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Review

Complexities in tetracycline analysis—chemistry, matrix extraction, cleanup, and liquid chromatography

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Abstract

The extraction and cleanup of commonly used tetracyclines (oxytetracycline, tetracycline, chlortetracycline, and doxycycline) from sample matrix, and their subsequent determination via liquid chromatography can be problematic. Many manuscripts report on various challenges encountered when developing a method for tetracycline antibiotics determination. These complexities often result in less than perfect recoveries or chromatograms and are based on the underlying chemistry associated with tetracyclines. This review compiles, compares, and discusses the results and observations found in published methods, while focusing on chemical principles in order to increase the practicing chemist's understanding of TCs to aid him/her in developing useful analyses.
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Keywords: Tetracycline antibiotics; Biological matrix extraction; Chromatography

Contents

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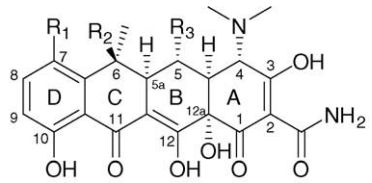
1. Introduction

Since their discovery in the mid-1900s, tetracycline antibiotics (TCs) have played a prominent role in sustaining health among physician and veterinarian. The therapeutics have been of particular interest for their use in food producing animals because of their broad spectrum activity and low cost. While quantities used worldwide are difficult to pinpoint, oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC), and doxycycline (DC) (Fig. 1) have been reported as high use TCs [1]. TCs are widely approved for use in food producing animals with tolerances ranging from 0.1 to 2 ppm in commonly consumed animal tissue and products [2,3]. Tolerances for less commonly consumed tissue reach 12 ppm [2].

The use of TCs within the veterinary community has prompted the publication of various reviews [1,4–8]. Some reviews have focused exclusively on TCs while others have included a variety of antibiotics. Most recently, Oka et al. [1] authored a review that includes an extensive set of tables listing conditions employed during the chromatographic analysis of TCs. Due to its breadth, the Oka review is an excellent resource for those seeking references regarding the analysis of particular TCs within a given matrix or utilizing a specific analytical column. However, this present review focuses on the underlying chemistry involved in the extraction, cleanup, and detection of TCs within biological/food matrices via column chromatographic analysis. It is not intended to include every analytical method published for TCs analysis. Rather, it will discuss general trends and problems, highlighting important physical and chemical properties associated with the analytical extraction and chromatographic detection of trace levels of TCs in biological matrices.



Oxytetracycline: R_1=H, R_2=R_3=OH
Tetracycline: R_1=R_3=H, R_2=OH
Chlortetracycline: R_1=Cl, R_2=OH, R_3=H
Doxycycline: R_1=R_2=H, R_3=OH

Fig. 1. Structures of selected tetracyclines.

2. Background chemistry

2.1. Impurities

TCs are biosynthetically produced, and as such, include a small percentage of impurities. In addition, degradation analogues, formed under certain isolation/analysis conditions, are another source of contamination (Fig. 2). Impurities common to the TCs being focused on in this review include epiTCs and anhydroTCs. EpiTCs, in which epimerization occurs at C-4, can be formed in aqueous conditions that are mildly acidic (pH 2–6) [9]. It has been reported that this isomer is not formed in significant levels at pH 4 during a same day extraction with McIlvaine buffer, nor when left in solution for up to 3 days at −20 °C in the absence of light [10]. A separate paper reports epimerization of CTC, but not OTC and TC, when extracting the residue from animal feed with pH 2 McIlvaine buffer [11]. EpiTCs can be reversed back to their active form under specific alkaline conditions in the presence of a complexing metal [12]. Under strongly acidic conditions anhydroTCs are formed by a loss of water (H on C-5a, HO on C-6) and proton transfer (O-11/O-12) thereby extending the aromatic nature of the D ring to include the C ring [13]. While most anhydroTCs are stable, anhydroOTC is unstable due to its C-5 hydroxyl, and quickly forms α- and β-apoOTC through a B-ring intramolecular reaction. OTC can contain another unique impurity, 2-acetyl-2-decarboxamidoOTC, as a fermentation byproduct [14,15]. CTC is particularly vulnerable to alkaline decomposition and forms isoCTC under alkaline conditions [16,17]. In addition CTC can contain 6-demethylCTC as a manufacturing impurity [18]. The above contaminants can also epimerize at C-4 to form epi- analogues. Without identifying the specific products, Onji et al. [19] report significant degradation (>90% loss) of TC in acidic (1 N HCl) conditions over the course of 4 days. Under the same conditions OTC is moderately degraded (40% loss) and CTC remains primarily intact (6% loss). Examination of the above degradation possibilities is important when considering analytical conditions from extraction through chromatography and detection. In addition, knowledge of the impurities can

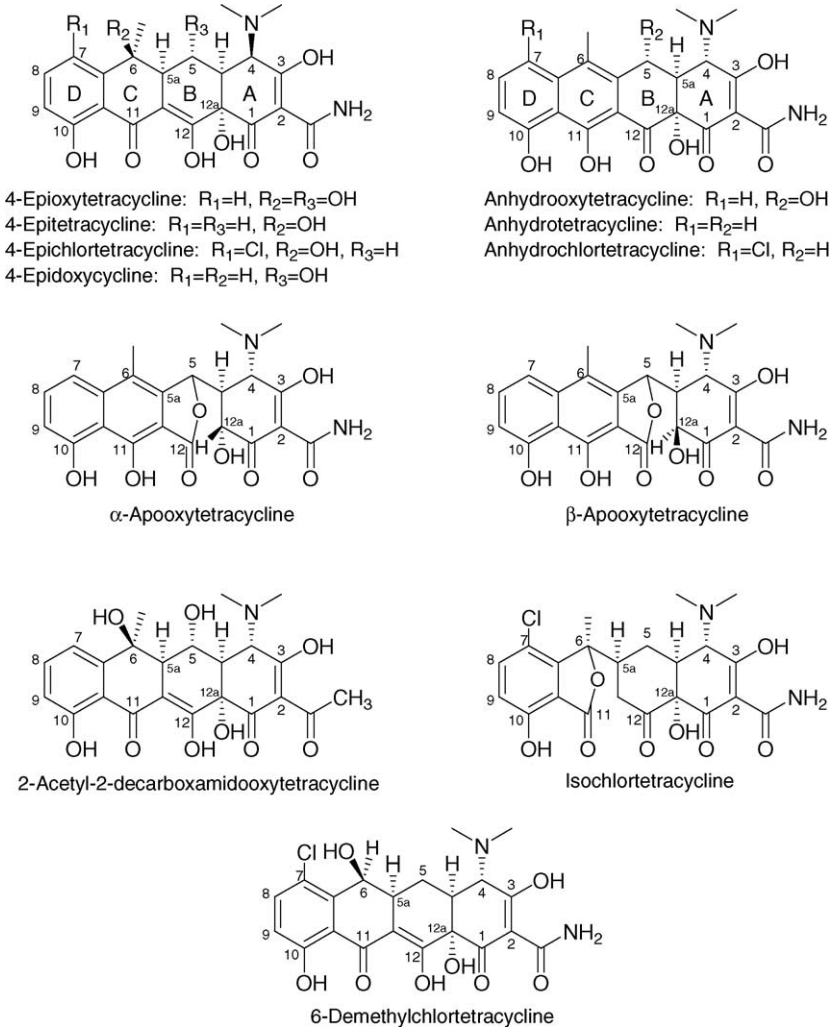


Fig. 2. Structures of selected tetracyclines antibiotic and degradation products.

be especially important during development of multi-residue screens.

2.2. Solubility, acid dissociation constants, and conformations

The solubility of TCs is greatest in alcohols such as methanol (MeOH) and ethanol, while varied in other organics like ethyl acetate (EtOAc), acetone, and acetonitrile (ACN) [20,21]. Aqueous solutions, which are often used for extraction, provide solubility in the low mg/mL range [21]. TCs are insoluble in saturated hydrocarbon solvents such as hexane. Table 1 lists the pK_a values for OTC, TC, CTC, and DC [22].

Table 1
Tetracycline antiobiotic pK_a values [22]

	pK_{a1}	pK_{a2}	pK_{a3}
Oxytetracycline·HCl	3.2	7.5	8.9
Tetracycline·HCl	3.3	7.8	9.6
Chlortetracycline·HCl	3.3	7.6	9.3
Doxycycline·HCl	3.0	8.0	9.2

The first pK_a is associated with the deprotonation of C3 hydroxyl. Loss of protons from O12 and dimethylammonium constitutes pK_{a2} and pK_{a3}, although the exact assignment of these dissociation constants remains controversial [22–24]. As indicated by their acid dissociation constants, the TCs contain localized charges across all pH values and only achieve an overall neutral state as zwitterions (Table 2). Under alkaline conditions, tetracyclines assume a conformation which allows for hydrogen bonding between N4 and OH12a. Under neutral and acidic conditions, the N4 position becomes protonated, disrupting the previous conformation, and a hydrogen bond interaction occurs with O3. TCs are also capable of assuming several other conformations depending on their environment [25].

2.3. Chelation

TCs form chelation complexes with multivalent cations [26]. Studies have indicated chelation can occur at the A ring (tricarbonyl) or BCD ring (phenolic β-diketone) systems (Fig. 3) [25,27–29]. Such mechanism is further supported by

Table 2
Tetracycline charge state continuum[a]

	pH < pK_{a1}	pK_{a1} < pH < pK_{a2}	pK_{a2} < pH < pK_{a3}	pK_{a3} < pH
O3 charge	0	–	–	–
O11–O12 charge	0	0	–	–
N4 charge	+	+	+	0
Overall charge	+	0	–	2–

[a] Continuum pK_a assignments made according to Leeson et al. [24].

the observed dramatic changes in spectral bands from both chromophoric regions of OTC upon interaction with complexing agents [28]. Zwitterionic OTC reportedly can exhibit intermolecular interactions, and self-association in the presence of cations [28]. Day et al. [30] postulate that this effect occurs more readily in nonpolar solvents; less so in aqueous solution because of the interaction of water with the complex. Multiple species of chelated TCs can co-exist in solution. The number and kind of species can change depending on pH or which metal is present, and different TCs can behave differently under the same conditions [28], further adding to the analytical complexity for this class of drugs. Additionally, ternary complexes of a TC + metal + ligand are possible, and can affect differences in TCs protonation states depending on choice of both metal and ligand present [25,27]. The formation of ternary complexes are favored by the structural flexibility of TCs and their ability to complex copper (for example) at multiple donor sites—hence the bioactivity of TCs for bacterial DNA [25].

Chelation complexes dramatically increase the fluorescence capabilities of TCs which, on their own, will weakly fluoresce under slightly alkaline conditions [30]. Molecular conformation influences fluorescence intensity, and is strongest when the BCD ring maintains a planar structure through rigid binding of the chelate at the diketone ring [30]. The fluorescence intensity of TC, in aqueous solution, increases with the addition of acetonitrile [31]. Tjornelund and Hansen [32] report increased fluorescence of TCs in organic solvents with the greatest intensification (34×) occurring in dimethylformamide. Separate studies performed on TCs with Mg^{2+}, Ca^{2+}, and Zn^{2+} have shown chelation increases with decreasing ionic radius [28,33]. Therefore, Mg^{2+} has the greatest complex formation while Ca^{2+} has the least when comparing the above ions. Likewise, the solubility of OTC in water is shown to increase across a broad range of pH values with the addition of Mg^{2+}. Calcium ions increase the water solubility of OTC from approximately pH 7–8 while zinc cations slightly increase solubility below pH 7 [28]. Chelation can also alter pK_a values, e.g. the pK_{a2} of TC is shifted to a slightly lower value in the presence of Ca^{2+} [34].

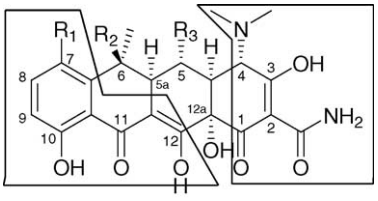


Fig. 3. Metal coordination and chromophoric of tetracyclines.

3. Extraction

3.1. Aqueous

Literature searches and published reviews reveal that aqueous based extractions are primarily employed, with a McIlvaine/ethylenediaminetetraacetic acid (EDTA) (pH 4) buffer system being most prevalent [1,7,10,35–43]. Aqueous based systems provide greater solubility over many organics, excluding alcohols, for TCs and are miscible with the biological matrices of interest. Kulshrestha et al. [44] report high molar solubility at pH 4 where the TCs are in equilibrium favoring the zwitterionic state over the fully protonated species. Succinic acid solutions (pH 4) have also been successfully applied to food based extractions [45–47]. While extractions are not limited to pH 4, we found that most aqueous based extractions were performed under acidic conditions (using HCl, perchloric acid, phosphoric acid) [1,4–8,19,48–52]. Of these alternatives it should be noted that Moats [51] found a 1 M HCl extraction suitable for OTC but not for TC due to stability. As stated in the background section, TC is more susceptible to acidic degradation, yet others report using 1 M HCl without incidence during the extraction of TC [48]. Blanchflower et al. [49], without further explanation, added glycine (0.1 M) to their 1 M HCl extraction solution when analyzing CTC. Acidic aqueous solutions have been successful in tetracycline antibiotic extraction without resulting in degradation of TCs. For instance, Pena et al. [10] report no loss of TCs in pH 4 McIlvaine buffer over the course of 3 days. One exception to the acidic extraction procedure, presented by Kawata et al. [53], utilizes an imidazole buffer (pH 7.2) system. While most extractions were facilitated using a vortex or mechanical shaker, Furusawa reports good success employing an ultrasonic homogenizer with egg and milk matrices [54,55].

3.2. Organic

Organic based extractions have been performed with EtOAc, ACN, and methanolic trichloroacetic acid (TCA) [56–58]. Iwaki et al. [58] found that for serum, methanolic

TCA was more effective than other organic and aqueous based extractions. While TCs are most soluble in alcohols, MeOH is rarely employed as a reaction solvent possibly due to difficulty of use during further cleanup (e.g. cannot be use as load solvent in most SPE applications) or its propensity to extract excessive matrix material. EtOAc was compared to aqueous based extractions in the evaluation of TCs in animal tissue and was found to be equally effective in both spiked and incurred samples [56]. ACN was used in conjunction with hexane for the extraction of OTC from chicken products [57]. Hexanes can safely be used for defatting/cleanup because TCs are insoluble in such hydrocarbons. Liquid/liquid extraction from an aqueous phase into an organic is difficult due to TCs continual isolated charge(s) and affinity for water over most organics. One successful example of a liquid/liquid cleanup utilized tetrabutylammonium as an ion pairing reagent, thereby facilitating the movement of OTC and TC from an aqueous buffer into dichloromethane [59]. Optimal extraction occurred at pH 8.2 where the TCs are primarily doubly deprotonated and carry an overall negative charge. A second example of liquid/liquid extraction was published, by Nelis et al. [60], in which a phosphate–sulfite buffer (pH 5.4) is added to bovine plasma and OTC is subsequently extracted with EtOAc/isopropyl alchohol (12:1). The authors submit that isopropyl alcohol increases the polarity of the organic phase and is therefore necessary for consistent extraction recoveries. Extraction into the organic phase is also aided by the overall neutral charge on OTC at pH 5.4 due to its zwitterionic state.

3.3. Complexes

When developing methods for the extraction and analysis of tetracyclines, it is important to consider their high propensity for forming chelation complexes. For instance, biological matrices contain divalent cations that can interfere with extraction. Disruption of these interactions is most commonly achieved through the addition of EDTA to the extraction solvent. EDTA has a greater affinity for the cations than TCs, causing improved recoveries of TCs when it is incorporated into the extraction solution. In addition to the McIlvaine/EDTA extraction combination, EDTA can be successfully applied with other buffers, like imidazole, as demonstrated by Kawata et al. [53]. Furthermore, EDTA was added during matrix solid phase dispersion isolation of TCs from milk to aid in disrupting metal–analyte chelation thereby improving recoveries [61]. On the other hand, ternary complexes may be exploited to aid in TC extraction, such as by making a normally hydrophilic TC now lipid-soluble [30].

3.4. Deproteination

Chemical deproteination within biological matrices is accomplished via acid, organics (i.e. ACN), and heat. When analyzing TCs, the first two approaches to deproteination are often selected. For milk samples, deproteination by acid is sometimes the only cleanup performed prior to analysis. For example, Furusawa [62] simply applied a 20% (v/v) TCA solution to milk in a 1.5:1 ratio, filtered, and injected. TCA deproteination is commonly employed in connection with the McIlvaine/EDTA extractions previously described [35,36,38,40,41]. ACN can either be used for extraction and deproteination, as demonstrated by Furusawa [57], or simply deproteination within aqueous extractions, as shown by Kawata et al. [53]. There does not appear to be concrete data which implicates one form of deproteination as superior to the other. As an alternative, molecular weight cutoff filters have been used in deproteination during the extraction/cleanup of TCs [10,54,63].

4. Further cleanup and concentration

4.1. Solid phase extraction

Solid phase extraction (SPE) is commonly employed to accomplish cleanup and concentration simultaneously. Due to their carbon backbone, aromatic region, and varied functional groups, TCs could theoretically be applied to wide range of SPEs. The non-polar regions of the ring system allow for cleanup using reversed phase (RP) C18 or polymeric sorbents, while phenyl SPEs take advantage of the D ring aromatic region. Aminopropyl as well as cyano SPEs allow for interactions with TCs' various carbonyl, hydroxyl, and amino sites. Although strong ion exchange SPEs are not likely to be used to adsorb TCs due to extreme pH values needed for desorption, strong anion exchange (SAX) cartridges have been used to remove matrix interference prior to loading on a RP SPE [64]. Table 3 contains typical conditions found in the published literature for TCs as applied to a range of SPEs. For RP SPEs, many authors have cited the polymeric Oasis HLB as being superior to the silica based C18 [35,36,47,65]. It is worthwhile to note that optimal recovery from the Oasis HLB sorbent have occurred when the TCs are loaded under acidic conditions where the pH is near or below the pK_{a1} value [65,66]. While it is possible to achieve success with untreated C18 cartridges [52], many have found it difficult to work with the octadecylsilyl sorbent due to TCs' affinity to adsorb onto silanols and form complexes with metal residues [39]. Pretreatment of the C18 SPE with EDTA [39], loading the analyte in a buffer containing EDTA [37,40–42], or SPE silylation [43] has improved results. Phenyl [38] and cyclohexyl [49] SPEs are among other sorbents used. Regardless of the SPE selected, one must be mindful of TCs' ability to associate with the sorbent through multiple interactions.

4.2. Metal chelate affinity chromatography

Metal chelate affinity chromatography (MCAC) exploits TCs' metal complexing properties to allow for additional cleanup. The sorbent is treated with aqueous copper (II) sulphate. The extract is loaded onto the column which retains

Table 3
Conditions for use of selected SPE cartridges

	Condition	Load	Wash	Elution	Special considerations
Polymeric (i.e. Oasis HLB) [64,65]	1. MeOH 2. Water 3. Aqueous acid (optional)	Aqueous acidic buffer	Water or water containing 5% MeOH	MeOH	Load at or below pK_{a1} for optimal recovery
C18 [37,39]	1. MeOH 2. Water 3. Buffer containing EDTA or saturated aqueous EDTA (optional)	Aqueous buffer containing EDTA	Water or buffer	MeOH	Conditioning and loading with EDTA may enhance recovery and provide consistent results
Phenyl [38]	1. ACN 2. Aqueous EDTA solution	Aqueous buffer containing EDTA	Water or buffer	ACN	Conditioning and loading with EDTA may enhance recovery and provide consistent results
Amino [69]	1. ACN	ACN	ACN	0.5 M buffer pH 7.5 containing 10% MeOH	Matrix extraction may require prior clean-up before SPE application

the TCs, according to previously described chelation mechanisms, until elution is prompted by a McIlvaine/EDTA buffer [45,67]. The copper ions provide for visual monitoring of the cleanup procedure (i.e. the TCs are found where the blue copper ions appear). Initially, the TCs are bound to the blue copper ions on the column until disruption by an EDTA containing buffer and elution of the copper ions, EDTA, and the analytes of interest. MCAC has been applied in an on-line LC system [56,68]. Here, the same principles described above apply except that elution of the TCs from the metal chelate column, following wash steps that are diverted to waste, flows directly onto an appropriate analytical column for separation and detection.

4.3. Evaporation

Conflicting observations concerning the stability of TCs during solvent removal (evaporation) are found in the scientific literature. Although a definitive explanation for some of these discrepancies is not available, it is prudent to be aware of the published findings. Mulders and Van De Lagemaat [43] and Ikai et al. [69] found significant loss of TC, most likely caused by increasing acid concentration, during the removal of a MeOH–oxalic acid solution. Findings, by Mulders and Van De Lagemaat [43], that TCs are stable during the removal of pure MeOH contradict reports of ∼30% loss by Onji et al. [19]. Most other discrepancies concerning evaporation involve EtOAc. Cooper et al. [56] and Nelis et al. [60] report no loss of TCs upon evaporation in straight EtOAc and EtOAc/isopropyl alcohol, respectively. In contrast, Rupp and Anderson [70] report loss of OTC during the removal of EtOAc. Long et al. [61] observe losses of TC, OTC, and CTC near 80% during solvent removal, performed in glass tubes, of pure EtOAc/ACN (1:3) solutions. A plausible explanation, binding of TCs to the glass, is offered by the authors. Interestingly, Long et al. noted that evaporating their milk extracts within glass adversely impacted TC recovery while leaving OTC and CTC unaffected. Based on the aforementioned contradictory findings, careful consideration should be given before employing evaporation during method development. Additionally, it should be confirmed that the evaporation step is not the cause of low recoveries.

5. Chromatography

5.1. Analytical columns

TCs are typically separated using a C18 or C8 RP analytical column. Many manufacturers and packings are cited in the literature, including both spherical and irregular packings, often noted with end-capping. A few authors have utilized a PLRP-S polymeric column [14,49], as well as a Novapak phenyl column [43], a Pursuit diphenyl column [70], or a Discovery amide C16 column [71]. Interaction of TCs with the silanols and trace metals present in silica packing materials significantly contributes to peak tailing and is a widely and often cited complaint of TC chromatography. The numerous double bonds and oxygen or nitrogen substituents of TCs provide for many sites of interaction. End-capping on reversed phase columns was preferred for its intrinsic ability to minimize silanol interactions. Owing to its non-silica backbone, the amide column would have no silanols to interact with TCs. Polymer columns were chosen to eliminate the interaction of silanols and trace metals altogether, although McCracken et al. [72] saw less satisfactory results with a polymer column versus a C8. Phenyl columns were chosen for their selectivity for the TC ring system, allowing for increased retention and separation power of TCs from their isomeric impurities or matrix peaks [70]. The diphenyl column was reported to provide a higher degree of retention (and thus room to adjust parameters) than a singly substituted phenyl column [70]. Column lengths of either ca. 250 mm or 150 mm were equally popular, but a column I.D. of ca. 4 mm and a packing of 5 μm were the most common. Several authors [35,42,61] reported

that flushing of the column with increased organic + water (or dilute acid) at the end of the day's runs was vital to maintaining performance. Column temperature was held equally at either room temperature or ca. 40 °C. A loss of peak intensity was noted for OTC as column temperature increased [48,70].

5.2. Mobile phase

The majority of methods report using an isocratic mobile phase, typically consisting of buffer and ACN. MeOH was used as the only organic modifier in a few cases [48,52,73] or occasionally in conjunction with ACN [36,40,43,60,65]. Khan et al. [14,18] reported using buffer with *tert*-butanol and THF on their polymeric column, or DMSO with perchloric acid solution on their C8 column. During method development the choice of one organic solvent versus another, the percent of that solvent versus the aqueous component, the molarity and type of the aqueous modifier, the column temperature, and the presence/absence of sample matrix extract all could exert significant chromatographic effects [70]. The possibility of TCs forming unexpected complexes with mobile phase components is not impossible and may need to be taken into consideration when evaluating chromatographic effects.

5.2.1. Parameters for ultraviolet detection

For ultraviolet absorbance determination, TCs are typically read at 350–365 nm in an acidic solution. Thus the preponderance of acidic mobile phases in the literature. The acid acts as a simple ionization suppression agent to minimize the occurrence of mixed separation mechanisms. Toward this end, phosphate buffer [14], acetate buffer [48], citiric acid [41], acetic acid [57], *o*-phosphoric acid [41,42], perchloric acid [18], or trifluoroacetic acid [65] have been used. However, the most common acids used are 0.01 M oxalic acid [35,36,38,40,43,60,61,71,74] and formic acid [66,73] for their additional ability to very effectively mitigate the effect of residual silanols on the stationary phase, and perhaps even scavenge residual metals [41]. Long et al. [61] report oxalic acid to permanently affect column selectivity, and suggest dedication of the analytical column to this analysis. Additional ion-pairing agents have been employed in a few cases, including use of 30 mM octane sulfonic acid (utilized to move interfering matrix peaks away from the OTC peak) [38], 0.04% heptafluorobutyric acid [74], 5 mM tetramethylammonium chloride [41], or 20 mM tetrabutylammonium sulfate [14]. The first two examples were used in conjunction with oxalic acid, but the other two with phosphoric acid. To block the effects of residual metal ions, the addition of ca. 0.01 M EDTA to the mobile phase is a common technique [14,41,57,74]. When using ultraviolet spectra as an identification tool, Walsh et al. [41] and Sokol and Matisova [40] warn that buffer and matrix residues will noticeably distort the spectra below 240 nm. The molar absorptivity (ε) of OTC at 357 nm is reported to be 12,589 M^{-1} cm^{-1} [60].

5.2.2. Parameters for fluorescence detection

For fluorescence determination, TCs are typically read in a neutral to slightly alkaline solution in the presence of solubilized metal cations such as Mg^{2+}, Ca^{2+}, Cu^{2+}, or Al^{3+}. Detection is accomplished at 380–390 nm excitation, and 490–520 nm emission. Fluorescent intensity is constant at pH 6.5–7.5 [48] and peaks around pH 7.5 [30]. At alkaline pH, TCs are in an ionic state, and as such are prone to tailing as described above. Post column reaction fluorescence methods use an acidic ACN–oxalic acid system for separation followed by the introduction of magnesium acetate in pH 9 boric buffer [35] or aluminum chloride solution [72]. Most fluorescence methods already have created the fluorescent species in the sample phase or on-column via the mobile phase. Various buffers have been reported to achieve a pH from neutrality to alkalinity, including 1.0 M imidazole pH 7.2 [37,53], 0.1 M glycine pH 12 [49], 0.1 M acetate pH 6.5 [48], and 0.5 M Tris pH 7.5 [70]. Although simple and available for the needed pH range, the typical phosphate buffer would not be useful due to its insolubility with metal cations. During their method development, Rupp and Anderson [70] reported an interference with the chelation effect when using ammonium acetate, a high fluorescence background with imidazole, and the need for a substantial molarity (and concomitant ionic strength) with Tris buffer in order to overcome secondary ion-exchange effects and produce an acceptable peak shape.

Blanchflower et al. [49] simply used the strongly alkaline glycine buffer to convert CTC to isoCTC to induce native fluorescence (at 340ex/420em). The other authors used Mg^{2+} or Ca^{2+} chlorides, or Mg^{2+} or Ca^{2+} acetates, at concentrations of 30 mM to 0.75 M. Curiously, some authors [37,48,53] added EDTA (ca. 10–25 mM) to their metal-containing mobile phases in the traditional manner to aid against peak tailing. However, they do not explain the counter-intuitive logic of this addition knowing that EDTA is a metal scavenger and would likely scavenge the fluorescence-inducing Mg^{2+} or Ca^{2+} from the mobile phase. In these cases the molar concentrations of EDTA were less than that of the Mg^{2+} or Ca^{2+}, and therefore no net effect was observed or documented. Conversely, Iwaki et al. [48] report that the EDTA actually enhances the fluorescence of the chelate, thus opening up questions as to the exact mechanism of EDTA's role in the system. Ternary complexes can enhance the fluorescence (with barbital sodium or L-tryptophan [30]) or magnitude of complexation (with polyethylene glycol [29]).

5.3. Separation of impurities

Developing multi-residue methods is difficult in general; but for TCs it is even more problematic due to their multiple impurities/degradation products. For example, OTC has about seven common impurities [14,70,73] each usually less than 2% of the parent, varying from one manufacturing lot to the next or with variations in sample/analytical handling. Their elution profile of course varies dramatically from one

chromatographic system to the next. Their elucidation in tissue methods is further confounded by interfering matrix peaks, not to mention the commercial unavailability of most of these impurities as reference standards. In the case of OTC, two of the impurities often elute close to or in tandem with the OTC peak, only separated with difficulty. EpiOTC is likely to elute just prior to OTC, whereas decarboxamidoOTC is likely to not be separated or give only partial separation [14,70]. Conversely, if only minimal separation is applied to the method, some of the impurities can co-elute with OTC and their distinct existence not even be evident. Recently, Lykkeberg et al. [73] reported complete separation of OTC from six of its impurities in pharmaceutical preparations using an Xterra C18 column with a MeOH–formic acid gradient (for LC separation prior to MS/MS-SRM detection). Consideration would need to be taken for application of their parameters to tissue matrix samples with light-based forms of detection.

6. Conclusions

Due to their efficacy, government approval, availability, and cost, TCs continue to be widely used. Although this class of compounds has been studied extensively with numerous detection methods currently published, researchers will undoubtedly continue to pursue analyses for these antibiotics. Table 4, which contains a sampling of methods published

Table 4
Selected methods recently published for TCs analysis

	Reference			
	[47]	[67]	[51]	[70]
Matrix	Pig tissue	Turkey tissue	Beef and Pork Tissue	Salmon
Sample size (g)	1.5–5 (tissue dependant)	1	15	2
Analyte	TC, OTC, CTC, DC	OTC	TC, OTC, CTC	OTC
Extraction/deproteination	0.1 M succinic acid (pH 4)	McIlvaine (pH 4), MeOH	Water–ACN, 0.1 M phosphoric acid, hexane–DCM cleanup	Na_2EDTA–McIlvaine buffer (pH 4)
SPE/other	Oasis HLB	Metal chelate affinity chromatography	NA	Strata X, Waters aminopropyl
Evaporation	At 50 °C under nitrogen	No	Partial evaporation at 40 °C	No
Column	PLRP-S (250 mm × 4.6 mm I.D., 8 μm)	LUNA C_{18} (150 mm × 4.6 mm I.D., 5 μm)	Prodigy ODS (4.6 mm × 150 mm, 5 μm)	Varian Pursuit Diphenyl (300 mm × 4.6 mm ID, 5 μm)
Mobile phase	1 mM oxalic acid–0.5% formic acid–THF	ACN–10 mM oxalic acid	4 mM oxalic acid–4 mM sodium decane sulfonate–ACN	$MgCl_2$ in tris(hydroxymethyl)-aminomethane (pH 7.5)–ACN
Detector	ESI MS	UV 355 nm	UV 370 nm	FLD
Spike range	50–1200 ng g^{-1}	50–1200 μg kg^{-1}	100–1000 μg kg^{-1}	100–1000 μg kg^{-1}
Recovery (%)	>80	>75 (in muscle)	>90 for TC, OTC	≥70
Precision (%)	<14.7	<3.9	<6	<9

	Reference			
	[54]	[71]	[35]	[36]
Matrix	Milk and eggs	Honey	Salmon	Bovine milk and muscle
Sample size	0.1 mL	3 g	5 g	5 g
Analyte	OTC	TC, OTC, CTC, DC, minocycline, methacycline	TC, OTC	TC, OTC, CTC, DC
Extraction/ deproteination	Ultrasonic homogenization with 0.1 M succinic acid solution (pH 2.5); ultrafiltration	0.1 M Na_2EDTA–McIlvaine buffer (pH 4)	Ultrasonication with Na_2EDTA–McIlvaine buffer (pH 4)–hexane (to remove fat)	Na_2EDTA–McIlvaine buffer
SPE	NA	Discovery DSC-phenyl	Oasis HLB	Oasis HLB
Evaporation	No	At 40 °C, 240 bar	Partial evaporation at 30 °C	Under nitrogen
Column	Mightysil® RP-4 GP (150 mm × 4.6 mm I.D., 5 μm)	Discovery RP-Amide C_{16}, 5 μm	Chromspher C_8 (100 mm × 3 mm I.D., 5 μm)	Hypersil C_8 (250 mm × 4.6 mm I.D., 5 μm)
Mobile phase	2.1 mM succinic acid solution (pH 3.6)	10 mM oxalic acid (pH 3)–ACN	10 mM oxalic acid (pH 2)–ACN	10 mM oxalic acid–MeOH–ACN
Detector	UV 267 and 354 nm	DAD 270 and 355 nm	FLD	DAD 365
Spike range	Milk 0.05–0.2 μg mL^{-1}; eggs 0.05–0.4 μg mL^{-1}	500 ng g^{-1} (15 ng mL^{-1} LOD)	50–200 μg kg^{-1}	50–150 μg kg^{-1}
Recovery (%)	≥84	>92	>83	>81
Precision (%)	≤2.3	<5.5	<7.2	<5.3

within the last 5 years, illustrates many of the current approaches being utilized for analysis. TCs' dynamics make it difficult to alter one condition or property without also affecting a secondary interaction. When developing an analytical scheme for TCs, one must be mindful of the properties and positions of functional groups, the acid dissociation constants and charge states, the nature of each solvent used, and the presence of metal chelation coordination and other possible ligands. This review examines the underlying chemistry and evolving trends in TCs chromatographic analysis for the benefit of future scientists.

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