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Review

Theory and methodology of antibiotic extraction from biomatrices

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Abstract

A short review on the basic theory and practices of the extraction and clean-up of agricultural antibiotics from biomatrices is presented. For the analysis of residues of ionophores, β-lactams, macrolides, chloramphenicol, aminoglycosides, tetracyclines and peptide antibiotics, the use of solid-phase extraction has become nearly ubiquitous as part of the basic extraction methodology. The majority of the methodologies for these compounds report recoveries greater than 70%, with relative standard deviations usually less than 15%. Each of the antibiotic classes, as well as antibiotics within each class, have unique chemistries that must be taken into account when developing a viable extraction method.

Keywords: Reviews; Extraction methods; Antibiotics

Contents

1. Introduction

Antibiotics are antibacterial agents derived from living organisms. In agriculture, selected members from several classes of these agents are available and licensed for use in animal husbandry. The main groups utilized are the β-lactams, tetracyclines, aminoglycosides/aminocyclitols, chloramphenicols,

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peptides, ionophores and macrolides [1]. Synthetic agents such as sulfonamides or nitrofurans are classified as antibacterial compounds, rather than as antibiotics.

The use of antibiotics in agriculture began in the 1950s with the use of oxytetracycline and chlortetracycline as feed additives [2]. Today, these and selected members of the other aforementioned antibiotic classes are commonly used as active disease treatment agents, prophylactics or growth promotants in agriculture. In the 1980s, it was estimated that at least 60% of all animals used for food were exposed to antibiotics at some point in their lives [3]. With current intense animal husbandry practices, this figure may be higher.

The widespread use of antibiotics in agriculture has resulted in the potential for residues of these compounds to be present in consumed foodstuffs. Monitoring of these residues is necessary to insure that they are not present at levels that may pose health risks to the public. Analytical methodologies have been successfully developed and established to identify and quantify these compounds. The scientific literature is rich in descriptions of the various methods available to perform such tasks. Several chapters in a recently published multi-author book [4–10] and article [11] have provided excellent reviews of the methodologies available for the analysis of antibiotics from biomatrices.

A quick overview of the published methods available for antibiotic analysis reveal that they have several steps in common. It is generally agreed that one of the first and most difficult steps required for antibiotic or any drug analysis is the extraction and clean-up of the drug from the biomatrix [12–16]. This is a prerequisite for all analytical methodologies, though the degree to which it is done varies widely. Screening methodologies (e.g., receptor and microbial screening tests) require only a minimal extraction and clean-up procedure. Quantitative/confirmative methodologies such as chromatographic or spectrometric assays, particularly of residues in tissue matrices, require extensive treatment of extracts before they are ready for analysis [13].

The commonly utilized techniques for the extraction and clean-up of antibiotics from biomatrices involve some form of liquid–liquid extraction (LLE) or solid-phase extraction (SPE). Supercritical-fluid extraction (SFE) has attracted interest with regards to its potential for drug analysis [17], but because of its requirement for specialized equipment, it has not found wide application. LLE was the first commonly utilized technique for drug extraction. Within the past two decades, SPE has gained prominence as being an integral part of most drug extraction procedures.

Several treatises exist that deal with both the theoretical and practical aspects of LLE and SPE. Theory on LLE is extensive [18], though it is based on ideal solutions rather than biological samples. SPE is based on chromatographic theory [19], though it has been noted [20] that it cannot explain all observed phenomena. Several chapters in the aforementioned multi-author book [4–10] reviewed briefly the methodologies employed in the extraction and clean-up of antibiotics from several matrices. Several literature sources provide descriptions of the theory and applications of extraction methodologies for antibiotics from biomatrices.

It is beyond the scope of this article to describe fully the chemistry and techniques for the extraction of antibiotics from biomatrices. Rather, the purpose is to provide a general overview of the basic theory behind extraction and clean-up methodology, as well as to provide a brief review of some of the current extraction methodologies that are utilized in the analysis of antibiotics from biomatrices.

2. Theory

2.1. Aqueous solubility

The model for the dissolution of a compound in solution is based on the concept of cavities in liquids [18]. Using this model, the solubility of a compound is determined by two separate phenomena: (1) removal or desorption of the solute molecule from its matrix, and (2) transfer of the solute molecule into cavities present in the solvent. In an ideal solution, the energy required for solution to occur can be described as:

$$W = w_{22} + w_{11} - 2w_{12} \quad (1)$$

where W is the overall energy required for dissolution to occur, w_{22} is the energy required for a solute molecule to be desorbed from the matrix, w_{11} is the

energy required to create a cavity in the liquid, and $2w_{12}$ is the energy released due to the interaction of solute–solvent molecules. Though overly simplified, this model provides the basis to the strategy an analytical chemist uses to create a working extraction method.

Desorption of drugs from a tissue matrix is dependent upon the molecular interactions occurring between the drug and matrix. The interactions that occur are hydrogen bonding, dipole–dipole interactions, ionic and covalent interactions [21]. Drug desorption/extraction from a biological matrix can be modelled according to a Freundlich sorption isotherm [22,23]:

$$\log C_b = \log k + mC_f \quad (2)$$

where C_b is the concentration of bound drug, C_f is the concentration of drug in the extracting solvent, k is the dissociation constant, and m is a measure of the availability of the drug for desorption. The utility of the parameters (especially m) in this equation is that they can give an indication of the ease with which drugs can be extracted from a matrix, and the requirements of the extraction protocol. This approach has been applied to the extraction of oxytetracycline (OTC) from bone, where it was shown that the optimal number of extractions necessary to satisfactorily extract OTC could be calculated [23]. The value of m is dependent upon the extracting solvent used, the acid/base properties of the drug, as well as the lipid content of the tissue [22]. The authors are not aware of any other formal studies that have investigated the application of this equation to other antibiotic extraction methodologies.

Selecting a solvent for antibiotic extraction is determined by two methods; empirical techniques, which are based on practical observations, and thermodynamic techniques, which are based on thermodynamic properties of the solvent and solute [24]. The major thermodynamic properties of interest in solvents with respect to its solvation abilities are its polarity, dipole–dipole interactions, proton-donor and proton-acceptor qualities [24]. A detailed list of such properties for a variety of organic solvents is provided by Snyder [25]. Additionally, lattice energy of the solvent itself will influence the solubility of a drug [18]. In a solute, the thermodynamic property of interest is its cohesive energy, which is a function of its composition as well as structure [26]. Calculation of the solubility of a chemical agent, especially when it is present as a residue in a biological matrix, on a purely thermodynamic basis may not be practical nor possible due to the complexity of the matrices, and thus empirical techniques are of greater use.

Salvatore and Katz [27] determined the solubilities of several antibiotics (erythromycin, oleandomycin, tylosin, hygromycin B, neomycin, streptomycin, spectinomycin, lincomycin, oxytetracycline, chlortetracycline, bacitracin, virginiamycin, bambermycins, monensin, novobiocin, nystatin, penicillin G) in several different organic solvents. Specific trends in solubilities of the compounds were noted by the authors. Solubility of macrolide antibiotics (erythromycin, tylosin), tetracyclines and the peptide antibiotics increased as solvent polarity increased. The aminoglycosides neomycin and streptomycin were poorly soluble in all solvents. Dimethyl sulfoxide (DMSO) and methanol were the best overall solvents, though DMSO's low vapour pressure makes it unsuitable for subsequent concentration processes. Salvatore and Katz [27] did not indicate the effect of pH on the solubility of the antibiotics in the solvents; such information for some antibiotics is available in the appropriate chemical encyclopedias.

Alteration of pH has a dramatic effect on the solubility of ionizable drugs. For example, raising the pH from 1 to 5 decreases the solubility of oxytetracycline from 31 g/l to 0.5 g/l [18]. Empirically, it is known that ionized compounds exhibit much higher solubility in polar solvents (especially aqueous solutions) than unionized compounds. Knowledge of the pK_a of ionizable compounds is taken into consideration when determining the solubility of a drug in solution. Equations for determining the solubility of a compound when parameters such as solubility of the ionized and unionized compound, pK_a and pH are known and can be found in any textbook on analytical chemistry.

Liquid–liquid partitioning in LLE methodologies are utilized for the separation of the drug of interest from other matrix components. A drug component is partitioned between two immiscible phases so as to bring about a favourable extraction of the drug or contaminants from one phase to another. The extractions are repeated to maximally extract the

compounds/contaminants from one phase to another in order to isolate the agent of interest in a cleaner extract for subsequent analysis. Partitioning can be described in thermodynamic terms, though knowledge of the maximum solubility of the drug in each phase is just as useful for determining the utility of partitioning for isolating an agent, and is inherently simpler to understand [18]. Values of the partition coefficients for drugs are the usual data that the analyst relies upon. These are ratios of the distribution of the drug between two immiscible phases (usually an organic and an aqueous phase). Octanol is commonly used as the organic phase when determining the partition coefficients. However, the use of other organic solvents may provide better estimates of phenomena of interest. For example, binding to proteins is better related to the log values of partition coefficients determined using isobutanol rather than octanol [18].

2.2. Solid-phase extraction

The advantages of SPE when compared to LLE are that it is faster, more reproducible, cleaner extracts are obtainable, emulsion creation is avoided and smaller sample sizes are needed [28]. It has also been stated that it may be cheaper when labour costs and the cost of disposing of used solvents are taken into consideration [14]. From an environmental point of view, a decreasing dependence upon the use of chlorinated hydrocarbons is also desirable. Additionally, SPE can be easily incorporated into automated analytical procedures, which can lead to greater accuracy and precision, as well as greater laboratory productivity [29]. As a result, SPE has gained popularity in analytical labs, whereas the use of LLE is waning.

There are four steps involved in the use of SPE sorbents [30]: (1) Conditioning of the sorbent, usually done to solvate and chemically prepare the sorbent bed for reproducible retention. The procedures will vary depending upon the SPE mechanism used. (2) Application of the sample to the sorbent, thereby allowing for selective retention of the analyte or contaminants in question in the cartridge while removing the other materials. (3) Rinsing the sorbent bed, usually with further extraction solvent, to remove unwanted materials while leaving the adsorbed material on the sorbent. Undesirable material chemically similar to the analyte may be preferentially removed by washing the sorbent bed with diluted solutions of the elution solvent. (4) Elution of the retained compounds, using a solvent that disrupts the analyte–sorbent interactions.

Four general extraction mechanisms are utilized in SPE; non-polar, polar, ion-exchange and covalent interactions [30]. Functional groups utilized in non-polar SPE are C_{18}, C_8, C_2, phenyl and cyclohexyl. Styrene–divinylbenzene copolymer (PRP-1) is also non-polar. Polar functional groups utilized are cyanopropyl, diol and aminopropyl; unfunctionalized silica and alumina are also polar in nature. Ion-exchange columns have two sub-classifications; cation- and anion-exchange. Cation-exchange groups are either strong exchangers (sulfonic acid derivatives) or weak exchangers (carboxylic acid derivatives). Anion-exchange groups are also strong (quaternary amines) or weak (primary, secondary, or tertiary amines) exchangers. Covalent interactions are dependent upon the chemistry of the analyte. Borate derivatives for the extraction of vicinal diol compounds is one example [30].

There is a general consensus that alkyl-bonded SPE sorbents, particularly C_8 and C_{18} sorbents, are the most popular [19,31]. However, the chemistry of these sorbents is not amenable to extract polar compounds from large sample volumes, and they are also subject to degradation. Additionally, the retention characteristics of the sorbents are dependent upon the manufacturer and lot [32–34], though improvements in manufacturing technology would undoubtedly reduce lot-to-lot variation. The major weakness of single-functional group SPE cartridges is that they cannot be readily used for multi-residue analysis, due to the widely varying chemistries of antibiotics as well as their metabolites. The use of multiple SPE cartridges utilizing different chemistries has been successfully applied for clean-up purposes [35], but at the cost of increasing analysis time. To compensate, manufacturers have created mixed-phase SPE cartridges, which combine non-polar and ion-exchange groups [36], thereby taking advantage of more than one chemical property of an analyte. The manufacturing technology for these columns seems to be behind that of the single-phase columns, as recent reports have indicated that use of multi-phase columns gave irreproducible extractions. Subsequent morphometric analysis of particles in the

cartridges revealed that they had a nonhomogenous distribution [36]. Other researchers, however, have reported recoveries with relative standard deviations (R.S.D.s) ranging from 5 to 15%, which is acceptable [37]. A somewhat new type of SPE cartridge based on the use of porous graphitic carbon (PGC) has appeared recently [19,38]. PGC, derived from heat-treated carbon blacks, has a homogenous particle distribution with a specific surface area of 250 m^2/g [19], which is similar to the surface area of 5 μm alkyl-bonded silica [39]. Particle sizes in silica-based SPE cartridges are 40 μm [30], indicating that they have a much smaller surface area and thus lower adsorbing capacity. PGC also appears to be multifunctional, retaining both polar and non-polar analytes with high affinity [19]. The main problem associated with PGC is that it adsorbs compounds very strongly, in some cases irreversibly.

SPE theory is based on chromatographic theory. The parameters that dictate the utility of SPE for a particular analyte are the breakthrough volume of the cartridge (V_B), the equilibrium volume of the cartridge (V_E), the capacity factor of the solute (k) and the concentration of the solute in the solvent (C_0). V_B refers to volume of solvent containing the analyte that can be percolated through a cartridge before the analyte is detected in the effluent. The capacity factor is defined as the number of moles of analyte adsorbed to the stationary phase of the cartridge relative to the number of moles in the make-up volume (V_M, also void volume of the cartridge). With these parameters, the amount of solute a cartridge can adsorb (n_s) is defined as:

$$n_s = V_M k C_0 \quad (3)$$

Adsorbent capacity is thus dependent upon capacity factor as well as the concentration. The adsorbed amount is linear until it begins to approach the total capacity of the solvent; however, in residue analysis, this usually will not occur [20]. Capacity factors usually correlate well with water–octanol partition coefficients for reversed-phase SPE sorbents. However, solvent properties such as proton donor/acceptor abilities and dipole–dipole interactions also affect the efficacy of solvent for both adsorption and desorption of an analyte to the sorbent [32]. No corresponding relationship appears to exist for multiphase adsorbents [38].

3. Methodologies utilized for the extraction of antibiotics from biomatrices

Extraction strategies needed for drugs depends upon the nature of the biomatrix. In LLE, the judicious use of multi-step procedures involving extraction and back-extraction into organic and aqueous phases with appropriate use of pH and ionic strength can remove desired compounds from matrices as well as reduce the amount of interfering contaminants in the final extract. Matrices such as urine do not need to be pre-treated prior to LLE or SPE of drugs. Plasma may require some degree of pre-treatment, primarily due to binding of drugs by the plasma proteins albumin, α-acid glycoprotein, lipoproteins and τ-globulins [11]. This usually includes a deproteination step involving the use of an organic solvent such as acetonitrile, or acids such as tungstic or perchloric acid. This can lead to drug loss due to occlusion of the drugs by the precipitating proteins. Milk is a further complex matrix due to the presence of a lipid emulsion. Disruption and extraction of the fat globules may be necessary. Tissue matrices are by far the most complex matrices for drug extraction. Distinct differences exist between, muscle, kidneys and liver. The liver is a fatty tissue that is the site of many metabolic enzyme systems. Levels of chloramphenicol are dramatically reduced in homogenized liver samples that have not had enzyme inhibitors added [11]. Other tissue sensitive drugs also exist. Extraction methodologies for tissue residues are regarded as the most difficult to develop [16].

Reviews of current methodologies in the analysis of antibiotics of importance to agriculture have been recently published [4–10]. As part of these reviews, extraction methodologies were also discussed briefly. The major points of these methodologies will be discussed in addition to developments that have occurred since then.

3.1. Ionophores

Ionophore or polyether antibiotics are chemically characterized by several cyclic ethers, a single terminal carboxylic acid group and several hydroxyl groups. Representative members of this class include salinomycin, monensin, lasalocid and narasin (Fig. 1). Salinomycin and narasin have virtually identical

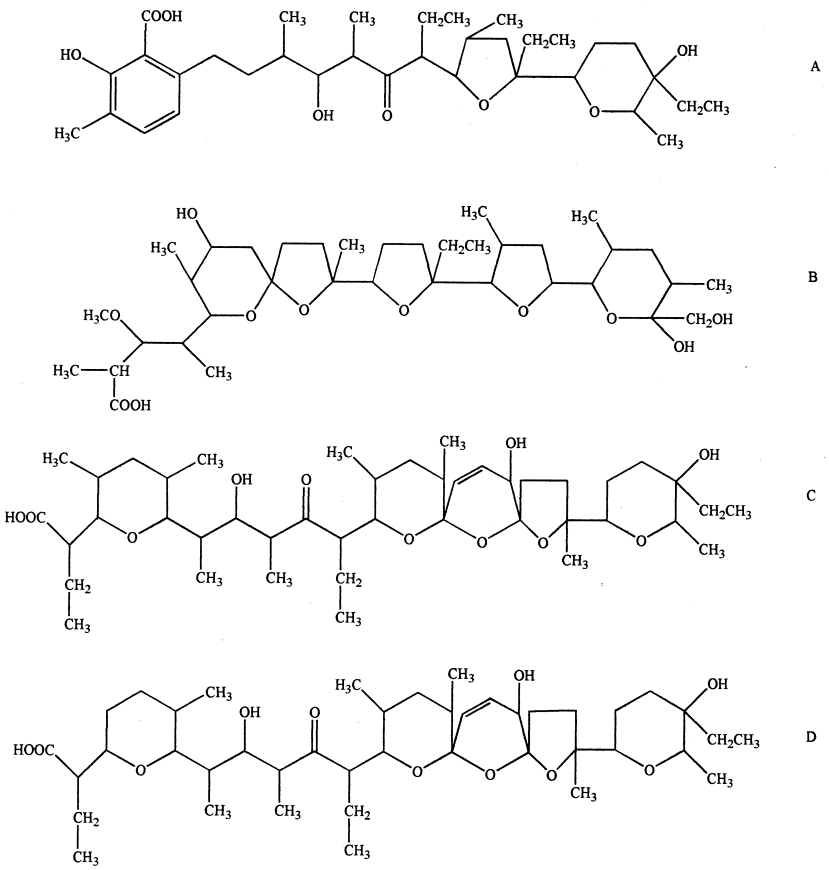

Fig. 1. Structures of the ionophore antibiotics lasalocid (A), monensin (B), narasin (C) and salinomycin (D).

structures; lasalocid consists of two cyclic ethers whereas the rest have six. The main chemical properties of interest in extraction methodology are their low polarities and their instability under acidic conditions.

Initial extraction of ionophores from biomatrices has traditionally been accomplished with the use of acetonitrile [4]. Recoveries close to 100% of all ionophores are possible with this extractant. The use of methanol or methanol–water mixtures is also effective, and the method seems to be fairly rugged with regards to the mixing ratio used [40] but tends to extract contaminants (e.g., fatty acids, polar compounds) as well. Coupling the extraction with alumina was found to be applicable to all ionophores except lasalocid; it was bound irreversibly to the sorbent [4]. Clean-up of salinomycin extracts from poultry tissue by liquid–liquid partitioning with hexane [41] gave recoveries of over 90% in all tissues except liver, where recovery was 80%. R.S.D.s were less than 5%. In contrast, Kennedy et al. [42] used a salt saturated water–acetonitrile (1:5, v/v) solution to extract lasalocid from poultry tissue liver with higher recoveries (80 to 100%) than from muscle (60 to 80%). R.S.D.s in both situations were greater than 15%. Other normal-phase extractions using less polar extractants such as isooctane and silica gel as the adsorbents have also been utilized [43]. Elution is then accomplished using a polar organic solvent (ethyl acetate or acetone), with average recoveries greater than 90% for monensin, salinomycin and narasin. Recoveries were independent of tissue source (beef or chicken). Chicken livers seemed to decrease recoveries slightly. In

contrast, lasalocid had an average recovery of 70%, with recovery in chicken liver higher. Asukabe et al. [44] reported that a wide variety of solvents (methanol, acetone, ethyl acetate, acetonitrile, benzene, hexane) are comparable for extracting most ionophores, but that the eluent composition must be sufficiently polar to effect a sufficient elution from normal-phase cartridges. Lasalocid was reported to have consistently lower recoveries than other ionophores.

3.2. Macrolides

Macrolides are chemically characterized by a macrocyclic lactone ring with isolated or conjugated double-bonds, attached to amino-sugars. Commonly used macrolides in agricultural practices consist of rings that are 12-, 14-, 16- or 17-membered structures. They are generally mixtures of more than one structural component. Representative members of the macrolide class of antibiotics are erythromycin (three components), tylosin (four components) and tilmicosin (two components). The major components of each antibiotic are illustrated in Fig. 2. The main chemical properties of interest to extraction methodology are their instability in acid and hydrophobic nature.

Direct extraction of macrolides from alkalinized liquid matrices or aqueous tissue homogenates (to suppress ionization of amino groups) directly into organic solvents (chloroform, dichloromethane, ethyl acetate, *tert*.-butyl methyl ether), with clean-up by back extraction into weakly acidic aqueous buffer, was the traditional method for macrolide extraction. Recoveries for these methods are close to 100%, with R.S.D.s less than 10% [5,45]. Hanada et al. [45] found that extracts from *tert*.-butyl methyl ether were generally better as they gave the cleanest extracts. Methanol and acetonitrile extracts of tylosin from feeds have been cleaned using acidic alumina [46], with tylosin recovery and R.S.D. near 100% and 10%, respectively. Extraction of tylosin and erythromycin by a methanol–acetone mixture (no clean-up) gave recoveries of over 90% for the two antibiotics. C_{18} silica cartridges have cleaned tylosin and tilmicosin from methanol and acetonitrile extracts of animal tissues (bovine and porcine muscle and kidney), with a recovery of 90% and R.S.D.$<$10% for tilmicosin, and a recovery of 80% and R.S.D.$<$ 8% for tylosin. Tissue effects were not apparent [47]. A chloroform extract of muscle for detection of several macrolides (tilmicosin, spiramycin, tylosin, erythromycin and josamycin) was cleaned by a diol column; recoveries were not reported [48].

3.3. Chloramphenicols

The best known member of this group is chloramphenicol (Fig. 3). Chloramphenicols are chemically characterized by a benzene ring and a substituted three carbon chain. They are highly polar and form glucuronates in the liver [6], requiring special sample handling techniques when this tissue is examined.

Extraction is usually performed with methanol, acetonitrile or ethyl acetate. Recoveries of chloramphenicol from fish were found to be the highest (ca. 90%) when ethyl acetate was used as the extractant, followed by acetonitrile (ca. 80%) and methanol (ca. 75%), R.S.D.s less than 10% [6]. Partitioning of the extract with a non-polar solvent is required to remove lipid material. Clean-up by SPE using both non-polar and polar phase materials have been performed. C_{18} provides the highest recovery (90 to 100%), though interferences are present [6]. Using silica gel and alumina, recoveries for chloramphenicol from Yellowtail fish extracts have been ca. 80 and 75%, respectively, with R.S.D.s less than 5% [48–50]. A recovery of less than 65% has been reported recently [51]. Munns et al. [52] had used a procedure similar to that of Nagata and co-workers [49–51] for extraction of chloramphenicol from shrimp except that SPE was not used. Virtually 100% recovery occurred, indicating that SPE by normal-phase cartridges may not be suitable for chloramphenicol.

3.4. β-Lactams

β-Lactams are chemically characterized by a β-lactam ring connected to a thiazolidine ring (penicillins) or dihydrothiazine ring (cephalosporins and cephamycins). Two representative members of this antibiotic class are penicillin G and ceftiofur (Fig. 4). They are unstable in aqueous acids and bases; penicillins are also unstable in methanol and are degraded by endogenous muscle enzymes [7].

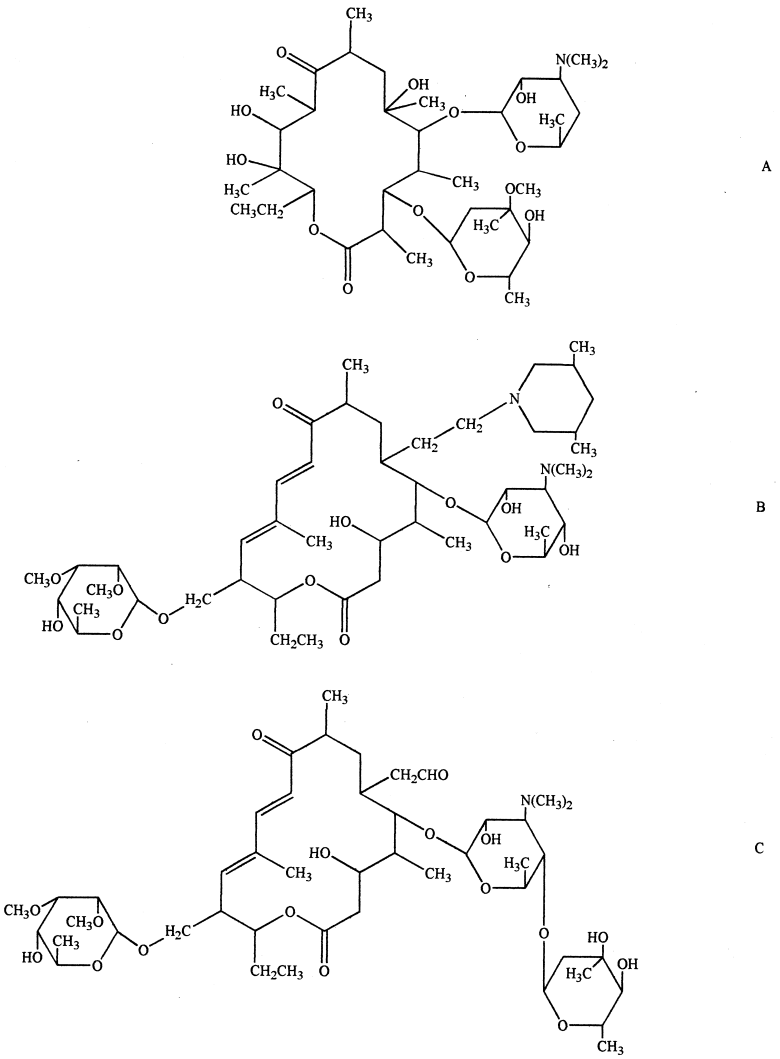

Fig. 2. Structures of the macrolides erythromycin A (A), tylosin A (B) and tilmicosin (C).

Traditional extraction methods have utilized aqueous extractions with protein precipitating agents to remove proteins prior to further treatments, in order to obtain cleaner extracts. Subsequent cleaning steps have utilized SPE (anion-exchange, diol and C_{18}) and immunoaffinity columns, with recoveries generally greater than 70% [7]. Recovery rates are dependent upon the tissue analyzed; Hong et al. [53] reported that most agricultural penicillins exhibited the highest recovery in kidney (80 to 100%), followed by liver (73 to 93%) and then serum (70 to 90%) following extraction with a phosphate buffer (pH 7)–acetonitrile solution and centrifugation. Gee et al. [54] reported similar recoveries from bovine liver, kidney and muscle using 3% NaCl–acetonitrile solution followed by a C_{18} SPE clean-up. The use of

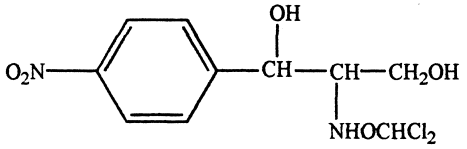

Fig. 3. Structure of chloramphenicol.

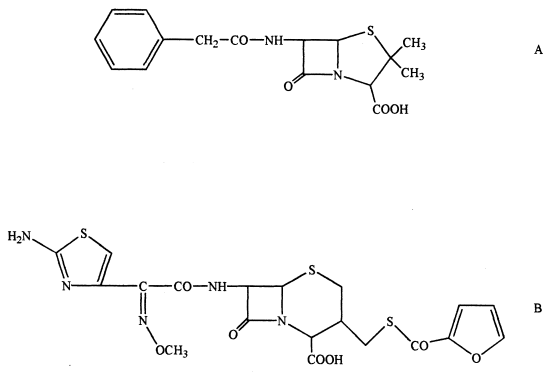

Fig. 4. Structures of the β-lactams penicillin G (A) and ceftiofur (B).

SPE for penicillin G does not seem to offer any advantage with regards to recoveries.

Ceftiofur undergoes rapid metabolism to form desfuroylceftiofur and furoic acid [54]; for meat and milk residues (except intramammary infusions) analytical methods are subsequently based on quantifying desfuroylceftiofur. In tissues, it is conjugated by means of a disulfide bond, necessitating the use of a reducing agent (usually dithioerythritol) in order to release it. Jaglan et al. [55] used a straightforward extraction with pH 7 phosphate buffer and dithioerythritol to extract the agent, and then centrifugation for clean-up. Recoveries are reported to be near 100%. Beconi-Barker et al. [56] went through a lengthy extraction process (extracting solution, dithioerythritol in borate buffer, followed by clean-up on C_{18}, anion- and cation-exchange SPE cartridges) and obtained recoveries of 70 to 85% in swine muscle, liver and kidney with R.S.D.s of ca. 15%.

3.5. Aminoglycosides

The aminoglycosides are chemically characterized by two or more sugars or amino sugars attached to an aminocyclitol ring [8]. Streptomycin and neomycin are two representative members of this antibiotic class (Fig. 5). Aminoglycosides are polar, resistant to acids, bases and heat, and are not extensively bound to proteins.

Aminoglycosides are chemically ideal for aqueous extraction. Extraction using buffers alone gave recoveries of approximately 50%, protein precipitation by acids increased recovery to 80%, NaOH digestion of proteins increased recovery to 90% [8]. Clean-up on normal-phase extraction columns (silica gel and cyanopropyl) have resulted in cleaner extracts. Weak cation-exchange columns have also been used successfully for clean-up of gentamicin from plasma and bacterial culture broths with recoveries over 80% [57]. Strong cation-exchange columns (i.e., sulfonic acid derivatives) appear to be detrimental to the absolute recoveries (less than 60%) of the aminoglycosides streptomycin and dehydrostreptomycin from tissue matrices, though R.S.D.s (less than 8%) are excellent [58].

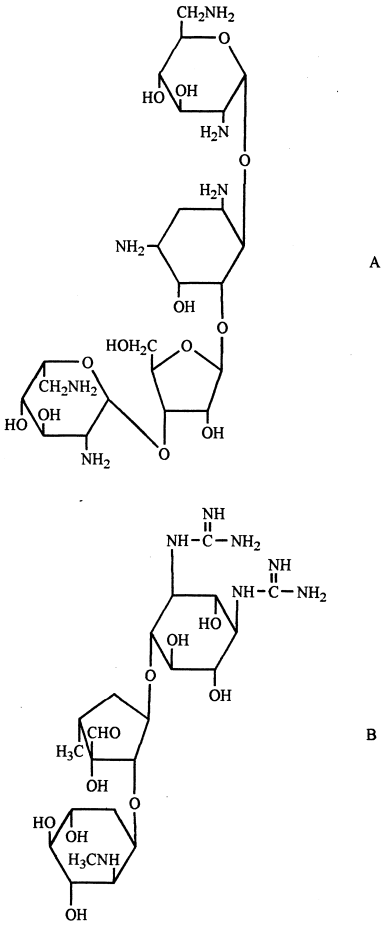

Fig. 5. Structures of the aminoglycosides neomycin B (A) and streptomycin (B).

3.6. Tetracyclines

Tetracyclines are chemically characterized by a partially conjugated four-ring structure with a carboxyamide functional group. Chlortetracycline (CTC), oxytetracycline and tetracycline (TC) are representative members of this antibiotic class (Fig. 6). They are amphoteric compounds soluble in polar and moderately polar organic solvents, and have the ability to form strong complexes with multivalent cations; it is the latter feature that is primarily taken into consideration when developing extraction methodologies for these agents [9].

Oka and Patterson [9] extensively reviewed the literature to 1995 on the methodologies utilized for the analysis of tetracyclines. In addition to the review by Shaikh and Moats [59], both treatises indicated that there is an enormous variety of extraction methodologies for tetracycline analysis. The one common factor in all of these methodologies is that the majority utilize aqueous solutions containing chelating agents to decrease the tendency for tetracyclines to bind to cations in the matrix. EDTA, oxalic and citric acids are the most commonly used chelating agents. McIlvaine's buffer, used in many extraction procedures [9] contains citric acid. The use of EDTA–McIlvaine's buffer, combined with SPE using alkyl-bonded silica cartridges for clean-up, was established by Oka et al. in 1985 [60] and appears to be the current standard for the extraction of tetracyclines from tissue matrices. The method utilized an oxalic acid methanol solution to elute the tetracyclines from the cartridges. The addition of oxalic acid was found to be necessary to effect reproducible elutions; this was attributed to the presence of contaminants and free silanol groups on the cartridges. Oka et al. in 1997 [61] utilized the same procedure but eliminated the use of oxalic acid, using instead an ethyl acetate–methanol mixture. Recoveries for OTC (75 to 80%), TC (70 to 80%) and CTC (55 to 70%) were less than that reported in 1985. R.S.D.s were also generally higher (3 to 7% versus 1 to 4%). As well, the use of oxalic acid eliminated the tissue effect on recovery. Without oxalic acid, extracts from liver and kidney gave lower recoveries of tetracyclines than muscle tissue extracts. However, exclusion of oxalic acid from the eluting solvent allows for its subsequent concentration, which in turns increases sensitivity of the assay. Recently, there is increased interest in using the chelating abilities of tetracyclines to their benefit when developing extraction methodologies [62]. The use of metal chelating affinity columns was used to clean-up succinate buffer extracts of porcine kidney and muscle, and bovine liver for tetracycline, oxytetracycline and chlortetracycline analysis. Recoveries varied from 40 to 70%, with no apparent effects due to the tetracycline type or tissue. This may be of some benefit, as Blanchflower et al. [63] showed that chlortetracycline is especially prone to epimerization in aqueous solutions, which may account for its low recoveries.

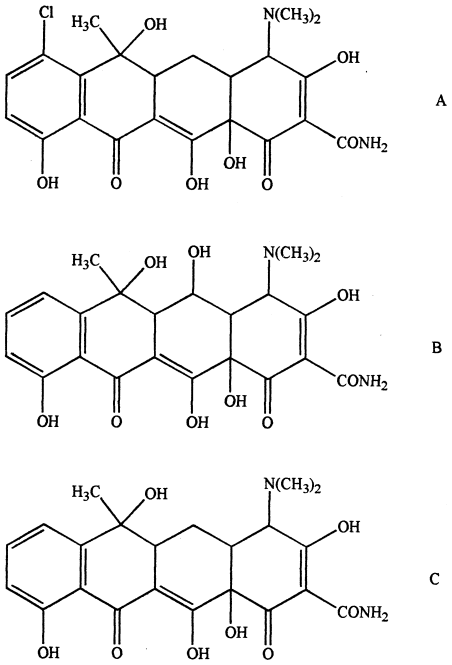

Fig. 6. Structures of the tetracycline antibiotics chlortetracycline (A), oxytetracycline (B) and tetracycline (C).

3.7. Peptide antibiotics

Peptide antibiotics are amino acid (L and D) containing compounds covalently linked to other chemical entities, and consist of more than one component [10]. Bacitracin and virginiamycin are two antibiotics of this class (Fig. 7). The main chemical characteristic of analytical interest is that

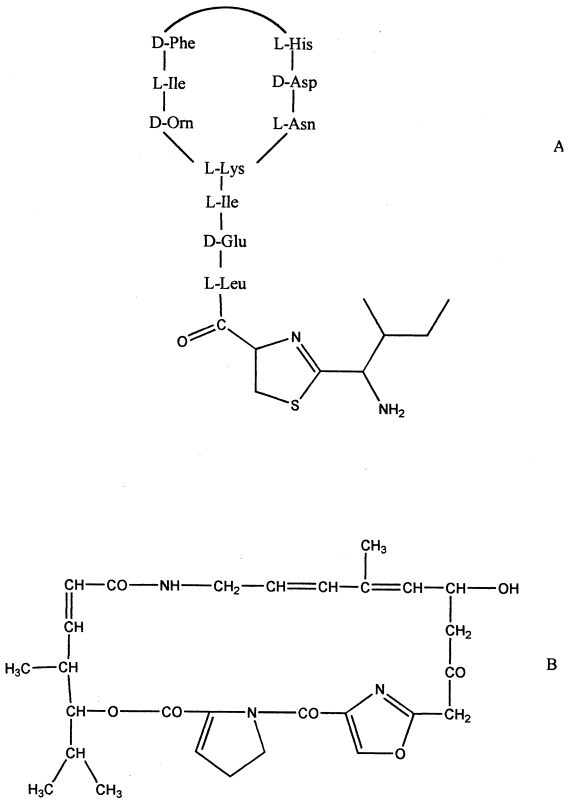

Fig. 7. Structures of the peptide antibiotics bacitracin A (A) and virginiamycin M1 (B).

they are similar to matrix components, which is one of the major reasons peptides are difficult to analyze in biological matrices. Additionally, metabolic pathways of the peptides have still not been elucidated, complicating development of extraction methodologies for metabolites.

To date, very few methods have been published on the analysis of bacitracin from biological matrices. Assays for bacitracin distribution in tissue have relied on ^{14}C-labelled materials [64]. These studies revealed that the majority of bacitracin doses are eliminated in the faeces, and that it is not significantly absorbed. Detection of bacitracin in the tissues was not possible. Ikai [10], using 0.5% aqueous sulfuric acid with clean-up on a C_{18} cartridge was able to get a 64% recovery of bacitracin A from beef muscle, R.S.D. 7%. It was not indicated whether extraction of incurred bacitracin from tissues was possible. In light of the data from Donoso et al. [64], it may not be practical to develop such a method.

As with bacitracin, the majority of administered virginiamycin is also excreted in the faeces [65]. After administration of ^{14}C-labelled virginiamycin to rats, turkeys and cattle, it was determined that metabolites of virginiamycin are present in the liver, with the majority of them covalently bound to the tissue. The extractable metabolites were unidentifiable. In contrast, Moats and Leskinen [66] had successfully extracted incurred virginiamycin M1 from swine. A 0.3 *M* ammonium phosphate–methanol (1:1, v/v) solution was used as the extractant. The extract was cleaned by partitioning with petroleum ether, and then virginiamycin was extracted into methylene chloride. Recoveries of the agent from spiked samples of swine muscle, kidney and liver were 94%, 85% and 86%, respectively, with R.S.D.s less than 6%. Moats and Leskinen [66] did not report the recovery of incurred virginiamycin residues, though the work of Gottschall et al. [65] would indicate that it would be low.

4. Conclusions

For all antibiotics, with the possible exception of the peptides, regulatory agencies must address the potential of antibiotic residues appearing in edible tissues. For tissue residue analysis methodologies, the first and most important step is the extraction of these compounds from the biological matrix. Techniques for residue analysis have changed as different technologies have become available. LLE has been largely supplanted by SPE. C_{18} is the predominant form of SPE utilized, though other emerging phases such as multi-phase columns and PGC offer distinct advantages. Techniques such as matrix solid-phase dispersion (MSPD), a physical variation of SPE cartridges whereby the initial liquid solubilization of the drug is replaced by the use of a solid support, have also been investigated.

Due to the unique chemistries of the antibiotic classes, and of different members within each class, diverse methodologies must be applied to extract these agents from the matrices within which they reside. An additional complicating factor is the tissue type. The recovery of some antibiotics (e.g.,

chloramphenicol) can be dramatically affected by the tissue to which an extracting scheme is applied. These and other reasons make antibiotic extraction from tissue matrices one of the most complicated steps in residue analysis.

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