluid from a tissue sample and placed on a plate of growth medium streaked with bacterial spores and incubated for 6 h. A purple zone surrounding the sample swab indicates the presence of antimicrobial agent(s) [17].

2.6. Delvotest P

This test is a qualitative color reaction test based on acid production by *Bacillus stearothermophilius* var. *calidolactis*. This changes the color of bromocreosol purple to yellow. If antibacterials are present, bacterial growth is inhibited and the purple color remains. Delvotest P is an AOAC Official Method for β-lactams in milk. Sensitivity for β-lactams is $\geqslant 0.005$ IU/ml milk. β-Lactam residue is confirmed using penicillinase [10]. It will also detect a wide range of antibiotics including TC at 0.2 μg/ml and OTC at 0.3 μg/ml [26]. Macaulay and Packard [29] reported 11% false positives with this test. Delvotest P is simple to run and the color change is easily evaluated as blue *vs.* yellow. A disadvantage is the 2.75-h analysis time.

2.7. Brilliant Black reduction test

The Brilliant Black reduction test is another qualitative color reaction test and can be used to detect antibiotic residues in milk and tissue. *Bacillus stearothermophilus* is the test organism used with an assay medium containing brilliant black indicator. The assay medium remains blue if bacterial growth is inhibited by antibiotic residues, but if no residues are present the growth of the bacteria reduces the indicator to a yellow color. Limit of detection of tissue extracts for OTC is 0.1 μg/ml [30].

2.8. Competitive receptor binding assay

Competitive receptor binding assay (Charm II test) is a competitive microbial receptor binding assay that can detect residues of seven classes of antibiotics. It is the only AOAC Official Method of Analysis for tetracyclines in milk [31]. Serum, urine, egg, honey and tisue extracts may also be used. In this method, microbial cells with specific receptor sites are added to milk or tissue extract containing added ^{3}H-labelled tetracycline. The [^{3}H]tetracycline competes with any residues of the tetracycline family present in the sample for the available bacterial receptors. Following centrifugation, the sample is decanted, the precipitate is resuspended, com-

bined with scintillation fluid and its activity measured using a scintillation counter. Sample activity is compared to a zero standard and the level of radioactivity is inversely related to the residue level of the sample. The level of radioactivity used ([^{3}H]tetracycline 0.5 μCi/umol, 0.052 μCi/test) is exempt from Nuclear Regulatory and Agreement State regulations [31]. Limits of detection (ng/ml) in milk are 3 (CTC), 6 (democycline), 100 (DC), 4 (MC), 5 (OTC) and 1 (TC). Serum, urine and egg LODs are 100 for TC [32]. Assay time is 12–15 min [33]. The receptor site of the Charm II binds a functional group of the drug, rather than a side chain, as with immunoassay tests. This allows detection of a class of antibiotics by binding at a single receptor site. Results of the Charm II test have been confirmed using MIT assays for chlortetracycline. Nine samples positive for tetracycline with the Charm II were confirmed positive using a MIT [34]. However, Collins-Thompson *et al.* [35] reported that 40 of 48 milk samples positive for tetracycline by the Charm II test were negative by disc assay, and 8 showed indistinct zones of inhibition. Increased sensitivity of the Charm II and a possible unknown interfering factor were suggested [35]. Charm and Chi [33] reported a 2.3% incidence of false positives for tetracycline in milk and Senyk *et al.* [36] reported no false positives for tetracyline in milk. Charm II has been evaluated as a confirmatory method for positives from microbial screening assays [34,37].

2.9. Thin-layer chromatography–bioautography

Various separation procedures combined with bioautography have been reported and are a blend of physicochemical and bacterial growth inhibition techniques. These include paper chromatography–bioautography [38] TLC-B [23,39–41], and electrophoresis–bioautography [42]. TLC-B is based on selective tissue extraction followed by TLC. The developed TLC plates are placed on a bacterial growth medium seeded with *B. subtilis*. The location of zones of inhibition are used to identify specific antibiotic residues. The sensitivity of the method can be adjusted and antibiotic residue recovery is quantitative. TLC-B provides a multiresidue detection method and can be used to identify individual antibiotics within a class of antibiotics [39]. It has been used in Canada since 1984 for the confirmation of positive in-plant tests [18]. Neidert *et al.* [39] reported minimum detectable amounts in fortified muscle samples (ng/g) as 15 (CTC) and 30 (OTC and TC) as determined by the minimum amount causing visible inhibition zones on 100% of tests at that level. MacNeil *et al.* [23] reported TLC-B lacked the sensitivity of HPLC for OTC but was of equal sensitivity with MIT. STOP had greater or lesser sensitivity than TLCB depending on the growth medium used [23].

2.10. Electrophoresis–bioautography

This procedure can be applied to milk and meat samples and is usually preceeded by a set of MITs. It is a qualitative test but can be made semi-quantitative and is unlikely to allow resolution of related tetracyclines. Antibiotic identification is based on initial MIT results, electrophoretic migration distance, and the appearance of the zone of inhibition [42].

2.11. Enzyme immunoassay

The Cite Probe tetracycline test is a screening test for residues of chlortetracycline, oxytetracycline and tetracycline in milk. It is a competitive immunoassay that visually compares the relative color intensity of a control spot with a sample spot. It is packaged as a self contained kit and can be easily run on-farm. Assay time is 5 min. The limits of detection of tetracyclines in milk are 40 ng/ml for CTC and OTC and 20 ng/ml for TC [43].

The tetracyclines have also been readily detected by other means. In this regard, the fluorescence of tetracyclines under UV light has been used as an indicator of previous tetracycline treatment. It has been used for the detection of OTC residues in bone and injection sites, but fluorescence is non-specific and persists in bone for an extended time after treatment [23,44].

3. METHODS OF ISOLATION

The tetracyclines are congeners of a naphthacenecarboxamide. They have minimal water solubility at pH 7 but will form soluble sodium or hydrochloride salts. The pK_a values of tetracycline itself are 3.3 (hydroxyl group at position 3), 7.5 (dimethylamino group at position 4) and 9.4 (hydroxyl group at position 12). Thus, tetracyclines can exist as zwitterions and are soluble in either dilute acid or

base [6,7]. The bases and hydrochloride salts of these compounds are relatively stable as dry powders. However, most of these compounds rapidly lose antimicrobial activity when in solution, a fact that must be taken into consideration in the design of methodology for their isolation, detection and quantitation. Tetracyclines are also prone to rapid degradation by exposure to light [45–47] and precautions to prevent losses by this route are essential. The tetracyclines are soluble in most alcohols but are quite insoluble in lower solvent strength organics, such as chloroform [6,7]. Their solubility in such solvents is enhanced by conversion to their anhydro- derivatives. However, the compounds doxycycline and minocycline are more lipophilic than their counterparts and are thought to be capable of penetrating mammalian cell membranes, giving them unique pharmacological properties [48].

Isolation of the tetracyclines from aqueous solutions as ion pairs can be conducted by classical counter-current organic solvent extraction of an alkaline medium [6,7]. Isolation from tissues and food products is, however, far more complex. One difficulty in isolating these compounds is associated with the propensity of the tetracyclines to form chelation complexes with metal ions [7,49] and to bind with sample matrix proteins. Indeed, the adsorption and therapeutic effect of the tetracyclines can be diminished by the presence of elevated levels of di- and/or trivalent metals in the diet [48]. Such complexes make the tetracyclines less sensitive to decomposition by light, however [49]. In terms of enhancing the extraction efficiency of a method applied to tissue or other food matrices for the tetracyclines, one should consider the inclusion of a competing chelating agent, such as ethylenediamine tetraacetate (EDTA), citrate or oxalic acid, in order to obtain satisfactory recoveries, especially for low (< 100 ng/ml) concentrations. Consideration must also be given to providing the appropriate conditions to minimize protein binding [4]. This may, of course, not be a concern in assays that are performed directly on the sample matrix itself, such as the use of microbial inhibition, immuno- and bacterial receptor assays for the determination of tetracyclines in milk, blood or urine. However, the possible creation of false negative or false positive results from such chelation, protein binding or possible sequestration of the tetracyclines during the performance of such assays has not been thoroughly examined.

3.1. Isolation from milk, urine and blood

The analysis of various tetracyclines occurring in milk, urine and blood, as well as tissues and other foodstuffs, has been accomplished by a number of direct ultraviolet (UV), fluorometric and biological assays [6,7,50,51]. However, these methods lack specificity, although providing a degree of screening capability and simplicity for the research setting, wherein the drug administered is known. Analyses have also been performed by the application of HPLC and TLC after the isolation of the drugs using the more classical countercurrent extraction methods [6,7] as well as a variety of solid phase extraction (SPE) techniques. Several methods have also utilized direct injection of sample supernatant or following protein precipitation [52]. Of further interest are two more recent methodologies that may offer some advantages in comparison to these approaches.

In 1986, Tyczkowska and Aronson [53] reported a multi-tetracycline drug residue isolation method for serum from a variety of animal species utilizing Centricon-30 molecular mass cutoff (30 000) filters. In this process OTC, MC, TC and DC were isolated with recoveries of 76–103% via treatment of the sample (500 μl) with 500 μl mixture (1:1) of LC mobile phase (Na_2HPO_4, phosphoric acid, methanol, acetonitrile, triethylamine and water) and 2% of 85% phosphoric acid in a microseparation system. The sample was then centrifuged for 50 min and assayed by HPLC. A similar approach has been used to isolate DC from bovine and swine serum and urine. Riond *et al.* [54] diluted serum or urine samples with acetonitrile–phosphoric acid–water (20:2:78) and centrifuged the samples through 30 000 (serum) or 10 000 (urine) molecular mass cutoff filters. This method was also applied to the isolation of OTC, TC and CTC from milk by Thomas [55], wherein samples were diluted with an EDTA–phosphate buffer, and filtered through a similar type of molecular mass cutoff (25 000) filter, as described above. After centrifugation for 60 min the milk solids were resuspended and centrifuged for an additional 40 min. Recoveries ranged from 89–97% over the range of concentrations examined (50–1500 ng/ml). Although Tyczkowska and Aron-

son [53] did not utilize chelating agents for serum, they are essential to the isolation process when using milk as a matrix.

A second approach for the isolation of the tetracyclines TC, OTC and CTC from milk was reported by Long *et al.* [56], and utilized a method called matrix solid-phase dispersion (MSPD). In this process the milk sample (500 μl) was blended with a mortar and pestle with 2 g of octadecylsilane (C_{18}, ODS) derivatized silica (40 μm, 60 Å pore size, 18% load, endcapped) and 0.05 g each of EDTA and oxalic acid. The blend was transferred to a column and the column was eluted with 8 ml of hexane and 8 ml of ethyl acetate–acetonitrile (1:1, v/v). The latter eluate was evaporated to dryness, suspended in LC mobile phase (0.01 *M* oxalic acid–acetonitrile, 7:3, v/v), centrifuged and filtered for LC analysis. Recoveries ranged from 64–93% over the range of concentrations examined (100–3200 ng/ml).

3.2. Isolation from tissues

Isolations of tetracyclines from various tissues have followed the classical approach of repeated homogenization of relatively large quantities of sample in the presence of an extracting and/or denaturing solvent. The resulting homogenate supernatant is then often put through a series of sample manipulations to remove co-extracting materials while retaining a high recovery for the target tetracycline(s). For example, Onji *et al.* [57], minced 20 g of sample (bovine muscle or fish) and homogenized with 100 ml of 1 *M* HCl for 5 min. The supernatant obtained after centrifugation was filtered through 5 g of Celite. The pellet was re-extracted with 50 ml of 1 *M* HCl, centrifuged, filtered and the combined filtrates poured onto an Amberlite XAD-2 column. The column was washed with 200 ml of water and then eluted with 100 ml of methanol. The methanol was reduced in volume and the filtered sample was analyzed by HPLC. Recoveries for TC, OTC and CTC were between 67 and 83%.

In a similar approach, Moats [58] homogenized 25 g of tissue (bovine or porcine muscle, liver and kidney) with 75 ml of 1 *M* HCl. An aliquot (8 ml) of the homogenate was mixed with acetonitrile (32 ml), allowed to stand and then decanted with filtration. An aliquot (20 ml) was then extracted with methylene chloride–light petroleum (b.p. 30–60°C) (20 ml each). The resulting water layer was isolated and its volume was adjusted to 4 ml prior to analysis by HPLC. Recoveries ranging from 71–106% for TC, OTC and CTC were obtained over the range of concentrations (1–10 μg/ml) examined.

Similarly, Oka and co-workers [59,60], have homogenized 5-g samples with three separate portions of extracting solvent (total of 50 ml). However, the supernatant obtained after centrifugation was further fractionated using a C_{18} cartridge followed by washing of the cartridge with 20 ml of water. The tetracyclines (TC, OTC, CTC and DC) were eluted with 10 ml of elution solvent and assayed by HPLC. Recoveries were from 68–95% for the various compounds examined at a level of 1 μg/ml in cattle and swine muscle, kidney and liver.

In a similar effort, Rogstad *et al.* [61] developed an SPE procedure for the isolation of oxytetracycline from fish muscle and liver. In this report, 5 g of minced tissue were mixed with 1 g of EDTA and 5 ml of hexane–dichloromethane (1:3). The samples were repeatedly (3X) homogenized in 20 ml of EDTA–phosphate buffer and the combined extracts were centrifuged. After addition of NaCl and a heating and cooling cycle the supernatant obtained after centrifugation was loaded onto a C_8 cartridge. The cartridge was washed with a water–acetone solution (4% acetone) and OTC was eluted with 5% and 10% water in acetone. Recoveries were in the 89–100% range for the tissues examined.

Bjorklund [62] similarly conducted several (3X) homogenization–extraction (citric acid and Na_2HPO_4 buffer) steps on 2 g of fish tissue (trout muscle or liver), sonicated the extracts and then filtered them through paper filters. The resulting filtrate was fractionated on a Bond-Elut C_{18} column by washing with water (30 ml) and eluting the drug with 10 ml of 0.01 *M* oxalic acid in methanol. Recoveries ranged from 77–96% for the isolation of OTC, TC, CTC and DC from salmon liver and muscle.

Reimer and Young [63] have reported a nearly identical SPE method for OTC, TC and CTC in salmon muscle tissue that is based on the reports by Oka and co-workers [59,60]. In this method 5 g of tissue are repeatedly (3X) homogenized with buffer (citric acid and Na_2HPO_4), filtered and loaded onto a Bond-Elute C_{18} column. The column is washed with water and the drugs are eluted using 9 ml of 0.01 *M* oxalic acid in methanol solution. However,

recoveries ranged from 45% (CTC) to 100% (OTC).

Riond *et al.* [54] have applied the use of molecular mass cutoff filters to the isolation of DC from bovine muscle, renal medulla and lung tissues. Minced tissue samples (0.1–0.3 g) were sonicated (30 min) with a solution containing methanol–acetonitrile–phosphoric acid–water (30:10:2:58) and applied to the filtration system, being centrifuged for 30 min. Recoveries ranging from 57% (lung) to 94% (muscle) were obtained for the various tissues.

Long *et al.* [64] have reported a method for the isolation of OTC from fish muscle tissue using MSPD. Muscle tissue, blended as described above for milk, was washed with hexane (8 ml) and the oxytetracycline was eluted with 8 ml of acetonitrile–methanol (1:1). An average recovery of 81% was obtained over the range of concentrations examined (50–3200 ng/g).

4. METHODS OF ANALYSIS

Methods designed to screen for the tetracyclines and to isolate them from foods of animal origin must work in conjunction with procedures designed to separate, detect and confirm the presence or absence of the compound in the sample. This requires that the sensitivity of the screening test and the determinative or confirmatory test be compatible. Consideration must also be given to the possibility that a response from an immunoassay or receptor assay could be the result of the presence of several drugs within a class and that the subsequent instrumental analysis aplied to such a sample would make this fact discernable. Several chromatographic methods have been developed that meet these criteria.

4.1. Thin-layer chromatography

Over the last forty years, numerous paper and thin-layer chromatographic methods for the detection and quantitation of tetracyclines have been reported [4,7]. Some of these reports involve the conversion of the tetracyclines to their corresponding anhydro derivatives [3], a dehydration reaction initiated by heating in 1 *M* HCl. This enhances the UV and fluorescence response of the tetracyclines and makes them more readily extractable from aqueous media at an acid pH. This method has been used by the FSIS for the determination of the nature of the tetracycline present in a sample suspected of being violative [2].

More recently, Oka *et al.* [65] have presented optimized methodologies for the TLC analysis of eight tetracyclines using silica gel high-performance and C_8 reversed-phase plates. Detection in the 0.1–0.03 μg range was accomplished using a diazonium salt solution containing Fast Violet B and scanning of the plates using a densiometer. However, this development was not applied to tissue extracts.

As previously mentioned, several TLC methods have been coupled with bioautography [23,39–41], wherein bacteria seeded media is adsorbed onto developed TLC plates to provide zones of growth inhibition at the R_F values of various antibiotics. Although requiring an overnight incubation, this process can be adapted to provide a generic detection method for antibiotics while being relatively specific, with identification being based on R_F values [39]. This same bioautographic approach has also been applied to sample extracts separated by electrophoresis [42].

4.2. High-performance liquid chromatography

This method of analysis is, perhaps, the one most often employed for the purposes of determining the identification and quantity of the various tetracyclines in the greatest variety of sample matrices. As such, several reviews have been published focusing on LC for the separation and detection of tetracyclines [4–8]. There are also legions of publications involving the chromatography of tetracyclines and their epimers in various pharmaceutic preparations, but these are not considered in detail here [66–69]. Nevertheless, the results of such studies and those relating to the analysis of the tetracyclines isolated from tissue matrices are often affected by the same factors and, thus, have relevance.

One of the factors seen consistently in the analysis of tetracyclines by HPLC is again related to their propensity to form complexes. The tetracyclines as a group are affected by the presence of metals and the presence of free silanols on silica-based solid supports used for HPLC, TLC or for SPE [4–8]. Complications involving free silanol content were more inherent in the derivatized solid support materials sold or prepared for use in studies during the late 1970s to mid-1980s and have been overcome to

a large degree by different manufacturing processes, such as end-capping, the inclusion of citrate, oxalate and/or EDTA in mobile phases, and the availability of all-polymer-based solid supports. Difficulties with metal chelation can be controlled by the use of chelating agents, as given above, or by intentionally complexing the tetracyclines with a metal to reduce competition for such binding. Such chelation can be used to the analyst's advantage since it may also enhance subsequent UV and fluorescence detection and can also provide a molecule that is more electrochemically active and detectable [3,4,7].

Since the tetracyclines have several pK_a values and can exist as zwitterions [6,7] one must consider the control of mobile phase pH in the development of an analytical method. The ability to undergo ion formation also makes the use of ion pairing reagents for the separation and analysis of the tetracyclines practical and several methods have been offered that utilize this approach [4–8]. The maximum and minimum allowable pH values for HPLC instrumentation, effects on column lifetime and that of related components, as well as the nature of the effect of pH on other co-extractants in the sample for analysis, all tend to control the pH that is determined to be optimal in such analyses. With tetracyclines, however, one must also consider the fact that these compounds are susceptible to epimerization over the pH range of 2–6 and that they are generally unstable at acidic and basic pH values [6–7]. Thus, the storage of samples for long periods of time on autosamplers in acidic or basic solutions, especially if unprotected from light, should be avoided.

The tetracyclines have been analyzed via separation on reversed-phase (C_8, C_{18}) derivatized silica solid supports, all-polymer- or resin-based non-ionogenic solid supports as well as ion-exchange solid supports. Of these, the reversed-phase systems, especially C_{18}, have found the most application for the widest range of tetracycline drugs and sample matrices (Table 1). Many of the methods presented are based on those developed by Oka and co-workers [59,60], and utilize a 0.01 *M* oxalic acid–acetonitrile–methanol mobile phase at pH 2.0. This simple isocratic system has advantages over those using gradients or requiring more complex mobile phase compositions in not requiring a re-equilibration time and eliminating the number of reagents in specific combinations required for adequate analysis.

Most HPLC methods for the analysis of tetracyclines have employed fixed, variable-wavelength or diode array UV detectors for sample monitoring. The relatively high levels of these drugs that are allowed to occur in tissues (0.1–4.0 μg/ml in the USA) makes this method of detection adequate for most applications. The extinction coefficients for the tetracyclines are relatively large and the monitoring of samples separated by HPLC at wavelengths ranging from 350–380 nm can give detection into the low tens- of nanograms/g or ml of sample range. The ability to monitor at this wavelength also endows such assays with a degree of specificity, depending on the nature of the matrix and the co-extractants present. This provides the ability to obtain relatively clean chromatograms, which in turn reduces signal-to-noise ratios and allows one to have relatively short analysis times (8–16 min/sample) without the need to conduct time and solvent consuming gradient analyses.

Although the tetracyclines possess the ability to be detected by fluoresence, few methods employing this technique have been developed. However, Blanchflower *et al.* [70] have presented a technique for the HPLC–fluorescence detection analysis of chlortetracycline in tissue samples. This approach is based on the conversion of the CTC to its more highly fluorescent iso derivative. An excitation wavelength of 340 nm and an emission wavelength of 420 nm were utilized. However, the conversion process required some 2.5 h after extraction of the sample and limits of detection were not significantly improved over those seen with UV.

The tetracyclines are also electrochemically active [7,71] but little in the way of application of this method of detection to these drugs as residues in food animal tissue or matrices has been published.

4.3. Gas chromatography

There are a limited number of publications involving the use of gas chromatography for the analysis of tetracyclines [72,73]. Tsuji and Robertson [73] were the first to apply trimethylsilylation to the tetracycline molecule, obtaining sufficiently volatile and stable derivatives for packed-column gas chromatography–flame ionization analysis. The method was also shown to be capable of performing quanti-

TABLE 1

REPRESENTATIVE METHODOLOGY FOR HPLC ANALYSIS OF TETRACYCLINES FROM VARIOUS SAMPLE MATRICES

grad. = Gradient analysis; ACN = acetonitrile; DMF = dimethylformamide; MeOH = methanol; THF = tetrahydrofuran.

Drug(s)	Matrix	Column type	Mobile phase	Detection	Analysis time (min)	Ref.
TC, OTC, CTC	Bovine tissues, Bovine, swine serum	PLRP-S, 5 μm	grad.; H_3PO_4–MeOH–ACN (30:50:20)	UV, 355 nm	22	58
OTC, MC, TC, DC	Serum	Phenyl Spheri-5 MPLC	ACN–triethylamine–Na_2HPO_4–H_3PO_4–MeOH (1.5:0.5:8.6:2:10)	UV, 267 nm	23	53
OTC	Fish	ODS Hypersil	$(NH_4)_2HPO_4$–diethanolamine–ACN–DMF–H_3PO_4 (8.1:0.05:1.9:0.6, pH to 2.5 using H_3PO_4)	UV, 365 nm	8	79
OTC, TC	Fish	Hypersil SAS, 5 μm	Citrate–Na_2EDTA–ACN (34.5:0.5:15)	UV, 370 nm	8	80
OTC	Fish	Supelcosil LC-18DB, 5 μm	Phosphate buffer (pH 2)–ACN–THF (81:10:9)	UV, 357 nm	10	61
OTC, TC, CTC, DC	Fish	ODS-Spheri-5	ACN–DMF–0.01 *M* oxalic acid (22:6:72)	UV, 355 nm	8	62
TC, OTC, CTC	Milk	NovaPak C_{18}	grad.; 0.01 *M* oxalate–MeOH–ACN (70:8:22)	UV, 360 nm	16	55
DC	Bovine, porcine tissues, body fluids	Phenyl Spheri-5	ACN–triethylamine–0.08 *M* Na_2HPO_4–H_3PO_4–MeOH (2.5:0.5:73.8:1.7:22.5)	Diode array, 235–380 nm	12	54
TC, OTC, CTC	Milk	MicroPak C_{18}, 10 μm	0.01 *M* Oxalate–ACN (70:30)	Diode array, 365 nm	8	56
MC, OTC, TC, CTC, Methacycline, Dimethyl-CTC	Bovine plasma, kidney	μBondapak C_{18}	Na_2HPO_4–ACN–DMF–ethanolamine (76:24:6:0.5)	UV, 254 nm	12	81
TC-OTC-CTC	Salmon	LiChroCART RP-18	0.01 *M* Oxalate–ACN–MeOH (73:17:10)	Diode array, 355 nm	15	63
CTC, OTC	Swine tissues	LiChrosorb RP-8	0.01 *M* Oxalate–ACN–MeOH (1:1.5:2.5)	UV, 350 nm	10	77
OTC, CTC, DC	Bovine, porcine tissues	LiChrosorb RP-18	0.01 *M* Oxalate–ACN–MeOH (1:1.5:2.5)	UV, 350 nm	12	59,60
OTC	Catfish muscle	MicroPak C_{18}, 10 μm	0.01 *M* Oxalate–ACN–MeOH (70:27.5:2.5)	Diode array, 365 nm	6	64

tative analyses, having good agreement with microbial inhibition assays. However, the formation of the derivatives was sensitive to a number of variables.

5. MASS SPECTROMETRY: CONFIRMATION OF TETRACYCLINES

There have been few articles published on the application of various mass spectrometric (MS) techniques to the detection, identification and confirmation of the tetracyclines. The various extraction and HPLC methods available make analysis of the tetracyclines by LC–MS a practical concept, but only two articles applying this approach to the confirmation of DC [74] and to OTC, TC and CTC [75] have been found by the authors. Nevertheless, such methods are presently being applied by governmental agencies for the confirmation of tetracycline positives that are found to be in violation of regulatory limits (personal observation).

One notable exception is the MS–MS method reported by Traldi *et al.* [76], for the analysis of OTC in milk and muscle tissues using a single-ion monitoring (SIM), collisionally activated decomposition (CAD), mass-analyzed ion kinetic energy spectrometric (MIKES) approach. In this method, crude extracts from fortified (1–10 ng/g or ml) milk or muscle, obtained by ethanol extraction and, centrifugation, evaporation and resolubilization in ethanol, were directly introduced into the instrument and analyzed by the SIM-CAD-MIKES-MS–MS method. Limits of detection were in the low ng/g range and adequate data were obtained to permit confirmation of identity of the species examined.

6. METHOD DEVELOPMENT AND EVALUATION CRITERIA

The choice of methods for a given application is driven by a variety of factors. For sample extraction these factors include the following;

(a) The sample size necessary to obtain a given limit of detection or determination for the analytical instrumentation available.

(b) The nature of the matrix and the availability of existing methods to deal with the isolation of the target molecules from the specific matrix.

(c) The necessary specificity of the isolation technique; *i.e.*, the ability to isolate a single compound within a drug class, several compounds within a drug class or to isolate several drug classes from a single sample.

(d) Sample numbers and turn-around time.

(e) The cost of the method, including supplies and disposables, time and overall labor involved and the costs of the instrumentation that will be applied.

All of these factors are interrelated and are dependent on what the analyst must or needs to accomplish. However, the simplest method of extraction is the one that requires no sample manipulation. These are the methods that extract the drug directly from the sample matrix by means of specific or selective antibodies or receptors. These assays, as described above, can be configured to simultaneously isolate, quantify and, in some cases, provide a preliminary identification of a variety of different compounds. Such tests are most applicable to aqueous solutions of the drugs, such as milk, urine, blood or blood fractions and to tissue homogenates or solubilized extracts from tissues or other food products. Despite their power, such approaches are, however, also prone to interference from naturally occurring compounds in the sample matrix and to the possible concentration of such interferences from extracts. Interferences that also bind to antibody or receptors can lead to false positive results. Such interferences may be naturally occurring compounds or other drugs or their metabolites that occur in the sample but are not recognized as having cross-reactivity in the assay. A further concern is that the drug may remain bound to sample proteins or be in complexes that do not bind with a given antibody for their detection, leading to false negative results or a reduced response from what is actually a higher than detected level. This is especially of concern for drugs like the tetracyclines that possess both high protein binding and complex formation potential.

For these reasons, and for purposes of performing more exacting quantitation and identification of the detected residues, one must often perform sample manipulations subject to the considerations listed above, even for immuno- or receptor assays. For the tetracyclines the following approaches have been delineated;

(a) Protein precipitation–direct HPLC injection;

mainly for aqueous samples, such as serum, but has also been applied to milk and urine. Organic solvents or acids used for precipitation must be removed prior to use of the supernatant in an immunoassay.

(b) Counter-current and/or homogenization–solvent extraction; classical solvent–matrix distribution conducted by extraction of an aqueous sample and/or by the homogenization of the sample in the presence of an extracting solvent. Emulsion formation can be a problem and centrifugation and repeated re-extraction of the pellet obtained from the samples is often required. Again, organic solvents and strong acids would interfere with the use of the isolate in an immunoassay format.

(c) Solid-phase extraction; except for aqueous sample matrices, requires pre-solubilization or extraction, as described above, prior to addition and fractionation on the column. Drugs isolated using organic solvents could be assayed by antibody methods after evaporation of the solvent and resolubilization in an appropriate assay buffer or reagent.

(d) Ultrafiltration; as above, also requires pre-solubilized sample extract if other than an aqueous sample. However, aqueous filtrates could be used directly in immuno or receptor assays.

(e) Matrix solid-phase dispersion; can be applied to aqueous or solid samples. Organic solvent used for elution would have to be removed before extract could be used in an antibody or receptor based assay.

All of these methods may prove useful for a given application. However, a further consideration that has obtained greater significance is the recognition that methods that generate large quantities of solvent or other wastes are becoming too expensive and too hazardous to the environment to perform. Thus, extraction methods that use large sample sizes and, thus, require large volumes of organic solvent to adequately perform residue isolation are becoming increasingly unacceptable. For this reason alone many of the classical homogenization–extraction methods will have to be eliminated and procedures such as direct injection, ultrafiltration, SPE and MSPD will have to be implemented.

Any method for the isolation of drugs must take these factors into consideration. For the tetracyclines one must also develop the analytical methodology to eliminate pH and light-induced decompositions or rearrangements as well as assure that complications from protein binding and chelation with metals is eliminated or controlled. The exclusion of light, the use of appropriate buffers to control pH and the ionization state of the drug and the inclusion of chelating agents such as citrate, EDTA and oxalate should provide appropriate control for these factors and provide adequate recovery and ability to analyze tetracycline containing samples.

These factors are not only the case in the extraction methodology but also in the methods applied to the quantitation and identification of the tetracyclines. As described, several of the immunoassays available in the market are quite specific and can be quantitative. The use of such tests for such purposes thus eliminates much of the sample manipulation and difficulties that arise as described above. Similarly, where the particular tetracycline to be quantitated is known, as in various tetracycline research scenarios, one may find simple UV or fluorescence monitoring of the sample to be adequate. Nevertheless, there will be the need at some juncture to provide a less equivocable analysis of the residue. For the tetracyclines the method of choice would be HPLC with UV detection, using a variable-wavelength or diode array detection system. Monitoring by UV at wavelengths between 350 and 380 nm provides adequate detection and a degree of selectivity over co-extractants that allows for excellent separation and short run times (8–16 min) for one or a number of tetracyclines in a single analysis and in a variety of matrices.

Although many HPLC techniques have been described over the years, one must exclude from consideration any strict adherence to LC methods reported prior to 1985. This fact is in large measure due to the very different nature of the solid supports available today when compared to those of the past. More recent methodology [Table I] has shown that a reversed-phase C_{18} column and an isocratic mobile phase at an acid pH (*ca.* pH 2), containing oxalic acid, acetonitrile and methanol can provide more than adequate methodology for the separation, quantitation and identification of the various tetracyclines and has been used to perform large surveys for tetracycline contamination in the food supply [77]. Such methodology can complement microbial or immunoassay tests in that they have

short analysis times overall and LODs in the same concentration range.

Although little is available in the literature regarding the confirmation of tetracyclines isolated from food matrices, it is a practical matter that gas chromatography [72,73] and LC–MS [74,75] are applicable to such endeavors. Gas chromatographic analysis can be accomplished by trimethylsilylation of the tetracycline molecule and, in combination with MS, especially in various selected ion monitoring modes, should provide more than adequate sensitivity and selectivity for performing confirmations for suspect samples. The CAD-MIKES-MS–MS method offered by Traldi *et al.* [76], provides a highly sensitive and specific technique for tetracycline analysis. However, this can be little more than an experimental MS curiosity since the availability of the instrumentation to perform such analyses is out of the reach of most laboratories.

7. EVOLVING ANALYTICAL METHODS

There is little doubt that the direction being taken for the determination of drug residue contamination and possible violations in foods of animal origin is that of the increased utilization of immuno and receptor based assays. These assays are finding increasing application to the direct screening of milk, blood and tissue transudate or homogenate for the more rapid identification of suspect or violative samples. This technology will be extended to include the development of and to make more readily available the use of immunoaffinity chromatographic columns or discs [78]. However, one cannot obtain adequate data to identify and, in some cases, quantify residues that produce responses in such assays alone. There will remain a need to provide techniques for the chromatographic fractionation and identification of the various residues. Where sample homogenization or disruption, coupled with a degree of fractionation, is needed methods based on MSPD, SPE and ultrafiltration can be applied. These methods, like the immunoassays, are also amenable to automation and are, thus, compatible in terms of sample through-put and turn-around time with the more rapid screening techniques. Advances in LC technology, in terms of in-line extraction, microbore columns and more sensitive and selective detectors, will also prove applicable.

8. CONCLUSIONS

While numerous methods for the isolation, separation, detection and quantification of the tetracyclines occurring as residues in foods of animal origin have been developed, they are rapidly being replaced by antibody-antigen based systems that perform simultaneous extraction, isolation, detection and, in some cases, quantitation. Such approaches are also replacing slower and less specific microbial inhibition assays that have been the backbone of many residue monitoring programs. The new assays must be supported by extraction methods that are capable of performing rapid and reasonably specific isolation techniques, providing rapid identification and/or a degree of confirmation of the residue in question. Depending on the sample matrix involved, there are analytical approaches available to perform such analyses for several possible tetracycline residues in a single sample simultaneously. Further, simple isocratic HPLC analysis methods for all or most of these compounds simultaneously are also available and can be conducted in a relatively short analytical time frame per sample. Thus, the combination of evolving immunoassay detection and screening technologies coupled with multi-residue extraction or sample preparation techniques and rapid HPLC analysis should provide the necessary speed and accuracy for the monitoring, regulation and control of tetracycline residues in foods of animal origin.

9. ABBREVIATIONS

ACN	Acetonitrile
AOAC	Association of Official Analytical Chemists
CAD	Collisionally activated decomposition
CAST	Calf antibiotic and sulfa test
CTC	Chlortetracycline
DC	Doxycycline
DMF	Dimethylformamide
EDTA	Ethylenediamine tetraacetate
FAST	Fast antibiotic screen test
FSIS	Food Safety and Inspection Service
grad.	Gradient analysis
HPLC	High-performance liquid chromatography
LAST	Live animal Swab test

LC	Liquid chromatography
LOD	Limit of detection
MC	Minocycline
MeOH	Methanol
MIKES	Mass-analyzed ion kinetic energy spectrometry
MIT	Microbial inhibition test
MS	Mass spectrometry
MSPD	Matrix solid-phase dispersion
OTC	Oxytetracycline
SIM	Single-ion monitoring
SPE	Solid-phase extraction
STOP	Swab test on premises
TC	Tetracycline
THF	Tetrahydrofuran
TLC	Thin-layer chromatography
TLC-B	Thin-layer chromatography–bioautography
USDA	United States Department of Agriculture
UV	Ultraviolet

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