Analysis of veterinary antibiotics and their degradation products in ground water using liquid chromatography tandem mass spectrometry (LC/MS/MS)

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Abstract

Antibiotics are widely used in agriculture as growth enhancers and for disease treatment and control in animal feeding operations. Concerns for increased antibiotic resistance of microorganisms have prompted research into the environmental occurrence of these compounds. Assessment of the environmental occurrence of antibiotics depends on development of sensitive and selective analytical methods based on new instrumental technologies. Liquid chromatography/mass spectrometry (LC/MS) with electrospray ionization (ESI) is a relatively new technology useful for sensitive and selective analysis of a wide variety of antibiotics and their degradation products. While ESI is a sensitive and versatile LC/MS source, variations in sample matrix composition affect ionization efficiency and detector response. Regular use of internal standards and surrogates may help compensate and correct for these variations, however, matrix effects remain a significant problem in quantitative analysis by LC/MS. Co-eluting compounds can lead to enhancement or suppression of either analyte or internal standard response. Use of solid phase extraction (SPE) with polymeric or reverse phase sorbents to concentrate antibiotics from water can provide method detection limits at low part per trillion levels. Modification of SPE procedures, such as using a more selective sorbent, and adding a wash step, may help minimize the effect of sample matrices on ionization efficiency and detector response. Extraction and analysis of fortified samples provides the best indication of matrix effects, however, this is not practical for large numbers of samples.

Liquid chromatography-tandem mass spectrometry, using multiple reaction monitoring (MRM), can improve quantitation by LC/MS. In MRM, target compounds are isolated and fragmented. Detection of characteristic reaction product ions (fragments) produces lower background noise and greater selectivity than molecular ion detection. The design of both ion trap and the triple quadrupole mass spectrometers permit MRM analysis. The triple quadrupole provides superior sensitivity and selectivity when compared to the ion trap. For example, instrument detection limits for a number of antibiotics measured with the ion trap ranges between 5-50 picograms (10^{-12} gm) while instrument detection limits of a triple quadrupole may be well below 1 picogram. Extraction and subsequent analysis of tetracyclines using the ion trap provides method detection limits near 0.2 μg/L for a 100 mL sample of groundwater, and near 2 μg/L in 20 mL of buffered wastewater. In comparison, method detection limits estimated using a triple quadrupole are as low as 0.010 μg/L(10 pptr) in ground water.

Finally, antibiotics used in animal feeding operations may be excreted unchanged or as degradation products. Depending on their chemical characteristics, both metabolite and parent compounds may be transported into the environment. Many antibiotic degradation products have been shown to have antimicrobial effects similar to the parent compounds. As in studies of the environmental fate of pesticides, it is often necessary to develop methods which include analysis of degradation products. Multi-residue methods often provide the maximum amount of information from the same time and effort as single component methods. Because chemical characteristics dictate extraction procedures and instrument operating conditions, however, it is necessary to develop multi-residue methods for groups of chemically similar compounds. Tetracyclines are particularly challenging in development of multi-residue methods, especially if transformation products are included. Reversible epimerization and their tendency to form metal complexes further complicates the analysis of these compounds. For example, LC/MS analysis of a standard of anhydrochlortetracycline produces 2 or 3 epimers corresponding to well-resolved chromatographic peaks that must be summed together for quantitation of this compound in samples. The relative proportion of epimers of a single compound can vary considerably between standards and samples, and over time in the sample solution. Thus, quantitative analysis of tetracyclines and their degradation products requires control of complexation reactions and must account for epimer formation during extraction and analysis.

Introduction

The use of pharmaceutical compounds is critical in maintaining today's agricultural standards and practices in animal husbandry, and has been the subject of much controversy for over 30 years. Antimicrobial drugs are a group of pharmaceuticals that exhibit selective toxicity to microorganisms. An antibiotic is an antimicrobial substance produced by a microorganism that inhibits the group or kills other microorganisms (Prescott, 2000). The use of antibiotics in raising livestock began over half a century ago, shortly after the discovery of tetracycline compounds. Chlortetracycline was first isolated from a culture of soil microorganisms, *Streptomyces aureofaciens*, by retired mycologist Benjamin J. Dugger in 1945 at Lederle Laboratories.

The discovery that antibiotics can act as growth promoting agents came shortly thereafter. Other investigators at this facility searching for a source of "animal protein factor" fed chicks dried fermentation mash of *Streptomyces aureofaciens*. Stokstad et al. 1949 reported that chicks fed fermentation mash grew faster and larger than those fed with other known growth promoters. Chlortetracycline was identified as the component of the mash responsible for stimulation and its ability to enhance growth rates was quickly confirmed in turkeys and swine. Thus began the wide spread use of antibiotics in livestock. The potential for economic benefits of this ability was immediately recognized and one of the lead investigators later reported (Jukes, 1986) on the widespread media coverage of the initial announcement of its discovery during the 1950 American Chemical Society meeting in Philadelphia, PA.

By the late 1960's, concerns over the potential for development of antibiotic resistance from the use of antibiotics in livestock lead to an inquiry by a British government appointed committee. This committee was formed because of outbreaks of antibiotic resistant infections in both livestock and humans in Great Britain and Central America. The Swann Report (Swann, 1969) recommended that antibiotics important in human medicine be withdrawn from use as growth promoters and low-dosage disease prevention. This report led to a decrease in the use of antibiotics for livestock in Europe, but had little effect in North America (Prescott, 2000).

Antibiotics are widely used today in agriculture for growth enhancement and for disease treatment and control in all sizes of animal feeding operations. By some estimates, half of all antibiotics produced in the United States are used in agriculture and the majority is used as growth promoters and for disease prevention in swine. According to a U.S. Department of Agriculture National Animal Health Monitoring System Survey (USDA, 1999) between 1990 and 1997, antibiotics were used in most phases of swine production and administered via injection, feed and water (Figure 1). Nearly 25% of small feedlot and 70% of large cattle feeding operations used antibiotics, and tetracycline antibiotics were some of the most frequently used feed additives for both swine and cattle. During this period nearly 60 million head of hogs and 100 million head of cattle were produced annually in the United States (USDA, 1999). The bulk of U.S. hog and cattle production occurs in a handful of agricultural states and may intensify the potential environmental impact of livestock production to these areas (USDA, 2004).

Analysis of veterinary antibiotics in ground water

Concerns for increased antibiotic resistance of microorganisms have prompted research into the environmental occurrence of these compounds. An accurate assessment of their occurrence depends on development of sensitive and selective analytical methods based on new instrumental technologies. Because some studies have suggested that antibiotics may be persistent in the environment (Kummerer, 2003), there is the potential for leaching and contaminating ground water. As has been shown with many pesticides, detection of these compounds and their transformation products can help verify which compounds are persistent and mobile in the environment. Analysis of antibiotics in groundwater and other matrices is challenging both because of the low concentrations likely to be found and because of their unique chemical properties. Table 1 lists several groups of antibiotics commonly administered to either swine or cattle together with some chemical and physical properties. In contrast to most pesticides used in agriculture, antibiotics tend to be quite hydrophilic and nonvolatile with multiple sites to donate and accept protons.

Many antibiotic classes, such as the β-lactams (penicillins) and macrolides, are unstable at high or low pH and are readily transformed, which limits the development of multi-residue methods which require adjustment of pH. Low level (ppb) analysis of tetracycline antibiotics in environmental samples is particularly challenging

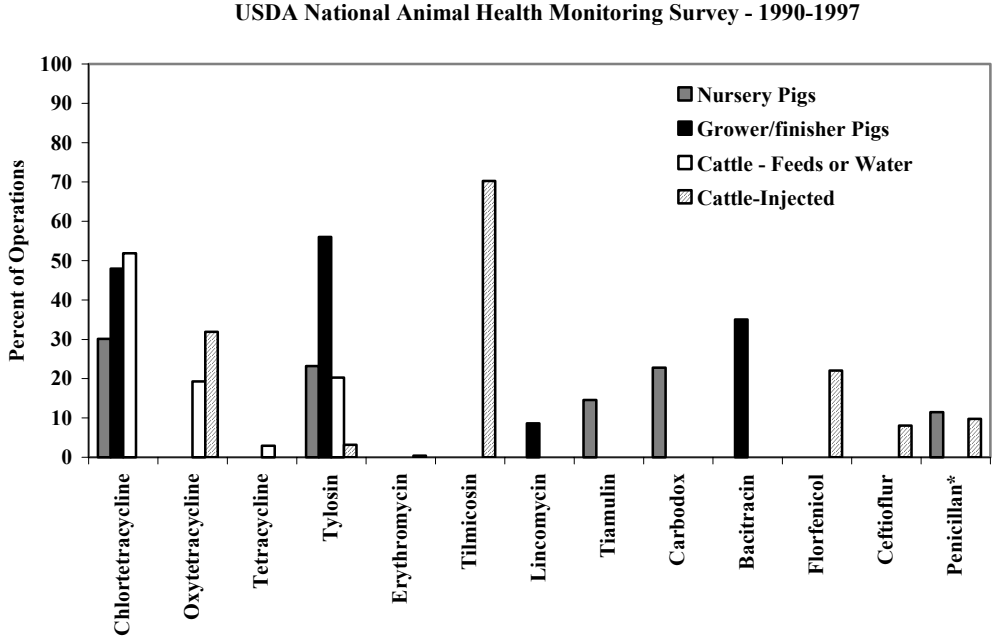

Figure 1. NAHMS Survey of antibiotic use in swine and cattle feeding operations (USDA, 1999)

because these compounds are strong chelators that readily bind to metals, proteins and silanol groups (Oka and Patterson, 1995; Tolls, 2001; Ternes, 2001). Tetracyclines are amphoteric and exhibit varying solubility with pH. Because antibiotic transformation products also exhibit anti-microbial properties, it is often desirable to include analysis of these compounds to better characterize the environmental occurrence of the parent compounds.

Group	Compound	Molecular Weight	Solubility (g/L)	Boiling Point (°C)		Proton Acceptors	Proton Donors	LogKow	pKa - Ac		pKa - Ba	Vapor Pressure (Torr)
Aminocyclitols		Spectinomycin	332.35	na	583	9	5	1.17	9.25	8.56	5.05E-16	
Aminoglycosides		Neomycin	614.64	na	927	19	19	-3.70	12.9	9.52	na	
Beta-lactams		Penicillin G	334.39	na	663	6	2	1.67	2.62		1.69E-18	
		Ampicillin	349.41	na	684	7	4	1.35	2.61	6.79	1.21E-19	
		Ceptiofur	523.57	na	na	12	4	0.54	2.62	2.9	na	
Chloramphenicols		Florfenicol	358.21	2.5	617	5	2	0.71	10.7		4.16E-16	
Fluoroquinolones		Enrofloxacin	359.39	130	560	6	1	2.53	2.74	7.11	2.10E-13	
		Danofloxacin	357.38	na	569	6	1	1.85	2.73	9.13	8.41E-14	
Lincosamides		Lincomycin	406.54	0.9	647	8	5	0.86	12.9	8.78	1.85E-19	
Macrolides		Tilmicosin	869.13	566	927	15	4	5.09	13.16	9.81	na	
		Tylosin	916.10	5.0	980	18	5	3.41	13	7.37	na	
Sulfonamides		Sulfadimethoxine	280.30	na	539	7	3	0.42	6.69	1.48	1.05E-11	
		Sulfamethazine	278.33	1.5	526	6	3	0.80	7.45	2.79	3.64E-11	
Tetracyclines		Oxytetracycline	460.43	1.0	839	11	8	-1.22	4.5	9.26	6.27E-30	
		Chlortetracyline	478.88	0.6	821	10	7	-0.04	4.5	9.68	1.57E-28	

Table 1. Chemical and physical properties of several groups and examples of veterinary antibiotics used for swine and cattle (CAS, 2004).

Analysis by liquid chromatography/mass spectrometry

Most antibiotics are polar and relatively nonvolatile so that analysis by gas chromatography is possible only after derivatization of these compounds to a more volatile form. Derivatization and gas chromatographic (GC) methods have been developed for many pharmaceuticals in aqueous samples (Ternes, 2001) and instrumentation based on gas chromatography with quadrupole mass spectrometers are readily available. However, the difficulties of attaining reproducible derivatization combined with the analytical complexities of veterinary pharmaceuticals have favored development of methods which utilize liquid chromatography (LC). While LC is well suited to analysis of polar organics such as antibiotics, the lack of suitable chromophores (light absorbing functional groups) in these compounds severely limits the sensitivity of ultraviolet detectors most often used in high pressure liquid chromatography (HPLC). Detection by liquid chromatography/mass spectrometry (LC/MS) provides the most promising method for analysis of these compounds at environmentally relevant levels.

GC/MS has been commercially available for almost 30 years and is a standardized and even routine instrumental technique in many environmental laboratories. In contrast, the challenge of providing a suitable interface between a liquid separation system and the vacuum of a mass spectrometer delayed the development of quantitative commercial LC/MS systems. There are many instrumental configurations available today, each with a unique set of advantages and disadvantages. The working principles and technical properties of each type of mass spectrometer coupled to an HPLC determine the sensitivity, selectivity and robustness of the analytical method (Lemière, 2001). Thus it is important to understand the differences and limitations of each type of LC/MS when comparing results of antibiotics determined by different laboratories.

Though there are numerous variations among LC/MS systems, most instruments consist of a: 1) separations module, 2) ion source, and 3) mass analyzer. In the majority of cases, the separations module (HPLC) is identical to that used in other liquid chromatography instrumentation. Often the LC pumps are configured for lower flow rates and have an auto-sampler designed for minimal carryover. Ion sources for LC/MS have gone through many changes over the past 20 years. The technical difficulties in developing an effective interface between HPLC and the mass analyzer are the main reason LC/MS has only recently become a tool in environmental analysis. Separation and detection of target compounds by mass spectrometry depends on efficient conversion and transport from a dissolved, often neutral form in the HPLC to an ionized gaseous form suitable for introduction into a mass analyzer. In contrast to earlier LC/MS ion source designs, most modern instrumentation use atmospheric pressure ionization (API) methods where ions are generated outside the vacuum region of the mass analyzer. The two main types of atmospheric pressure ion sources now available for LC/MS are electrospray ionization and atmospheric pressure chemical ionization.

Electrospray ionization (ESI) is perhaps the most versatile and "softest" ion source for LC/MS. In ESI the effluent from the HPLC is sprayed across a high electrical potential from a needle and evaporation of the mobile phase is enhanced with a stream of heated nitrogen gas. As evaporation occurs, the ions are transformed from a dissolved phase into a gaseous phase immediately prior to introduction into the vacuum region of the mass spectrometer. Because ESI is a "soft" ionization technique, it generally produces either molecular ions or charged complexes (adduct ions) with little if

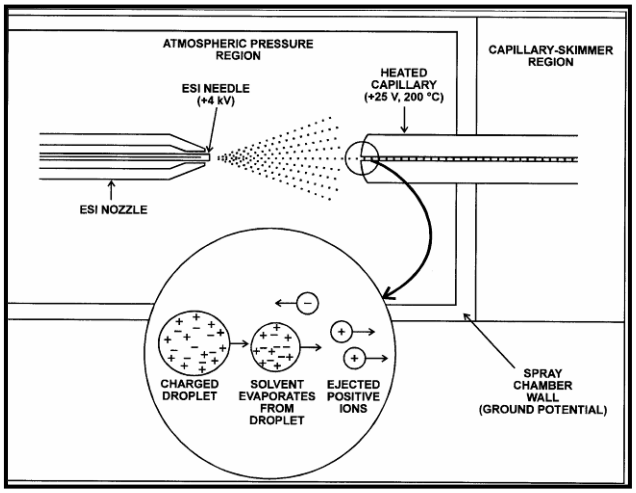

Figure 2. Schematic diagram of ionization process in positive ion mode (Finnigan, 1996)

any fragmentation. Many compounds are ionized by electrospray and detection using this interface is affected by other substances in the matrix. Ion efficiency in electrospray is affected by change in spray composition when compounds co-elute. Even compounds that do not ionize may affect ionization by preventing transfer of dissolved ions into gas phase ions during the evaporation stage. In spite of the difficulties associated with the "matrix effect", ESI remains the most widely-used ion source in LC/MS primarily because of its versatility and sensitivity.

Atmospheric pressure chemical ionization (APCI) is a more energetic API ionization method for LC/MS and uses a corona discharge (plasma) in combination with heating and evaporation to produce ions. In contrast to ESI, ionization occurs in the gas phase and often results in the formation of fragment ions as well as molecular ions. APCI is better suited to lower molecular weight and less polar organic compounds. APCI is less susceptible to matrix effects than electrospray primarily because it is a gas phase ionization process.

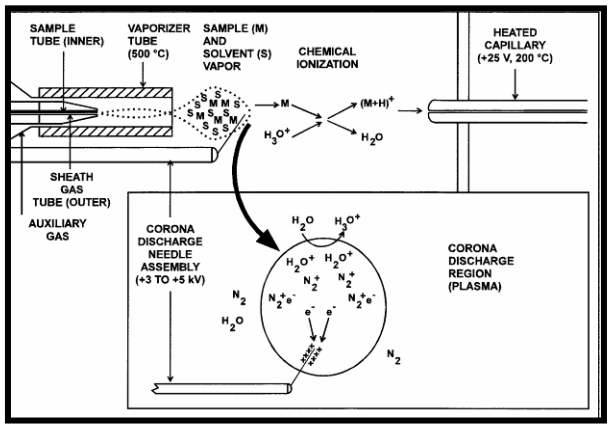

Figure 3. Schematic diagram of atmosphic pressure ionization (APCI) source (Finnigan, 1996).

The design of the mass analyzer used is the major factor determining LC/MS method sensitivity, selectivity and robustness. Unit resolution (1 amu) mass analyzers are most commonly used for quantitive analysis in LC/MS because of their versatility, lower cost, and ease of use. These include: 1) single quadrupole 2) ion trap, and 3) triple quadrupoles.

A single quadrupole provides the simplest, least expensive, and most commonly-used mass analyzer in LC/MS (Lemiere, 2001). Radio frequency (RF) and direct current (DC) potentials are applied to 4 rods (quadrupoles) to create a controllable electrostatic field. By varying the ratio of RF and DC potentials, ions with known mass/charge ratios (m/z) pass through the field and are detected. The lower limit of detection for single quadrupole LC/MS systems is in the range of 5-50 picograms (10^{-12} gm), and is comparable to the specifications for a quadrupole GC/MS in chemical ionization mode. For comparison, a 25 µL injection of a 1 ppb test solution contains 25 picograms (pg) of analyte and would be near a single quadrupole LC/MS instrument detection limit. It should be noted that ionization efficiency and thus sensitivity will vary widely among chemicals and between instruments of the same design. Quadrupoles detectors, sometimes referred to as "mass filters", are capable of rapid scanning or selected monitoring of a few ions. Instrument sensitivity is usually much less when scan mode is used than selected ion monitoring mode.

Another common type of LC/MS analyzer is the quadrupole ion trap. The ion trap uses an electrostatic field to collect or "trap" ions of differing masses over a specified time period. Ions of selected m/z are then ejected from the trap and detected by varying the RF voltage. Helium is added as a damping gas to reduce the loss of ions from collisions during storage. Ion trap LC/MS systems offer about the same level of sensitivity as quadrupoles, but since all ions are collected and detected, ion traps can provide more complete spectra at lower levels than quadrupole LC/MS systems. Ion traps can also be used for tandem MS detection by fragmenting ions as they are held in the trap. Two big advantages of tandem MS/MS detection are reduction in background (noise) and better selectivity, thus improving both sensitivity and confidence level in compound detection. Thus liquid chromatography with tandem mass spectrometry (LC/MS/MS) is preferred for analysis of samples with complex matrices.

While an ion trap can provide MS/MS detection in time, a triple quadrupole provides tandem mass spectrometry in space. The triple quadrupole LC/MS employs two quadrupole mass analyzers separated by a third operated in only in the RF mode that serves as a collision cell. As with the ion trap, the collision cell is filled with a damping gas used to impart kinetic energy on ions and generate fragments of target compounds that are then separated and detected using the final quadrupole. The triple quadrupole is more expensive than a single quadrupole or ion trap, but offers the lowest background and best precision of any unit resolution LC/MS and is

much less expensive than higher resolution instruments. The reduced background combined with increased ion transmission efficiency in reaction monitoring mode provide instrument detection limits well below 1 pg depending on the compound.

Sample Preparation

LC/MS may be used to determine concentrations of polar organics in a wide variety of matrices without any sample preparation. In contrast to gas chromatographic methods, aqueous samples are quite compatible with the mobile phases typically used in reverse phase HPLC. Instrument detection limits for many systems are in the low ppb range, and in many cases all that is needed is to filter and inject a sample to obtain results. However, because the concentration of antibiotics and other pharmaceuticals occur at concentrations much lower than this, pre-concentration is required in order to obtain the required sensitivity. In addition, because many co-eluting compounds will affect ionization, some form of clean up is usually necessary to minimize matrix effects.

Solid phase extraction (SPE) offers the simplest and most flexible method for pre-concentrating and purifying antibiotics from aqueous samples. A tremendous array of forms, phases, and cartridges are available from a variety of manufactures, and often tailored to specific protocols, analytes, and samples. New sorbents are constantly developed to improve recovery and selectivity of extractions. Because of the wide variety of sorbents now available, method development often requires a comparison of the extraction efficiency of different phases and similar phases from different manufacturers.

Most methods for extraction of antibiotics from aqueous matrices employ some type of hydrophobic, or reverse phase packing. Sorbents can include bonded silica such as octadecyl (C-18), graphitized carbon, or a macroporous polymer such as polystyrene divinylbenzene (PS-DVB). These phases are among the most hydrophobic available and are historically the most widely used SPE packings (Thurman and Mills, 1998). PS-DVB resins were among the first used for trace enrichment of organic contaminants from water in the 1960's, and experienced a resurgence in use through improved manufacturing methods and the modification of polymer functional groups in the 1990's. For example, Weigel et al. (2004) recently compared the recovery of 15 human pharmaceuticals and hormones from 7 different polymeric sorbents including three non-functionalized PS-DVB, two forms of functionalized PS-DVB, and two hydrophilic-lipophilic co-polymers. While all packings were satisfactory for some compounds, overall recoveries were highest and most consistent using the co-polymers.

Co-polymer packings, such as the Waters Oasis HLB product line have been commercially available since 1997 when they were first used to extract pharmaceuticals from biological fluids. The mixture of N-vinylpyrrolidone and divinylbenzene functional groups can provide stronger retention and greater selectivity for a wide range of polar organic compounds. The co-polymer sorbents have a higher capacity for the same amount of packing as C-18 bonded silica and recoveries are not affected by drying.

Tetracyclines – A Case Study

Tetracycline antibiotics are widely used in agriculture as growth enhancers and for disease treatment and control in animal feeding operations. Concerns for increased antibiotic resistance of microorganisms have prompted research into the environmental occurrence of these compounds. Tetracyclines are relatively stable in vitro and a large

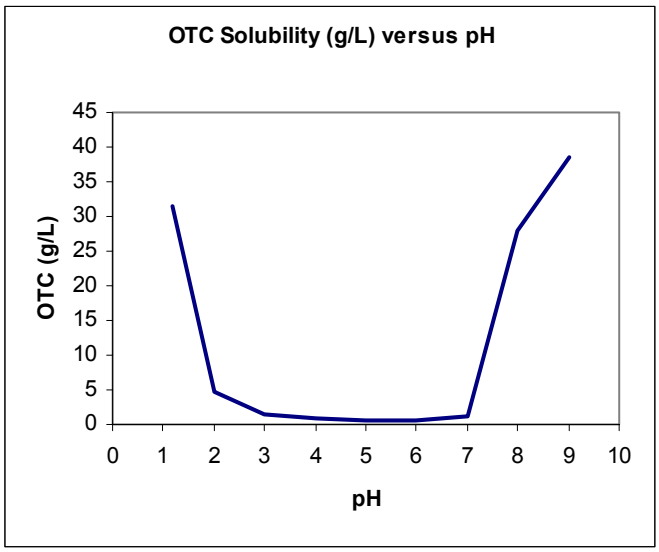

Figure 4. Variation of oxytetracycline solubility in water versus pH (Mitscher, 1978)

fraction of these compounds are excreted from animals unchanged.

Depending on the pH, these antibiotics can be extremely soluble in water (Figure 4). However, because these compounds are strong chelators that readily bind to metals, proteins and silanol groups, complexation and sorption very likely limits their environmental mobility (Tolls, 2001; Ternes, 2002). These chemical properties can also complicate their analysis.

The reversible formation of C4-epimers, which are identical in molecular composition but chemically and structurally quite different, further complicates quantitative analysis of these compounds in both water and soil samples. The rate of epimerization is controlled by sample pH and affected by the presence of dissolved metals. Reproducible extraction of tetracyclines from complex matrices requires control of these characteristics using a strong complexing agent (citrate, EDTA, and/or oxalic acid) and pH buffer (Hamscher et al. 2002; Lindsay et al. 2001; Snow et al. 2003; and Zhu et al. 2001). Quantification of tetracyclines must account for formation of the epi-tetracycline forms (Oka and Patterson, 1995).

Besides epimerization, dehydration of the functional groups in these compounds can occur at very high or very low pH to produce the anhydro-forms of these compounds. The dehydrated form of oxytetracycline is relatively unstable and decomposes rapidly to form α- and β-apo-tetracyclines. Many anhydro- and epi-tetracyclines have been shown to have antimicrobial effects similar to the parent compounds (Halling-Sorensen et al. 2002). Other degradates that may be environmentally persistent include terrinolide.

Several methods for analysis of tetracyclines in ground water have been reported in the literature using solid phase extraction LC/MS. Zhu et al. (2001) compared recovery of 4 commonly-used tetracycline antibiotics using 1 gram C-18 bonded silica and 200mg Oasis HLB cartridges in ground water and phosphate buffered waste water samples. While recovery and consistency was comparable between both packings, it was also necessary to add a buffer to the eluting solvent for complete recovery from the C-18 bonded silica. The difficulty in eluting tetracyclines from bonded silica was attributed to the chelating behavior of these compounds.

Lindsey et al. (2001) describe a similar method for extraction and analysis of 5 tetracyclines and 6 sulfonamide veterinary antibiotics using Oasis HLB cartridges. Samples were fortified with EDTA to help minimize the chelating activity of tetracyclines. A comparison of tetracycline and sulfonamide recovery for two sizes (60 and 500 mg) of Oasis HLB packings, one 150 mg PS-DVB, and a 500 mg C-18 bonded silica suggested the smaller HLB cartridge produced the best overall recovery. Hamscher et al. (2002) used a 200 mg PS-DVB cartridge to extract tetracyclines from citric acid buffered water with recoveries similar to that reported by Zhu et al. (2001) and Lindsey et al. (2001). Table 2 compares recoveries reported for three common tetracycline antibiotics for these three papers.

SPE Method	**Hamscher et al. (2002) 200 mg PS-DVB citric acid buffer**	**Lindsey et al. (2001) 60 mg HLB EDTA buffer**	**Zhu et al. (2001) 200 mg HLB phosphate buffer**
	Average recoveries ±σ (%) at 1 ppb extracted from water		
Oxytetracycline	77±9	100±14	80±8
Chlortetracycline	90±9	89±13	79±11
Tetracycline	86±14	98±13	90±8

Table 2. Tetracycline recoveries reported for SPE of ground water using PS-DVB and HLB cartridges

Lindsey et al. (2001) utilized a quadrupole, while Hamscher et al. (2002) and Zhu et al. (2001) used an ion trap LC/MS for tetracycline detection. Columns and mobile phases were similar, with all investigators recommending high purity reverse phase silica columns to minimize interaction between tetracyclines and the column. The protonated molecular ion $[M+H]^+$ was used for quantitation by Lindsey et al. (2001), while Hamscher et al. (2002) and Zhu et al. (2001) utilized multiple reaction monitoring by selecting and fragmenting the molecular ion of each target compound, using a characteristic product (fragment) ion for quantitation.

Ground water detection limits among the three analytical methods were in the range of 0.1 to 0.3 μg/L depending on the compound and the method used to make this estimation.

The method by Zhu et al. (2001) has been modified to include dehydrated degradation products and optimized for analysis on a Waters Micromass triple quadrupole instrument. An improvement in method sensitivity was obtained using the triple quadrupole versus the ion trap LC/MS/MS analysis (Table 3).

The results of quality control samples analyzed between 2002 and 2004 indicate and overall improvement in method accuracy and precision. During the analysis of 120 samples over a 24 month period, the average difference between laboratory duplicates (range) decreased from 0.230, 0.131, and 0.010, to 0.013, 0.009, and 0.009 μg/L for oxytetracycline, tetracycline and chlortetracycline respectively using the triple quadrupole. Surrogate (doxycycline) recovery in samples averaged 80±16% in samples analyzed using the triple quad in comparison to 120±40% using the ion trap system. Recoveries of oxytetracycline, tetracycline and chlortetracycline in fortified blanks improved slightly and averaged 104±7%, 94±10%, 110±20% using the triple quadrupole compared to 88±16%, 90±10%, 108±16% using the ion trap. Recovery of tetracyclines in sample matrices showed a similar trend. Tetracycline recovery in samples fortified at the time of collection was not as consistent as those fortified immediately before extraction suggesting that there may be some loss either through degradation or sorption.

Compound	Triple Quad Method Detection Limit (ppb)	Ion Trap Method Detection Limit (ppb)
Minocycline	**0.010**	**0.20**
Oxytetracycline	**0.004**	**0.09**
Tetracycline	**0.005**	**0.06**
Chlorotetracycline	**0.007**	**0.11**
β-apo-oxytetracycline	**0.010**	**0.10**
Anhydrotetracycline	**0.016**	**0.05**
Anhydrochlortetracycline	**0.008**	**0.12**

Table 3. Comparison of method detection limits (MDL=st_{n-1}) for triple quadrupole and ion trap LC/MS/MS detection of tetracyclines and some degradation products.

Even with the improved sensitivity and accuracy, tetracycline analysis with the triple quadrupole must address epimer formation and matrix effects. Figure 5A illustrates how epimer formation is especially problematic with the anhydro degradation products of tetracyclines. The proportion of epimer species within a compound varies even within the same solution, and cannot be corrected by assuming a constant amount. Epimerization of the internal standard is especially problematic in this analysis.

The effect of sample matrices will affect quantitation of these compounds as long as interferences cannot be separated. In an experiment where demeclocycline (internal standard) was constantly infused during an injection, figure 5B shows how the intensity of product ion varied considerably while a ground water sample extract was being analyzed. Internal standards are added to samples and extracts to correct for variation in recovery and detector response in LC/MS analysis. As illustrated in figure 5B, however, changes in ionization efficiency due to sample matrix may not be constant during analyte detection. Thus it becomes even more important to selectively extract and separate target compounds from the matrices which can cause both positive and negative interferences.

One approach is to use 2 types of cartridges in tandem, such as a strong ion exchange resin coupled with a polymeric phase, to selectively remove ionic interferences. Blackwell et al. (2004) recently reported a sensitive method for analysis of 3 veterinary antibiotics utilizing HPLC with UV detection with a strong anionic exchange cartridge in tandem with a polymeric cartridge. Detection limits ranged from 0.2-0.4 ppb and were comparable to methods utilizing LC/MS. Improved selectivity of sorbents may also help reduce problems associated with sample matrices.

Another approach to improving methods for analysis of antibiotics involves synthesis of a customized sorbent such as a molecularly imprinted polymer (MIP). The creation of an MIP is performed by combining a given target analyte (e.g., tetracycline) with polymerizable functional monomers. After polymerization is initiated, this forms a rigid cross-linked and macroporous polymer that now contains binding sites complementary to the target analyte. The target is then removed and the MIP can then be used for the isolation of this or related compounds from samples. MIPs have recently been used for the solid-phase extraction of various drugs, nicotine, triazines, nitrophenol, bentazone, and chlorinated phenoxyacids. Although MIPs have not been used directly with LC/MS/MS or in the study of emerging contaminants, recent reports have appeared in which these were prepared for antibiotics such as penicillin G, oxacillin, ampicillin, chloramphenicol, cyclosporine A, erythromycin A and oleandomycin.

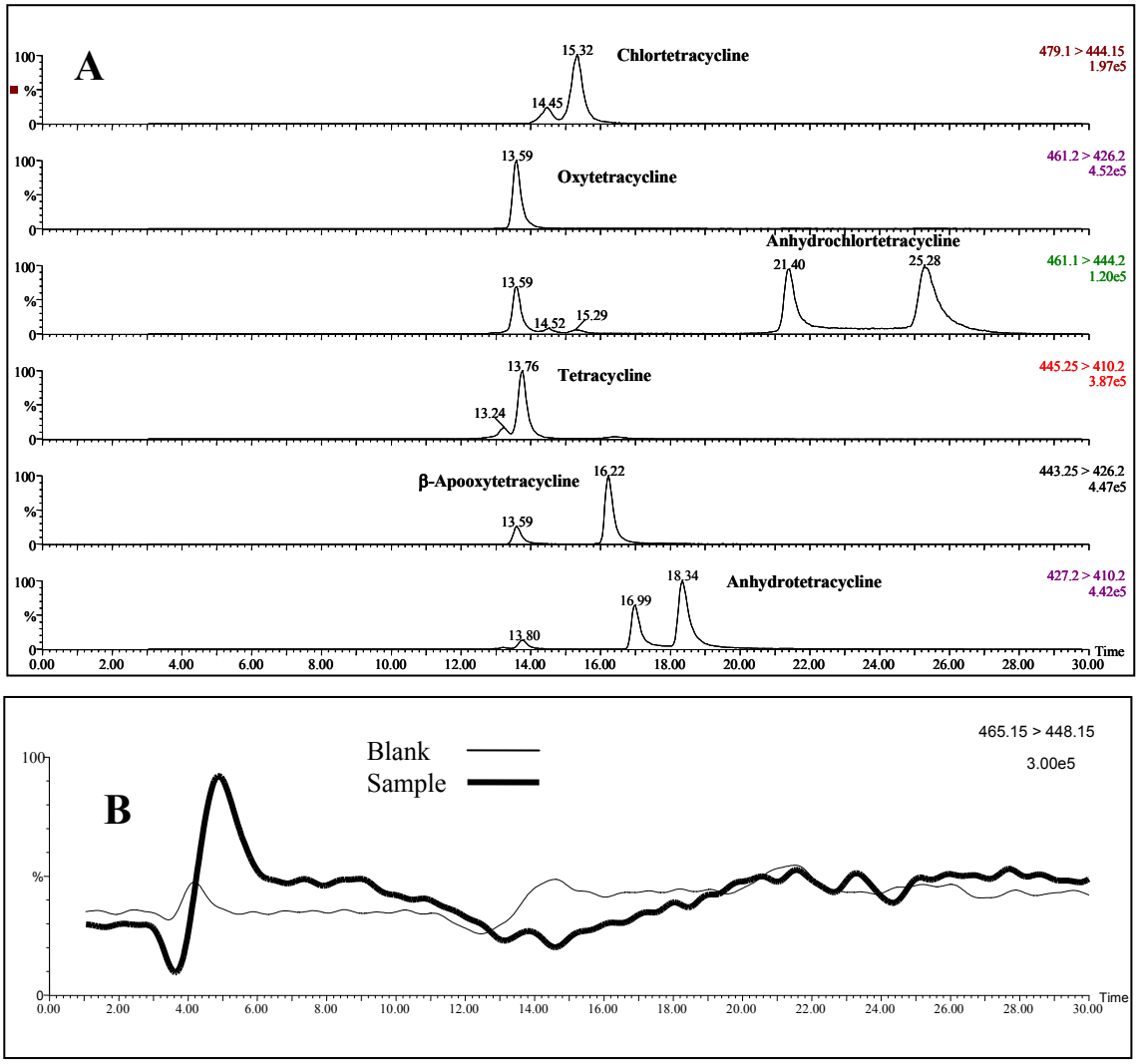

Figure 5A. LC/MS/MS multiple reaction monitoring chromatogram for the major tetracyclines and their degradation products for the triple quadrupole. Note multiple peaks and retention differences in epimers of tetracycline degradation products.

Figure 5B. Variation in demeclocycline detection during infusion while analyzing a solvent blank and a complex ground water sample extract.

Conclusions

While antibiotics have used widely used in raising livestock for over half a century, this use is not without controversy. A complete understanding of the environmental occurrence and potential impact of these compounds requires the development of sensitive and selective analytical methods. Methods based on detection by liquid chromatography tandem mass spectrometry (LC/MS/MS) offer the most sensitivity and selectively for analysis of these compounds in environmental samples. LC/MS with a single quadrupole or LC/MS/MS with an ion trap are tremendously sensitive methods, as well, but are more prone to problems resulting from sample matrices. Ionization and detection of these compounds from complex matrices can still be affected by co-extracted and co-eluting interferences no matter which detection method is used. Thus, improved separation techniques will be required to accurately understand the environmental fate and occurrence of veterinary antibiotics.

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