ANALYSIS OF ANTIBIOTIC DRUG RESIDUES IN BIOLOGICAL MATRICES, AFTER EVALUATION OF VARIOUS EXTRACTION METHODOLOGIES AND DETERMINATION PROCEDURES

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Declaration

I hereby declare that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed Marie Mc Grane Date 21-9-00

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TABLE OF CONTENTS

LIST OF FIGURES

List of tables

Abstract

This study involves the development and validation of methods for the analysis of antibiotic drug molecules. Techniques for the extraction of residues of these drugs from biological matrices were investigated and subsequently validated. Extraction of sulphonamide residues from porcine muscle was investigated using on-line dialysis, followed by trace enrichment. Direct UV detection and post-column derivatisation was investigated and methods validated. Matrix solid phase dispersion was investigated for the extraction of penicillin residues form porcine muscle. HPLC separation coupled with UV detection of derivatives of the extracted residues was performed. The determination of aminoglycoside residues from milk and porcine liver was investigated. The chromatographic behaviour of these analytes was studied, followed by analysis of aminoglycoside residues using ion-exchange and immunoaffinity column chromatography. Finally, a HPLC method for the separation of aminoglycosides and related compounds was developed, using MS detection.

CHAPTER 1

INTRODUCTION TO ANTIBIOTIC

DRUG RESIDUE ANALYSIS

1.1 ANTIBIOTIC RESIDUES IN FOOD

Food safety, which assures that food is "safe" for human consumption, involves the determination of various substances present that are likely to cause a toxic effect. Chemical residues may be naturally occurring or be present as a result of human intervention. In agriculture, selected antibiotics are available and licensed (under EU and national regulations) for use in animal husbandry. The main groups utilised are β-lactams, tetracyclines, sulphonamides, aminoglycosides, and macrolides[1]. Originally, naturally occurring antibacterials were classed as antibiotics, whereas synthetically produced antibacterials were classified as chemotherapeutics. More recently, the term antibiotic includes naturally occurring and synthetic molecules, which are used for the treatment of bacterial infections. In the 1920's, the discovery of penicillin transformed the world of medicine and veterinary science, and was the first of a wide range of antibiotics that could be used for treating previously fatal bacterial infections.

1.1.1 Reasons for antibiotic use and consequences of residues in food

While antibiotics are required to treat infections in individual animals (therapeutic use), they are also used prophylactically, to prevent the occurrence and spread of infections in intensive production systems such as pig and poultry. Prophylactic antibiotic administration, through the use of medicated feedstuffs, has resulted in increased resistance to bacterial diseases and improved performance in animals. The use of antibiotics as growth promoting agents also results in improved performance as a result of suppression of mild, non-diagnosed infections, increased absorption efficiency of nutrients and elimination of toxins produced by microbes. The presence of antibiotic drug residues in animal products such as milk, meat and eggs can present hazards for public health,

industry and the environment. The undesirable effects can be classified as toxicological, development of resistant strains of bacteria and allergic responses.

A major public health concern is the increasing occurrence of strains of bacteria resistant to antibiotics. Some antibiotics are beginning to loose their effectiveness, thereby making it difficult to treat some common illnesses. This is due to over-exposure of humans to antibiotics. This phenomenon has resulted in the removal, or restriction on the use of certain antibiotics. The use, and misuse, of antibiotics in treating diseases in humans is the prime cause for development of resistant bacteria. Continuous use of antibiotics can result in the emergence of a new generation of organisms, which possess genes with resistance to certain antibiotics. Therefore, the use of antibiotics in animal production can result in animals becoming carriers of resistant bacteria that cause diseases which are difficult to treat; for example, resistance is common in *Salmonella* strains originating from veal[2]. Prolonged exposure to antibiotics as a result of consuming residues contained in food is also suspected to contribute to antibiotic resistance. The question as to whether ingestion of antibiotic contaminated food is capable of suppressing the natural microflora in the digestive tract is still controversial. The microflora protect the body from colonisation by pathogenic organisms within the stomach and intestines, and are necessary for the body's natural defence mechanisms.

The presence of antibiotic residues in food is of major industrial importance. A fermentation step is frequently employed for the manufacture of food products, such as cheese and yoghurt, from milk. Milk contaminated with antibiotic residues, and

subsequently employed in the manufacture of cheese or yoghurt, can result in the inhibition of culture development, due to elimination or reduction of the micro-organism necessary to allow fermentation.

While most antibiotics are not toxic at residue levels, some do elicit toxic responses. Chloramphenicol[3] and penicillin[4] antibiotics may exhibit toxicological effects. These antibiotics have been responsible for triggering intense immune reactions in susceptible individuals. Nitrofuran antibiotics can also elicit toxicological effects, when they persist as bound residues that are released as toxic substances[5]. For most other classes of antibiotics, residue levels likely to occur in foods are insufficient to cause acute toxic effects.

1.1.2 Factors responsible for antibiotic drug residues occurring in foods

A number of factors may be responsible for the persistence of antibiotic drug residues in foods. The main reason reported is failure to observe withdrawal times. Following antibiotic administration, withdrawal times are specified, after which time the animal or animal products are fit for human consumption (residue levels should be below the maximum residue limit (MRL) set by regulation). Early slaughter can result in the occurrence of residues in animal tissue. Biological factors pertaining to each animal's metabolism can also cause residues in foods. Impaired liver or renal function can result in incomplete elimination of the parent compound or its active metabolites from the animal's system. Administration of a number of drugs in a short time period can affect drug elimination from the body, due to inhibition of hepatic enzymes essential for drug

metabolism. Improper injection can result in deposition of the antibiotic, and the rate of elimination from the body is reduced. Also, in intensive farming systems where antibiotics are administered in drinking water or medicated feed, carry-over can result in the presence of residues in the finishing feed[6].

1.1.3 Control of antibiotic residues

EU member countries are required to test for the presence of antibiotics and other veterinary drug residues in foods of animal origin[7]. Maximum Residue Limits (MRLs) are the levels of drug legally permitted and recognised as acceptable in a food. The MRL is the maximum concentration of residue, resulting from the correct use of a veterinary drug, which should occur in food. MRLs are based on the type and amount of residue considered to be without any toxicological hazard for human health as expressed by the acceptable daily intake and an additional safety factor. MRLs give an indication of food safety and provide trading standards. Minimum withdrawal periods, post administration, are established, which ensure elimination of the drug and/or metabolites from edible tissues to levels at or below the MRL, at milking or slaughter.

1.1.4 Matrices examined for antibiotic drug residues

The first step in residue analysis is to decide on a suitable sample. For the analysis of residues of permitted antibiotics, edible tissues are analysed to check whether any residues present are at or below the MRL. Antibiotic levels are frequently determined in milk, eggs, meat (muscle, liver, kidney), blood, urine and animal feedstuffs. Body fluids such as

urine, milk or plasma are easily available and can be used for analysis when animals are not intended for slaughter. Determination of residues in urine can be used as an indication of the levels likely to exist in tissue. Antibiotic monitoring in milk is common, as antibiotics are frequently employed in the therapeutic treatment of diseases affecting the lactating animal, such as mastitis. For residues of prohibited antibiotics, a target matrix may be used. This is the matrix where the drug residues are most likely to persist. Urine, liver and kidney are target matrices due to their role in metabolism and excretion. The influence of tissue matrix on antibiotic detection limits was investigated by Okerman *et al.*[8]. The Four-plate test was employed for the analysis of pork, beef and chicken muscle samples. Tetracyclines, sulphonamides, aminoglycosides, β-lactams, quinolones, and macrolides were tested. Tetracyclines were the only antibiotics detected with similar efficiency both in the presence and absence of matrix. Some beta-lactams and aminoglycosides were not detected in spiked muscle tissue at concentrations five-fold higher than the detection limits without tissue.

Animal feedstuffs are also analysed for antibiotic residues. Animal finishing feed, that which is fed to animals in the period before slaughter, should be certified free of veterinary drugs and a specific withdrawal period is set for each antibiotic. The antibiotics commonly administered through animal feeds are the macrolides and polypeptides which are used in growth promotion and the ionophoric polyether antibiotics, used to improve feed efficiency and for the treatment of coccidiosis. In a recent study by Lynas *et al.* on the analysis of feedstuffs in Northern Ireland, the authors concluded that of the contaminated feedstuffs detected, chlortetracycline was detected in 50 % of the samples.

Sulphonamides were the next most frequently detected category[9]. Cross-contamination during milling is the prime cause of unintended antibiotic residues in feedstuffs.

1.1.5 Method development for analysis of antibiotic residues

In the following section, areas of particular importance in the chemical analysis of veterinary drug residues are discussed. In addition, terminology frequently employed in drug residue method development, and employed in the chapters to follow is addressed.

Quantification: Quantification is the determination of the amount of antibiotic residue present. The amount of analyte present in the sample is determined by comparison with a range of standards, in the form of a standard curve.

Validation: Once a method has been developed for the determination of a residue(s), it must then be validated. This is the process used to confirm that the analytical procedure employed is capable of determining residues between specific concentration limits with specified accuracy and precision. In the validation of methods for veterinary drug residues, the percentage recovery is determined at various concentrations of analyte and the variation in the recovery assessed over different assays. In the validation of a quantitative analytical method for the determination of antibiotic residues in biological matrices, a number of important parameters must be clarified, such as sensitivity, specificity, accuracy, precision, linearity, and the detection and quantification limits.

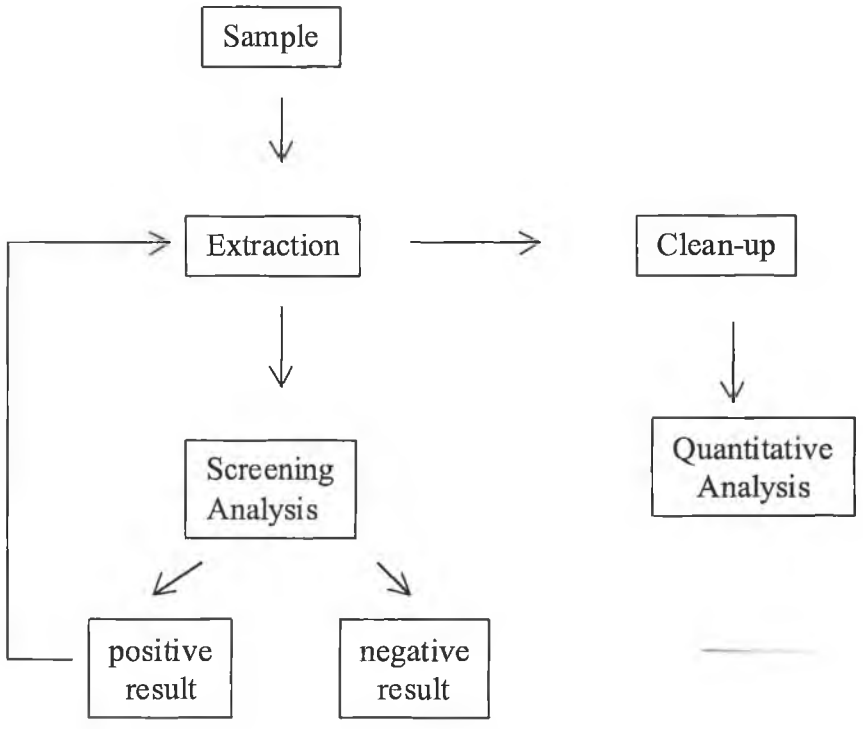
Limit of detection/quantification: To be of use, the method chosen must be capable of determining the analyte at levels below the MRL. The limit of detection is defined as the smallest concentration of analyte that produces a signal that exceeds the signal to noise

ratio by three times. It is also necessary to establish the smallest concentration of analyte that can be determined in a sample, with a specified degree of accuracy and within laboratory reproducibility. This level is defined as the limit of quantification of the method. These limits can be expressed in terms of concentration or absolute mass; however, for veterinary drug residues concentration is employed, and expressed in terms of the amount of sample used for analysis (e.g. ng g^{-1} or ppb).

Interferences: An interference is any constituent of a sample that acts in such a way as to hinder the process of assaying the analyte[10]. Interferences may be multiplicative or additive. Multiplicative interferences do not themselves produce signals of their own, but alter the signal produced by the analyte. They can act in a number of ways. One example is by decreasing the availability of the specific form of the analyte responsible for producing the measured signal, such as inhibiting derivatisation. Multiplicative interferences may also affect the physical process by which the signal is generated (for example, during fluorescence detection, quenching of the signal can occur), or may alter the efficiency with which the signal produced by the analyte is transmitted to the detector (e.g. emitted luminescence may be absorbed by other sample constituents, preventing it from being detected). Additive interferences are species that produce signals of their own, which add to that of the analyte. These interferences are also apparent in the control sample. The presence of interferences can increase the limit of quantification of the method. When the residue concentration is well in excess of the interferences, their presence may go undetected, however; as the residue concentration approaches the limit of detection of the method, the analyte signal detected is much lower and so other background signals play a much greater role. Therefore, the method may not be considered quantitative/qualitative at analyte concentrations affected by interferences.

Precision and accuracy: The accuracy of a measurement is "the closeness of agreement between the true or assigned value and the mean result obtained by applying the experimental procedure a very large number of times", as defined by ISO. The accuracy of a procedure may be evaluated by applying it to the measurement of a certified reference material or by determining the concentration of the analyte by an alternative independent technique. In addition, a measure of accuracy may be made by the analysis of fortified samples. The precision is "the closeness of agreement between the results obtained by applying the experimental procedure several times to the same sample under prescribed conditions". The precision is the repeatability within one assay (intra-assay) and may be extended to several assays (inter-assay).

1.1.6 Overview of the protocol employed for antibiotic residue testing



The flow chart shows the steps typically involved in antibiotic residue analysis. Similar to most residue procedures there are two main areas; sample preparation followed by determination. The sample preparation step may be divided into extraction of the drug from the biomatrix, followed by clean-up of the extract although, in some cases, there is overlap between the two categories. The most difficult challenge to the residue analyst is that of extracting the analyte from a complex matrix, with a minimum of matrix co-extractives. The extent to which the extract requires clean-up varies, depending on the method of determination and the matrix under analysis. Sample pre-treatment is usually more extensive for tissue samples than body fluids and the presence of co-extractives can result in higher limits of determination. Screening methods ideally require minimal sample preparation, therefore, the clean-up step is frequently omitted, whereas quantitative/confirmatory methods generally require more extensive sample extraction and clean-up.

Methods for determining the presence of antibiotic residues in foods usually fall into one of two categories, screening or quantitative/confirmatory methods. When large numbers of samples are presented for analysis, they are often put through an initial screening process and those samples found to give a positive result are subjected to further, more specific and accurate quantitative analysis. While chemical assays are more specific and quantitative than microbial inhibition assays, they usually require considerably more sample preparation. Changes in EU legislation and advances in consumer awareness have increased the need to develop more specific and sensitive methods for antibiotic analysis. With trends towards multiresidue analysis on the increase, a reliable separation technique is essential. In addition, the sample extract presented for final determination contains

matrix co-extractives, therefore the quantitative technique must be capable of separating/distinguishing the analyte from any matrix present. A wide range of determination techniques are available for antibiotics and advances are being made in areas such as chromatography and spectroscopy.

1.2 SCREENING ASSAYS

Screening tests are usually less expensive and less time consuming than the more specific quantitative or confirmatory methods. In general, they do not require very laborious sample pretreatment steps. They may detect the presence of an antibiotic residue or a class of antibiotics and usually allow high sample throughput. Biologically based assays, frequently employed for screening purposes, include microbial inhibition assays, immunoassays, enzyme and receptor assays. Microbial inhibition assays tend to be non-specific in nature, therefore they are used as screening methods to identify samples which may contain antibiotic residues and these query positive samples are then subjected to further analysis. They are commonly employed for routine, qualitative analysis, due to their speed and ease of use. Receptor-based assays also allow high sample throughput. Immunochemical assays may offer increased specificity and high sample throughput but are generally inferior to chemical methods for quantitative analysis.

1.2.1 Microbial inhibition assays

The agar diffusion assay can be carried out as a tube or plate assay[11]. An agar gel capable of supporting the growth and multiplication of micro-organisms is inoculated uniformly

with a suspension of a test organism that is sensitive to the antibiotic(s) under test. When an aqueous solution is placed in contact with the gel, any antibiotics present may diffuse from the solution into the gel. Therefore, a tissue extract or milk solution containing antibiotics will behave similarly and diffuse into the gel. The antibiotic will inhibit the growth and multiplication of the micro-organism and so a clear zone will appear in the agar around where the sample was applied. This is termed the zone of inhibition[11]. The size of the zone is proportional to the concentration of the antibiotic present. The disc-assay is a form of agar diffusion test, where a filter paper disk is soaked in an antibiotic solution or sample extract containing antibiotic, and the disc is placed on the agar that is inoculated with antibiotic sensitive micro-organisms. Penicillins, tetracyclines, aminoglycosides and cephalosporins have all been detected using this method[12].

The Four-Plate Test, alternatively known as the Frontier Post Test, is a popular choice for antibiotic screening[13]. As the name infers, four agar plates are required, three using *Bacillus subtilis* at pH 6.0, 8.0, and 7.2 and the fourth using *Micrococcus luteus* at pH 8.0. The sample (in the form of a disk of tissue) is incubated on the plates at 37°C for 24 hours. The *B. subtilis,* pH 6.0 plate is sensitive to penicillins and tetracyclines, while the pH 8.0 plate responds to aminoglycosides and the pH 7.2 plate is for the detection of sulphonamides. The *M. luteus* plate responds to macrolide antibiotics. The Four-Plate Test is not sufficiently sensitive to all antibiotics (for example sulphonamides, chloramphenicol) to ensure that they are below the MRL values. The test is also incapable of determining which antibiotic of a certain class is present and false positive and false negative results have been reported. Okerman *et al.*[14] used the Four-Plate Test to screen meat samples from retail outlets within the European Union. Positive samples were

confirmed by enzyme immunoassay, thin layer chromatography and receptor assays. The authors concluded that the method was not capable of detecting samples containing sulphonamides and quinolones at EU MRLs of 100 ppb and 50 ppb, respectively, and reported a likelihood of false positives using plates seeded with *M. luteus*. A derivation of the Four-Plate Test, the one plate test, has been described by Koenen-Dierick *et al.*[15]. Various antibiotic classes including β-lactams, tetracyclines, aminoglycosides, sulphonamides, macrolides and quinolones were included in the study. The method was based on the detection of antibiotic residues by growth inhibition of *Bacillus subtilis* in agar medium at pH 7. The medium contained trimethoprim to aid detection of sulphonamides and β-glucuronidase to aid detection of chloramphenicol residues. The authors compared the performance of this One-plate test with the Four-Plate Test and tested its ability to detect residues at the MRLs established in the EU. They concluded that the One-plate assay was relatively similar in sensitivity for detection of β-lactams, sulphonamides and aminoglycosides. The One-plate was less sensitive to chloramphenicol and macrolides than the Four-plate test. In general the One-plate test was less sensitive for the detection of the tetracyclines and less sensitive to flumequin yet more sensitive to enrofloxacin.

1.2.2 Charm test

The Charm II tests are microbial receptor or antibody assays for the detection of antimicrobial drugs. Separate tests for the antibiotic drug families β-lactams, macrolides, sulphonamides, tetracyclines and chloramphenicol are available. The tetracyclines and chloramphenicol assays are antibody assays and the remaining are microbial receptor

assays. A binding reagent (a microbial cell with specific receptors for the antibiotic in question or, for tetracyclines and chloramphenicol, an antibody specific to the antibiotic) is added to the sample. If an antibiotic is present it will bind to the receptors on the microbial cell (or antibody) and prevent the binding of radiolabelled antibiotic, which is added subsequently. Therefore, the more radiolabelled antibiotic detected the lower the concentration of antibiotic in the sample. Korsrud *et al.*[16] described the detection of antimicrobial residues in suspect meat samples using the Charm II receptor assay. Sulphonamides, streptomycin and erythromycin were the antibiotics of interest. They compared the results with those obtained using thin layer chromatography or liquid chromatography, and confirmed the Charm II test to be an acceptable alternative, with a lower limit of detection. The results showed the incidence of false positives to be higher, using the Charm II test, however, this may be due to the lower sensitivity of the Charm II assay. The detection sensitivities of the Charm II test assay, estimated by Korsrud *et al.* were 10 ppb for penicillin G (which has an MRL of 4 ppb in milk), 200 ppb for gentamicin (which has an MRL of 100 ppb in milk), and 300 ppb for tetracycline (which has an MRL of 100 ppb in milk).

Other bioassay kits available include the Cite Probe (β-lactam) kit[12], which is based on the binding of β-lactam antibiotics by a penicillin binding protein on a membrane. An absorbance reader is used to assess the colour change which indicates if the sample is positive or negative. The Lactek (β-lactam) kit[17] is based on the principle of enzyme linked immunosorbent assay (ELISA), where antibodies are coated inside a tube and response measured as optical density. Rapid tests which have been developed include the "Delvotest", based on microbial inhibition, enzyme inhibition assays, such as "Penzyme",

and bacterial metabolism inhibition assays[18]. Korsrud *et al.*[19] recently evaluated a large number of microbial inhibition kit assays. They concluded that while the ideal test kit does not exist, due to limitations in sensitivity, specificity and ruggedness, they ranked the New Dutch Kidney Test (One-plate test) and the "Swab Test On Premises" (STOP)[19] highest with respect to cost, labour, and specificity.

1.2.3 Biosensors

Biosensors have been used extensively in clinical and environmental monitoring. However their application in antibiotic drug residue analysis has been limited. They utilise biological molecules, such as enzymes, or antibodies, capable of recognising specific target analytes. The molecules are coupled to a transducer that responds to the reaction between the analyte and the bound biological molecule. An automated flow injection immunoanalysis system was described for the determination of cephalexin in milk. Protein G was immobilised on the surface of an immuno-reactor serving as a matrix for the polyclonal anti-cephalexin antibody[20]. A conjugate was added to the sample, which bound in a competitive manner to the antibodies in the immuno-reactor. Aminophenol was enzymatically generated and was detected at a carbon electrode. The assay required 16 min per sample and had a limit of determination of 1 ng ml^{-1} in milk. A recombinant *Escherichia coli* sensor for the detection of tetracycline antibiotics has been described by Korpela *et al.*[21]. The sensor is based on genetically engineered bacteria, which emit visible blue light in the presence of tetracyclines. This is due to the presence of luciferase genes, which were activated in the presence of tetracyclines. The sensor was specific only to tetracycline antibiotics. Optimal induction of luminescence occurred

within 90 min in the presence of tetracycline antibiotics and detection sensitivities were approximately 10 ng ml^{-1}. β-lactam antibiotics were detected using a biosensor containing immobilised penicillinase and amidohydrolase. The enzymes were covalently bound to pH membrane electrode material, resulting in a biosensor with a shelf life of 2 months. Response time was 2.5 min and 30 ppb β-lactam could be detected[22].

Application of molecular imprinted polymers to sensor technology has been reported; chloramphenicol antibiotic residues in serum have been determined using a biosensor containing a molecular imprinted polymer[23]. Various polymers were investigated in the development of the sensor. The main interactions between the polymer cavity and the analyte were identified as the two hydroxyl groups of chloramphenicol. An optical detection system was developed based on displacement of chloramphenicol-methyl red by chloramphenicol from the sensor and linearity was observed over the range 1 to 1000 ug ml^{-1}.

1.2.4 Chemical screening methods

Absorption spectrophotometry involves the detection of an absorption signal at a specific wavelength, characteristic for the analyte under study. UV absorption detection can also be employed, where the analyte is not directly measured, but a product of analyte activity is detected. This may serve to increase the non-specificity of UV detection for antibiotic residues. An example of this is the spectrophotometric assay described by Everest *et al.*[24] for the determination of β-lactams in kidney tissue. The assay utilises the inhibition of the enzyme carboxypeptidase by β-lactam antibiotics. In the absence of β-lactam residues,

the action of this enzyme results in the release of alanine and H_2O_2. The H_2O_2 oxidises a chromogen, also present, to a coloured compound that absorbs at 512 nm. When β-lactam residues are present in the sample extract, the enzyme action is inhibited resulting in decreased absorption intensity. Derivative spectrophotometry has been applied to the screening of β-lactam antibiotics. In this method, the spectrum of the sample, acquired under a specific set of conditions (such as in acidic solvent) is subtracted from the spectrum of the sample acquired under a different set of conditions (such as alkaline solvent). Absorption bands can be considerably sharpened and the method can be used to discriminate between the analyte and matrix. Third derivative spectrophotometry has been used to determine mixtures of penicillins in commercial injectables[25], but no methods have been reported for the analysis of residues in animal tissues.

Thin layer chromatography (TLC) methods are also employed for antibiotic screening, as they can provide high sample throughput and relatively high detection sensitivities. Silica and cellulose acetate plates are commonly employed and a variety of solvents and mixtures of solvents used for analyte separation. Various detection techniques have been employed. Detection may require the addition of a visualisation agent, such as Fast Violet B salt to the plate, with heating, to create a coloured product. Direct UV scanning of the plate is frequently employed. Derivatisation of the analytes on the plate by fluorescent dyes provides a rapid, inexpensive method for detection. Thin layer chromatography-bioautography is a popular method for the analysis of antibiotic residues. Following separation by thin layer chromatography, the analytes are detected by a microbial inhibition assay. The chromatography plate may be incubated against an agar plate seeded with sensitive micro-organisms. This method of screening was one of a few screening

techniques employed by MacNeil *et al.*[26] for the determination of penicillin residues in veal tissue. The authors reported better sensitivity when penicillin residues were detected in liver and kidney than similar concentrations in muscle tissue. TLC has been applied to a large range of antibiotic residues including sulphonamides[27], aminoglycosides[28], macrolides[29], and β-lactams[30]. TLC-mass spectrometry has recently been reported as an antibiotic residue screening method. The method can be performed manually or with on-line detection. Manual detection requires removal of the spot from the TLC plate followed by solvent extraction of the analyte, and this solution is subject to MS determination. Alternatively the TLC plate, once developed, can be placed on a stage inside the mass spectrometer. The plate can be scanned or the spots imaged. Extensive sample preparation is generally not required, relative to that required for HPLC determination. The method is applicable to an extensive range of compounds, yet the unavailability of commercial interfaces allowing direct detection of analytes on the TLC plate currently presents a problem. TLC-MS determination of tetracycline residues has been reported[31]. The TLC plate was developed using non-volatile solvents (oxalic acid, Na_2EDTA). Employment of similar solvents for the LC-MS of tetracycline residues resulted in clogging of the interface and deposits in the ion source. Using TLC-MS, the solvents remain on the plate and are not introduced into the mass spectrometer. The TLC plate was developed and the tetracycline spots were condensed. The FAB matrix was applied to the condensed tetracylines on the TLC plate. The stationary phase containing the condensed zone was mulled with FAB matrix and subject to MS detection. Sensitivity of 0.1 μg per spot was reported, when a condensation technique was employed to concentrate the spot on the TLC plate.

1.3 EXTRACTION METHODS

Fedeniuk and Shand recently described the energy theory for transfer of an analyte from matrix to the extraction solvent[1]. The first step involves removal or desorption of the solute molecule from its matrix. The second step involves dissolution of the solute in the extraction solvent. The energy required for the extraction process to occur can be described by the following equation:

$$E_{Total} = E_{Desorb} + E_{Cavity} - E_{solute\text{-}solvent} \qquad (1.1)$$

where E_{Desorb} is the energy required for the analyte to desorb from the matrix, E_{Cavity} is the energy required to create a cavity in the extraction solvent and $E_{solute\text{-}solvent}$ is the energy released when the analyte interacts with the extracting solvent. Desorption of the analyte depends on the strength of the molecular interactions between the analyte and the matrix (ionic, covalent, hydrogen bonding or dipole-dipole interactions). The ease with which these analyte-matrix interactions can be broken can be calculated using the following equation:

$$\log C_b = \log k + mC_f \qquad (1.2)$$

where C_b is the concentration of bound drug, k is the dissociation constant, C_f is the concentration of drug in the extracting solvent, and m is a measure of the availability of the drug for desorption. The value of m depends on the functional groups of the drug, the extracting solvent used and composition of the matrix (fat, water content). This equation is known as the Freundlich sorption isotherm. This approach has been applied to calculate the optimal number of extractions necessary for determination of oxytetracycline in bone, or sulphonamides in fish[32]. The authors describe the application the sorption isotherm to estimate the degree of analyte binding to matrix. They concluded that repeated extraction

can increase the liberation of tetracycline residues from calcium rich tissues, and that sulphonamide tissue-binding is reduced as the pH of the extraction medium approaches the pKa of the sulphonamide.

1.3.1 Homogenisation

If the sample for analysis is a solid, an homogenisation step is employed. This serves to create a uniform sample and also disrupts the sample structure, thereby assisting in the release of any residues contained. Homogenisation techniques using a blade blender or mechanical probe are the most common methods. The first step in the extraction procedure is sometimes included with homogenisation. The sample may be homogenised in the presence of a solvent (deproteinising agent), or alternatively, the sample may be homogenised first to provide an increased surface area for contact with the solvent.

1.3.2 Solvent extraction

Following homogenisation (if necessary), the sample is mixed with the extraction solvent. This serves to dissolve the antibiotic residues and other matrix co-extractives and frequently the solvent facilitates deproteinisation of the sample. Organic solvents are particularly advantageous in antibiotic residue analysis as they enable extraction of protein associated drugs, with simultaneous deproteinisation of the sample. Some examples of extraction solvents are summarised in Table 1.1. The most important factor in selecting an extraction solvent are its thermodynamic properties, such as polarity and ability to interact with the analyte through proton donation or acceptance. The solubility of an analyte in a

solvent can be calculated (see section 1.4.1), but the solubility of an analyte in the presence of matrix cannot be calculated, due to the complexity of biological matrices. Salvatore and Katz[33] studied the solubilities of several antibiotics in various organic solvents. They reported methanol and dimethylsulfoxide to be the most widely applicable. The authors reported employing organic solvents to partially separate antibiotics in animal feeds, based on the solubilities of the different antibiotics in different solvents. Different combinations of antibiotics resulted in the extracts when different solvents were employed, therefore qualitative identification of the antibiotics contained was made. The extracts were then subject to paper electrophoresis and bioautography determination[34].

Water miscible organic solvents such methanol, ethanol or acetonitrile are often used in antibiotic residue extraction, due to the polar nature of most antibiotics. Water immiscible organic solvents, used in extracting chloramphenicol and tetracyclines, may require a slightly more labour intensive extraction method to ensure the matrix (with the analyte of interest) is exposed to the solvent. Proteins are generally insoluble in organic solvents, so they precipitate out, and in doing so, residues can be released from protein binding sites. Therefore, the precipitating agent should be one in which the analyte is soluble, to prevent co-precipitation of the analyte with the protein. Due to the toxic properties of many organic solvents and the problems of disposal of solvents, trends towards low solvent or solvent free methods are encouraged.

Since the majority of residue determination methods use reversed phase chromatography, organic solvents often have to be removed, otherwise the analytes may not be retained on

the column due to the high elution strength of the sample solvent. Evaporation of the solvent allows the analyte to be reconstituted in mobile phase allowing optimum interaction between the analyte and the stationary phase and providing a rapid pre-concentration step. Mechanical methods such as vortexing, centrifugation and sonication are also employed at the initial extraction step to disrupt tissues, assisting solvent exposure to the matrix. For aqueous extraction, varying the pH of an extraction solvent can render the extraction solvent more polar so that it may have greater capacity for solubilising a polar antibiotic.

Strong mineral acids have been used for the extraction of tetracyclines as these compounds are not acid labile[35]. Trichloroacetic acid has also been used as a protein precipitant for the extraction of kanamycin from tissue[36], oxytetracycline from fish tissue[40] and tobramycin from kidney[37]. Due to the zwitterionic nature of proteins, they are usually positively charged (cations) in strongly acidic environments and negatively charged (anions) in strongly basic environments. Acids form insoluble protein salts with the cationic proteins, and so they are commonly employed as extractants and protein precipitants. Shaikh *et al.*[38] used acidic buffer and heat to extract neomycin from tissues. Acid extraction is unsuitable for analysis of analytes prone to hydrolysis, such as penicillins.

Table 1.1 Extraction solvents used for antibiotic residue analysis.

APPROACH	MATRIX	ANTIBIOTIC
Mineral Acids (e.g. HCl, $HClO_4$)	Meat, Fish, Blood	Tetracyclines[35]
Tungstic acid	Tissue	Penicillin[39]
Trichloracetic acid - with HCl - with NaOH	Tissue Fish Kidney	Kanamycin[36] Tetracycline[40] Tobramycin[37]
Buffer	Liver, Fish	Tetracycline[41] Neomycin[38]
Water-immiscible organic solvents (for example, hexane, ethylacetate)	Tissues Milk	Tetracyclines[42] Chloramphenicol[43] Sulphonamides[44]
Water-miscible organic solvents (for example, methanol, acetone)	Milk	Penicillins[45]

A relatively new form of solvent extraction is that of microwave-assisted solvent extraction[46]. The extraction solvent can be microwave-absorbing (possess a high dielectric constant) or non-microwave-absorbing (possess a low dielectric constant). Using a microwave-absorbing solvent such as water, radiation heats the solvent to a temperature higher than its boiling point and the hot solvent provides a rapid extraction of analyte under moderate pressure (a few hundred pounds per square inch). In the case of non-

microwave-absorbing solvent, such as chloroform, the solvent does not become hot, while the sample (which usually contains water or compounds with a high dielectric constant) absorbs the microwave energy and releases the heated analytes into the cool surrounding solvent, in which the analyte is soluble. Microwave-assisted solvent extraction has been applied to the analysis of salinomycin antibiotic residues with recoveries in the range of 78-100 %[47]. The authors claim the method to be rapid, accurate, environmentally friendly and cost-effective. Tissue samples were irradiated in ethanol for 9 sec, centrifuged, and the pellet irradiated in an ethanol/propanol solution. The combined supernatant was evaporated, the residue was reconstituted and subject to HPLC determination.

1.3.3 Supercritical fluid extraction

Supercritical fluid extraction (SFE), is becoming an increasingly popular technique in the field of residue analysis. Supercritical fluids are advantageous for the extraction of drugs at residue levels by virtue of their enhanced solvating power. In addition, concentration of the extracted analyte can be readily achieved by removal or evaporation of the extracting solvent, which is more readily accomplished with supercritical fluids than with an ordinary solvent. However, for analysis of antibiotic residues in animal tissue, the solvating properties of supercritical fluids result in the extraction of tissue fats and other non-polar matrix components. The polarity of the supercritical fluid can be varied by changing its density through altering pressure. The procedure can be carried out with on-line or off-line analyte collection (trapping). Supercritical carbon dioxide is the most popular fluid used, though others such as pentane, diethyl ether, methanol and tetrahydrofuran have been used. However, carbon dioxide is sometimes too non-polar to

extract many antibiotic residues efficiently, so modifiers are employed, which increase the polarity of the extracting fluid and its solvating power. Typical modifiers are organic solvents such as methanol, acetonitrile, acetone or toluene. Sulphonamides were extracted from incurred pork tissue using carbon dioxide modified with 10-20 % methanol[48]. Recoveries in the range 52-97 % were reported for various sulphonamide residues. Maxwell *et al.*[49] applied supercritical fluid extraction to the analysis of sulphamethazine, sulphadimethoxine and sulfaquinoxaline in chicken, beef and pork tissues. On applying the method to beef and pork liver, the authors reported reduced recoveries due to matrix binding of the sulphonamide residues. A variety of pre-SFE treatments were investigated. The pH of the liver was adjusted with sodium hydroxide and phenylbutazone was added to compete with the sulphonamide residues and displace them from the matrix binding sites. Recoveries of 80-107 % were obtained for samples fortified at 100 ng g^{-1} with the three sulphonamides.

1.3.4 Matrix solid phase dispersion

Matrix solid phase dispersion (MSPD) involves the dispersion of the sample (usually tissue) onto a solid sorbent or dispersant. The technique involves blending the tissue sample with a tissue dispersant and the resulting mixture is then packed into a column, allowing the mix to be washed with various solvents, similar to conventional solid phase extraction. Octadecylsilane (C18) is the most popular tissue dispersant used but diatomaceous earth[50] and silica[51] have also been used. Blending of sample and C18 material is performed using a pestle and mortar. The C18 material acts to disrupt the tissue cells, thereby exposing the cell contents and rendering any residues extractable.

C18 material consists of a silica base onto which long carbon chains, which are non-polar and hydrophobic in nature, are bonded. The cell membrane is constructed of a lipid bilayer. The hydrophobic C18 will therefore solubilise these lipid regions exposing their hydrophilic, polar regions where most drug residues are located. The method uses small sample size, thereby limiting sensitivity but has many advantages relative to the more classical methodology. It provides a rapid, simple method of extraction that involves low solvent usage and does not require sophisticated equipment. MSPD is discussed more fully in Chapter 3 where its application to the extraction of penicillin residues from muscle tissue is described. Other antibiotics extracted using this technique include chloramphenicol[52], tetracyclines[53], sulphonamides[54] and cephalosporins[55]. Long *et al.*[56] described the determination of oxytetracycline in fish tissues using MSPD extraction followed by HPLC determination. A hexane wash removed non-polar substances, such as lipids. Antibiotic elution was effected using an acetonitrile/methanol solution containing butylated hydroxyanisole, which prevented oxidative loss of oxytetracycline during extract preparation. Recoveries of 67 to 86 % were reported with standard deviation of 5 to13 % for samples fortified in the range 50-3200 ng g^{-1}. Van Poucke *et al.*[57] used MSPD for the analysis of sulphonamide residues in milk samples. The method described involves mixing of the C18 material with the milk sample within the syringe barrel, followed by washing and elution. Using HPTLC with fluorescence detection, recoveries of 83-91 %, with standard deviations between 2 and 4 %, were obtained for milk samples (2, 5 or 10 ml), when 2 g C18 was employed.

1.3.5 Membrane-based extraction methods

Table 1.2 outlines the membrane techniques available for drug residue analysis and the principles by which separation/extraction occur. These techniques are relatively new to sample preparation, particularly in the area of antibiotic residue analysis. There are a range of formats for membrane techniques, many of which are easily incorporated in an on-line system. In addition, they are low on organic solvent consumption and some techniques do not require very sophisticated equipment.

Table 1.2 Membrane based extraction techniques

Technique	*Principle*	*Driving force*
Filtration	Size Exclusion	Pressure difference
Dialysis	Size Exclusion	Concentration difference
Electrodialysis	Size Exclusion & Selective Ion transport	Potential difference
Membrane Extraction	Partitioning	Concentration difference

1.3.5.1 Filtration

Filtration of samples and sample extracts is used routinely in sample preparation. Disposable membranes are employed, in the form of filter paper, syringe filters or ultrafiltration devices. Recently 96 well filter plates have become available[58]. Separation takes place under gravity (through filter paper), or by the application of external pressure

in the form of a vacuum, centrifugal force or positive pressure (in hand held devices). Large molecular size matrix co-extractives remain on the upper-side of the membrane while the analytes and small molecules pass through to the under-side. Prior to chromatographic determination, samples are frequently filtered to remove any non-solubilised material. Ultrafiltration was applied to β-lactam determination in serum[59]. Ultrafiltration differs from ordinary filtration in terms of the size of the particles that are separated and in the application of centrifugal force as a driving force for the separation process. Ampicillin was separated from serum prior to LC determination using ultrafiltration[60]. A two-chambered centrifugation tube was used. The diluted serum was placed in the upper chamber, which was separated from the lower chamber by the filter membrane. Centrifugation forced the low molecular weight components through the micro-porous membrane filter and into the lower chamber, while high molecular weight proteins were retained on the filter membrane. Thomas[61] achieved recoveries of near 100 % for the determination of tetracycline residues in whole milk, with direct injection of the filtrate. This method is useful for the analysis of analytes sensitive to classical deproteinisation agents (acids or organic solvents).

1.3.5.2 Dialysis

Dialysis has traditionally been overlooked in residue analysis as it was regarded as a lengthy technique, resulting in a dilute sample requiring concentration and with poor recovery. However, the advent of automated dialysis has rendered this technique much more convenient. Dialysis has been widely used to separate proteins from small molecules, often for purification of the protein. In trace analysis, the reverse approach is

adopted where the protein is eliminated and the dialysate recovered. The majority of trace analysis applications using dialysis are performed using commercial dialysis block, (depicted in Figure 1.1). This approach has been employed for the analysis of nitrofurans in milk, eggs and meat[62]. In order to achieve good recoveries in a short time, two long channels of minimum depth are employed, separated by a membrane. The sample is pumped into the channel on the upper-side (donor) of the membrane. The molecules of large molecular weight (proteins) remain on the upper-side while the residues of interest migrate to the under-side (acceptor). Most membranes employed for this technique have a molecular weight cut-off in the region of 15,000 daltons, while most drug residues are in the region of 200-500 daltons. Membranes of smaller pore size would increase dialysis times and are likely to become blocked.

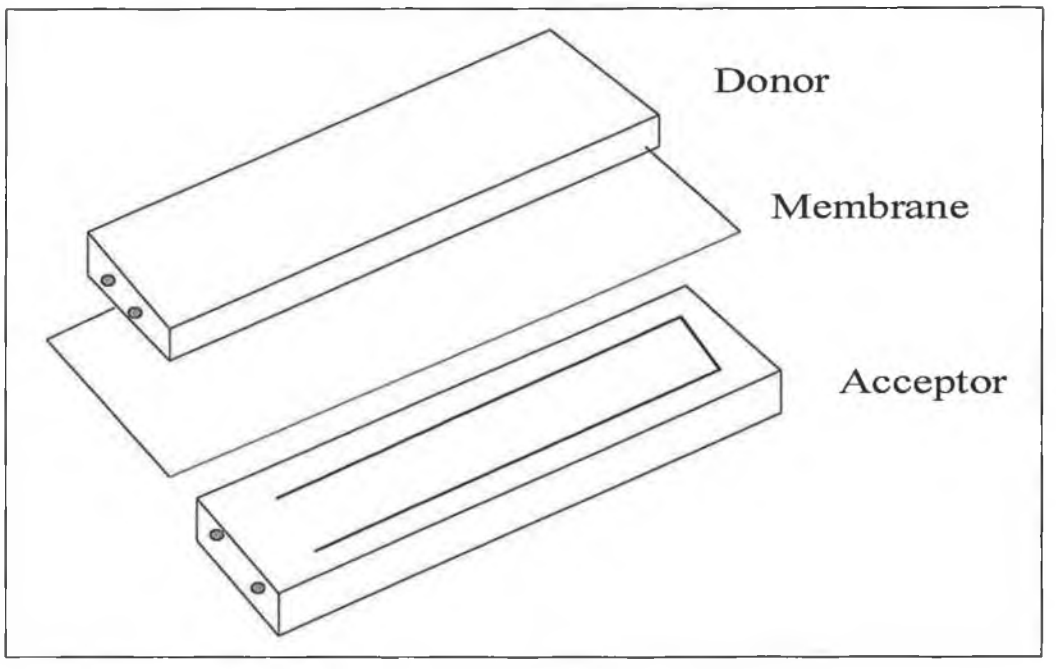


Figure 1.1 *Dialysis block, showing donor moiety, membrane and acceptor moiety.*

As dialysis is a diffusion process, to increase the efficiency of the process the solvent on the under-side of the membrane must be renewed continuously to avoid equilibrium being

established, where both sides of the membrane hold a similar concentration of residue. This results in a dilute solution, consequently membrane dialysis is followed by a concentration step. On-line trace enrichment has been employed to facilitate this requirement. The dialysate is directed onto a small cartridge, which concentrates the analyte(s), prior to HPLC. In most analytical applications, the acceptor channel is continuously being renewed, and this form of the technique is termed continuous dialysis. There is a trade-off between dialysis time and recovery, as the longer the period of time the sample is allowed to dialyse, the greater the recovery. The size of the dialysis channel on the upper-side of the membrane limits the size of the sample, thereby determining the sensitivity of the method. The sample can be introduced into the channel in one injection (stagnant), in more than one aliquot (pulsed) or by continuous injection (continuous flow). The membrane is regenerated in preparation for the next sample by solvent washing. This step should also be sufficient to prevent sample carryover. Dialysis involves aqueous extraction, thereby eliminating the co-extraction of fats, which contribute to fouling of the analytical column.

The analysis of sulphonamides by this technique is discussed in Chapter 2. Agasøster[63] described an automated dialysis method for the analysis of oxytetracycline antibiotic residues in muscle, liver, milk, and egg. Dialysis was followed, after concentration on an enrichment cartridge, by direct automatic injection onto a HPLC column, followed by post-column derivatisation. Two fifteen kD molecular weight cut-off cellulose acetate membranes were employed in series, the first capable of dialysing 370 µl and the second capable of dialysing 100 µl. A polystyrene packed enrichment column was employed to concentrate the extract before HPLC analysis. Recoveries were generally high (66-100

%), relative to dialysed standards, from all matrices with limits of determination of 1 ng g^{-1}. Another antibiotic residue, mitomycin C[64], has been extracted from human blood and urine with a limit of determination of 1 ng g^{-1}. Dialysis was also followed by concentration on a C8 pre-column before direct HPLC separation. Dialysis is commonly employed on-line with liquid chromatographic, gas chromatographic[65], and electrophoretic[66] techniques.

1.3.5.3 Electrodialysis and membrane extraction

Electrodialysis is an extension of dialysis, where the membrane is coupled to an electrophoretic separation mechanism. Typically a dialysis block is used with electrodes inserted in the donor and acceptor channels. The concentration gradient together with the electric potential difference cause the transport of charged compounds through the membrane. Positively charged compounds migrate towards the negative electrode, while negatively charged compounds migrate towards the positive electrode and neutral compounds only undergo passive diffusion through the membrane. Therefore, analytes are separated on the basis of molecular size and their charge. On-line electrodialysis has been employed for the determination of acids, bases and quaternary amines in water samples[67]. However, the application of electrodialysis to the analysis of veterinary drug residues has not been reported. Most studies are explorative and no routine methods have been reported.

As with dialysis, membrane extraction typically involves a planar membrane, between a donor and an acceptor phase. Membrane extraction may be considered under three types:

1) supported liquid membranes, 2) silicone rubber membranes and 3) micro-porous membrane liquid-liquid extraction (MMLLE). In the supported liquid membrane, the membrane is impregnated with a water-immiscible organic solvent. In the donor phase, the pH is such that the analytes are uncharged and readily extract into the membrane liquid, or silicone polymer (in the case of the silicone rubber membranes). The acceptor phase pH is such that the analytes are charged upon entering, to prevent back extraction into the organic/silicon membrane. In the MMLLE membrane, organic solvent is present in both the acceptor phase and membrane pores. This technique involves a single liquid extraction, as opposed to an extraction followed by a back-extraction, for the other membranes. To obtain high recoveries, the analyte must exhibit high solubility in the membrane material. Hydrogen bonding reagents are frequently added to the membrane to facilitate the extraction of polar analytes into the membrane. Ion-pair reagents may be added which can selectively bind to specific functional groups, thereby aiding extraction of the analyte. Transport across the membrane can also be "facilitated", where a carrier in the membrane, which is insoluble in the donor and acceptor phases, interacts with the analyte at the interface between the donor phase and the membrane and transports it into the acceptor phase. The technique has been applied to the analysis of penicillin G in fermentation media[68], carboxylic acid[69], amines[70], and organic acids (citric acid, lactic acid). Penicillin G was determined in a continuous flow system using a biosensor consisting of a bilayer lipid membrane[71]. In this membrane, enzymes were immobilised into a lipid matrix, so no organic solvent was used. On reaction of the enzyme with the analyte, hydronium ions were produced which caused a fluctuation in current, relative to the concentration of analyte detected.

As with electrodialysis, application to veterinary drug residue analysis has not yet been reported. Supported liquid membranes have been applied to the analysis of herbicides and pesticides in drinking and ground water[72,73]. Analysis of the anabolic steroid, bambuterol, has also been performed in plasma, using a liquid membrane[74] and a silicon membrane was applied to the analysis of triazines in vegetable oil[75].

1.4 CLEAN-UP METHODS

The requirement for sample clean-up in residue analysis depends on the detection system used and the matrix under analysis. HPLC with UV detection is a very popular determination method for analysis of drug molecules. However, most sample matrices contain endogenous compounds that have UV-absorbing properties, so a clean-up step to remove these interferences is necessary. The types of interferences and their effects are discussed in section 1.1.5. Prior to detection, a separation step is usually employed and extracts may need to be cleaned-up to avoid fouling of the components used for this separation, such as the chromatographic column. Another important feature of sample clean-up techniques is their ability to pre-concentrate extracts which is often very necessary in residue analysis to improve the limit of determination.

1.4.1 Liquid-liquid partitioning

Convenience, ease of use and the availability of highly purified organic solvents have contributed to the widespread use of this clean-up technique in residue analysis. The antibiotic is partitioned between two immiscible phases so as to bring about favourable

extraction of the drug into one phase, while the unwanted matrix co-extractives partition into the other phase. Multiple extractions are frequently employed to ensure maximum recovery of the analyte. The analyte may be selectively extracted from a large solvent volume into a much smaller volume of solvent, or for volatile solvents, evaporation of the solvent serves to concentrate the analyte. The degree of extraction is dependent on the distribution co-efficient K_D where

$$K_D = [A]_o / [A]_a \qquad (1.3)$$

when $[A]_o$ is the molar concentration of the analyte [A] in the organic phase and $[A]_a$ is the concentration of the analyte in the aqueous phase[76]. K_D is dependent on the type of organic solvent used, the pH and the ionic strength of the aqueous phase, so the procedure must be optimised for each analyte. The organic solvent selected is that which affords maximum analyte extraction efficiency, with the minimum extraction of interferences. The solvent chosen is usually of the minimum polarity necessary to extract the residue so that the more polar interferences are not extracted. Liquid-liquid partitioning using hexane is common in antibiotic residue analysis in tissues. This non-polar solvent dissolves lipophilic molecules (fats) and so acts as a clean-up solvent. The pH of the aqueous phase can also be adjusted to allow extraction of endogenous compounds with a view to their elimination. This was employed by Moats *et al.*[77] in the analysis of tylosin in muscle. Acidic buffer was added to the muscle extract to remove interferences, and methylene chloride was then added to extract the tylosin.

Liquid-liquid extraction can be difficult for residues that are soluble in water at all pH values. In this case, "salting out" has been employed, where the addition of a salt to an

aqueous solution increases its ionic strength and its polarity, thereby increasing the affinity of the more polar residues for the aqueous phase and the non-polar residues for the organic phase. Malisch and Huber[78] found that many compounds partitioned into the organic layer on the addition of a salt (or methylene chloride) to acetonitrile filtrates. However, this method does not work for very polar analytes. In the analysis of tetracyclines, Moats[79] described the recovery of tetracyclines from the water layer formed when a methylene chloride solution was added to a filtrate containing hexane. The water was immiscible with the methylene chloride and hexane, resulting in the formation of a water layer. The analytes partitioned from the least polar hexane into the more polar methylene chloride and into the most polar water. Another method for the liquid-liquid extraction of highly polar ionic residues is to convert the analyte into a neutral ion-pair complex, by the addition of an excess of suitable ions of opposite charge and to extract this complex into an organic solvent. This was demonstrated by Modin and Schroder-Nielsen[80] for the extraction of penicillins from fermentation media. The ion-pairing agent, a tetrabutylammonium ?, was added as a cation, and complexed with the penicillins present as anions, allowing their extraction into organic solvent. In residue analysis from complex matrices, liquid-liquid back-extraction is often employed. After the initial extraction into an organic solvent, the analyte can be back-extracted into an aqueous phase with pH adjusted so the analyte ionises and remains in the aqueous phase.

However, liquid-liquid partitioning is a relatively time-consuming technique. Losses may occur due to degradation of analytes on evaporation, emulsion formation or adsorption of the analytes to glassware. Also, the trend towards reducing organic solvent usage is

forcing analysts to look to more environmentally-friendly methods of extraction. In addition, the technique does not lend itself well to automated methods.

1.4.2 Gel permeation chromatography

Gel permeation chromatography, also known as molecular exclusion chromatography, size exclusion chromatography or gel filtration, separates molecules on the basis of size. The stationary phase packings are generally gels or resins consisting of beads with small pores that can be penetrated by small molecules but not by large molecules. The columns have a high loading capacity, however selectivity is generally reduced in comparison to conventional chromatographic packings. The liquid phase carries the sample onto the column and through the gel phase. The small molecules are delayed on the column as they pass in and out of the gel particles, while the larger molecules stream past and are excluded from entering the gel particles. No chemical interactions occur between the analyte and the stationary phase, unlike other forms of chromatography. Biancotto *et al.*[81] have assessed the suitability of gel permeation chromatography for the determination of sulphonamides, nitrofurans and growth promoters in animal feedstuffs. The method described allows simultaneous analysis of a wide range of analytes, exhibits high recoveries (98-100 %) and performs effectively in removing matrix co-extractives. However, the method was only tested on samples containing relatively high levels of analyte (1000 ppb). Gel permeation was employed on-line, for the analysis of gentamicin, amikacin and tobramycin in serum[82]. A Sephadex column was employed for extraction followed by reversed-phase, ion-pair chromatography with post-column derivatisation. Recoveries were in the range 89 to 100 % with between day coefficients of variation in the range 3 to 4 %. Petz and Meetschen[83] conducted a study using gel permeation as a clean-

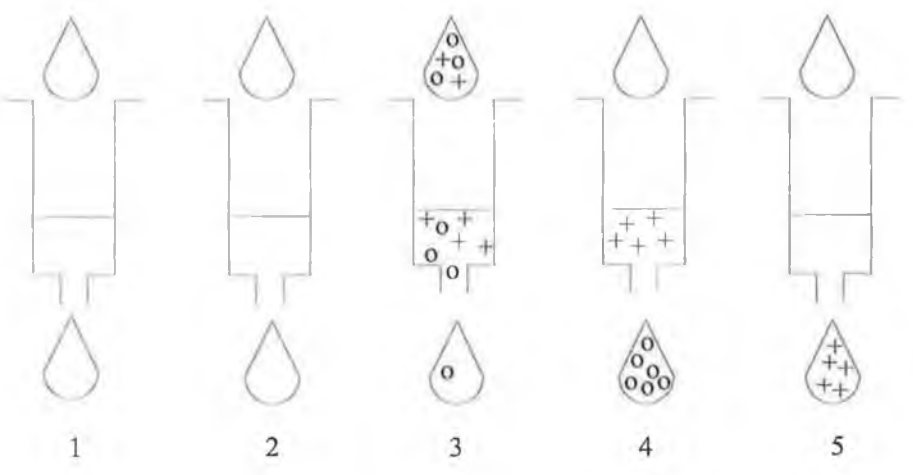
up procedure for the analysis of 40 veterinary drugs including sulphonamides, nitrofurans, tetracyclines, oxytetracycline, penicillin G and tylosin. Residues were determined in liver, kidney, muscle, egg and milk, and UV detection was employed. The authors concluded that similar results could be achieved when silica SPE columns were used for clean-up, with final determination being by HPLC with UV detection.

1.4.3 Solid Phase Extraction

Solid Phase Extraction (SPE) is a technique used in residue analysis to clean-up samples prior to determination and/or to concentrate samples to improve determination limits. It is a fast, easy to use method offering efficiency and reproducibility.

The first step in an SPE procedure is the selection of an appropriate sorbent; for example octadecylsilane is commonly used for non-polar residues, and ion-exchange materials are used for strongly acidic or strongly basic drugs. The typical steps involved in the use of SPE are shown in Figure 1.2. A large number of SPE stationary phases exist, as shown in Table 1.3, many of which are bonded silica-based materials. Traditionally, reversed-phase, normal phase and ion-exchange columns have found wide application for the analysis of antibiotic residues. A number of new stationary phases have been developed, such as mixed-mode sorbents, porous graphite carbon, polymeric columns, restricted access columns and metal-loaded columns. For multi-residue analysis, mixed-mode cartridges are available. A large number of drugs contain nitrogen atoms in a form that may be readily protonated. Therefore, mixed-mode sorbents have been developed that contain reversed-phase alkyl chains in the same column as ion-exchangers, resulting in a

mixed mode column. An SPE column containing C18 and cation-exchange groups was employed for the analysis of gentamicin in tissue and whole milk[84]. Very clean chromatograms resulted, free of matrix interference. The column was prepared in neutral buffer, so that the amino groups on the analytes were uncharged. The analytes were retained on the column due to ionic interactions and other matrix co-extractives were retained through hydrophobic interactions. Therefore the matrix components could be removed using a methanol wash, which was followed by analyte elution using a basic solution.



1. Cleaning and activation of the sorbent.
2. Conditioning of the sorbent with a solvent of similar nature to the sample extract.
3. Loading of the sample, containing the analyte (+) and matrix co-extractives (o) at approximately 1 ml min^{-1}.
4. Washing of the cartridge to remove the unwanted matrix components.
5. Elution of the analytes by disruption of the analyte-sorbent interactions.

Figure 1.2 *Protocol involved in the SPE procedure*

Graphite carbon columns exhibit higher efficiency for trapping polar analytes than the classical C18 column. Retention mechanisms include hydrophobic, ionic and other non-classified electrostatic interactions; therefore, both polar and non-polar analytes are retained with high affinity. The packing consists of homogeneous particles of relatively small diameter (5 μm), which provide a large surface area for high binding capacity. Elution problems have been reported when using porous graphite carbon columns. Elution with methanol or acetonitrile has resulted in low recoveries and methylene chloride or tetrahydrofuran are employed[85]. Rose *et al.*[86] employed porous graphite carbon SPE for the determination of penicillins in liver and muscle. The authors claim that the flat crystalline surface of the SPE packing, in combination with the reversed phase mechanism, was ideally suited to the clean-up of the highly conjugated organic analytes and allowed elution of the penicillins in acetone, which could be easily concentrated. Polymeric resins in the form of SPE columns have been reported for the analysis of tetracyclines[35]. These columns are frequently employed for trapping polar analytes, particularly those with strong electron withdrawing moieties. The retention mechanism was reported to be a combination of adsorption and partitioning[87].

Recently, a 'hydrophilic-liphophilic balanced' (HLB) sorbent has been introduced commercially. This polymeric column is capable of trapping basic, acidic and neutral analytes. The resin contains two monomers, allowing for both hydrophilic and lipophilic retention. Unlike traditional C18 sorbents, the need for column activation in methanol is omitted, the column can run dry without adverse effects on performance and the non-polar analytes can also be retained. Oasis® HLB columns (from Waters, Milford, MA, USA) were employed for the analysis of tetracycline in plasma and high recoveries resulted,

even when the packing was allowed to run dry during the SPE procedure[88]. High specific surface area, together with the presence of polar hydrogen groups, is responsible for the universal retention possible.

Restricted access SPE sorbents have become popular for on-line SPE methods. These sorbents combine size exclusion of matrix co-extractives of high molecular mass with enrichment of low molecular mass analytes at the inner pore surface. Therefore, direct injection of biological samples without the traditional deproteinisation step is possible. In addition to their size exclusion properties, the sorbent particles are coated with a hydrophilic protein or polymer to avoid adsorption of proteins and clogging of the SPE column. Internal surface reversed-phase sorbents (ISRP) are the most popular form of restricted access SPE. ISRP packing was employed by Ibach *et al.* for the analysis of oxacillin residues in milk[89]. After protein precipitation using acetonitrile and fat removal, samples were subjected to on-line SPE-HPLC. The method was compared with that described by Petz and Meetschen[90], where samples were deproteinated using acid and acetonitrile and extracted using complicated liquid-liquid extraction procedures. Results of both methods agreed well, demonstrating the validity of the rapid ISRP method.

Antibiotic residues have also been derivatised to promote retention on SPE. Agarwal[91] employed this approach for the analysis of gentamicin residues in beef. The tissue samples were deproteinated by immersion in boiling water followed by centrifugation, and the extracted residues were cleaned-up using silica SPE. The analytes were loaded on the column in the presence of ortho-phthalaldehyde, which derivatised the gentamicin

residues. The derivatives were eluted in ethanol, which could be easily evaporated to concentrate the extract. Alternatively, ion-pairs have been employed to promote retention. Lou *et al.*[92] employed sodium pentanesulphonate to assist retention of ampicillin and amoxicillin residues on a C18 SPE column. Solid phase extraction is based on chromatographic principles, the only difference being the much increased capacity factor when the analyte is retained on the column and interferences washed off, or the much decreased capacity factor when the analyte is eluted in the void volume leaving the interferences behind on the cartridge. If necessary, vacuum or positive pressure can be used to increase the rate of passage of the sample through the sorbent but in general the longer the residence time of the sample on the cartridge, the better the retention of the analyte and the higher the recovery.

Conditions on the SPE column are optimised to maximise the retention of the compounds of interest while minimising retention of interfering substances, often by introducing controlled pH conditions. As with liquid-liquid extraction, the strongest wash solvent, which does not elute the drug residue, is used to remove matrix interferences and the weakest solvent to give complete recovery of the residue is used in the elution stage, to avoid eluting more strongly retained interferences.

Table 1.3 Table showing the various SPE packings available and their application in antibiotic residue analysis.

	Sorbent	*Analyte*
Reversed Phase	Butyl (C_4)	Quinolones[93]
	Octyl (C_8)	Aminoglycosides[94],
	Octyadecyl (C_{18})	Quinolones[95], Tetracyclines[96]
	Cyclohexyl (C_6H_{11})	Chlortetracylcine[97]
	Phenyl (C_6H_6)	Tetracyclines[98], Quinolones[99]
	Cyano (CN)	Macrolides[100]
Normal *Adsorbents*	Silica (SiOH)	Sulphonamides[116]
	Florisil (Mg_2SiO_3)	Tetracyclines[101]
	Alumina (Al_2O_3)	Sulphonamides[102], Ionophores[103]
Bonded phases	Diol (COHCOH)	Macrolides[104],
	Aminopropyl (NH_2)	Sulphonamides[105]
Ion Exchange *Anion sorbents*	Amino (NH_2)	Nitrofurans[106]
	Quaternary amine (N+) SAX	Sulphonamides [107],Cephalosporins[108]
Cation sorbents	Carboxylic Acid (COOH)	Aminoglycosides[109],
	Sulphonic Acid (HSO_3)	Sulphonamides[110], Aminoglycosides[111]
	Propyl Sulphonic Acid ($(CH_2)_3SO_3H$)	Chloramphenicol[112]
Size exclusion	Sephadex	Aminoglycosides[82], Cephalosporins[113]
Polymeric	XAD	Tetracyclines[35]
Mixed-mode SPE	C18 & Cation-exchange	Aminoglycosides[84]
Restricted Access	Reversed-phase	Penicillins[89], Sulphonamides[114]
Carbon Base Sorbents	Porous graphite carbon	Penicillins[86]

SPE is well suited to automation due to the repetitive nature of the operations involved. The technology requires dedicated instruments and relies on computer support to provide systems capable of prolonged unattended operation. On-line SPE is used in conjunction with chromatographic determination procedures. The technique is automatically controlled by means of valve switching, and the analyte(s), when eluted, is directed to the final detection system. On-line methods are becoming increasingly popular, allowing reproducible analysis, largely unattended. On-line SPE has been described for the analysis of sulphamethazine in plasma[115]. SPE, in the form of C18 disposable extraction cartridges, was employed allowing automatic sample preparation followed by on-line injection of the sample extract into the HPLC system. Analyte recoveries of 90 % and greater were obtained and typical RSD values were 0.7 % (within day) and 2.0 % (between day).

Two SPE cartridges can be used in tandem for the analysis of residues from complex matrices, one reversed-phase and the other normal phase. This can serve to remove both polar and non-polar interferences. This was demonstrated by Cooper *et al.*[116] in the determination of sulphonamide residues in porcine kidney. Following deproteinisation using acetonitrile, clean-up was effected by a two stage SPE method using reversed phase (C18) and normal phase (silica) cartridges. The first clean-up, on C18, removed very hydrophobic matrix components that might be irreversibly retained on the HPLC column, and removed very polar matrix co-extractives that might interfere with the second SPE step. SPE on silica allowed further removal of matrix components and the analytes eluted in a relatively small volume (2.5 ml) methanol-dichloromethane solution.

Solid phase extraction disk technology has been growing in popularity over recent years[46]. SPE disks resemble membrane filters, usually 1 mm or less in thickness, with diameters ranging from 4 to 96 mm. The disks are usually polytriflouroethylene (PTFE) material, embedded with the stationary phase sorbent. The relatively large cross-sectional area and thinness enable higher flow rates and eliminate channelling, which can result in losses in packed bed columns. The number of stationary phases currently available for SPE disk technology is more limited than for SPE cartridge technology. One of the newest formats of SPE disk technology is the 96 well plate format[117]. This is used mainly for high sample throughput in the pharmaceutical industry, usually in an automated system. A major advantage of SPE disk technology is the low volume elution required, but low sample capacity has hindered its application in veterinary drug residue analysis.

1.4.4 Immunoaffinity column chromatography

Immunochemical methods for the isolation of veterinary drug residues are becoming increasingly popular. The methods display specificity, sensitivity, and high sample throughput. These benefits, combined with the versatility of the column format, have resulted in the development of immunoaffinity column chromatography. In immunoaffinity column chromatography, antibodies raised against the analyte(s) of interest, are immobilised onto a sorbent support, which is packed into a syringe barrel. The poor availability of antibodies is the major reason for the limited use of immunoaffinity chromatography. In developing an immunoaffinity method, a number of parameters can be varied to achieve ideal conditions. The nature of the support, the coupling mechanism for immobilisation of the antibody on to the solid support and the

nature of the antibody (monoclonal or polyclonal), all play a role in determining the characteristics of the method. Following preparation of the immunoaffinity column, optimum sample loading, washing and elution procedures must be determined. Since the success of the method depends on the ability of the antibody to bind the analyte, it is necessary to work under conditions optimal for antibody/antigen interaction, which are as close as possible to physiological conditions. This feature limits the application of immunoaffinity to water-soluble drugs, which includes most antibiotics. Extremes of pH, heat, salt concentration and organic solvent content can result in denaturation of the antibody. During sample loading, conditions must be conducive to the formation of the antigen-antibody complex. The complex must be unaffected by washing solvents and elution conditions require dissociation of the antigen-antibody complex. In addition, dissociation of the complex should ideally be reversible so that the antigen-antibody complex can easily be reformed, allowing re-use of the immunoaffinity column. Washing procedures should be optimised so that sample carryover is prevented. Dietrich *et al.*[118] described the production and characterisation of monoclonal antibodies against ampicillin and their applicability for the preparation of a multiresidue immunoaffinity column for β-lactams. They reported on the isolation of one group of antibodies that showed no major cross reactivities with other penicillins, except for amoxicillin, and one group that had marked cross reactivities with many other β-lactams. They coupled the latter antibody and an antibody against cloxacillin to CNBr activated Sepharose. The capacity of the resulting column was 6.6 and 5.4 $\mu g\ ml^{-1}$ for ampicillin and cloxacillin, respectively, and recoveries for six different penicillins were in the range 67-100 %.

1.4.5 Molecular imprinted polymers

Molecular imprinting involves the preparation of polymers with specific recognition sites for certain analytes. Briefly, a number of monomers are assembled around a template molecule (the analyte) and the template is removed, leaving an 'imprint' or cavity into which the analyte fits. The polymer can be packaged into a syringe barrel, and imprinted polymers have also been used as a stationary phase for liquid chromatographic separation[119]. Preparation of solid phases for thin-layer chromatography[120] have employed MIPs and they have been employed in receptor assays, as an alternative to the more traditional antibodies, as the specific binding reagent[122]. Desorption of the analyte is the main problem encountered, due to the high affinity of the polymer for the analyte. Conditions required to elute the analyte can result in leakage of the molecules responsible for the imprint or deformation of the site, preventing more than once off use. To ensure capture of the analyte by the imprint, samples must be applied in organic solvent. Therefore, the analyte must be extracted from biological samples into organic solvent prior to application onto the polymer. However, research into the development of non-covalent molecular imprints is ongoing, allowing polar solutions (aqueous samples) to be applied to the column[121]. There are no reports published for the application of MIPs in antibiotic residue analysis. An MIP was used in a solid phase extraction format for isolation of the herbicide, atrazine, from beef liver[122]. The authors reported high non-specific binding with aqueous solvents, caused by the partitioning of the lipophilic atrazine into the organic polymer. Complete retention on the column occurred with chloroform and elution was achieved with acetonitrile. Sample preparation prior to MIP SPE, was limited to shaking and centrifugation in chloroform. Molecularly imprinted

polymers have also been employed for the determination of theophylline in serum and plasma[119].

1.4.6 Liquid chromatography fractionation

Liquid chromatography (LC) has been applied as a clean-up step in addition to its use for residue determination. The technique is used to separate analytes from each other or from interferences in the sample matrix. Using LC, a fraction of the mobile phase eluate, containing the analyte is collected, based on the retention time of the peak of interest. This technique is employed as a clean-up step in residue analysis when followed by another quantitative techniqueS (such as immunoassay or HPLC). A drawback of the technique is the dilution factor, which results, for example, 100 µl sample injected and a 1 ml fraction collected due to the width of the peak as the analyte elutes off the column. However, there are many advantages to employing LC fractionation as a clean-up step. Operating costs for LC fractionation are low, once the necessary equipment is present and most residue laboratories are well equipped with LC instrumentation. The clean-up is highly reproducible and can be fully automated and carried out unattended. Tarbin *et al.*[123] described the analysis of penicillin G in milk using LC fractionation followed by LC determination. For fractionation clean-up, 1.5 ml sample extract was loaded into an injection loop installed before the column. The sample was focussed by bracketing the injection on either side with phosphoric acid. The extracts were chromatographed and a 2 ml fraction collected. 1 ml of this fraction was re-chromatographed, for quantification, again using focussing at the top of the column to achieve the desired sensitivities. The same column could be used for both LC steps, or use of two similar columns allowed

automatic injection of the cleaned-up extract into the analytical column. Zomer *et al.*[124] used LC fractionation followed by microbial receptor detection for the analysis of β-lactams in milk. The method combined the advantages of liquid chromatography with the selectivity and sensitivity of a microbial receptor assay. After SPE, the extracted residues were separated by isocratic HPLC and the fractions analysed using the Charm II test. The resulting LC-receptogram gave added confidence to immunoassay results because of the assurance of a specific retention time coupled with a specific microbial receptor reaction.

1.5 RESIDUE SEPARATION TECHNIQUES

Following extraction and clean-up, analytes are subjected to separation from each other or any matrix co-extractives and finally detected. Therefore, quantification involves separation of the extracted residues, followed by detection. These latter steps are usually described as one system e.g. HPLC separation followed by UV or fluorescence detection. Chromatographic methods are the most widely used methods for quantitative residue analysis offering sensitivity and versatility. Chromatographic analysis combines separation capabilities with a variety of sensitive detection techniques. Final detection may be by a confirmatory technique e.g. mass spectrometry (MS).

1.5.1 Liquid chromatography

Liquid chromatography has emerged as the method of choice for antibiotic determination due to the polar, non-volatile nature of most antibiotic molecules and the adequate sensitivity of the detection technologies available. Advances in instrumentation allow

liquid chromatography separation with detection at very long and short wavelengths, including visible, ultra-violet and fluorescence. Another advantage is that procedures can be easily linked to form partially or completely automated systems. Pre- and post-column derivatisation techniques enhance sensitivity and allow detection at wavelengths that are less affected by matrix interferences.

The term "high performance liquid chromatography" evolved with the availability of column packings with small particle diameters and is used to distinguish HPLC from the original, more classical glass column liquid chromatography methods. For antibiotic drug residue analysis, non-polar, reversed-phases are most popular. Some of the SPE packing materials already discussed are also employed for HPLC stationary phases in liquid-solid chromatography. The advent of column end-capping has made reversed-phase chromatography more efficient for the analysis of polar antibiotic residues. The presence of silanol groups on reversed-phase columns traditionally resulted in broad, tailing peaks for polar analytes. The advent of base-deactivated columns, and polymeric columns assisted in overcoming this drawback. Internal surface reversed-phase chromatography (ISRP)[125], where the column packing allows direct injection of samples rich in protein, is available, but the highly complex nature of many sample matrices, has hindered the application of this column type. Ion-exchange packings have also been employed for antibiotic separation. Ion-exchange columns possess stationary phase resins with functional groups attached. Analyte ions, possessing affinity for these functional groups, are retained on the stationary phase. Walker and Coates[126] employed a cation-exchange column for the determination of gentamicin in plasma and urine. Pre-column derivatisation was employed, where fluorescamine reacted with the amine group of

gentamicin to produce a carboxyl group. This allowed the use of a cation-exchange column with a low mobile phase pH to suppress ionisation of the carboxyl group and to effect separation on the basis of protonated amino groups.

1.5.2 Gas chromatography

Gas chromatography (GC) is also used in the determination of antibiotic residues, but to a much lesser extent than HPLC methods. Most antibiotics are polar, non-volatile compounds, therefore derivatisation is necessary in order to use GC analysis. The high temperatures necessary for GC analysis often exceed the temperatures at which many antibiotic molecules are stable. Separation using GC offers some desirable properties for antibiotic residue analysis. The high sensitivity of detection techniques, such as electron capture, may justify the time and inconvenience of derivatisation to enhance the volatility of antibiotic residues. Allen[127] has reviewed the GC detection of chloramphenicol with detection limits in the range 1-10 ng g^{-1}. GC analysis has been applied to penicillin residues by Petz and Meetschen[128]. After isolation of several penicillins from animal tissue, the residues were methylated, cleaned-up further on diol cartridges, evaporated and dissolved in cyclohexane. A derivatisation step was required to form volatile methyl esters, which improved thermal stability and polarity, allowing separation on a fused-silica capillary column. Detection limits of 2 ng g^{-1} were reported.

1.5.3 Immunoassays

Immunoassays may be applied as rapid screening methods, or as semi-quantitative determination techniques. Immunochemical detection is based on the ability of antibodies

to bind specifically with antibiotic residues. Various forms of this technique are in use, such as radioimmunoassay (RIA) and enzyme immunoassay (EIA), the main difference being in the method of labelling. Competitive assays are frequently used for the analysis of antibiotics. These methods are based on the competition of free antigen and labelled antigen for a limited amount of antibody. The assay response represents the amount of bound labelled antigen, which is inversely proportional to the concentration of the analyte. Immunochemical tests may be of microtitre plate format, for routine applications, or membrane based dip-stick tests for rapid screening[129]. The use of RIA for analysis presents problems such as the requirement for expensive scintillation counters, the disposal of radiolabelled waste and the commercial availability of labelled antigens. However, the technology provides good sensitivity, suitable for residue level detection, and can be applied to routine analysis of large numbers of samples. Enzyme linked immunosorbent assay (ELISA) is a popular immunological assay used in screening and determination of antibiotics in muscle and kidney. ELISA assay kits are commercially available for a wide range of antibiotics. Small amounts of sample are required relative to that required for some other analytical or microbiological techniques. ELISAs using a microtitre plate format have been described for streptomycin[130] and sulphonamides[131]. Detection limits in the range of 1-10 pg ml^{-1} can be achieved. Chloramphenicol has been detected by a microtitre plate assay hyphenated to HPLC. The sample extract was chromatographed and the fraction of interest subjected to immunoassay. This LC-immunogram technique resulted in a highly selective, reliable method with good detection limits[132]. This antibiotic was also detected by enzyme-linked immunofiltration assay (ELIFA). A nylon membrane was coated with polyclonal antibodies against chloramphenicol to which the sample was exposed. A developer was then added and

colour intensity was inversely proportional to the amount of antibiotic present. The results were read visually[133].

1.5.4 Capillary electrophoresis

Capillary electrophoresis (CE) is a rapidly growing separation technique that involves the migration of solutes in an electric field. The technique allows separation of analytes on the basis of charge. The analytes, present as charged ionic species, move at different velocities, depending upon their charge, through an activated capillary column. Optical detection can be made at the column outlet end. The technique is low on organic solvent consumption, can be easily automated, has efficiencies similar to capillary gas chromatography and requires small amounts of sample. The small sample permitted, lack of simultaneous multi-sample analysis capabilities, in addition to the requirement for highly charged ions have hindered the application of capillary electrophoresis in antibiotic residue analysis. Flurer[134] has described the analysis of twelve aminoglycoside antibiotics by CE. A fused silica capillary was operated at 34°C with borate buffers and UV detection at 195 nm. The analysis of amikacin in human plasma, by CE, has been described by Oguri and Miki, with sample preparation limited to ultrafiltration[135].

1.5.5 Supercritical fluid chromatography

Supercritical fluid chromatography (SFC), often described as a hybrid of gas and liquid chromatography, has been used in antibiotic residue analysis. It offers some of the best features of gas and liquid chromatography and avoids the use of organic solvents. The

mobile phase is a supercritical fluid, which possesses physical properties intermediate to those of gases and liquids. The universal detectors suitable for gas-liquid chromatography are applicable to this technique. Open tubular columns are the most popular choice. Because of the low viscosity of supercritical fluids, columns can be much longer than those used in liquid chromatography, thereby leading to greater efficiency. SFC is still a relatively new technique and its impact on the field of residue analysis has not yet been fully realised. However, the low solvent consumption and applicability to non-volatile, thermally unstable compounds that do not possess easily detectable chromophoric groups, will result in developments of the technique in the area of residues. Packed column SFC with UV detection has been evaluated for the determination of sulphonamides in kidney extracts[136]. Both silica and amino bonded stationary phases were examined and each provided different selectivities for the sulphonamides under study. The amino packing was more sensitive to modifier (methanol) concentration and the authors claim that other modifiers are of limited value for sulphonamide analysis by SFC.

1.6 DETECTION TECHNIQUES

The final step of antibiotic residue analysis, the detection method, has a large impact on the amount of sample preparation required. In addition, the nature of the separation step employed can often limit the detection steps applicable. The conjugated ring structure of most antibiotic molecules allows detection by UV spectrophotometry. However, for antibiotic residue analysis, UV detection may be undesirable, as many materials absorb appreciably in this spectral region, presenting matrix interference problems. Derivatisation techniques are frequently employed to enhance detection sensitivity and to

overcome the problem of matrix co-extractives, which can mask the presence of the residue. Derivatisation can allow detection at wavelengths beyond that at which most substances absorb or can result in the formation of a molecule with strong absorption properties, producing a high intensity signal. Alternatively, it can allow a completely new detection technique to be employed. The addition of a fluorophore, allowing fluorescence detection is frequently employed for antibiotic residue analysis, both in pre-column and post-column mode. Fluorescence detection is more specific and generally offers enhanced detection sensitivity. Pre-column derivatisation can affect the separation, either positively or negatively, if the moiety added dominates the chromatographic properties. Derivative stability is another feature to be considered when employing derivatisation in the pre-column mode. The number of samples in a run may be limited to the stability time of the derivative. For post-column derivatisation, additional equipment is required and additional optimisation is necessary with respect to mobile phase and reagent flow rates, mixing coil length and diameter. Post-column derivatisation can also result in increased base-line noise due to the pumping action of the reagent pump.

Electrochemical detection can be employed for antibiotic residues containing amines (penicillins), thiols and hydroxyl groups (aminoglycosides). This non-confirmatory technique does not require derivatisation but detects analytes directly. Its high sensitivity requires relatively long equilibration times and requires the presence of salts in the mobile phase, to assist ionisation of the residue molecules. Oxidation of the analyte, at the surface of an electrode causes a change in current flow and chromatograms are obtained by plotting current against time. Carbon paste and gold electrodes have been employed for aminoglycoside residue detection[137]. Polarimetric detection has been used for the

detection of antibiotic residues that exist as isomers. Specific rotation detection has been applied to the study of erythromycin in milk[138], allowing identification of various forms of the same drug, which possess minor structural differences. Refractive index detection has also been employed for aminoglycoside residue detection[139] with a detection limit of 1 mg ml^{-1}. This method of detection is often less sensitive than the more classical detection techniques but is more specific. Currently, since not all veterinary preparations are required to be chirally pure, the requirement for methods capable of detecting optically active substances is limited.

Mass spectrometric detection of antibiotic residues is becoming increasingly popular, as analysts require confirmatory techniques for determining the presence of antibiotic residues. Briefly, mass spectrometric detection involves the ionisation of molecules, resulting in a pattern of molecular ions and fragments that are specific to that antibiotic. Mass spectrometric detection can theoretically provide unequivocal identification for all antibiotics. The high cost and requirement for skilled operators initially hampered the application of this technique to veterinary drug residue applications. However, LC-MS is now routinely employed for the determination of antibiotic residues with most MS systems now being commercially available as bench-top LC detectors. The difficulty in obtaining diagnostic molecular ions has led to the existence of a wide range of ionisation techniques. Ease of operation and cost have resulted in quadrupole instruments becoming most popular. Many LC-MS methods reported in the literature employ thermospray interfaces; however, the mass spectrometric analysis of antibiotics has significantly benefited from the development of electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI). The ionisation method employed is influenced by a variety

of parameters such as mobile phase flow rate and composition, analyte thermal stability, volatility, and ionisation state. Criteria that define the performance of confirmatory methods for residues have been established. For application of mass spectrometry in regulatory residue analysis, monitoring of four ions is required and ion ratios in samples must be within 20 % of the ion ratios determined in standards.

1.7 CONCLUSION

In the past ten years, the science of veterinary drug residue analysis has moved towards less labour intensive, more automated assays. For antibiotic residue analysis, rapid screening methods are frequently employed, most of which employ the principle of microbial inhibition. Immunodiagnostic techniques, such as EIA have also featured, offering increased specificity and sensitivity, relative to microbial inhibition methods. In chemical analysis, HPLC remains the method of choice for antibiotic residues. A wide range of detection techniques are used, including UV, fluorescence and electrochemical detection. The employment of confirmatory detection for routine analysis has increased significantly. The on-line combination of LC-MS has become a popular technique. The ease of operation and robustness of newly-developed interfaces has resulted in the routine employment of MS for detection and simultaneous confirmation.

In sample preparation, trends have been towards more automated, environmentally friendly methods, exhibiting high sample throughput. Currently, SPE is the sample preparation technique most widely employed in antibiotic residue analysis. Liquid-liquid

partitioning has been largely superseded by this rapid, efficient technique. Reasons for this include the ease with which SPE procedures can be fully automated and automated systems are now readily available commercially. SPE is relatively low on solvent consumption and does not make huge demands on operator time, when compared with liquid-liquid partitioning. Supercritical fluid extraction has also featured in sample preparation, however, its requirement for specialised equipment has hindered its employment.

Sample preparation is the rate-limiting step in many veterinary drug residue methods. Despite trends towards rapid methods with high sample throughput, many of the methods reported in the scientific literature still employ techniques that have been in use for many years. In recent years, much research has been directed at developing the ideal confirmatory detection technique, and attention has been focussed on making MS detection more widely applicable and more sensitive. However, there have been some moves towards the development of automated sample preparation systems, such as automated dialysis, or on-line SPE. Nevertheless, even these methods require considerable sample preparation, prior to employing an automated procedure. Therefore, sample preparation techniques in general remain labour intensive and demand a considerable proportion of the analyst's time.

Trends in sample preparation are to develop methods that are less labour intensive and that are amenable to automation. The objectives should be to increase the reliability of existing methods and eliminate clean-up steps, by employing more selective extraction

procedures. Ideally, a universally applicable extraction method is desirable, possessing reliability, specificity, rapidity and simplicity. The development of multiresidue methods is increasing with on-line enrichment to improve sensitivity. The ideal method would incorporate sample preparation on-line with the entire analytical procedure and produce a confirmative result. Future trends for antibiotic residue analysis are likely to involve methods that may be used on-site, without the need for extensive instrumentation and skilled operation, and yet produce a reliable result.

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CHAPTER 2

THE ANALYSIS OF

SULPHONAMIDE DRUG RESIDUES

IN PORK MUSCLE USING

AUTOMATED DIALYSIS

2.1 INTRODUCTION

Sulphonamides are a class of veterinary drug that find use in the treatment and prevention of various bacterial infections in farm animals. They are commonly administered for the treatment of respiratory diseases in animals, such as pneumonia, for intramammary infections, such as mastitis, and for urinary and intestinal tract infections. Sulphamethazine is the most commonly used member of the class. As discussed in Chapter 1, the use of these antimicrobial agents may leave residues in tissues or milk, which can pose a risk to consumer health. A high level of exposure of some micro-organisms to these drugs may manifest itself in the development of drug resistant strains[1]. The use of sulphonamides has been approved by regulatory agencies, provided sufficient withdrawal periods are adhered to, allowing elimination of the residues from edible tissues. Maximum Residue Limits (MRLs) of 100 ng g^{-1} (ppb) have been set by the EC for sulphonamides in tissue[2].

2.1.1 Mechanism of action

Sulphonamides, first discovered in 1935, are bacteriostatic chemotherapeutics, which act by inhibiting the growth of new bacteria. They act by preventing the production of folic acid (needed for DNA production) through competition with the folic acid precursor, *p*-aminobenzoic acid. Diaminopyrimidines, such as trimethoprim, are frequently administered alongside sulphonamides, as they possess a synergistic relationship with sulphonamide antibiotics, resulting in a bactericidal effect.

2.1.2 Physical/chemical properties

Chemically, sulphonamides are substituted aromatic amines, substituted at the N-1 position as shown in Figure 2.2. Sulphanilamide, shown in Figure 2.1, depicts the basic chemical structure for the sulphonamide class. Sulphonamide antibiotics are amphoteric, possessing an NH_2 group and an SO_2 moiety. The various members of the class have a wide range of pKa values, which can make analysis difficult. In general, they are insoluble in water, diethyl ether and chloroform, but readily soluble in polar organic solvents such as acetone. All members of the class possess UV absorption properties at 254 nm or 270 nm.

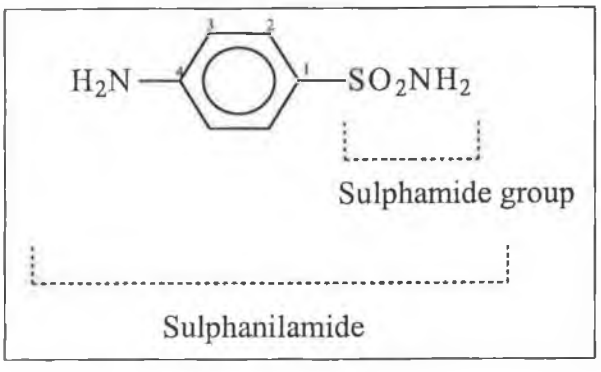

Figure 2.1 *Basic chemical structure for sulphonamide antibiotics.*

Generally sulphonamides are stable compounds, but a few members of the class are sensitive to UV degradation. *In vivo*, the aromatic amine becomes acetylated resulting in the production of N-acetyl-sulphonamide. Stability of sulphonamide residues in the presence of matrix components has been studied. Thomas *et al.*[3] studied the effects of frozen storage on the stability of sulphonamide antibiotic residues in fortified porcine liver. They examined the effects of storage at -20°C on sulphamethazine, sulphathiazole, sulphadimethoxine, sulphachloropyridazine and sulphaquinoxaline. They concluded that

all the analytes degraded during storage. Sulphaquinoxaline degraded most rapidly (half-life 271 days) and sulphadimethoxine was the most stable (half-life 567 days). This study demonstrates the need for analysis of samples close to time of slaughter as the residue content in samples which have been held in frozen storage for extended periods will not be indicative of the residue content when the animal was slaughtered. Hasset *et al.*[4] concluded that temperatures reached during frying, roasting, grilling and boiling of meat were insufficient to degrade sulphonamide residues.

Various studies have been reported on identification of the target organ for sulphonamide residue analysis. Randecker *et al.*[5] reported higher levels of sulphamethazine in liver than kidney after administration of medicated feedstuffs. They also suggested that sulphamethazine serum and urine levels could be indicative of tissue levels. The Sulpha-On-Site (SOS) test, used by the USDA, for regulatory control of sulphamethazine, determines the level of analytes in the urine, as an indication of the concentration in tissues. DePaolis *et al.*[6] administered radiolabelled sulphamethazine to pigs and found levels of radioactivity to be greater in liver than in kidney. However, it is possible that the activity measured is not just indicative of levels of the parent compound but also that of metabolites present. Shearer *et al.*[7] reported highest levels of sulphamethazine in plasma, followed by kidney, liver and muscle. Two trials were included in the study; in the first trial, pigs were fed sulphamethazine treated feed up to the day of slaughter. In the second trial, pigs were fed sulphamethazine treated feed and then fed unmedicated feed prior to slaughter. Samples were collected at slaughter and analysed. The distribution of sulphamethazine was in the ratio 1:2:3:6 for muscle:liver:kidney:plasma.

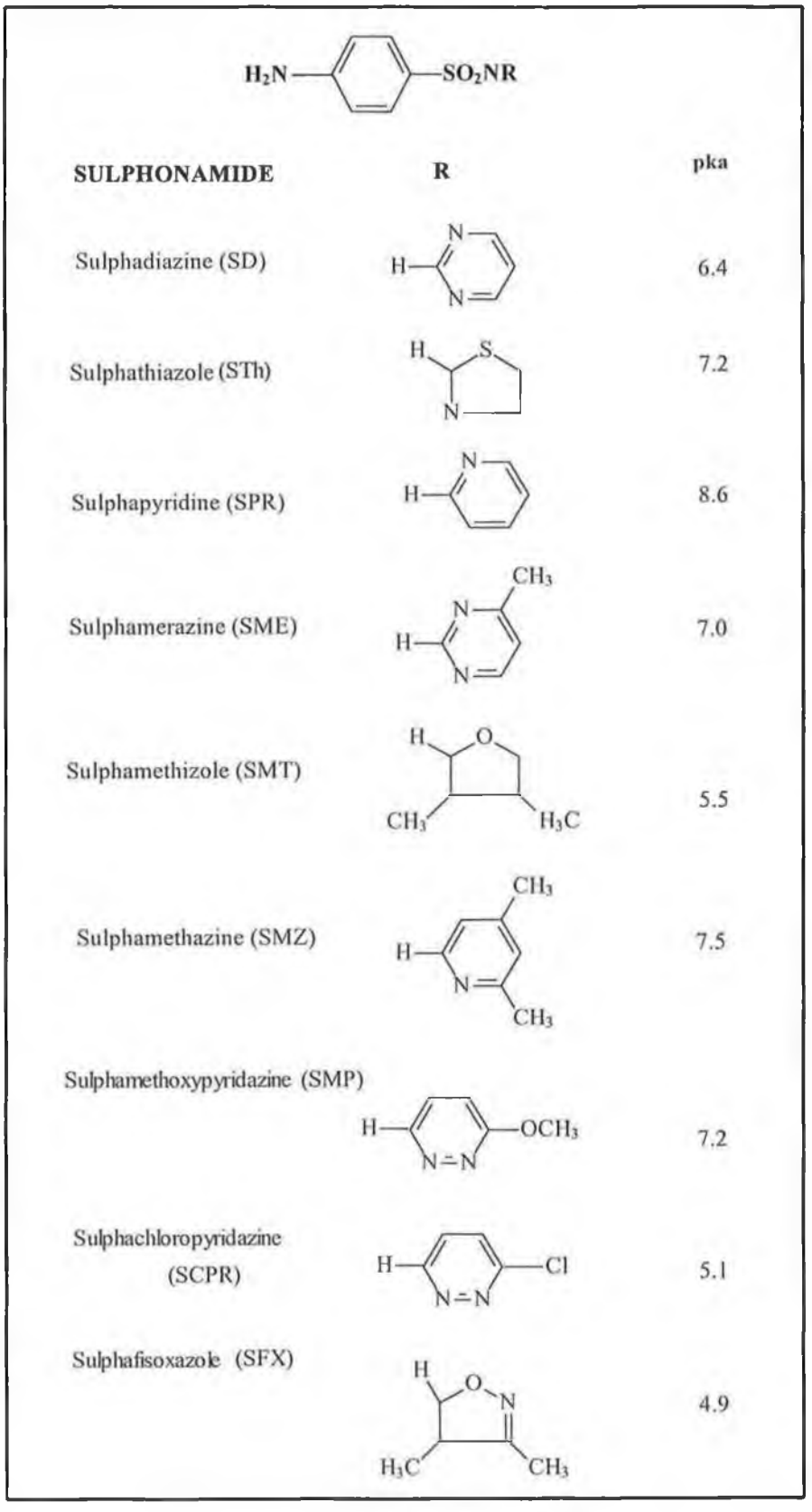

Figure 2.2 *Chemical structures and pKa values of the sulphonamides under study*

2.2 METHODS FOR THE ANALYSIS OF SULPHONAMIDE RESIDUES IN TISSUES

2.2.1 Sample preparation

As discussed in Chapter 1, a critical step in residue analysis is sample preparation, designed to extract the residues while eliminating the problem of matrix interferences. Traditionally, organic solvent extraction methods, using several extraction solvents, were employed followed by centrifugation, pH adjustment and back extraction. These steps may result in a laborious, time-consuming method, allowing a limited number of samples to be analysed per day. Due to the number of sulphonamide drugs employed in veterinary medicine, multi-residue methods are desirable, which are capable of determining a number of residues simultaneously. However, this task can be difficult, due to the varying pKa values within the group (5.1-11.5). Most methods reported follow the general protocol of homogenisation in an extraction solvent, mixing to achieve extraction, centrifugation, clean-up using solid phase extraction, or liquid-liquid partitioning with various washes and pH adjustments, and finally chromatographic determination.

2.2.1.1 Solvent extraction and liquid-liquid partitioning

Chloroform, ethyl ether, acetone and methylene chloride are commonly used for extraction of sulphonamides. Acetonitrile has been used for deproteinisation. Smedley and Weber[8] described a method for the analysis of ten sulphonamide residues in milk at 10 ppb, based on chloroform-acetone extraction followed by dissolution of the residues in potassium phosphate buffer and removal of fat with hexane. Tsai *et al.*[9] used acetonitrile

for extraction and deproteinisation of milk samples for the analysis for six sulphonamides. The sulphonamides were derivatised prior to HPLC separation and fluorescence detection. For the determination of sulphamethazine in porcine tissue, Thomas *et al.*[10] extracted tissue with ethyl acetate and back extracted into methylene chloride. Emulsions were minimised by inclusion of a centrifugation step. Sulphamethazine analysis in muscle by Boisson and Keng[11] involved initial extraction with chloroform followed by back extraction into alkaline sodium chloride solution in a separatory funnel. Haagsma *et al.*[12] extracted sulphamethazine from porcine tissue by ultrasonication in the presence of dichloromethane. Petz[13] used a liquid-liquid partitioning method for the determination of sulphanilamide and sulphaguanidine in muscle and liver. The residues were extracted with acetonitrile at pH 8.5, and the aqueous portion of the extract was separated by adding sodium chloride. The extracted residues were subsequently partitioned between aqueous methanol and hexane. The residues partitioned into the aqueous methanol layer and this was used for chromatographic determination.

Ion-pair extraction has been described for the extraction of sulphadimethoxine from fish tissue[14] and sulphapyridazine from serum[15]. Tetrabutylammonium hydroxide was employed in both studies. The addition of the ion-pair rendered the sulphonamides more extractable by chloroform or dichloromethane, as it complexed with the negatively charged sulphonamide, resulting in a neutral molecule, which partitioned into the relatively non-polar organic solvent.

2.2.1.2 Solid phase extraction

Sulphamethazine, sulphadimethoxine and sulphamonomethoxine were determined in animal tissue and egg by Hori *et al.*[16]. The method of analysis for these drugs involved an initial acetonitrile deproteinisation followed by a hexane wash to remove lipids. An SPE clean-up step was employed with Bond-Elut™ C18 from which the sulphonamides were eluted using acetonitrile, containing triethylamine (TEA). The TEA acted as an amine modifier competing with unbonded silanols on the silica back bone. Recoveries in the range of 81-98 % were obtained for residues at 0.1-0.2 μg g^{-1} tissue. Haagsma and De Water[17] described a method for the rapid determination of sulphanilamide, sulphamethazine, sulphaquinoxaline, sulphadoxine and sulphadiazine in porcine kidney and muscle, with a detection limit of 50 ng g^{-1}. Following an initial chloroform-acetone extraction, an ion-exchange clean-up step was used. An aromatic sulphonic acid cation-exchange column was employed. This allowed the retention of the sulphonamide drugs (of various solubilities and pKa's) from an acidified extract. Elution could be achieved with alkaline buffer, but this was not suitable for subsequent HPLC analysis, so ammonia vapour was passed through the column, followed by methanol elution. The authors postulated that ammonia deprotonates the sulphonamides thereby breaking the bond with the sulphonic acid column and permitting elution in a small volume of solvent.

A method for the determination of sulphamethazine in milk using SPE was carried out by Unruh *et al.*[18] with recoveries of 96 to100 % over the range 0.5 to 15 ng ml^{-1}. Two clean-up columns were arranged in tandem. An initial C18 SPE column retained the sulphamethazine and sulphabromomethazine (internal standard), when applied in phosphate buffer, pH 5.7. The sulphonamides were found to elute off the C18 sorbent in

methanol, but this eluate contained riboflavin, lipids and other compounds, which interfered with the subsequent chromatographic analysis. Therefore, aluminium oxide and cation-exchange resin (AG MP-1) were arranged in a tandem column arrangement to clean-up and isolate sulphamethazine and sulphabromomethazine. The aluminium oxide removed the interfering fluorescent riboflavin and free fatty acids from the extract. Sulphamethazine and sulphabromomethazine were eluted off the AG MP-1 resin with an acidic acetone solvent, which could be easily applied to determination by TLC.

Silica SPE has been employed for the clean-up of four sulphonamide drugs and their N^4 metabolites in porcine muscle[19]. Cooper *et al.*[20] also employed silica SPE for the determination of sulphadimethoxine, sulphaquinoxaline and sulphamethazine in porcine kidney. Suhren and Heeschen[21] employed aromatic sulphonic acid cation-exchange SPE for the determination of eight sulphonamide residues in milk. This allowed the milk to be deproteinated using acid and the acidic extract could be applied directly onto the SPE column.

Cox *et al.*[22] have used SPE columns packed with XAD-2 polymer for clean-up of samples containing sulphamethazine. A phosphate buffer extract (pH 6.8), resulting from liquid-liquid extraction, was applied to the column and sulphamethazine eluted using methanol. Aerts *et al.*[23] also used XAD-2 and XAD-4 microcolumns for sulphonamide multi-residue analysis. They described an automated dialysis system for the extraction of sulphonamides from milk, meat and eggs, followed by clean-up and concentration on C18, Corasil or XAD material.

2.2.1.3 Matrix Solid Phase Dispersion

Matrix solid phase dispersion (MSPD) has been employed for single and multi-sulphonamide residue analysis in biological fluids and tissues. The basic principles behind the method are discussed in Chapter 1. Chapter 3 describes the isolation of penicillin residues using this method. It has been employed in combination with thin layer chromatography, to provide a simple, rapid method for the determination of six sulphonamide residues in tissue and milk[24]. Matrix interferences were removed using a hexane wash and the sulphonamide residues were eluted using dichloromethane. Long *et al.*[25] extracted eight sulphonamide drug residues from pork tissue using this technique. Sulphonamides were eluted from the C18-tissue blend using dichloromethane, yielding extracts clean enough for HPLC separation with UV detection at 270 nm. Recoveries were in the range 70-95 % with a limit of determination of 62.5 ng g^{-1}. A hexane wash removed lipid material and neutral chromophores that interfered with UV detection. Dichloromethane elution allowed elimination of highly polar compounds, which were retained on the column.

Shearan *et al.*[26] have compared MSPD with a standard solvent extraction method for sulphamethazine in pork tissue. The MSPD protocol was similar to that described by Long[25] and residue content of extracts were determined by HPLC and TLC. The authors concluded that MSPD, linked with the TLC system described required improvement, as the detection system was not sensitive enough to measure levels of sulphamethazine at or below the MRL. Increasing the quantities of tissue and packing material, in an effort to improve sensitivity, would prove expensive, so the authors recommended the use of more concentrated extracts. They also described an alternative approach to evaporation of

methylene chloride resulting from MSPD, which was back extraction of sulphamethazine into 500 µl 0.01 N sodium hydroxide. Solvent extraction in an ultrasonic bath was employed, followed by SPE clean-up, providing extracts suitable for TLC and HPLC detection systems at levels as low as 50 ng g^{-1}. However, this method was inferior in terms of time and ease of use and recoveries were no better than with MSPD.

The same authors have published an on-site method for sulphamethazine determination in pork carcasses[27]. It links MSPD with a micro-column concentration step, prior to determination by a modification of the TLC based "Sulpha-On-Site" test. The method overcomes the requirement for sophisticated instrumentation. MSPD elution was effected using methylene chloride. Petroleum spirit was added to the methylene chloride extract to aid sulphamethazine retention during clean-up on a silica micro-column, similar to that used by Unruh *et al.*[18]. Sulphamethazine was eluted with 150 µl methanol, which was applied to TLC and residues were detected using fluorescence. Multi-residue MSPD extraction has been applied to the determination of five sulphonamide residues in salmon muscle[28]. C18 sorbent was employed, fat was removed using a hexane wash, and analytes were eluted in dichloromethane, which could be easily concentrated.

2.2.1.4 Immunoaffinity column chromatography

Immunoaffinity column chromatography provides a simple, quick method for sample clean-up. The production and characterisation of polyclonal antibodies to sulphamethazine and their potential for use in immunoaffinity column chromatography has been reported by Crabbe *et al.*[29]. After characterisation of the antibodies in an ELISA, the IgG fractions were coupled to CNBr activated Sepharose and employed for the

immunoaffinity column clean-up of urine samples. The column had a high capacity for sulphamethazine (1900 ng ml^{-1} gel), and showed high cross reactivities for the major metabolites of sulphamethazine present in urine. Mean recovery from urine samples fortified at 100 ng ml^{-1} was 96 % (CV = 5 %).

Immunoaffinity column chromatography was employed by Martlbauer *et al.*[30] for sulphonamide analysis. Monoclonal antibodies developed for sulphamerazine and sulphadiazine capture, were mixed with activated Sepharose and loaded onto a syringe barrel. Milk was defatted, by centrifugation, and diluted with saline prior to loading onto the immunoaffinity column. The eluted analytes were determined by LC with UV detection. The sulphadiazine antibodies showed cross reactivity with sulphamerazine, with 84-96 % of the two drugs being bound to the column.

2.2.1.5 Supercritical fluid extraction

As previously mentioned, many sulphonamide residue analysis methods employ solvent extraction steps, which are time-consuming and use relatively high volumes of organic solvents. Therefore, the application of supercritical fluid extraction for the analysis of sulphonamide residues is appropriate. Supercritical trifluoromethane and methanol modified trifluoromethane were compared with supercritical carbon dioxide and methanol modified carbon dioxide for the extraction of sulphonamides from fortified chicken liver[31]. Supercritical trifluoromethane and methanol modified trifluoromethane had higher solvating powers and extraction selectivity for the extraction of sulphamethazine, sulphadimethoxine and sulphaquinoxaline. This was likely due to the strong hydrogen

bonds formed between the basic analytes and trifluoromethane. For both fluids, methanol modification was necessary to achieve recoveries of greater than 67 %. Mean recoveries of 100 %, 86 % and 31 % with RSD values of 4 %, 12 % and 14 % were obtained for sulphamethazine, sulphadimethoxine and sulphaquinoxaline, respectively, for chicken liver samples fortified at 5 ppm. Maxwell and Lightfield[32] have described a multiresidue method for the determination of sulphamethazine, sulphadimethoxine and sulphaquinoxaline in chicken liver. Unmodified carbon dioxide was employed, with in-line trapping on neutral alumina. Determination was by HPLC. Recoveries of 72-98 % were achieved with standard deviations in the range 1.2 to 10.2, for samples fortified between 50 and 1000 ppb.

2.2.2 Screening methods

Screening techniques commonly used in antibiotic drug residue analysis are discussed in Chapter 1 and many of these have found application for sulphonamide residue screening. Microbiological inhibition methods including the Four Plate Test, the new Dutch kidney test, swab test on premises (STOP), Calf Antibiotic and Sulfa Test (CAST) and Fast Antibiotic Screen Test (FAST) have been used.

2.2.2.1 Microbial inhibition assays

The STOP test has been used for the analysis of sulphonamide residues in kidney. A cotton swab was inserted into the tissue sample and then incubated overnight, on agar medium containing *Bacillus subtilis*. Johnston *et al.*[33] compared STOP results with standard microbiological inhibition plate assays and reported 94 % agreement. The CAST assay is very similar in method protocol and sensitivity to the STOP assay, with overnight

incubation at 44°C. The FAST procedure is also similar in protocol but the growth medium contains a purple dye that becomes yellow in the presence of bacteria (due to acid production during bacterial metabolism); the change in colour from purple to yellow indicating the absence of antimicrobial residues. Results are available more rapidly using FAST screening, as only a six hour incubation is required. The Delvotest works on the same principle as the FAST assay, and requires shorter incubation (around 2 h). Bugyei *et al.*[34] have used the Delvotest for the analysis of sulphamethazine in chicken liver and kidney. They compared the results obtained with those obtained for samples analysed by a standard plate assay. Limits of determination were lower using the Delvotest, however, a greater incidence of false negatives occurred. The Brilliant Black Reduction Test uses *B. Stearothermophilus* as the test organism and also works on the basis of a change in colour of the medium. The limit of determination for most sulphonamides is 1 ppm using this test, however, sensitivities for the various members of the class vary; for example, 100 ppb sulphadimethoxine may be detected[35].

2.2.2.2 Bacterial receptor assays

Receptor based assays, such as the Charm™ test, have been reported for sulphonamide residue screening[36]. The method has been applied to the analysis of residues in liver, kidney and muscle. Limits of detection are in the low ppb range. In this assay, microbial cells possessing specific receptor sites for the sulphonamide residues are added to the sample. Radiolabelled drug standard is also added, and the labelled drug competes with any residues from the sample for the receptor sites. Further sample treatment involves centrifugation and the precipitate (containing the receptor-bound radiolabelled drug standard) is measured for radioactivity. The activity measured is inversely proportional to

the amount of residue in the sample. The test is only specific for a particular family of antibiotics, such as sulphonamides. A relatively high rate of false positives have been attributed to screening using Charm kits, and the test has different sensitivities for different members of the same family. Zomer *et al.*[37] described a method for the determination of ten sulphonamide residues in milk. Liquid chromatography was employed to isolate the analytes and detection was by the Charm II test. The limit of determination of the assay was between 1 and 5 ppb for the various sulphonamides under study. Samples fortified at the minimum determination level for each sulphonamide were analysed and variation in recovery of 7.6 to 24 % resulted.

2.2.2.3 Immunoassays

Immunoassays have been employed as screening methods for sulphonamides in milk and tissues. It has been reported that immunoassay methods require greater sample preparation than many other screening methods employed, such as microbial inhibition and receptor assays[38]. Garden and Sporns[39] described a method for the analysis of sulphamerazine in milk using an EIA. Sample dilution with water was the only sample preparation step required and the method had a limit of determination of 0.15 ppb. Jackman *et al.*[40] reported the analysis of raw milk using an EIA. The assay proved linear between 0.02 and 40 ppb, for twelve sulphonamide residues. Cross reactivities of other sulphonamides with each antisera were less than 1 %, with four exceptions at 55, 33, 13 and 3 %. Dixon-Holland and Katz[41] described an EIA for the detection of sulphamethazine in porcine urine and muscle. Urine samples without any clean-up, or muscle extracts, were analysed and detection was by absorbance measurement using 2,2'-azino (3-ethyl-benzothiazole) sulphonic acid as a chromogen. Sulphamethazine was

detected at concentrations of 20 ng g^{-1} in muscle and 10 ng ml^{-1} in urine. A similar competitive EIA for the specific determination of sulphamethazine in plasma was developed by Singh *et al.*[42] for the concentration range 10-1000 μg kg^{-1}; thirty-six sulphonamides were assayed for cross reactivity and only one, sulphamerazine, showed cross reactivity of 12 %. Ram *et al.*[43] developed an EIA for sulphamethazine determination at 1-5 ng ml^{-1} porcine plasma and serum using an automated technique.

Crooks *et al.*[44] have developed an immunobiosensor for the detection of sulphamethazine and sulphadiazine residues in porcine bile. The method described employed an optical biosensor (BIAcore™) for routine screening. For this instrument, antibodies were immobilised onto sensor chips and the sample pumped over the surface of the chip for 5 min. The false positive results were 0.14 % for sulphamethazine and 0.34 % for sulphadiazine, which were lower than those achieved using the classical enzyme immunoassay. Biosensor analysis showed no false negatives and provided a rapid, simple screening technique.

2.2.2.4 Thin layer chromatography

Thin layer chromatography has been widely used for the determination of sulphonamide residues in foods. The use of selective detection reagents such as the Bratton-Marshall reagent, *p*-dimethylaminobenzaldehyde or flourescamine have provided sensitive methods. Bieganowska *et al.*[45] compared the chromatographic behaviour of some sulphonamides on normal phase and reversed-phase HPTLC plates. They investigated the influence of ion-pair reagents on the retention of sulphonamides on reversed-phase TLC

plates and also the influence of different modifiers on normal phase plates. They reported that Florisil and polyamide were more selective adsorbents than silica and concluded that sulphonamides were more strongly retained on reversed-phase adsorbent as the concentration of organic modifier in the mobile phase was increased. Reimer *et al.*[46] have reported a TLC based screening method for five sulphonamides in salmon muscle with a limit of determination of 40 ppb for sulphamethazine, sulphadimethoxine, sulphadiazine, and sulphapyridine and 100 ppb for sulphamerazine. The sulphonamides were detected on silica gel HPTLC plates after derivatisation with fluorescamine.

Van Poucke *et al.*[24] detected sulphonamide residues in milk at a concentration of 4 $\mu g\ kg^{-1}$ using TLC with fluorescence derivatisation and a three-phase multiple development chromatographic system. Shearan and co-workers[26] described the determination of sulphamethazine at 0.4 $\mu g\ ml^{-1}$ from direct application of urine on a TLC plate. Unruh *et al.*[47] described the quantification of sulphamethazine in pork tissue by TLC where silica gel plates were run, dried, dipped in fluorescamine solution, dried and sprayed with borate buffer. The plates were then stored at approximately -20°C for five minutes before scanning at 366 nm (excitation) and 400 nm (emission). This cooling step overcomes the problems of heat lability of fluorescent derivatives.

The "Sulfa-On-Site" (SOS) test is a commercially available TLC based kit for the analysis of sulphamethazine residues in porcine urine. It was designed for residue control at meat slaughter plants, so skilled technicians are not required. Using this method, residues may be visually detected under UV light. The migration distance (R_f value) is used for presumptive identification and the intensity of the fluorescence allows approximate

quantitation. The intensity of the sample spot is compared with the intensity of the spots for two standard solutions of 0.4 and 1.3 μg ml^{-1}. Where the intensity of the sample spot exceeds that of the 0.4 μg ml^{-1}standard, it is considered that the liver contains sulphamethazine residues in excess of the MRL (0.1 μg ml^{-1}), and where the sample spot intensity exceeds that of the 1.3 μg ml^{-1} standard, the carcass (muscle) is considered to contain sulphamethazine in excess of the MRL (0.1 μg g^{-1}).

2.2.2.5 Spectrophotometric assays

The highly sensitive Bratton-Marshall colour test forms the basis of the spectrophotometric method used by Schwartz[48]. In this method, the free aryl primary amine (N^4, see Figure 2.1) is converted to a diazonium salt, using nitrous acid, and after removal of the excess acid, the diazonium salt is coupled to a chromogen to form an azo dye with a detection wavelength of 545 nm. The method is not highly specific, so other primary amines interfere. The non-specificity of the reaction in feeds has been overcome by anion-exchange extraction, in the method described by Schwartz[49]. Sulphamethazine, on reaction with Bratton-Marshall reagents, yielded a pink coloured compound. Schwartz used this method to detect sulphamethazine in milk at low ppb levels. The quantification was carried out spectrophotometrically at 540 nm. He also used this detection system for sulphathiazole in honey.

2.2.3 Quantitative methods

Quantitative residue methods for sulphonamides are based on chromatographic methods, particularly HPLC, and less frequently GC. Aside from UV detection, most other methods require derivatisation.

2.2.2.1 High performance liquid chromatography (HPLC)

Reversed-phase liquid chromatography has proven to be the most popular method for sulphonamide drug residue analysis. A review of various HPLC methods was prepared by Agarwal[50]. Numerous studies have been developed for single and multiresidue methods. Most sulphonamide methods use C18 or C8 columns. Horii *et al.*[16] used a Nucleosil C18 column for sulphamethazine, sulphadimethoxine and sulphamonomethoxine separation. A C2 column was employed by Alwai and Russel[51] for the separation of sulphamethazine and sulphadiazine in milk extracts. Weiss *et al.*[14] used a μ-Porasil column for the analysis of sulphadimethoxine. Capacity factors could be easily influenced by varying the concentration of ammonium acetate in the mobile phase buffer. Normal phase chromatography with a silica stationary phase was employed for the analysis of sulphadiazine and ormethoprim in fish tissue[52]. A cation-exchange column was employed by Rychener *et al.*[53] for the chromatographic separation of thirteen sulphonamide residues in muscle, liver and kidney extracts. However, two separate HPLC mobile phases were necessary, as it was not possible to separate all residues in one chromatographic run. Brewster *et al.*[54] evaluated restricted access media for the HPLC analysis of sulphonamide residues. Three commercially available columns were tested for their suitability for multiresidue sulphonamide analysis. The authors reported matrix problems when bovine serum samples were directly injected and only one column allowed a multiresidue

method. Micellar liquid chromatographic separation of twelve sulphonamides was described by Yang and Khaledi[55]. Sodium dodecyl sulphate micelles were employed with an endcapped C18 column. The retention behaviour and selectivity pattern of the sulphonamides were examined under various concentrations of sodium dodecyl sulphate and organic modifier. Direct on-column injection was used to separate twelve sulphonamide residues extracted from human urine and cow's milk. The method showed repeatable retention times with standard deviation values in the range 0.005 to 0.035. Micellar liquid chromatography has also been described by Symanski and Szczepaniak for the analysis of six sulphonamides[56]. The method is based on the reversed-phase separation of the analytes with the anionic surfactant sodium dodecyl sulphate as the micellar mobile phase. The pH and micelle concentration were the two most important parameters affecting retention of the analytes. Good peak symmetry and resolution were obtained. Reproducible retention times were noted (RSD values of 0.26 to 0.86 min). UV detection was employed, allowing limits of detection between 0.02 and 0.2 $\mu g\ ml^{-1}$. Micellar liquid chromatography has been applied to the analysis of five sulphonamide residues in serum. Azo dye pre-column derivatisation was employed and the derivatives were separated on a C18 column with SDS-pentanol mobile phase[57]. The method proved linear between 1 and 20 $\mu g\ ml^{-1}$. Baseline resolution was not achieved for all five analytes in one run.

The retention behaviour of sulphonamides on reversed-phase columns is dependent not only on the polarity of the molecules, but also on the extent of ionisation of the functional groups. Therefore, the pH of the mobile phase is important. Mobile phases generally contain a high percentage of aqueous buffer (phosphate or acetate buffered between pH 2 and 5 and acetonitrile or methanol modifier. Sulphonamides possess a UV absorbance

band at approximately 270 nm, which allows HPLC with direct UV detection. Typical sensitivities achieved for UV detection of sulphonamides are in the region of 2 to 10 ng. Photodiode array detection has been commonly applied to sulphonamide analysis. Cooper *et al.*[20] employed diode array detection for the analysis of sulphonamides in porcine kidney. This allowed the detection of sulphamethazine at 299 nm, sulphadiazine at 251 nm and sulphaquinoxaline at 256 nm. Alternatives to direct UV detection are precolumn derivatisation and post-column derivatisation. These techniques are employed to improve the specificity of sulphonamide detection. For sulphonamide residue analysis, several derivatisation techniques employing a fluorescent tag have been described. Fluorescamine and *p*-dimethylaminobenzaldehyde (DMAB) are widely used as derivatisation reagents for sulphonamide analysis with HPLC. Pre-column derivatisation with fluorescamine was used by Takeda and Akiyama[58] for the detection of sulphonamides in meat. A limit of detection of 10 ng g^{-1} was reported. Sulphonamides in the presence of fluorescamine, at pH 3, yielded highly fluorescent compounds measurable with excitation at 405 nm and emission at 495 nm. Gehring *et al.*[59] have used fluorescamine for post column derivatisation of fourteen sulphonamide residues in salmon tissue. Fluorescamine is an expensive reagent, which only remains stable for 48 hours in a cooled solution. It reacts with the free N^4-amino group of sulphonamide molecules.

DMAB fluorescence derivatisation is the method employed in this study and is dealt with in greater detail in the discussion section. It was developed initially as a spray reagent for TLC[60] and later adapted as a HPLC post-column derivatisation technique by Stringham *et al.*[61]. They compared it to the Bratton-Marshall colorimetric method and found derivatisation with DMAB to be safer, quicker, less demanding in terms of glassware and

requiring fewer clean-up steps due to the longer wavelength used for detection of DMAB derivatives. Tsai *et al.*[62] used fluorescamine derivatisation followed by post-column reaction with TDPO (bis[2-(3,6,9-trioxadecanyloxycarbonyl)-4-nitrophenyl] oxalate) and hydrogen peroxide, resulting in limits of detection around 1 ppb. Derivatisation of sulphonamide residues using *o*-phthaldialdehyde in the presence of mercaptoethanol was described by Vinas *et al.*[63]. The method was applied, post-column, to the determination of residues in meat, fish and eggs. The limits of determination were in the range 11 to 19 ppb with a maximum RSD of 0.7 %, using a level of fortification of 0.4 ppm.

Electrochemical detection has also been applied in sulphonamide residue analysis. Alwai and Russel[51] also used electrochemical detection in the determination of sulphamethazine and sulphadiazine in milk. Sample preparation was limited to chloroform extraction of milk samples and the authors reported electrochemical detection to be more sensitive than UV detection at 263 nm. The limit of detection was 10 ppb. Parks[64] described the analysis of sulphanitran, amongst other nitro-containing drugs in chicken muscle using HPLC with amperometric detection. A glassy carbon electrode was used with an applied potential of –0.8 V. Adsorptive stripping voltammetry has been described for the determination of sulphamethazine in milk samples[65]. Milk was extracted using solvent extraction procedures. The residue was diazotized and coupled with 1-naphthol, to form a stable compound. The diazotized sulphonamide accumulated on the mercury electrode by electrochemical adsorption and was stripped cathodically. The method had a limit of determination of 3.8 ppb.

2.2.3.2 Gas chromatography

Gas chromatographic methods for sulphonamide analysis have the advantage of being more sensitive than HPLC. The low volatility and high polarity of these drugs necessitate derivatisation. Manuel and Steller[66] reported a method for determining six sulphonamides in bovine and porcine tissues. The residues were methylated at the N^4 position by diazomethane, separated on a packed column and detected by electron capture detection. The methylation of sulphamethazine by diazomethane was found to be approximately 90 %. However, methylation with diazomethane was reported to produce N-methyl and ring-methyl derivatives and the latter failed to elute off the GC column[38]. Matusik *et al.*[67] modified the method of Manual and Steller for the determination of sulphamethazine and two of its metabolites in incurred animal tissues. Later, they extended the method to include sulphachloropyridazine, sulpadimethoxine, and sulphathiazole[68]. Holtmannspotter and Their[69] described a capillary GC method with flame ionisation detection for sulphonamide residue analysis. The method could be applied to the analysis of six sulphonamides in eggs, milk and tissue. However, the limit of detection was 10 ppm and MRLs for the sulphonamide residues in tissues are 0.1 ppm.

2.2.3.3 Supercritical fluid chromatography

Supercritical fluid chromatography (SFC) with UV and mass spectrometric detection was employed by Perkins *et al.*[70] for nine sulphonamides. Packed-column SFC separation on silica or amino-bonded silica was reported, using CO_2 with methanol modifier, as the mobile phase. Tissue fortified at approximately 3 mg kg^{-1} was used to test the method. The authors investigated the effects of various modifiers on the separation of

sulphonamides, using both columns, and reported the amino bonded column to be much more sensitive to modifier variations. Separation of the nine drugs on silica was possible, but it was not satisfactory due the requirement for low pressures, resulting in long run times with broad peaks. The application of an amino-bonded phase allowed higher pressures and higher modifier concentration resulting in sharper peaks in a much shorter time.

2.2.3.4 Capillary electrophoresis

Sulphonamide residues extracted from serum and urine were analysed by capillary electrophoresis[71]. Residues were analysed using on-line SPE employing columns packed with styrene/divinylbenzene. Samples were loaded onto the columns without any pre-treatment. Elution was effected using acetonitrile and the analytes were separated using a fused silica capillary column with phosphate buffer of pH 7. RSDs values were between 6 and 9 % and between 7 and 10 % for the analysis of serum and urine, respectively. The same authors analysed serum and urine for sulphonamide residues using on-line dialysis coupled to capillary electrophoresis[72]. Following removal of particulates and proteins by automated dialysis, the extracted residues were concentrated on a C18 SPE column. The analytes were desorbed and subsequently injected into the capillary electrophoresis system. Using UV detection, the method had a limit of determination of 0.05 to 0.3 μg ml^{-1} for the range of sulphonamides. RSD values were between 3 and 8 % for serum and between 2 and 6 % for urine samples fortified at five times the limit of detection (between 50 and 100 ng ml^{-1} for urine, and 100 and 300 ng ml^{-1} for serum). Sixteen sulphonamide residues were determined in porcine muscle using capillary electrophoresis, by Ackermans *et al.*[73]. Sulphonamide residues were extracted by stomaching in acetonitrile, followed by

centrifugation and filtering, and 39 nl were pressure injected into the capillary. UV detection at 254 nm was employed and the limit of detection was between 2 and 9 ppm for the various sulphonamides.

2.2.3.5 Mass spectrometry

Mass spectrometric (MS) detection of sulphonamides has been employed using direct MS, HPLC-MS and GC-MS. Most methods employ positive ion monitoring and the protonated molecular ion $[M+H]^+$ as the base peak, with the exception of sulphanilamide, for which the base peak is $[M+NH_4]^+$. Pleasance *et al.*[74] reported the separation and identification of twenty-one sulphonamides by reversed-phase HPLC with ion-spray MS. Tandem mass spectrometry provided structural information. Henion *et al.*[75] described the detection of three sulphonamide residues in urine by means of LC-atmospheric pressure chemical ionisation MS. The protonated molecular ion was primarily detected using single ion detection. HPLC-atmospheric pressure chemical ionisation MS of eight sulphonamides was described by Combs *et al.*[76]. In full scan mode, 0.8 ng analyte could be detected and selected ion monitoring allowed detection of 50 pg for most of the analytes. The method was applied to the determination of residues extracted from chicken liver, by supercritical fluid extraction.

Mass spectrometry with GC separation has been reported. Detection limits of 1 $\mu g\ kg^{-1}$ sulphonamides from pork tissue were achieved by Carignan and Carrier[77] using LC clean-up. Subsequent N^4-methylation followed by GC-MS with electron ionisation in the multiple-ion detection mode was employed. GC-MS determination of sulphamethazine in porcine tissues has been described by Cannavan *et al.*[78]. Samples were extracted with

chloroform-acetone, followed by two SPE clean-up steps, prior to organic solvent partitioning. The extracted residue was methylated and silylated to allow GC analysis with selected ion-monitoring MS detection. Recovery values from muscle, liver and kidney ranged from 86 to 114 % with RSD values between 2.8 and 9.0 %. The assay had a limit of detection of 10-20 ppb in the various matrices. The authors reported that the methyl/trimethylsilyl derivatives exhibited better chromatography than the commonly used *N*-methyl-derivatives.

Mass spectrometric detection of residues, without the application of a chromatographic separation technique has proved popular for sulphonamide analysis. Brumely *et al.*[79] applied collision-induced dissociation-mass analysed ion kinetic energy spectrometry (CID-MIKES) to the identification of sulphonamide residues in tissues. Extracts were introduced directly with a solid probe and spectra obtained by chemical ionisation with helium being used as the collision gas.

2.2.4 Summary

This review of the literature highlights the large number of methods available for the determination of sulphonamide residues. However, most methods employ a number of sample preparation steps that are lengthy, labour intensive and consume relatively large volumes of organic solvents. Few methods employ automated techniques and most methods are applicable to only one to three sulphonamide residues. Despite the strong UV absorbance properties of the sulphonamides, most HPLC methods require derivatisation to achieve the desired specificity, as the commonly used sample preparation techniques result in co-extraction of excessive matrix interferences. Methods that do not employ HPLC

with derivatisation are frequently outside the detection range of interest for veterinary drug residue analysis. Therefore, there is a need for a rapid, automated, multiresidue sample preparation method.

This study investigates the application of dialysis, followed by trace enrichment, for extraction of sulphonamide drug residues from porcine tissue. The Gilson ASTED system (Automated Sequential Trace Enrichment of Dialysates) was employed in the development and validation of the method. The procedure described investiagted nine sulphonamide drug residues using reversed-phase HPLC as the method of determination. Muscle samples were blended in saline, centrifuged, and the harvested supernatant was filtered before being subjected to dialysis for an optimised time of 11 min. The resulting dialysate was concentrated on a reversed-phase trace enrichment cartridge (TEC) prior to HPLC analysis. This sample preparation method is highly automated, is confined to three sample preparation steps (homogenisation, dialysis and trace enrichment) and has low consumption of chemicals.

The developed method was evaluated by carrying out intra- and inter-assays on fortified porcine muscle. Both UV detection at 280 nm and post column derivatisation were investigated. The derivatisation reaction involved reaction of the sulphonamides with DMAB to yield a coloured derivative with an absorption maximum at 450 nm. Mean recoveries were high (56 - >100 %) using both methods of determination. Using UV detection at 280 nm, the detection limit was found to be 30 ng g^{1}, while post column derivatisation gave detection limits of 20 ng g^{-1}.

2.3 EXPERIMENTAL

2.3.1 Introduction

In this study, on-line dialysis was performed in the pulsed mode where the sample was held static in the donor stream (on the upper side of the membrane) and the recipient stream flowed continuously, at a fixed rate, along the under-side of the membrane. Small molecules, such as sulphonamides, passed through the membrane pores into the recipient stream while larger molecules such as lipids and proteins failed to dialyse across the membrane and were flushed to waste. The resulting dialysate was then concentrated on a reversed-phase sorbent, and, when the maximum amount of dialysate was loaded on the enrichment cartridge, the concentrated extract was backflushed onto the analytical column for residue determination.

Sulphonamide antibiotics were determined in pork muscle samples at concentration levels of 40 to 200 ng g^{-1}. Both UV detection at 280 nm and post column derivatisation using DMAB with detection at 450 nm were investigated. Figure 2.2 shows the chemical structure of the drugs studied, with the drugs being listed in order of elution from the C18 analytical column.

2.3.2 Chemicals and reagents

All solvents were of analytical HPLC grade and purchased from Merck Ltd., Dorset, UK. Sulphanilamide (SA), Sulphaguanidine (SG), Sulphadiazine (SD), Sulphathiazole (STh), Sulphapyridazine (SPR), Sulphamerazine (SME), Sulphamethizole (SMT), Sulphamethazine (SMZ) Sulphamethoxypyridazine (SMP), Sulphachlorpyridazine

(SCPR) and Sulphafisoxazole (SFX) were purchased from Sigma-Aldrich Company Ltd., Dorset, UK. Stock solutions of 1 mg ml^{-1} were prepared in 100 % methanol and dilutions were prepared in water:acetonitrile (80:20). The HPLC mobile phases used were sodium acetate buffer (0.05 M, pH 4.6):acetonitrile (30:70), for UV detection at 280 nm, and sodium acetate buffer (0.05 M, pH 4.6):acetonitrile:methanol (80:10:10), for post-column derivatisation. The pH was adjusted using glacial acetic acid, added drop-wise while stirring. Both mobile phases were vacuum filtered through a 0.45 μm filter and degassed in a ultrasonic water bath for 20 min. The mobile phase flow rate was set to 1 ml min^{-1}. Aqueous sodium azide solution (0.5 %) and acetonitrile (75 %) were prepared monthly and used as system maintenance solvents. 500 ml of post-column derivatising reagent was prepared by dissolving 7.5 g DMAB (Sigma-Aldrich Company Ltd., Dorset, UK) in 400 ml HPLC grade water and slowly adding 100 ml phosphoric acid (85 %). This was stirred continuously for 30 min prior to degassing in an ultrasonic water bath for 30 min. The reagent solution remained stable for 48 hours when protected from light.

2.3.3 Equipment

A Gilson ASTED™ XL system was employed, which was equipped with two Gilson 401C dilutors. A Waters 510 HPLC pump was used to deliver the mobile phase. A Waters 501 HPLC pump was used to deliver the post column derivatising reagent, at a flow rate of 0.5 ml min^{-1}. The derivatisation reagent was combined with the column eluate in a stainless steel mixing tee and reacted in a 5 foot stainless steel coil of 0.5 mm internal diameter, prior to detection at 450 nm. A Jones Chromatography column heater was used to maintain the column temperature at 25°C (for UV detection at 280 nm) and 30°C (for post-column derivatisation). The analytical column was a Waters Symmetry C18 column,

5µm, 100 Å, 4.6 x 250 mm. A Waters 486 tunable absorbance detector set at 280 nm (for UV detection at 280 nm) or 450 nm (for post-column derivatisation) was used. Chromatograms were acquired and processed using Millennium software, version 2.15. Two dialysis blocks were used: Gilson ASTED™ 100 µl dialysis block for UV detection at 280 nm and Gilson ASTED™ 370 µl dialysis for post-column derivatisation. The cellulose acetate membranes used in the blocks (100 µl, 15 kD membrane for UV detection at 280 nm and 370 µl, 15 kD membrane for post-column derivatisation) were mounted on inert polytriflouroethylene frames. Membranes were changed after approximately 50 samples. Trace enrichment cartridges (TEC) were Gilson ODS packed, Prelute 3 (70 mg, 10 mm) from Anachem Ltd., Luton, UK.

2.3.3.1 ASTED maintenance

When not in use, the membrane (over and under) and the sample lines were flushed with the sodium azide solution to prevent microbial growth. The sample lines were flushed with the acetonitrile solution to remove protein deposits, prior to storage. The trace enrichment cartridge was flushed with acetonitrile (100 %) which was used as a storage solvent.

2.3.4 Sample preparation

A 10 g quantity of homogenised porcine muscle was weighed out, which was fortified by spiking the tissue with 50 µl of a known concentration of drug solution (containing the nine sulphonamides of interest). In all assays, samples fortified at a high level of fortification and at a low level of fortification, and a drug free sample were included. 20 min after fortification, the samples were blended with saline solution (0.9 %, 20 ml) in a

stomacher for 30 min. An aliquot (10 ml) of the resulting mix was centrifuged for 30 min at 3500 rpm. 1 ml of the supernatant was filtered through a 0.45 μm syringe filter into a vial for ASTED™ extraction (dialysis and trace enrichment) and HPLC determination.

2.3.5 Dialysis and trace enrichment conditions

Sodium acetate buffer (50 mM, pH 4.6), was used as donor and recipient solutions. A sample extract (100 μl for UV detection at 280 nm, 370 μl for post-column derivatisation) was dialysed for 11 min into a 2 ml volume of recipient solution. The donor was held in static mode while the recipient was in pulsed mode (i.e. the recipient was drawn continuously at a fixed rate along the under-side of the membrane until 2 pulses of 1 ml volumes had travelled the under-side of the membrane and had passed onto the TEC). The trace enrichment cartridge was prepared by flushing with acetonitrile (500 μl) followed by water (500 μl) and finally sodium acetate buffer (50 mM, 500 μl). Following TEC loading, the analytes were removed and carried onto the analytical column by backflushing the column with HPLC mobile phase.

2.4 RESULTS AND DISCUSSION

2.4.1 Principle of automated dialysis

Dialysis is a technique capable of separating macromolecules from small molecular compounds. The technique has been applied to a variety of biological matrices including serum, plasma, whole blood, milk, egg and tissue homogenate[80,81,82,83]. Dialysis is becoming increasingly popular in residue analysis as the technique can be easily automated and is less demanding than the more traditional methods[84,85].

Figure 2.3 depicts how the ASTED™ system operates. The sample was removed from the ASTED sample rack (by means of an injection needle, powered by dilutor 1) and delivered onto the donor side of the dialysis block, via the injection port and the right hand side six port valve (A). The recipient stream was continuously renewed at a fixed rate for 11.1 min, during which time analytes and other small molecules diffused across the cellulose acetate membrane and were loaded onto the trace enrichment cartridge. The analytes were trapped on the reversed-phase material of the TEC, while other dialysates were flushed to the waste reservoir. Following concentration on the enrichment cartridge, the left hand side valve (B) switched, allowing the mobile phase to backflush the enrichment cartridge and sweep analytes onto the analytical column. The dialysis membrane was then regenerated by flushing, over and under, with water, followed by dialysis solvents. 1 ml of each was found to be sufficient to avoid sample carry-over. Sample carry-over was also avoided by bracketing the sample in the needle with air segments. This reduced dispersion of the sample along the needle. The TEC was

reconditioned by rinsing with water (500 µl), followed by acetonitrile (500 µl), water (500 µl) and finally dialysis recipient solvent (sodium acetate buffer, 500 µl).

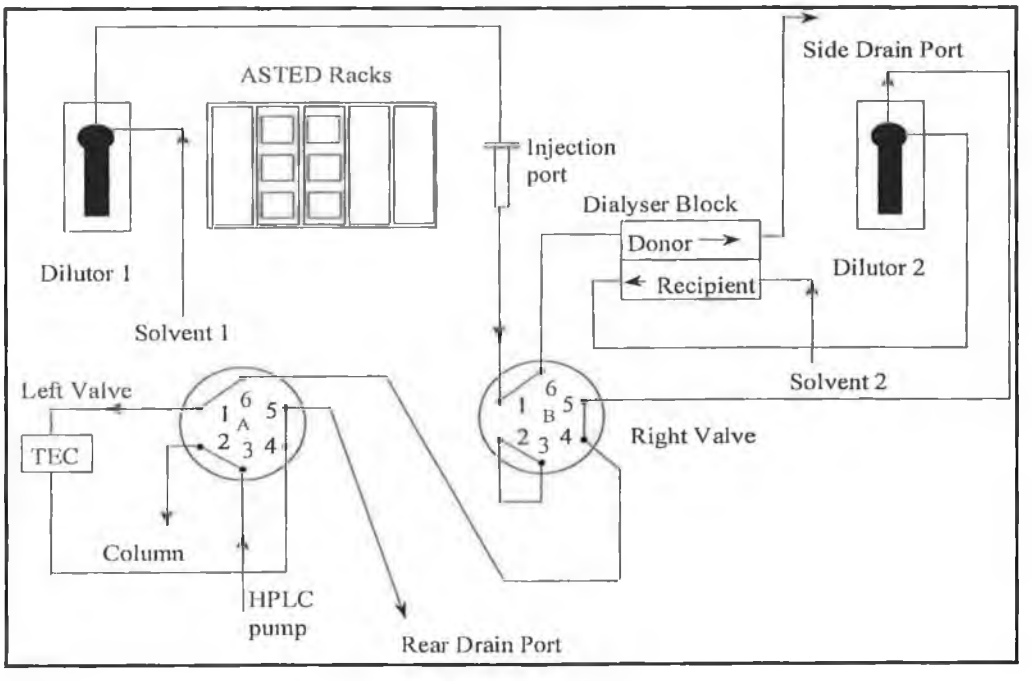

Figure 2.3 *Schematic of the ASTED™ system.*

2.4.2 Optimisation of dialysis and enrichment

Optimisation of dialysis involved investigating the nature and volume of dialysis solutions. The buffer component of the mobile phase proved to be the best donor and recipient solutions, possibly due to more selective removal of the analytes from the trace enrichment cartridge using buffered mobile phase. Mobile phase buffer (sodium acetate) was prepared at various pH values and employed as dialysis solutions. Figure 2.4 shows the detector response for sulphamethazine recovery to be greatest when the mobile phase buffer (pH 4.6) was employed as dialysis solutions. Sulphamethazine was frequently chosen as the test analyte, as this is considered the most important sulphonamide from a

residue viewpoint. In addition, it was considered to be representative of medium polarity for the analytes under study.

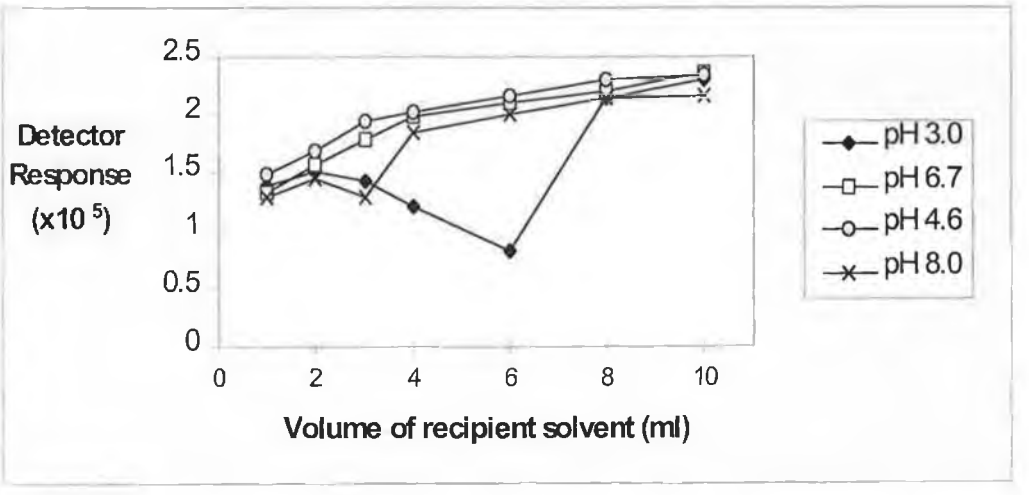

Figure 2.4 *Optimisation of dialysis solution pH, for sulphamethazine.*

Optimisation of the time allowed for dialysis involved optimising the aspiration rate of the recipient solution. The recipient solution was continuously renewed, since, as new recipient solution was aspirated in, the current solution was pushed out of the membrane onto the trace enrichment cartridge. The trace enrichment cartridge contained C18 as the packing material and therefore, it acts in a similar fashion to the analytical column, where the less polar analytes are retained and the more polar analytes elute more rapidly. As the recipient dialysis solution continuously sweeps the TEC, the analytes make their way from the head of the cartridge to the end. At a certain point, the volume of recipient solution that has swept the cartridge becomes large enough to elute the more polar analytes off the cartridge. This is termed the breakthrough volume and this volume limits the dialysis time permissible. The longer the dialysis time, the greater the volume of recipient solution.

The breakthrough volume of the TEC for the nine sulphonamides was found to be 2 ml, therefore the recipient solution was set to two pulses of 1 ml volume. The graph in Figure 2.5 shows the effect of dialysing into various volumes of dialysis recipient solution. 2 ml was chosen as the optimum volume to allow sufficient enrichment of the more non-polar analytes, without complete loss of the polar analytes. The graph shows sulphaguanidine and sulphanilamide to be non-retained, even when 1 ml of dialysis recipient solution was employed. This is due to the polar nature of these analytes. They possessed low capacity factors on the analytical column, so they were lost in the matrix front, under all recipient volume conditions, in the presence of matrix.

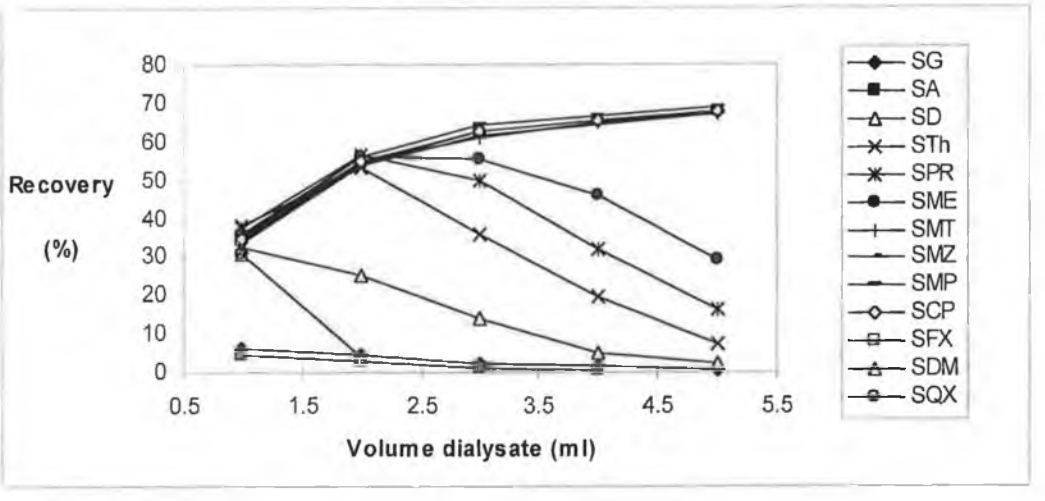

Figure 2.5 *Optimisation of TEC breakthrough volume.*

To achieve maximum dialysis time, the aspiration rate of the recipient solution was set to the minimum value possible (0.18 ml min^{-1}). Therefore, optimum dialysis time, consisting of the time to dialyse a total volume of 2 ml was 11.1 min. Figure 2.6 demonstrates that recovery was greatest, at this time, for most analytes. Longer dialysis times resulted in larger volumes of dialysate, which exceeded the breakthrough volume of the TEC.

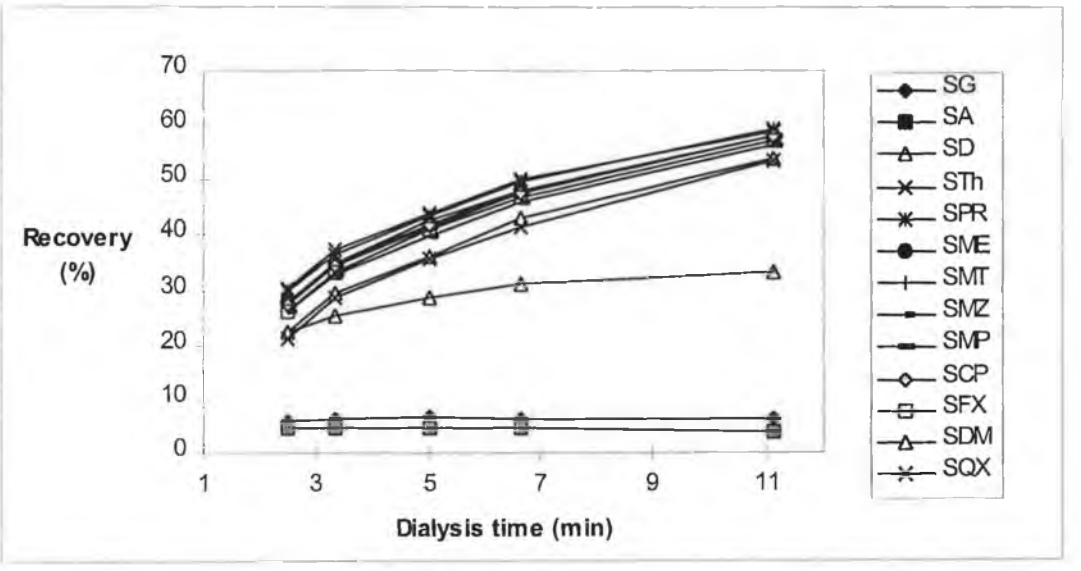

Figure 2.6 *Optimisation of dialysis time.*

It was suspected that creation of a temperature gradient might exhibit an effect on the concentration gradient that exists during dialysis. The effect of varying the recipient solution temperature (6°C, 20°C, and 41°C), while maintaining the donor solution constant at 20°C, and, conversely maintaining constant recipient solution temperature with varying donor solution temperature was evaluated, but found not to have any marked effect on recovery. A larger dialysis block, capable of dialysing 370 µl was employed for post-column derivatisation. This allowed greater sensitivity and brought the limit of determination down from 30 ng g^{-1} to 20 ng g^{-1}.

During the investigation, good trace enrichment was obtained using C18 TECs without extensive interference effects. However, Aerts *et al.*[23] stated that trace enrichment on C18 material was inadequate and resulted in too many interferences. Their chromatograms displayed matrix interferences up to 60 min and they concluded that an additional clean-up step or more specific detection was necessary. Various TEC washing procedures were

investigated, with UV detection at 280 nm being employed, as a means for removing matrix co-extractives. Flushing the cartridge with water, or with sodium acetate or sodium phosphate buffers did not remove matrix co-extractives without removing some of the retained analytes also.

2.4.3 Chromatographic separation

The electron withdrawing sulphoxide group (SO_2) on the sulphonamide molecules results in the presence of acidic NH linkages (Figure 2.2), with pKa values in the range 4.79-8.56, for the analytes under study. The pH of the mobile phase is therefore important and the mildly acidic mobile phase (pH 4.6) gave good separation for most of the sulphonamides investigated. Buffers of various pH values between 4 and 7 were investigated, to achieve optimum resolution of thirteen sulphonamides (sulphaguanidine, sulphanilamide, sulphadiazine, sulphathiazole, sulphapyridine, sulphamerazine, sulphamethizole, sulphamethazine, sulphamethoxypyridazine, sulphachloropyridazine, sulphafisoxazole, sulphadimethoxine, and sulphaquinoxaline). At a pH of greater than 5.0, retention time for some analytes increased and baseline resolution was not achieved for many analytes. Various columns were also investigated with respect to peak resolution and peak shape, and the Waters Symmetry™ column proved best. With the chromatographic conditions described, certain sulphonamides such as sulphaguanidine and sulphanilamide eluted very close to the solvent front, most likely due to the presence of free amine groups on these molecules, while other sulphonamides such as sulphaquinoxaline and sulphadimethoxine were seen to elute at 45-50 min, likely due to the presence of two aromatic rings. Mobile phase composition was varied in an effort to increase the retention of the more polar analytes and to decrease the retention of the less polar drugs. An isocratic method could

not be achieved which eluted all analytes, within 30 min, without a loss of resolution between many analytes, or poor retention of the polar sulphaguanidine and sulphanilamide. Gradient elution was not desirable, when post-column derivatisation was investigated, as fluctuations in mobile phase composition would result in baseline drift. When the anlaytes were analysed in the presence of matrix, sulphaguanidine and sulphanilamide, the early eluting drugs, were severely affected by matrix interference, therefore they were removed from validation studies. The late eluting sulphadimethoxine and sulphaquinoxalone exhibited broad peak shape, resulting in poor sensitivity. When these two analytes were removed, it was possible to shorten the run time to 30 min. Further studies, therefore, were limited to the nine sulphonamides shown in Figure 2.2.

2.4.4 Sample pre-treatment

Saline of pH 7 was used to blend samples. Various solutions differing in pH (4 to 8) were investigated. It was suspected that the pH of the extraction solution may affect the polarity of the sulphonamide residues, thereby influencing their partitioning into the extraction solution, or their ability to associate with tissue proteins. pH was not seen to have any significant effect on the extraction of the nine sulphonamides. Various deproteinisation reagents were investigated, such as trichloroacetic acid (TCA) and ammonium sulphate. Ammonium sulphate failed to give a clear supernatant following centrifugation, while TCA treatment gave a clear supernatant and firm pellet following centrifugation. Addition of 2 ml of a 50 % TCA solution to 20 ml saline, prior to stomaching with tissue, produced a clear extract and did not affect the recovery of the analytes after dialysis. However, it failed to remove chromatographic interferences and increased the size of the early eluting matrix front. Therefore, saline at pH 7 (unadjusted)

was employed as the extraction solution. Various volumes were investigated. Using 10 g of porcine muscle, a volume of less than 20 ml resulted in a non-homogenous mix, requiring longer stomaching times.

A stomaching time of 30 min was found necessary to extract the antibiotic residues. Figure 2.7 shows that longer times resulted in no significant increase in recovery and shorter times resulted in lower recoveries of analytes. A centrifuge time of 30 min resulted in a supernatant clear enough for direct injection into the ASTED system, following filtering through a 0.45 μm filter. Shorter time resulted in a more cloudy supernatant, which clogged the dialysis membrane over time.

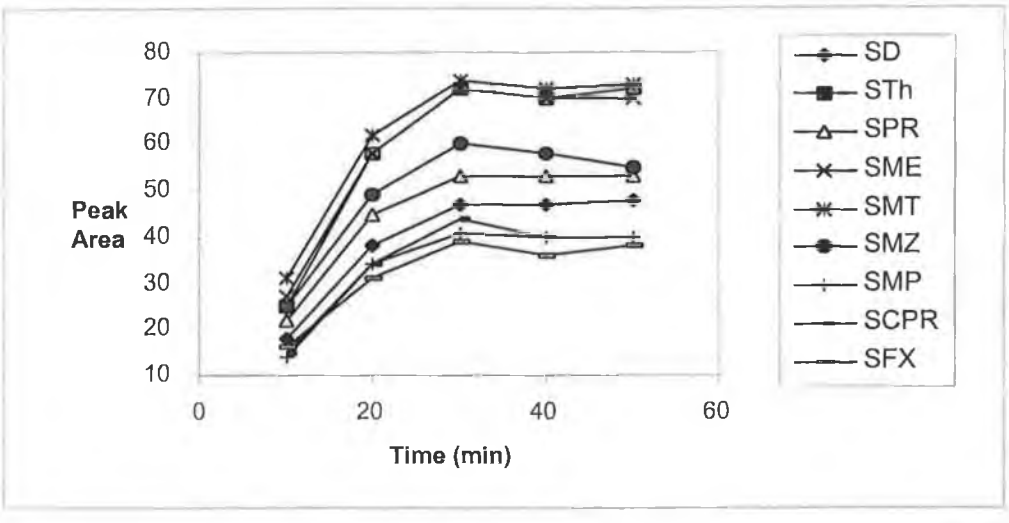

Figure 2.7 *Optimisation of stomaching time required for sulphonamide extraction.*

2.4.5 Quantification

Recovery of the method can be addressed in two ways, absolute or relative. Absolute recovery refers to the quantity of sulphonamides that are recovered after dialysis and enrichment. This can be assessed by comparing the response (peak areas) of standards chromatographed directly by HPLC, to that of standards prepared by ASTED-HPLC (i.e. dialysis, trace enrichment and HPLC). Table 2.1 shows the absolute recoveries for the nine sulphonamide residues using a 0.05 μg ml^{-1} standard drug solution. Relative recovery is the recovery of sulphonamides from the fortified sample, relative to that of the standard, both of which are prepared by ASTED-HPLC. Therefore, relative recovery reflects the capacity of the entire extraction and clean-up method to recover the sulphonamide residues from porcine tissue. As standards are processed through dialysis and trace enrichment similarly to samples, the relative recovery was used for quantitation.

Table 2.1 Absolute recoveries for nine sulphonamide drugs at 0.05 μg ml^{-1} (n=3).

Drug	*Mean Recovery (%)*	*Drug*	*Mean Recovery (%)*
SD	36.1	SMZ	54.8
STh	63.7	SMP	52.1
SPR	61.5	SCPR	49.0
SME	57.3	SFX	41.8
SMT	48.7		

Sulphonamides: Sulphadiazine (SD), Sulphathiazole (STh), Sulphapyridine (SPR), Sulphamerazine (SME), Sulphamethiazole (SMT), Sulphamethazine (SMZ), Sulphamethoxypyridazine (SMP), Sulphachloropryidazine (SCPR), Sulphafisoxazole (SFX)

Linearity of dialysed standards was compared with that of standards chromatographed directly by HPLC. Standards in the range 0.005 μg ml^{-1} to 0.5 μg ml^{-1} were prepared and linearity was comparable for both methods. R^2 values of 0.9995 were obtained for the direct HPLC measurement of the standards. ASTED-HPLC analysis gave R^2 values of 0.9992.

2.4.6 Determination with UV detection at 280 nm

The limit of determination for the nine sulphonamides was 30 ng g^{-1} (equivalent to a tissue extract concentration of 0.01 μg ml^{-1}) using UV detection at 280 nm. The method was evaluated by carrying out intra- (within day) and inter- (between day) assays on muscle samples fortified at 40, 120, and 200 ng g^{-1}. A five point calibration curve in the range 0.005 μg ml^{-1} to 0.32 μg ml^{-1} was employed. The curve proved linear between these points with an R^2 value of 0.99. Intra-assay variation was based on the results for five different fortified samples, all chromatographed in the same assay. The results are presented in Table 2.2. All recoveries were high (71-117 %). The standard deviation is relatively higher for those drugs affected by interferences (sulphadiazine, sulpamerazine). This was particularly evident at the lower levels of fortification (40 and 120 ng g^{-1}). Figure 2.8 shows chromatograms resulting for a fortified tissue sample and a residue free tissue sample (control), where matrix interference is clearly evident, affecting the sulphadiazine response. Inter-assay variation was based on the results for five different fortified tissue samples, chromatographed in five separate assays. The results are presented in Figure 2.9. Mean recoveries were in the range 80 to 131 %. Standard deviations (depicted by the error bars) were somewhat higher than those of the intra-assay and were relatively higher for sulphonamides affected by matrix interferences. For example, samples fortified at 40

ppb, resulted in mean recovery, for sulphadiazine, of 116 % with SD of 45 %, at 120 ppb, recovery was 117 % with SD of 29 %, and at 200 ppb, recovery was 130 % with SD of 33 %. Sulphonamides not affected by matrix showed high recovery and relatively low variation; for example recovery for sulphamethazine at 40 ppb was 90 % with SD of 12 %, at 120 ppb, recovery was 80 % with SD of 2 % and at 200 ppb, recovery was 91 % with SD of 9 %.

Table 2.2 **Intra-assay variation for the determination of sulphonamides in muscle using automated dialysis-HPLC with UV detection at 280 nm (n = 5).**

	Recovery of sulphonamides (Mean ± SD, %)		
Fortification level:	**40 ng g^{-1}**	**120 ng g^{-1}**	**200 ng g^{-1}**
SD	110 ± 26.5	117 ± 19.0	101 .± 3.7
STh	81 ± 10.1	84 ± 8.0	78 ± 3.9
SPR	81 ± 3.7	97 ± 9.4	87 ± 1.2
SME	86 ± 3.0	94 ± 13.5	88 ± 0.6
SMT	83 ± 6.1	93 ± 18.5	81 ± 1.8
SMZ	83 ± 17.5	93 ± 15.4	92 ± 2.1
SMP	80 ± 20.0	82 ± 5.8	80 ± 3.2
SCPR	57 ± 11.5	90 ± 6.6	71 ± 3.7
SFX	102 ± 8.9	94 ± 9.6	90 ± 4.2

Sulphonamides: Sulphadiazine (SD), Sulphathiazole (STh), Sulphapyridine (SPR), Sulphamerazine (SME), Sulphamethiazole (SMT), Sulphamethazine (SMZ), Sulphamethoxypyridazine (SMP), Sulphachloropryidazine (SCPR), Sulphafisoxazole (SFX)

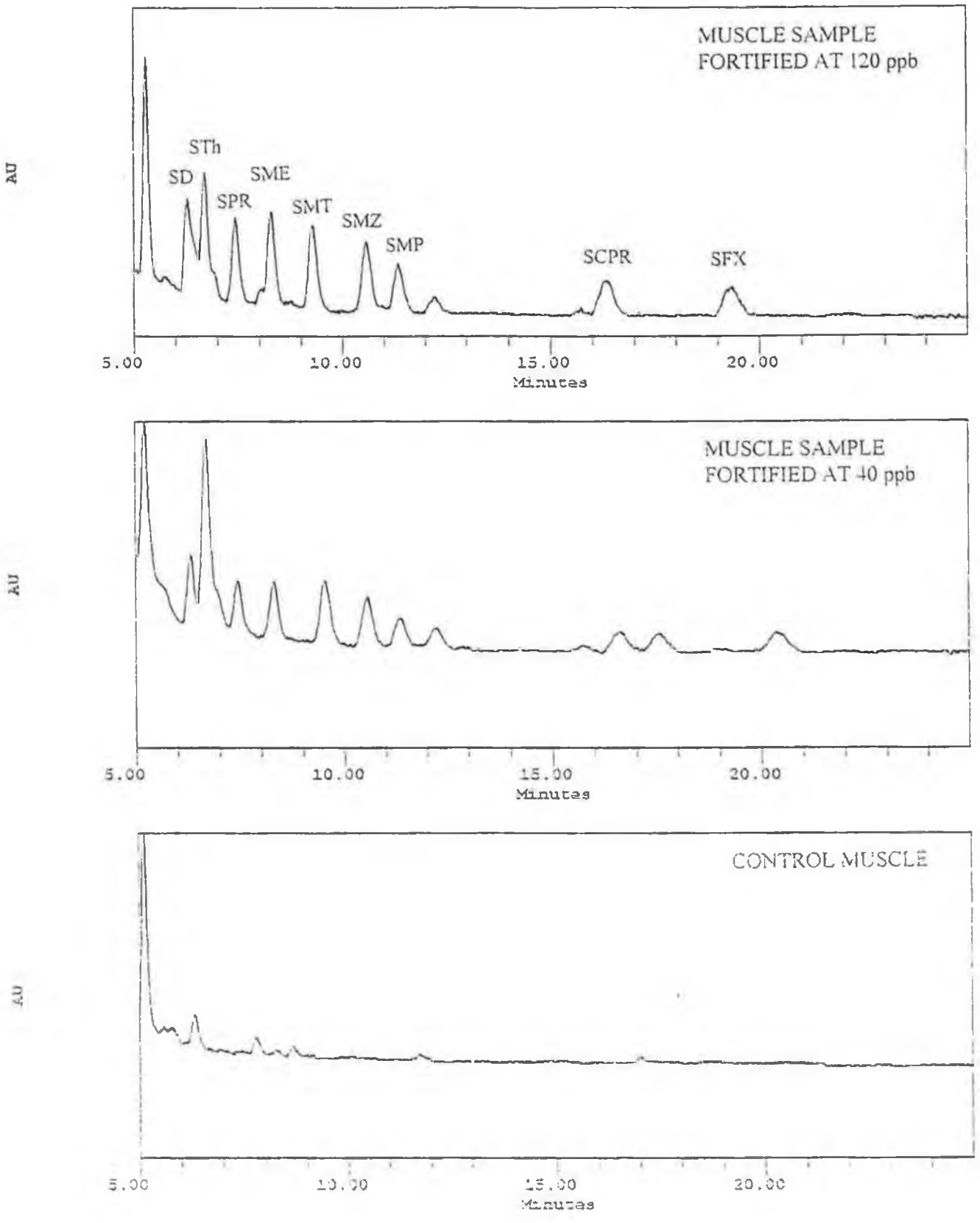

Figure 2.8 *Chromatogram showing control muscle and muscle samples fortified at 40 and 120 ppb, using direct UV detection at 280 nm.*

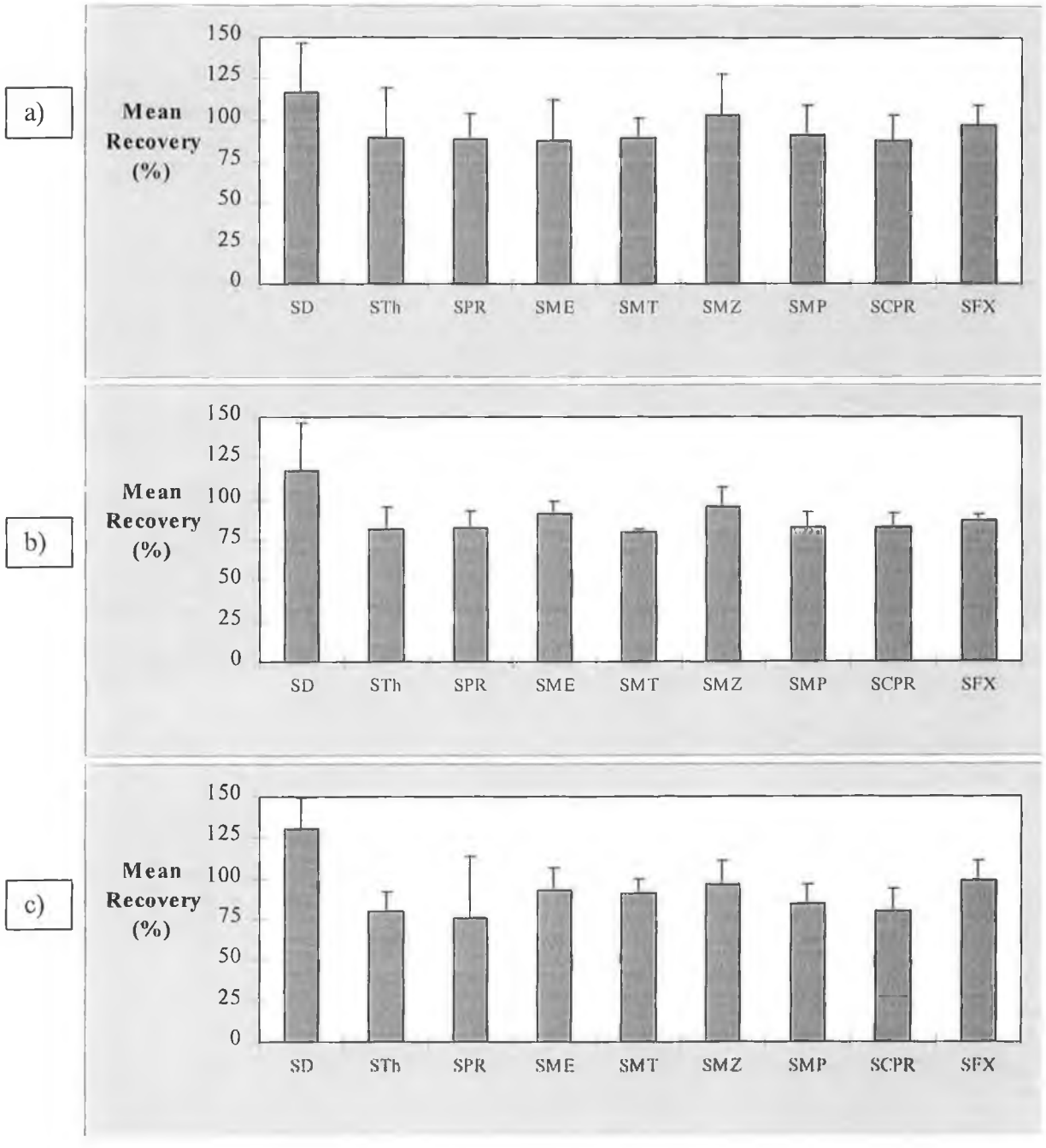

Figure 2.9 *Inter-assay variation for the determination of sulphonamides in muscle, with detection by UV at 280 nm. Bar charts show the mean recovery and standard deviation (error bars), for the nine drugs in tissue samples fortified at a) 40 ppb b) 120 ppb and c) 200 ppb in five separate assays.*

Sulphonamides: Sulphadiazine (SD), Sulphathiazole (STh), Sulphapyridine (SPR), Sulphamerazine (SME), Sulphamethiazole (SMT), Sulphamethazine (SMZ), Sulphamethoxypyridazine (SMP), Sulphachloropryidazine (SCPR), Sulphafisoxazole (SFX)

2.4.7 Post-column derivatisation

Initial attempts to overcome matrix effects were directed to the sample preparation prior to ASTED extraction. Various extraction methods were investigated including enzymatic degradation of tissue, blending using an homogenising probe and protein precipitation. The enzyme-catalysed degradation, using *Subtilisin A*, resulted in a solution of small proteins/peptides capable of dialysing across the 15 kD dialysis membrane. Blending in a stomacher was compared to blending using an homogenising probe and, since no differences were found, stomaching was chosen because of its ease of use.

Post-column derivatisation resulted in a more specific detection system. The post-column derivatisation reaction used was that recommended by Aerts *et al.*[23] and is a modification of the reaction performed according to Stringham *et al.*[61]. It was necessary to establish the optimum length and diameter of the reaction coil, so that the derivatisation reaction had adequate time to occur and non-ideal mixing of the derivatisation agent with the column eluent was prevented. In this reaction, the nucleophilic amine group common to all sulphonamides (Figure 2.1) attacks the carbonyl group on the DMAB, resulting in a nucleophilic substitution reaction. Under the acidic conditions provided by the mobile phase and the phosphoric acid in the derivatising agent, a conjugated product is formed, having absorbance at 450 nm. A coil of similar bore to that used by Aerts *et al.*[23] (0.5 mm i.d.) proved to give the best peak resolution, allowing adequate mixing of the column eluent with the derivatising reagent. Non-ideal mixing resulted in a noisy baseline, which was probably due to the high viscosity of the phosphoric acid in the derivatising agent. Aerts *et al.*[23] used a coil length of 25 ft.; in this study, the coil length was limited to 5 ft. as a longer coil resulted in poor resolution between sulphadiazine and sulphathiazole. Using

this derivatisation configuration and employing a modified mobile phase it was possible to operate at a column temperature of 30°C which was found to be more controllable than the lower temperature of 25°C used for UV detection at 280 nm.

The limit of determination for the nine drugs, using post-column derivatisation was 20 ng g^{-1}. Standards in the range 0.007 μg ml^{-1} to 0.1 μg ml^{-1} were used and a residue-free tissue sample was included with each assay. Table 2.3 shows the intra-variation for five samples fortified at two levels (40 and 100 ng g^{-1}) and assayed by post-column derivatisation, with detection at 450 nm. Figure 2.10 shows the chromatogram for a porcine muscle sample fortified at 100 ng g^{-1} and assayed using post-column derivatisation. Results using post-column derivatisation showed high mean recoveries, with inflated values for sulphadiazine. The standard deviations within a single assay were lower, compared to those when direct UV at 280 nm was used. However, using the derivatisation conditions described, it was necessary to clean the detector cell with an acidic solution regularly (between runs) to achieve an acceptable baseline, the system required an equilibration time of approximately 90 minutes, and this resulted in a method less robust than that using UV detection at 280 nm. Use of peek fittings (mixing tee and reaction coil) may assist in overcoming the problems encountered with derivatisation over time.

The method proved successful initially, but attempts to evaluate the method by carrying out an inter-assay variation study showed the system to be non-reproducible. Baseline instability and detector fouling presented problems over time. Baseline instability may be due to lack of a consistent back-pressure on the pump delivering the coloured derivatisation reagent. The flow cell in the detector appeared to become contaminated

over time due to precipitation. The precipitant may be the product formed when the DMAB reacts with the sulphonamide or may be simply due to the derivatising agent itself precipitating when combined with the 50 mM buffered mobile phase. Stringham *et al.*[61] reported baseline instability problems when using this method. They also reported that use of a deuterium lamp contributed to baseline instability due to the dramatic drop off in lamp energy output at increased wavelengths. A deuterium lamp was employed in this study. In a later publication[80] they halved the derivatisation agent concentration to 0.75 % and provided a list of possible causes for baseline noise, which included pump pulsations, leaking valves, detector lamp instability and air bubbles in the detector or reagents. Aerts *et al.*[23] reported a reduction in the limit of determination using post-column derivatisation in comparison to detection at 280 nm, due to baseline noise.

Table 2.3 **Intra-assay variation for the determination of nine sulphonamides in muscle by automated dialysis-HPLC with post-column derivatisation and detection at 450 nm (n = 5).**

	Recovery (%) of sulphonamides (Mean ± SD)	
Fortification level:	**40 ng g^{-1}**	**100 ng g^{-1}**
SD	173 ± 10.6	160 ± 10.3
STh	86 ± 4.1	82 ± 5.
SPR	100 ± 13.2	99 ± 6.3
SME	97 ± 4.4	99 ± 5.3
SMT	75 ± 4.3	74 ± 5.5
SMZ	93 ± 4.9	79 ± 3.4
SMP	97 ± 4.9	69 ± 5.0
SCPR	53 ± 6.6	56± 5.8
SFX	74 ± 5.3	94 ± 6.7

Sulphonamides: Sulphadiazine (SD), Sulphathiazole (STh), Sulphapyridine (SPR), Sulphamerazine (SME), Sulphamethiazole (SMT), Sulphamethazine (SMZ), Sulphamethoxypyridazine (SMP), Sulphachloropryidazine (SCPR), Sulphafisoxazole (SFX)

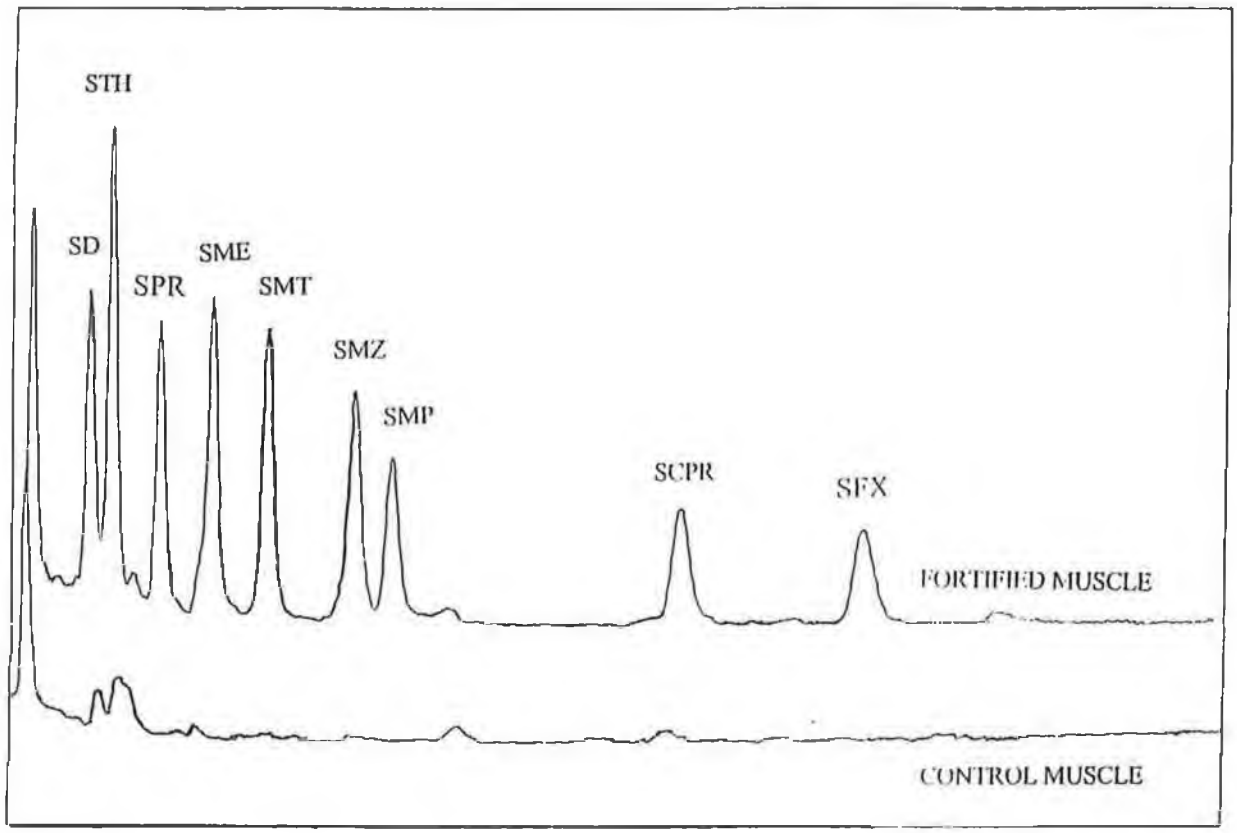

Figure 2.10 *Chromatogram for a muscle sample extract, fortified at 100 ng g^{-1} and detected, following post-column derivatisation, at 450 nm.*

2.5 CONCLUSION

Aerts *et al.*[23] analysed sulphonamide antibiotics in milk, meat and egg using dialysis and trace enrichment. They concluded that dialysis followed by trace enrichment was not suitable when using direct UV detection and found it necessary to investigate post-column derivatisation using DMAB. In the procedure described by the authors, 8 ml of sample was required for dialysis. In this study, it was observed that large sample volumes resulted in poor dialysis performance for the next sample injected, as the washing protocol was insufficient to remove the non-dialysed material on the surface of the membrane. The method developed here has been validated, to assess and confirm its ability to perform reproducibly over time, and recoveries for nine sulphonamides are presented. In this study, once the sample has mixed in the stomacher, it is subject to 11.1 min dialysis, followed by backflushing for 1 min. While the sample is being chromatographed, the next sample is dialysed and enriched. In the method of Aerts *et al.* the dialysis and trace enrichment procedure required 29 min per sample (24 min dialysis and 5 min backflushing following trace enrichment). Therefore, it can be concluded that this method offers significant advantages over previously reported dialysis methods for sulphonamides.

These results demonstrate that the ASTED™ system is a suitable sample preparation procedure for determination of sulphonamide residues in tissue. The system allows for high sample throughput, while minimising the use of solvents and operator time, when compared to other separation methods. The success of the system lies in the capacity to apply dialysis as a separation tool to remove proteins, lipids and other cell debris, without the need for extensive off-line sample preparation steps, and still result in an adequately

concentrated sample for direct injection onto a chromatographic column. Dialysis is a non-specific technique, allowing movement of all molecules below a particular size limit, and so is not specific for the analytes of interest. Despite its non-specificity, dialysis followed by trace enrichment and on-line HPLC with UV detection provided a method capable of determining nine sulphonamide residues in porcine tissue, with good recovery. Application of post-column derivatisation to detection of the extracted residues served to increase the specificity of the method, when compared with direct UV detection at 280 nm. The small improvement in the limit of determination reported using post-column derivatisation is a result of employing a dialysis block capable of dialysing a larger volume of sample extract. However, operational problems arose with this highly automated arrangement, therefore UV detection at 280 nm proved more successful.

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CHAPTER 3

MULTI-RESIDUE ANALYSIS

OF PENICILLIN RESIDUES

IN ANIMAL TISSUES

3.1 INTRODUCTION

Alexander Fleming was responsible for one of the greatest advances in medicine, when, in 1929, he discovered penicillin. He isolated a compound from the mould of *Penicillium*, which exhibited toxic effects on bacteria. Since the introduction of penicillin as an antibiotic in the 1940s, there has been a dramatic decline in the number of deaths due to pneumonia, tuberculosis and other previously fatal infections.

Penicillins are one of three naturally occurring classes of compounds belonging to the group of antibiotics collectively referred to as β-lactams. This group is comprised of penicillins, cephalosporins and cephamycins, all of which posses a β-lactam ring, the cyclic amide shown in Figure 3.1. The primary distinguishing structural feature of penicillins is the thiazolidine (five membered) ring coupled to the β-lactam ring to form what is referred to as the penam ring structure.

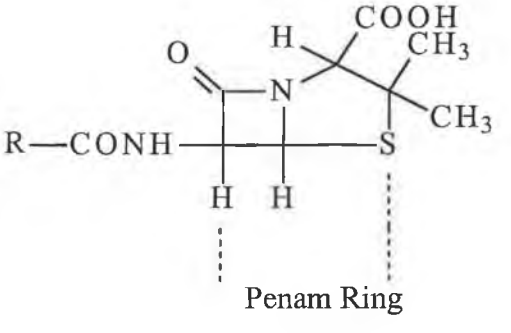

Figure 3.1 *Penicillin central structure*

Penicillin G (benzylpenicillin) and penicillin V (phenoxymethylpenicillin), produced metabolically by *Penicillium* moulds, are the natural penicillins and the amino (amoxicillin and ampicillin) and isoxazoyl (cloxacillin and dicloxacillin) derivatives are semisynthetic. Penicillins are administered by various methods for the treatment of mastitis in cows and for treating respiratory and gastrointestinal tract infections in all animals. They are also used prophylactically at subtherapeutic dose levels by addition to pig feed and poultry drinking water.

3.1.1 Mechanism of action

β-lactams elicit their antibiotic effect by preventing cell wall synthesis in bacteria. They bind to the enzyme peptidoglycan transpeptidase, thereby inhibiting its cross-linking role in building new cell walls[1]. This causes poor cell rigidity and cells undergo lysis. Over-exposure or misuse of penicillins is thought to bring about bacterial resistance, whereby the antibiotic is prevented from reaching its target (the infection site). The bacteria can modify their penicillin binding sites, thereby preventing or reducing their interaction with the antibiotic. Also they can produce enzymes, such as penicillinase, capable of modifying or lysing the drug molecule.

Allergic responses to penicillin residues have been widely documented, but very few incidences have been linked to the presence of the analytes as residues in food[2]. Anaphylactic reactions are observed only in individuals hypersensitive to penicillins. *In vivo,* degradation of the penicillin molecule occurs rapidly after administration, by rupture of the β-lactam ring. The resulting product reacts easily with proteins in the body, and the

protein-drug complex must be recognised by specific antibodies initiating the classical immune response suffered by individuals hypersensitive to penicillin.

3.1.2 Physical/chemical properties

Penicillins, as members of the β-lactam family, are polar in nature and therefore generally readily soluble in water. They are frequently formulated as salts, assisting their water solubility. β-lactam antibiotics exhibit limited stability in aqueous solutions, where they are susceptible to nucleophilic attack. They degrade rapidly at extremes of pH and are most stable in neutral pH media. Samples must be stored at very low temperatures (-76 °C) to prevent degradation[3]. The presence of the β-lactam ring causes the penicillins to be susceptible to methanolysis, where breaking of the ring results in the formation of penicilloic esters. Other organic solvents (acetonitrile, ethanol) also promote degradation of the molecules, but at a slower rate.

3.2 ANALYTICAL METHODS FOR DETERMINING PENICILLINS

As with most other classes of antibiotic, routine analysis of penicillin residues is usually performed by microbial inhibition tests. As discussed in Chapter 1, microbial inhibition tests suffer from a number of limitations, being suitable only for initial screening and, therefore, chemical methods of analysis have become more prominent. The majority of chemical methods reported have focussed on the determination of penicillins in milk. More recently, penicillin residues have been analysed in a wide range of matrices, such as animal tissue and body fluids, eggs, milk, yoghurt and cottage cheese. There are a number

of spectrophotometric and liquid membrane based methods that will not be included in this discussion as they have only been applied to the analysis of pure drug solutions[4,5].

Kidney is the target tissue for penicillin residue analysis, as residues tend to accumulate there during metabolism. Muscle, liver and spleen are also analysed, in addition to serum, plasma and milk samples. As with many other veterinary drugs, residues persist in the injection site long after they are depleted from other body tissues[6]. Depletion rates of penicillin residues from blood, muscle and kidney were examined and detectable levels persisted for up to 36 hours after administration[7]. The persistence of penicillin residues in tissues also depends on the dosage forms. Penicillin in the non-salt form degrades rapidly in the blood stream. Veterinary preparations of penicillin frequently employ a salt to retard the release of the drug in the body. Of the preparations employing a salt, sodium and potassium preparations are released most quickly and so are used as a rapidly acting treatment. Procaine penicillin, which is used intramuscularly, can be detected in the blood stream at levels in excess of the MRL for up to 24 hours after administration for therapeutic purposes. Benethamine penicillin, which is rarely used, persists for a number of days and benzanthine penicillin is retained markedly longer, exhibiting activity for seven days.

Storage of samples prior to analysis is an important factor in penicillin residue analyses. The presence of the β-lactam ring causes molecular instability. Weise and Martin[8] reported depletion of penicillin G in fortified bovine plasma after one month storage at –

70°C. Boison[9] recommended blending of muscle samples only on the day of analysis and prior storage of the unblended sample should be at –80°C.

3.2.1 Screening techniques for penicillin analysis

The "Penzyme" assay is used routinely for the determination of β-lactam antibiotics in food[10]. This uses the enzyme carboxypeptidase which releases alanine from a synthetic peptide substrate. Hydrogen peroxide and pyruvic acid are produced and the hydrogen peroxide oxidises a colourless chromogen to a coloured compound. Penicillin inhibits the carboxypeptidase enzyme, therefore no coloured compound is produced where penicillin residues are present. Other enzyme assays use β-lactamase or penicillin acylase. Medina *et al.*[11] described the screening of liver and kidney samples for β-lactams (penicillins and cephalosporins) using commercially available kits that were based on these enzymatic reactions. The assays were quick and easy to perform, with little sample preparation, but subsequent chemical determination showed a number of false negatives. While bioassay procedures can distinguish penicillin residues from other antibiotics by use of enzymes such as penicillinase, they cannot distinguish penicillins from one another, so bioassay techniques are commonly employed as an initial screening step, and chemical assays used for more specific analysis.

Penicillin residues in milk and tissue have been traditionally analysed by microbial inhibition tests. These techniques, similarly to the enzyme based methods, are incapable of distinguishing between the various members of the penicillin class, therefore they can only be used as screening techniques. The Four-plate test has been employed as a

screening test for the analysis of penicillin residues. This method has a limit of determination of 30-60 ppb for benzylpenicillin, ampicillin and amoxicillin and 300 ppb for oxacillin and cloxacillin[12]. Ang *et al.*[13] reported a study to establish the correlation between a liquid chromatography (LC) method with pre-column derivatisation allowing fluorescence detection and a microbial inhibition method for the analysis of amoxicillin residues in incurred and fortified fish muscle. They measured the percentage recovery and percentage co-efficient of variation for the two methods. They concluded that there was no statistically significant difference in the percentage recovery or variation in recovery between the two methods, but LC offered increased sensitivity and specificity. Zomer *at al.*[14] described a method for the identification and quantitation of β-lactams in milk using HPLC with detection by microbial receptor assay. Recoveries in the range 50-80 % were obtained and β-lactams were quantified at 10 ppb. This was achieved by collecting fractions from HPLC and analysing them using the Charm II test. Moats[15] also described the use of commercial test kits for the analysis of β-lactams in milk samples. He described a procedure where a penicillinase enzyme and a β-lactamase enzyme could be used to distinguish between penicillins and cephalosporins. The presence of cephalosporins will not be detected by penicillinase and will be detected by β-lactamase, as the penicillinase enzyme is only capable of hydrolysing the 6-aminopenicillanic acid ring of penicillin molecules and is inactive towards the 7-aminocephalosporinanic acid moiety of cephalosporin compounds. Several rapid screening tests have now been described for detection of penicillin residues. Table 3.1 demonstrates the determination limits for some screening methods employed for penicillin analysis[16].

Table 3.1 Determination limits for some penicillin antibiotics in milk by various microbial inhibition and enzymatic screening procedures.

	Test Sensitivity (ng ml^{-1})			
Antibiotic (MRL)	***LacTek***[17]	***Delvo test***[17]	***Penzyme***[17]	***Charm II***[16]
Penicillin G (5 ppb)	5	3	5	2
Ampicillin (10 ppb)	8	10	10	5
Cloxacillin (10 ppb)	8	30	80	35

ND = Not detectable

In 1981, Johnston *et al.*[18] published a screening method, the Swab Test On Premises (STOP), for the determination of antibiotic residues in meat and poultry tissue, with a determination sensitivity of 12.5 ng g^{-1} for penicillin G. The STOP test requires overnight incubation while many other screening methods allow results to be available in a few hours. STOP is based on the classical microbial inhibition test system.

Baker[19] described a spectrophotometric method for the determination of benzylpenicillin, methicillin and ampicillin. The method was developed for the determination of penicilloic acids in pharmaceutical preparations; these acids are reported to be responsible for the allergic response of many individuals to penicillin. The penicillin molecules were reduced to penicilloates, which are capable of reducing copper(II) to copper(I) more rapidly than penicillin. The copper(I) formed a complex that was readily detectable at 450 nm. Everest *et al.*[10] described a spectrophotometric screening method for the determination of β-lactam residues in kidney samples. The limit of determination was 50 ppb for penicillin

G. Recoveries of 84 % (with standard deviation of 4.0) were obtained. This enzyme-based assay utilises the inhibition of carboxypeptidase enzyme by β-lactam antibiotics. Sample preparation was limited to homogenisation and centrifugation.

An enzyme immunoassay (EIA) has been described for the detection of isoxazoyl penicillins (oxacillin, cloxacillin and dicloxacillin), using polyclonal antibodies that were raised against these antibiotics. Direct and indirect competitive assays were investigated using horseradish peroxidase and glucose oxidase as labels. 10-30 ng ml^{-1} were detectable in milk. Penicillin G has also been detected by EIA with a determination limit of 10 ng ml^{-1} in milk[20]. Wal and Bories[21] reported on the application of a radioimmunoassay method for the determination of penicillin G in milk, serum and urine. Antibodies were raised against the penicilloyl groups formed, on rupture of the β-lactam ring in the penicillin molecule.

3.2.2 Sample extraction/residue isolation methods

For the analysis of residues in biological matrices, the preliminary step in extraction methods is deproteinisation. Protein binding for penicillins has been reported as being significant, primarily to albumin. Binding is estimated to be 60 % for penicillin G, 20 % for ampicillin and amoxicillin and in excess of 90 % for isoxazoyl penicillins[9]. However, the absorption and elimination characteristics of penicillins in food animals indicate that penicillins would be found mainly in a non-covalently bound state in the tissues.

Organic solvents, such as methanol and acetonitrile, have been used to free non-covalently bound penicillins from macromolecules. Moats reported methanol to be less effective for deproteinisation, as β-lactams can undergo methanolysis[16]. He used acetonitrile to deproteinate milk and found the technique to be unsuitable for amphoteric penicillins (amoxicillin and ampicillin) but it gave 90-100 % recovery for monobasic penicillins (penicillin G and cloxacillin). Further clean-up steps were necessary to allow HPLC-UV detection. Trichloroacetic acid (TCA) was used by Verdon and Couëdor[22], and Ang and Lou[23], for protein precipitation in the analysis of ampicillin in milk. The use of aqueous-based precipitation reagents proved beneficial for analysis of whole milk samples as the fatty layer remaining after centrifugation could be removed from the supernatant surface. Boison *et al.*[24] used an aqueous extraction method for the analysis of penicillin G residues in liver, kidney and muscle tissue. C18 solid phase clean-up was necessary to allow pre-column derivatisation and HPLC determination. Boison[9] reported that buffer extraction is more efficient for removing unwanted matrix components than organic solvents, as only the water-soluble, polar components are partitioned into the aqueous phase, as opposed to both polar and non-polar endogenous components being partitioned into an organic solvent. This is due to the fact that most organic solvents have a wider distribution of partition constants. Using organic solvent extraction, further clean-up is required. This has been demonstrated by Hormazabal and Yndestad[25], who used acetone for the extraction of penicillin G and other β-lactams from plasma and tissues. The authors found it necessary to add 0.15 % trichloroacetic acid to the acetone to influence extraction of the residues from tissue. In addition a further SPE step was necessary to clean-up the extracts prior to LC-MS determination.

Sodium tungstate and sulphuric acid have been used as deproteinising agents in the extraction of penicillin residues. In the analysis of milk, Moats[26] reported the loss of monobasic penicillins due to binding to proteins when using tungstic acid as the precipitant, but minor losses occurred if acetonitrile was used in a 2:1 ratio of acetonitrile:milk. Recoveries of 85-100 % were achieved. The author favoured the use of acetonitrile due to the fact that extremes of pH are avoided, which may cause penicillin degradation. Back extraction of the penicillins into a buffered solution was necessary to allow a clean-up step. Boisson *et al.*[27] used sulphuric acid and sodium tungstate to deproteinise tissue for the analysis of penicillin G. They avoided the use of organic solvents in the extraction step and reported recoveries of 85-91 %. They employed an SPE clean-up technique and pre-column derivatisation.

Zomer *et al.*[14] used heat to deproteinise milk samples prior to the determination of penicillin residues. Ultrafiltration was used by Voyskner[28] for protein elimination in the analysis of penicillin G, cloxacillin, ampicillin and amoxicillin in milk. This extraction technique required no clean-up step prior to HPLC separation with UV photodiode array detection, but samples were fortified at high levels and the assay had a limit of determination of 100 ng g^{-1}. The same authors reported the recovery of penicillin G from milk to be less than 50 % when water or aqueous buffer was used for extraction, without addition of organic solvents, and reported a recovery of 80 % using an aqueous mixture of acetonitrile (40 % v/v) and methanol (20 % v/v)[29].

Munns *et al.*[30] used enzymatic hydrolysis of the β-lactam ring to form penicilloic esters of eight neutral penicillins in the determination of penicillin residues in milk. The authors described the hydrolysis of penicillin molecules to their ester derivatives to expand the number of analytes that can be separated under similar chromatographic conditions. Hydrolysis of the β-lactam ring can be easily achieved using sodium hydroxide, but the authors reported low recoveries using this method due to binding of analytes to proteins. They employed penicillinase to hydrolyse the β-lactam ring, which operates at the normal, slightly acidic pH of milk, and protein binding was not significant at this pH. Acetonitrile was used to precipitate proteins, and lipids were removed using methylene chloride. Since the formation of fluorescent derivatives was desired, it was necessary to liberate the side chain that has a terminal aldehyde, to allow its participation in the derivatisation reaction. This was accomplished by reacting the ester with mercuric chloride, following extraction.

3.2.3 Clean-up methods for penicillin analysis

Since the aqueous or organic extract resulting after deproteinisation may be very dilute and contain co-extractives, which inhibit analyte detection, a combined concentration and clean-up procedure is usually necessary. As previously mentioned in Chapter 1, liquid-liquid partitioning and solid phase extraction are frequently employed to clean-up sample extracts prior to chromatographic analysis.

Solid phase extraction has been the most popular clean-up technology applied to the analysis of penicillins. Nagata *et al.*[31] have used Florisil columns in the clean-up of

ampicillin from fish tissues. A methanol extraction step preceded SPE. The normal phase (silica) SPE column allowed the amphoteric analyte to be retained while non-polar interferences were removed using acetonitrile and diethyl ether. Munns *et al.*[30] used solvent extraction followed by silica gel clean-up for the extraction of eight neutral penicillins from milk. C18 SPE has been the most widely used packing in penicillin SPE analysis. Terada and Sakabe[32] extracted ampicillin, penicillin V and penicillin G, by the passage of undiluted milk through a C18 SPE column. Analytes were eluted using methanol. Boison *et al.*[33] used Bond Elut™ C18 columns whereas Terada *et al.*[34] used Sep-Pak™ C18. The latter columns had less than 14 % carbon loading and the Bond Elut™ had a carbon loading of 18 %. Boison *et al.* found that a minimum carbon loading of 17 % in the packing bed was necessary to achieve greater than 70 % recovery using reversed-phase SPE. This is an important point to consider when applying C18 SPE to penicillin residue analysis.

Ion-exchange chromatography was employed by Petz and Meetschen[35] for the clean-up and concentration of derivatised penicillins extracted from liver and kidney tissues. The authors employed GC determination for the analysis of seven penicillin residues. Solid phase extraction disk columns were employed by Ishida *et al.* for the extraction of ampicillin from serum[36]. Styrene divinylbenzene was the sorbent employed, which possesses both reversed-phase and ion-exchange characteristics. Ibach and Petz[37] employed SPE with restricted access packing for the determination of the isoxazoyl penicillins in milk. Alkyl-diol silica was used, with the internal surface of the particles being C18 and so capable of trapping the analytes while the external surface was

hydrophilic. Size exclusion interactions with this sorbent also assisted in excluding matrix co-extractives.

Liquid-liquid partitioning has also been reported for clean-up of penicillin residues. Verdon and Couëdor[38] used a phosphate buffer/isooctane medium while homogenising muscle tissue. Following centrifugation, the lower aqueous layer, containing the penicillin residues, was subject to C18 SPE clean-up, while the fats and any other non-polar tissue constituents were removed in the organic layer. A similar approach was taken by Fletouris *et al.*[39] for the analysis of milk. Dichloromethane was employed as the organic solvent to remove the non-polar constituents, while the penicillin residues partitioned into phosphate buffer. The authors report further clean-up resulting when the penicillin anions were converted to ion-pairs with tetrabutylammonium (TBA) cations, since the formed pairs were readily extracted into chloroform. Recoveries of 82.9 % were determined with relative standard deviation between 3.8 and 6.6 % for penicillin G, penicillin V, oxacillin, and cloxacillin. Blanchflower *et al.*[64] also used tertabutylammonium hydrogensulphate to assist the partitioning of extracted residues from buffer into dichloromethane. Partitioning into dichloromethane was most efficient when samples were acidified, to suppress the ionisation of the carboxylate moiety. However, at low pH values, degradation of the β-lactam ring occurred, therefore it was necessary to reduce the polarity of the molecules using an ion-pair. Moats[41] also reported increased recoveries when tetraethylammonium chloride was added to milk extracts containing ampicillin and amoxicillin. Moats[40] employed liquid-liquid partitioning in the clean-up of beef and pork tissue, during the analysis of penicillin G and cloxacillin residues. Acetonitrile was used to precipitate

proteins and the resulting extract was partitioned between dichloromethane and pH 2 buffer followed by partitioning into pH 7 buffer.

Automated liquid chromatographic clean-up has been described by Moats[41] for the determination of amoxicillin and ampicillin in milk. Milk samples were deproteinated using acetonitrile prior to LC fractionation on a reversed-phase column. The collected fractions were concentrated prior to HPLC analysis using a similar column, but with ion-pair chromatography and UV detection.

3.2.4 Quantitative techniques

In addition to the non-specific spectrophotometric techniques already discussed, chromatographic techniques have been the most widely applied residue quantitative techniques for penicillins. Capillary electrophoresis has been employed by Tsikas *et al.*[42] for the separation of six penicillin and four cephalosporin residues in pharmaceutical preparations. Isotachophoretic analysis was employed to achieve separation and concentration. No capillary electrophoresis methods have been reported to date for the analysis of penicillin residues in milk or animal tissue.

Thin layer, gas and liquid chromatography have all been reported for the analysis of penicillin drug residues in biological matrices. HPLC has proved to be the most popular method; using HPLC, the drugs can be directly detected due to their physiochemical

properties while GC analysis requires the formation of volatile thermally-stable derivatives.

3.2.4.1 Gas chromatographic techniques

Petz and Meetschen[35] reported a gas chromatography (GC) method using nitrogen phosphorus detection. The penicillins were extracted from milk using acetonitrile and extensive clean-up was performed by partitioning between buffer and organic phases prior to methylation with diazomethane. The method had sensitive detection limits (2 ppb), but it could not be used to determine amino penicillins, such as ampicillin and amoxicillin (which are used in veterinary medicine[43]), due to problems associated with their derivatisation. Mineo *et al.*[44] have published a GC method with flame ionisation detection to determine ampicillin, amoxicillin, penicillin G, penicillin V, oxacillin, cloxacillin and dicloxacillin, in addition to 15 other antibiotics in tissue. This method requires the formation of trimethylsilyl derivatives and many analytes co-eluted under various GC conditions. The limit of detection was 100 ppb for the penicillin residues. Tissue samples were extracted using trichloroacetic acid and extracts cleaned-up using Amberlite XAD-2 SPE.

3.2.4.2 Thin layer chromatography techniques

Planar chromatography has been described for the quantitative determination of ampicillin residues in milk and muscle[45]. Sodium hydroxide hydrolysis of the β-lactam ring was carried out which allowed a reaction with mercuric chloride, resulting in a fluorescent

compound. This compound was extracted from the matrix and concentrated on a silica column before being deposited on a silica TLC plate. The method allowed determination of ampicillin residues in milk and muscle fortified at 4 and 50 ppb, respectively. Salisbury *et al.*[46] have described a multiresidue TLC method coupled with bioautography for the determination of various antibiotic residues including penicillin G in animal tissue. A multi-step solvent extraction method was employed. The authors reported on growth inhibition of the micro-organism with some antibiotic media. Penicillin residues were readily identifiable based on the R_f value and the characteristic serrated-edge zone of inhibition. Milk was spotted directly onto TLC plates for penicillin analysis, without prior extraction[47]. Silicone TLC plates were employed and bioautographic determination was achieved by overlaying the TLC plates with agar seeded with *B. stearothermophilus* spores, followed by incubation.

3.2.4.3 Liquid chromatographic techniques

Liquid chromatography has been the method of choice for determination of most antibiotics, and β-lactams are no exception. Detection methods employed include direct detection of the native analyte, such as by UV, amperometric, conductimetric and spectrometric detectors, or fluorescence detection, which requires pre- or post-column derivatisation. Most HPLC methods employ reversed-phase C8 or C18 columns for separation of the analytes from each other and from matrix co-extractives. The presence of the carboxyl group (COOH) on all the analytes and the amino groups on the amphoteric penicillins render them polar analytes, suitable for reversed-phase separation. Elution is frequently achieved using buffers ranging from pH 2 to 7, in the presence of organic

modifiers (methanol and acetonitrile). The pKa of the acidic groups is in the region 2.6-2.76 for all penicillins and the pKa of the amino group is 7.25. Therefore, at pH's below 7 the amino groups, when present, are protonated, and the carboxyl groups are unprotonated, conveying polarity to the molecule resulting in earlier elution.

Moats[52] described a method employing a polymeric column for the HPLC determination of penicillin G in milk. The same column was used for LC fractionation clean-up. Various techniques were applied in an attempt to alter the retention times of analytes relative to other matrix co-extractives present. The nature and concentration of the modifier had little effect. Changing pH to convert the compound from the salt form to the acid form delayed retention. Inclusion of an ion-pair in the mobile phase also increased retention. Moats[41] also used ion-pair to achieve adequate chromatography during the analysis of amphoteric penicillins in milk. Tetrabutylammonium hydrogen sulphate[39] has been employed as an ion-pair for the isocratic resolution of penicillin G, penicillin V, oxacillin and cloxacillin. The inclusion of ion-pairs was investigated when attempting to develop a multiresidue, isocratic method that avoided mobile phase buffers of low pH, which can cause instability problems for penicillin analysis. Blanchflower *et al.*[64] used ion-pair chromatography to minimise the differences in retention of standards relative to sample extracts. Takeba *et al.*[48] used ion-pair chromatography for the determination of five penicillin residues in milk. To achieve retention of amoxicillin on a reversed-phase column, the authors tested the performance of five different alkylsuphonate salts and reported that decanesulphonic acid gave optimum retention. Ion-pair chromatography has been described by Voyksner *et al.*[49]. The authors used a variety of alkylsulphonates for the separation of a number of penicillins extracted from bovine milk. Both C8 and C12

alkylsulphonates were used individually or in combination. The choice and concentration of ion-pair reagent affected the sensitivity of the method. Photo diode array (PDA) detection was used, the authors reporting that sensitivity using UV-PDA was superior to thermospray MS.

HPLC with direct UV detection between 200-285 nm has been well documented for penicillin residues with detection limits in the region of 30-50 ppb[50, 51, 52, 53]. However, the chromatograms presented in many papers are relatively complex with a number of matrix peaks evident. Moats[52] concluded that it was necessary to inject the equivalent of 1 ml of milk to achieve the required sensitivity (10 ppb), using HPLC-UV. This demanded an assay with concentration and clean-up capabilities. An additional factor in UV detection of penicillin residues is the form in which the antibiotic is administered. As discussed earlier, penicillin G is frequently administered as procaine penicillin, to provide a slow release mechanism. Procaine is a ring structure, capable of UV absorption, therefore, 2 peaks are observed in the UV spectrum, one from procaine and one from the active form of the drug.

Liquid chromatography combined with electrochemical detection of penicillins in milk has been described by Kirchmann *et al.*[54]. Pulsed amperometric detection (PAD) using a gold electrode resulted in a detection limit of 6.6 ppm for penicillin G. Similar sensitivities were obtained for ampicillin, amoxicillin, cloxacillin, dicloxacillin, methicillin, nafcillin, oxacillin and penicillin V. Pulsed electrochemical detection has also been described by LaCourse and Dasenbrock[55] following reversed-phase chromatography

for the direct detection of β-lactams and lincomycin. Detection limits were typically 10 ppb and the method was applied to the analysis of chicken feed and pharmaceutical preparations.

Many HPLC detection techniques for penicillins minimise matrix interferences in an attempt to enhance detection sensitivities by employing derivatisation techniques that allow UV detection at longer wavelengths or fluorescence detection. Derivatisation may be pre- or post-column, and result in the formation of stable derivatives with strong absorbance properties. The most commonly used pre-column technique for penicillin residues is the formation of mercaptide complexes of penicillanic acids. This method was employed in the study reported in this chapter and involves the use of mercuric chloride and 1,2,4-triazole to form mercury mercaptide derivatives with UV absorption at 325 nm. Pre-column derivatisation with 1-hydroxybenzotriazole is a slight modification of this reaction in which benzoic anhydride is used instead of acetic anhydride for initial acetylation of the amino containing penicillins. This method has been employed by Shah *et al.*[56] in fermentation media analysis. Most methods reported using this form of derivatisation employ EDTA or sodium thiosulphate as ion-pairs in the mobile phase to promote chromatographic efficiency.

Complexing with copper(II) acetate has been used to determine benzylpenicillin in pharmaceutical preparations. Copper reacts in a stochiometric ratio of 2:1 to form a green complex with a maximum absorbance at 650 nm. However, this method has not been employed routinely for the analysis of penicillin residues in food, as the complex is only

stable for up to 25 min. Askal *et al.*[57] described a method in which 2,3-dichloro-5,6-dicyano-ρ-benzoquinone (DDQ) or 7,7,8,8-tetracyanoquinodimethane (TCNQ), acting as electron-acceptors, react with several penicillins which behave as electron donors to produce highly coloured radical ions. With DDQ an orange-red complex is formed with a strong absorbance at 460 nm or with TCNQ a complex with an absorbance maximum of 842 nm is formed. This method has not been reported for determining penicillin residues in food.

Pre-column derivatisation frequently allows fluorescence detection of penicillin residues. Reaction of penicillins with dansylaziridine results in highly fluorescent derivatives and has been used to detect eight neutral penicillins in milk by Munns *et al.*[30]. In this procedure, the β-lactam ring was hydrolysed to form a penicilloate that reacted with mercuric chloride to liberate a penicilloaldehyde product, which was extracted into methylene chloride. In this medium, it reacted with dansylaziridine forming a fluorescent compound, which was measured at an excitation wavelength of 254 nm and an emission wavelength of 500 nm. The method had a limit of determination of 25 ng g^{-1} but showed low recoveries (13 to 34 %) for cloxacillin and dicloxacillin due to incomplete hydrolysis. Coefficients of variation were high (21 to 34 %) due to variation in the hydrolysis step.

Pre-column derivatisation with 9-fluoroenylmethyl chloroformate in borate buffer has been applied to the analysis of penicillins containing an amino side chain. This method was employed by Shah and Adlard[58] for detecting penicillins in fermentation media, with determination limits of 10-50 μg ml^{-1}. Fluorescent detection of amphoteric penicillins in

milk, those containing an amino group, has been described by Ang and Luo using formaldehyde[59] and using salicaldehyde[60]. The degradation products of the penicillins using these aldehydes have fluorescent properties when excitation wavelength of 354 nm and emission wavelength of 445 nm were used and limits of detection of 1 ng ml^{-1} were reported.

Berger and Petz reported a method that uses 4-bromomethyl-7-methoxycoumarin (BrMmC) to form stable esters using 18-crown-6-ether as a catalyst for the determination of several penicillins in milk[61]. In non-aqueous media, 18-crown-6-ether catalyses the esterification of the carboxyl group on penicillin molecules. The ether reacts with the penicillin molecule, rendering the carboxylic acid group very reactive and unstable. This subsequently reacts with BrMmC to yield a product that can be detected fluorimetrically with an excitation wavelength of 320 nm and an emission wavelength of 400 nm. The reaction goes to completion when a 3-fold excess of BrMmC is present, so samples of very high residue concentration required dilution.

3.2.4.4 Liquid chromatography with mass spectrometric detection

Fundamental studies have been conducted to characterise the fragmentation pattern of penicillins using various MS ionisation techniques[62]. Using thermospray MS, β-lactams show intense ions with little or no fragmentation, as opposed to electrospray MS where fragment ions are easily induced[1]. A characteristic ion is frequently observed ($[M + H - 26]^+$), which is attributed to the thermally induced hydrolysis of the β-lactam ring, followed by loss of CO_2.

LC-MS has more recently been reported for the analysis of penicillin residues in milk and tissue. Voyskner *et al.*[49] were the first to report a method for the determination of amoxicillin, ampicillin and cloxacillin in milk. These authors described a conventional HPLC method employing ion-pair in the mobile phase for the determination of native analytes in milk. Development of a confirmatory procedure using thermospray–MS necessitated the introduction of an alternative LC method that was compatible with MS detection. The authors also reported problems with the MS detection of ampicillin due to matrix interference effects, resulting in the inclusion of an additional clean-up step, which allowed determination down to 200 ppb. The LC-MS method developed was further applied to investigate the degradation products of cloxacillin in various organic solvents[63].

Electrospray MS has been described by Blanchflower *et al.*[64] for the analysis of penicillin G, penicillin V, oxacillin, cloxacillin and dicloxacillin in muscle, kidney and milk. Liquid-liquid extraction was employed followed by negative ion, full scan electrospray MS confirmation. In contrast to many other MS methods for antibiotic residue analysis, the authors reported lower sensitivities when positive ion monitoring was employed. Single ion monitoring was used and chromatograms were relatively free from interference for all analytes except penicillin V. However, the authors do report ion-suppression, where buffer ions present compete with the extracted analyte ions for ionisation, causing a decrease in the signal. Nevertheless, determination limits were in the region of 25 ng g^{-1} in meat and 2 ng g^{-1} in milk for penicillin G and oxacillin, and 50 ng g^{-1} and 5 ng g^{-1} for penicillin V and cloxacillin in meat and milk, respectively. Hurtaud *et al.*[65] used particle beam LC-MS with negative ion chemical ionisation (NICI) for the confirmation of oxacillin, cloxacillin and dicloxacillin residues in bovine muscle tissue. The authors

concluded that NICI was more sensitive than electron impact, and single ion monitoring allowed detection sensitivities of 40 ppb for oxacillin and 50 ppb for cloxacillin.

3.2.5 Summary

There are a number of methods reported for the determination of benzylpenicillin in tissues and biological fluids. However, there is a lack of multi-residue methods, particularly methods capable of determining the amphoteric (ampicillin, amoxicillin), isoxazoyl (cloxacillin, dicloxacillin) and natural penicillins (penicillin G, penicillin V) in one analytical procedure. The methods that are available frequently employ labour intensive techniques, that use large quantities of organic solvents. For many of the methods published, no validation data is presented. Therefore, the aim of this study was to investigate the development of a multi-residue method for the determination of penicillin antibiotics used in veterinary medicine. MSPD provided a simple extraction procedure, which was low on chemical consumption and did not require elaborate equipment. The resulting extract was cleaned-up using SPE, which allowed fast and efficient extract clean-up and concentration. Pre-column derivatisation was employed, which increased the sensitivity and specificity of the method, and allowed reversed-phase chromatography without the need for ion-pair reagents. The present study incorporates five penicillin residues (penicillin G, amoxicillin, ampicillin, cloxacillin and dicloxacillin) and a method was developed for their determination in porcine tissue. The method was validated at levels below the MRL for the residues in muscle tissue.

3.4 EXPERIMENTAL

3.4.1 Introduction

The aim of this study was to develop a multiresidue method for the analysis of penicillins in animal tissue. Chromatographic systems were initially evaluated on the basis of multiresidue separation, peak profile and sensitivity. Pre-column derivatisation methods were investigated using both UV and fluorescence detection. A pre-column derivatisation technique using 1,2,4-triazole was chosen. Gradient elution was necessary to elute amoxicillin, ampicillin, penicillin G, cloxacillin and dicloxacillin derivatives from a C18 reversed-phase column using a phosphate buffer/acetonitrile mobile phase. The structures of these drugs are illustrated in Figure 3.2. Initial studies focussed on optimising the derivatisation reaction. Extraction of the five drug residues was performed using MSPD. The resulting extract contained interferences that prevented derivatisation of the residues present, therefore, a clean-up step was required. C18 SPE proved to be the most beneficial method, applying the sample to the column using phosphate buffer and eluting in acetonitrile.

3.4.2 Chemicals and materials

All solvents were of analytical HPLC grade and purchased from Merck Ltd., Dorset, U.K.

a) Preparation of standard solutions

Benzylpenicillin (Pen G), Ampicillin (Amp), Amoxicillin (Amox), Cloxacillin (Clox) and Dicloxacillin (Diclox) were purchased from the Sigma-Aldrich Company Ltd., Dorset, U.K. Stock solutions of 1 mg ml^{-1} were prepared monthly in HPLC grade water and

stored at -20°C. Dilutions were prepared daily in 100 % HPLC grade water. Buffer salts were of Analar grade and purchased from Merck Ltd., Dorset, U.K. These were prepared with HPLC grade water as required.

b) MSPD materials

Isolute® bulk end-capped C18 MSPD grade sorbent with mean particle size of 40-70 μm was used (International Sorbent Technology, Hengoed, Mid-Glamorgan, U.K.). The sorbent was prepared by washing with three volumes of hexane followed by three volumes of methanol in a 25 ml plastic syringe barrel. Pestles and mortars (external diameter 75 mm; capacity 70 ml), used for MSPD extraction, were made of glass and obtained from Fisons Scientific (Loughborough, UK). The syringe filters employed for filtration of the tissue extract in buffer were made from cellulose and were 0.45 μm porosity and 25 mm diameter (Sartorius, Göttingen, Germany).

c) SPE materials

For SPE, Bond-Elut™ C18, 3 cc, 500 mg columns were used (Varian, Harbor City, CA, U.S.A.). 0.2 M phosphate buffer was prepared by dissolving 6.23 g $Na_2HPO_4.2H_2O$ and 10.405 g $NaH_2PO_4.H2O$ in 300 ml HPLC water. This was stirred to dissolve the salts and made up to a final volume of 500 ml. The pH of this buffer was 6.5 on preparation.

d) Derivatisation reagents

0.2 M acetic anhydride was prepared by making 200 μl 98 % acetic anhydride (Sigma-Aldrich Company Ltd., Dorset, U.K) up to 10 ml with acetonitrile. 1 M 1,2,4-Triazole (Fluka, Dorset, UK) was prepared by dissolving 6.89 g in 25 ml water, 10 ml 10^{-3} M mercuric chloride (0.136 g in 50 ml HPLC water) was added, the pH adjusted to 9.0 with 5 M NaOH and the solution made up to 50 ml with water.

3.4.3 Instrumentation

A Waters 616 pump (Waters Chromatography, Milford, MA, U.S.A.) was used in gradient mode to deliver the mobile phase through a Waters Symmetry® C18, 5μm, 100Å, 4.6 x 250 mm analytical column. A Waters 486 tunable absorbance detector was set at 325 nm. A Waters 717 autosampler was used to inject samples and standards. A Jones Chromatography column heater (Hengoed, Mid Glamorgan, U.K.) was used to maintain the column temperature at 30°C. Chromatograms were acquired and processed using Waters Millennium® software, version 2.15. A water bath set at 60°C was employed for derivatisation.

3.4.4 Methods

Sample fortification, extraction and clean-up procedures are described in the following sections. Derivatisation and chromatographic details are also described and the protocol undertaken for method validation is outlined. A schematic presenting a brief overall view of the method is presented in Figure 3.3.

3.4.4.1 Tissue fortification

All muscle samples were stored at -20°C and thawed to 4°C prior to analysis. Muscle tissue from animals certified as not being treated with antibiotics was used for fortification. After weighing the tissue (1.0 g), samples were fortified by pipetting an aqueous solution of the drugs (20 μl) onto the tissue. The samples were held at room

temperature for 15 min prior to extraction to ensure the sample had absorbed the fortification solution.

3.4.4.2 MSPD extraction

1.0 g of fortified pork muscle tissue (see section 3.4.4.1) was weighed out into a glass mortar and 3 g of pre-washed C18 sorbent (see section 3.4.2 b) was added. The muscle and sorbent were blended using a glass pestle for 30 sec. The resulting mixture was removed from the mortar using a stainless steel spatula and transferred to a 10 ml plastic syringe barrel containing two filter paper discs. The syringe barrel was tapped to settle the mixture and two filter paper discs were placed at the head of the column. The mixture was compressed to a volume of 7.5 ml with a syringe plunger (from which the rubber seal has been removed). A 100 µl pipette tip was attached to the syringe outlet to control flow of eluate off the column. The column was washed with 10 ml hexane. After the solvent had passed through the column, a positive pressure was applied with a pipette bulb to remove any remaining solvent. Penicillins were eluted from the column using 12 ml methanol. The pipette bulb was used as before to initiate the flow and to subsequently remove any remaining methanol when the flow had ceased. The methanol extract was centrifuged at 15,000 rpm for 15 min at 4°C. The supernatant was evaporated under nitrogen at 40°C.

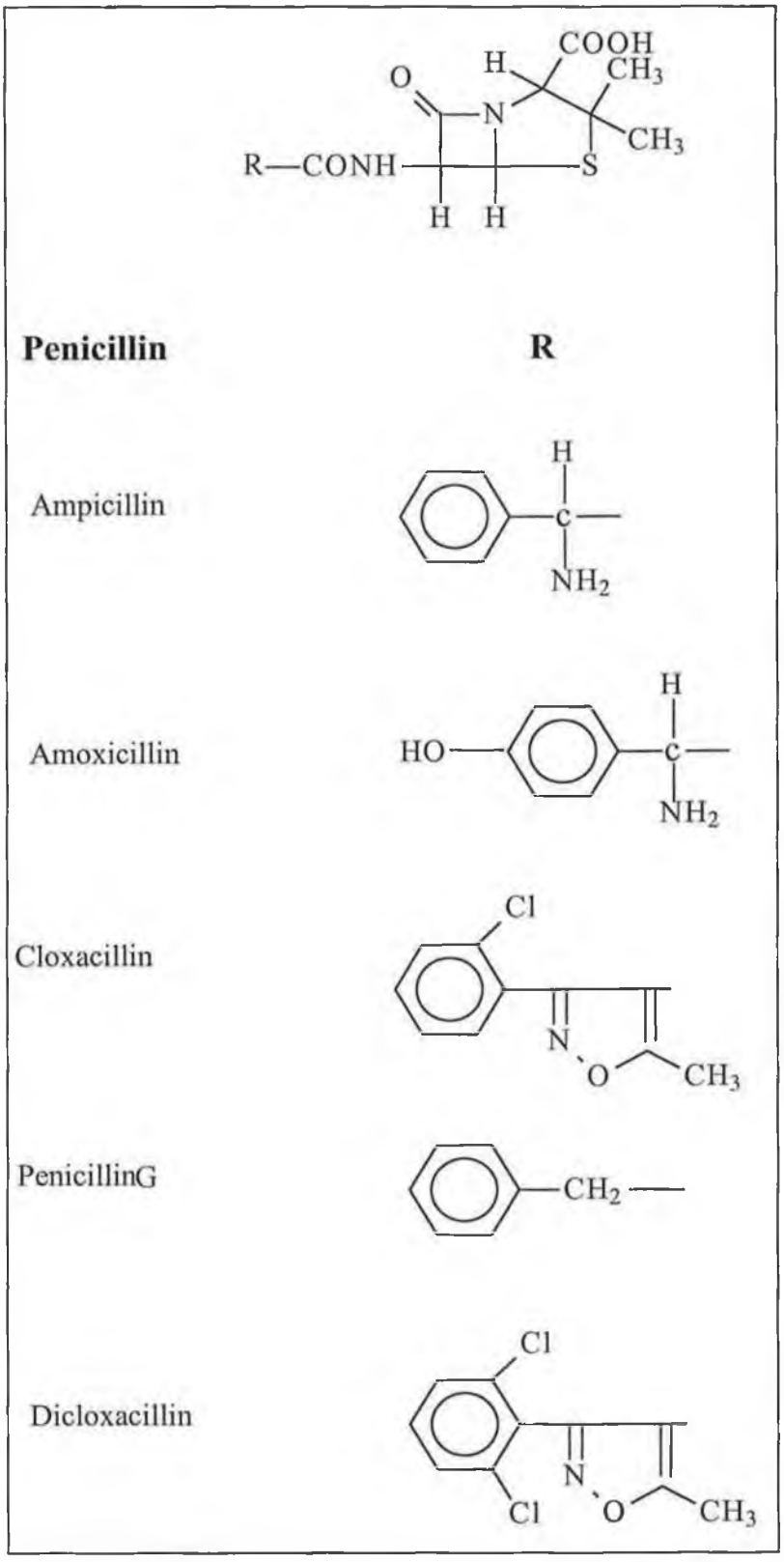

Figure 3.2 *Structural features of the penicillins.*

3.4.4.3 C18 SPE procedure

The residue was reconstituted in 6 ml phosphate buffer (see section 3.4.2 c) by vortexing for 30 sec and sonicating for 5 min to ensure complete dissolution of the residue. After filtering through a 0.45 μm syringe filter, this solution was applied to the C18 column (see section 3.4.2 c) which had been conditioned previously with 2 ml methanol, 2 ml water and 2 ml phosphate buffer (0.2 M). Initially the extractant was allowed to flow through the column under gravity for approximately 60 min after which time a slight vacuum (100 mm Hg) was applied. Following sample application, a water wash of 3 ml was applied to the SPE column, after which the penicillins were eluted using 4 ml acetonitrile. The acetonitrile was evaporated under nitrogen at 40°C prior to reconstitution in 400 μl water.

3.4.4.4 Derivatisation conditions

20 μl acetic anhydride (see section 3.4.2 d) was added to 400 μl standard or sample. This solution was vortexed for 20 sec. After 5 min, 400 μl of the derivatisation agent (see section 3.4.2 d) was added, the tube vortexed again for 20 sec before being incubated for 150 min at 60°C.

3.4.4.5 Chromatographic conditions

Phosphate buffer was prepared as follows: 0.075 M monobasic sodium phosphate (11.70 g $NaH_2PO_4.2H_2O$) and 0.035 M dibasic sodium phosphate (6.229 g $Na_2HPO_4.2H_2O$) containing 0.0157 M sodium thiosulphate (3.896 g $Na_2S_2O_3.5H_2O$) were dissolved in 600 ml of HPLC grade water. This was stirred to dissolve the salts and then made up to 1000

ml with water. Buffer and acetonitrile were vacuum filtered through 0.45 μm filter and degassed for 20 min in an ultrasonic bath. Buffer was replaced with freshly filtered and degassed HPLC water for system washing.

The following gradient system was used:

80 % buffer/20 % acetonitrile up to 13 min ; 75% buffer/25% acetonitrile up to 25 min ; 80 % buffer/20 % acetonitrile up to 60 min. The flow rate was 1.0 ml min^{-1}. A 100 μl aliquot of sample or standard was injected and a run time of 60 minutes employed. Re-equilibration time of 15 minutes was allowed before the next sample was injected. The column temperature was set at 30°C and the detector wavelength was set at 325 nm.

3.4.4.6 Validation

The linearity of the method was assessed between 0 and 0.8 μg ml^{-1}. The method was tested for intra- and inter-assay variation. The limit of detection of the method was based on the lowest standard detectable using the derivatisation technique employed, with a coefficient of variation of 3 %. The limit of determination was established at twice the lowest standard detectable. For method validation purposes, samples were fortified at twice the limit of detection and ten times the limit of detection. This provided validation data for samples fortified at a high level of fortification and a low level of fortification. For the intra-assay variation study, five samples were fortified at two different levels and each level analysed within the same assay. For the inter-assay variation study, samples were fortified at two different levels and each level was assayed over five separate assays. The percentage recovery was calculated using a six point standard curve. Standards were

prepared in water and derivatised and chromatographed alongside samples. The variation in percentage recovery was calculated at each level of fortification, within one assay (intra) and over several assays (inter).

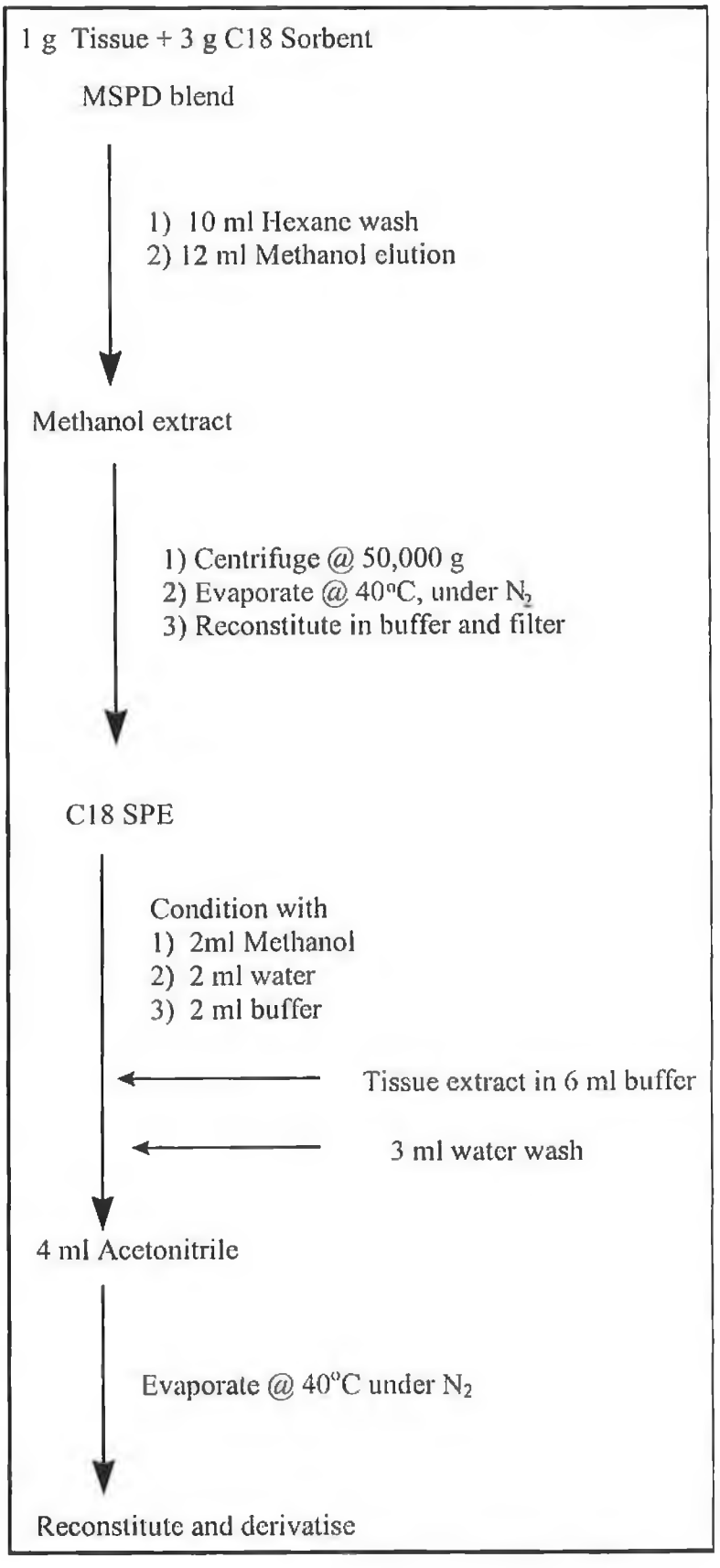

Figure 3.3 *Schematic of procedure developed.*

3.5 RESULTS AND DISCUSSION

The results for the extraction, clean-up, derivatisation and chromatographic techniques employed are presented in this section. Method optimisation is discussed and the results of method validation are presented.

3.5.1 MSPD extraction

The dispersion of tissue onto the C18 sorbent involves a mechanism whereby the tissues are disrupted by two methods. Firstly, mechanical forces resulting from the abrasive action of the C18, physically disrupts the tissue structure thereby dispersing the cells on the sorbent material. The tissue cells are also disrupted by hydrophobic forces. The sorbent material consists of long chains of carbon atoms, therefore the material is non-polar and hydrophobic in nature. Electron microscopy studies show that tissue cell membranes have a structure composed of a polar lipid bilayer (Figure 3.4). The bilayer is fluid because the hydrocarbon, non-polar tails consist of a mixture of saturated and unsaturated fatty acids that are fluid at body temperature. The integral proteins of the membrane have hydrophobic amino acid groups on their surfaces that allow the proteins to associate in the central hydrophobic portion of the bilayer. The peripheral proteins, on the other hand, have hydrophilic groups on their surfaces that are bound by electrostatic attraction to the hydrophilic polar lipid heads in the bilayer. No covalent bonds are known to exist between lipid molecules of the bilayer or between the protein components and the lipids. The absence of strong covalent forces allows the membrane to be disrupted more easily. The peripheral proteins are thought to float on the surface of the bilayer.

Therefore, the grinding movement of the tissue and the sorbent, using a mortar and pestle, disrupts the structure allowing the non-polar, hydrophobic C18 material access to the non-polar, hydrophobic bilayer and integral proteins, thereby unfolding the tissue cell structure. The more hydrophilic peripheral proteins and lipid heads now face inwards, with the hydrocarbon lipid tails extending outwards into the C18 sorbent. Adequate blending results in a uniform distribution of cellular components allowing the non-polar lipid constituents to be selectively eluted with hexane prior to eluting the penicillins with methanol.

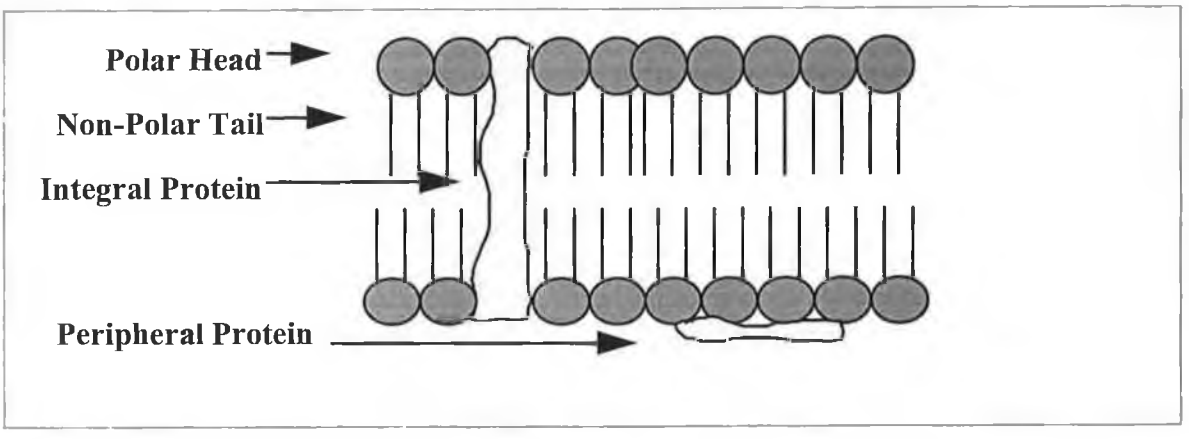

Figure 3.4 *Diagrammatic representation of the cell membrane.*

3.5.1.1 MSPD elution

Initial investigations carried out by Barker *et al.*[66] on the extraction of various classes of drug residues, including penicillin and ampicillin, using MSPD, involved evaluation of a range of elution solvents, such as hexane, benzene, ethyl acetate, and methanol. Application of similar solvents in this work to the elution of the five penicillins, at a concentration of 4 ppm, showed elution of analytes in both ethyl acetate and in methanol,

but at apparently very low recoveries. Attempts to elute the analytes in just one of these solvents continued to result in very poor measurable recoveries (2-10 %), with methanol proving to be very slightly better. Results for extracts spiked after the entire MSPD procedure also showed similar very low recoveries, demonstrating that matrix components were inhibiting the derivatisation reaction. Therefore, it was necessary to investigate a clean-up step. The inclusion of a C18 SPE step proved appropriate for removing derivatisation inhibitors.

Penicillin elution off the MSPD C18/tissue blend was also investigated using acetonitrile. Using MSPD extraction followed by derivatisation (that is exclusion of any clean-up step), recovery of penicillin G, cloxacillin and dicloxacillin was better using acetonitrile than when methanol was employed, but recoveries were only in the range of 31 to 49 %. Extracts that were spiked and then derivatised after the entire MSPD procedure (with elution in acetonitrile) showed recoveries of 30 to 55 % for penicillin G, cloxacillin and dicloxacillin. This proved that acetonitrile was less likely to elute the matrix components responsible for inhibiting the derivatisation reaction, but some inhibition still occurred. Therefore, similar to methanol elution, using acetonitrile MSPD elution, derivatisation of the extracted penicillins was not possible without the inclusion of a clean-up step. Investigation of MSPD elution followed by SPE clean-up resulted in recoveries that were consistently higher using methanol as the MSPD elution solvent. Table 3.2 shows that methanol promoted better recovery of the penicillins. This is likely due to its higher polarity and higher solvating power, compared with acetonitrile. The SPE clean-up step had not been optimised fully at this stage of the study, thereby accounting for the low

recovery of the analytes, but conditions for each sample were consistent so the results in Table 3.2 are comparable.

Table 3.2 Recovery (%) from samples fortified at 4 μg g^{-1} using methanol and acetonitrile as the MSPD elution solvents followed by SPE, (n=2).

	Mean Recovery (%)				
	Amox	***Amp***	***Pen G***	***Clox***	***Diclox***
Methanol	10	25	50	50	49
Acetonitrile	6	10	36	34	32

Various buffers were also investigated as MSPD elution solutions. This would allow the evaporation step after MSPD to be omitted and analytes could be applied directly onto the SPE column following SPE. Phosphate buffer at pH 2 resulted in a heavy white precipitate, which gave low analyte recoveries (15 to 19 % for the amphoteric penicillins and less than 10 % for the others) and blocked the SPE column. At pH 7, less precipitate was obtained, but amoxicillin was not recovered, ampicillin was recovered very poorly (3 %) and recoveries for penicillin G, cloxacillin and dicloxacillin ranged from 37-59 %. In addition to poor recoveries, buffer elution was lengthy and the SPE columns tended to become blocked.

Recovery from the standard MSPD procedure alone (that is, with no SPE step included) was assessed by developing a gradient LC method for separation of non-derivatised

standards with UV detection at 210 nm. The MSPD extract was evaporated as usual, reconstituted in mobile phase and chromatographed. Recoveries from tissue samples fortified with the 5 drugs at 4 $\mu g\ g^{-1}$ are presented in Table 3.3. Results are not presented for amoxicillin and ampicillin as these peaks were hidden among matrix interferences. Recoveries for penicillin G and the isoxazoyl penicillins from MSPD extraction were reasonably high.

Table 3.3 Recovery (%) from a tissue sample fortified with five drugs at 4 $\mu g\ g^{-1}$ and extracted by MSPD with no further clean-up, (n=2).

Mean Recovery (%)				
Amox	***Amp***	***Pen G***	***Clox***	***Diclox***
ND	ND	81	88	83

ND : Not detectable due to matrix interferences

Figure 3.5 illustrates optimisation of the elution of penicillin G, cloxacillin and dicloxacillin from the tissue-sorbent blend, using methanol (with UV detection at 210 nm). Because amoxicillin and ampicillin are more polar than the other penicillins, due to the presence of NH_2 groups (Figure 3.2), lower volumes of methanol would be required to elute them from the C18-tissue blend. Therefore, it was assumed that 6 ml methanol would be sufficient to elute all five penicillin residues. Initial MSPD investigations were conducted using 0.5 g tissue with 2 g sorbent. Subsequently, it was found necessary to

employ 1 g of tissue and 3 g sorbent so the MSPD wash and elution volumes optimised were increased to account for the larger void volume.

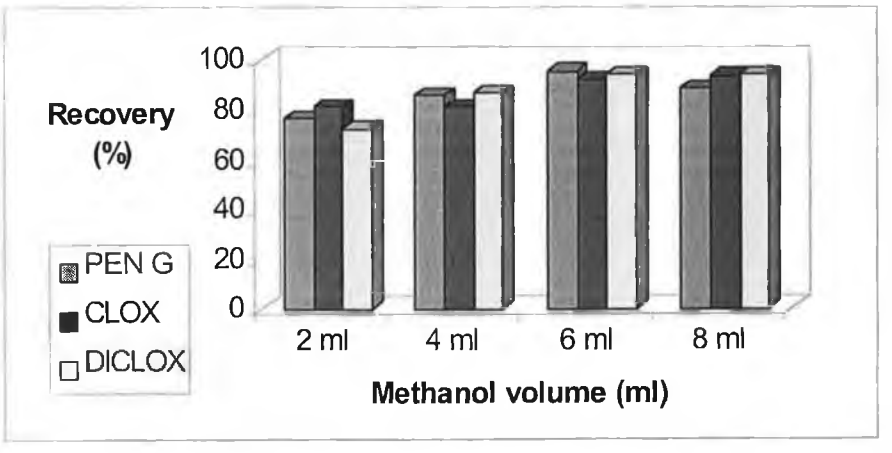

Figure 3.5 *Optimisation of the elution volume required to elute analytes from MSPD.*

3.5.1.2 MSPD wash

Initially, hexane, ethyl acetate and diethyl ether were investigated for use as MSPD wash solvents. Ethyl acetate proved capable of eluting some of the residues and when diethyl ether was employed as a wash solvent, very low recovery resulted. Hexane proved best for removal of non-polar fats. The volume of hexane used to wash the tissue blend was varied. 6 ml hexane was employed as volumes less than 6 ml resulted in lower recoveries and volumes greater than 6 ml showed no significant increase in recovery or removal of matrix. A water wash (4 ml) prior to methanol elution resulted in a visually much cleaner methanol extract but ampicillin and amoxicillin were not recovered and recoveries were reduced for the other analytes, possibly due to the elution of analytes in the polar water wash.

3.5.2 Solid Phase Extraction (SPE) clean-up

The MSPD extract was seen to contain white proteinaceous material. This protein was likely to react with the 1,2,4-triazole preventing it from reacting with the penicillin residues extracted. Also, the mercuric chloride necessary for the derivatisation reaction was likely to react with the protein. Initial clean-up investigations were concerned with the removal of these proteins using trichloroacetic acid (TCA) and ammonium sulphate, following MSPD. Using TCA, dicloxacillin was not recovered and no significant improvement was observed for the other analytes. Using ammonium sulphate, ampicillin and penicillin G were not recovered. These reagents also altered the pH of the extract to a more acidic extract, which was undesirable for derivatisation. The most commonly used clean-up step previously reported, C18 SPE, was investigated.

The method was investigated initially using control samples spiked following the MSPD extraction procedure. Following MSPD, the residues were in methanol. Due to the polar nature of this solvent it was not a suitable matrix in which to apply the extracted residues onto a reversed-phase SPE column. Therefore, the methanol was evaporated at 37°C under nitrogen. While higher temperatures resulted in quicker evaporation of the solvent, some loss of analytes was noted, particularly when the temperature was increased to 60°C. Degradation of penicillin molecules on evaporation has been reported by Moats[15]. Table 3.4 shows the recovery resulting from the evaporation of 8 ml methanol spiked with 20 µl of a 10 µg ml^{-1} standard drug solution. Penicillin G and cloxacillin appeared to be the most resistant to degradation, while ampicillin was the most susceptible. It is also worth noting that evaporation of this methanolic solution was likely to proceed more quickly than when matrix was present, due to increased water content, therefore losses in the

presence of matrix may be even greater. To avoid losses on evaporation, the process was carried out until "near" dryness.

Table 3.4 Recovery (%) of penicillins resulting from the evaporation of methanol, spiked with a standard drug solution, at 37°C, (n=3).

Mean Recovery (%)				
Amox	***Amp***	***Pen G***	***Clox***	***Diclox***
81	79	90	90	81

Following evaporation of MSPD extracts, samples were reconstituted in buffer in preparation for SPE. Initially potassium phosphate buffer (0.25 M, pH 7.0) was employed, similar to that used by Moats[41]. Sodium phosphate buffer (0.156 M)[27] was subsequently found to give higher recoveries. This buffer was not pH adjusted and had a pH value of 6.5 on preparation. It has been postulated that potassium ions perform better than sodium ions at suppressing any residual silanol groups that exist on the C18 column[67]. Therefore, when sodium phosphate was employed, the silanols may have played a role in retaining the analytes on the column. Figure 3.6 compares the recovery from tissue samples fortified at 4 $\mu g\ g^{-1}$ using the two buffers in the SPE step. Moats and Romanowski[68] reported instability problems when potassium phosphate buffer, pH 6.0 was employed during LC fractionation and found the addition of sodium phosphate buffer improved stability.

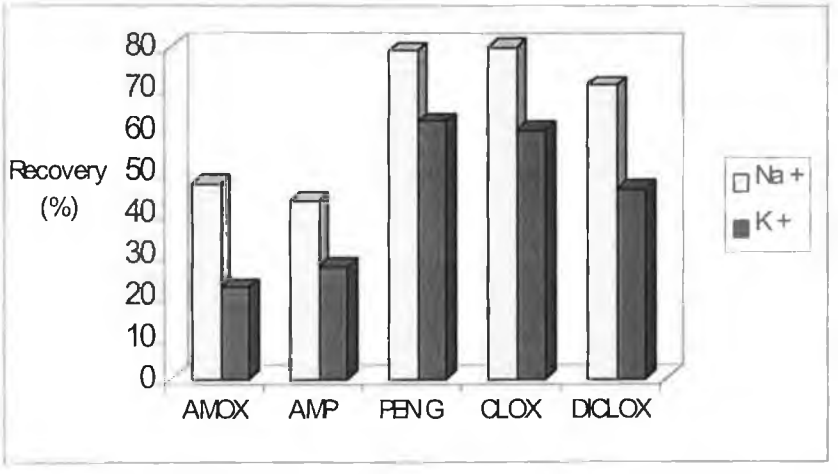

Figure 3.6 *Recovery of the ß-lactams from tissue samples fortified at 4 $\mu g\ g^{-1}$ and reconstituted in different buffers before SPE.*

The optimum pH of the SPE buffer was established by reconstituting blank tissues extracted using MSPD, in sodium phosphate buffers of various pH values between 2 and 8. Samples were spiked with all five analytes after reconstitution and prior to SPE. Columns were conditioned in the appropriate buffer prior to loading. Figure 3.7 demonstrates that pH 6.5 buffer gave highest recovery for all the analytes. This was the pH of the buffer on preparation, therefore no adjustment was necessary. At a pH value of 2.0, the acidic environment may cause degradation of the penicillin molecules resulting in the low recoveries. At pH 4.0 the low recoveries for amoxicillin and ampicillin may be due to protonation of the amino groups causing these analytes to be removed in the water wash; recoveries for penicillin G, cloxacillin and dicloxacillin were at 80 % approximately, as the environment was not so acidic as to cause degradation of the lactam ring. At pH 6.5, the pH approaches the pK_A of the amino group (~7.0), amoxicillin and ampicillin become more non-polar and are retained on the non-polar stationary phase. pH

6.5 was observed to be sufficient to retain the residues, as increase in buffer pH, to pH 8.0, did not result in increased recovery.

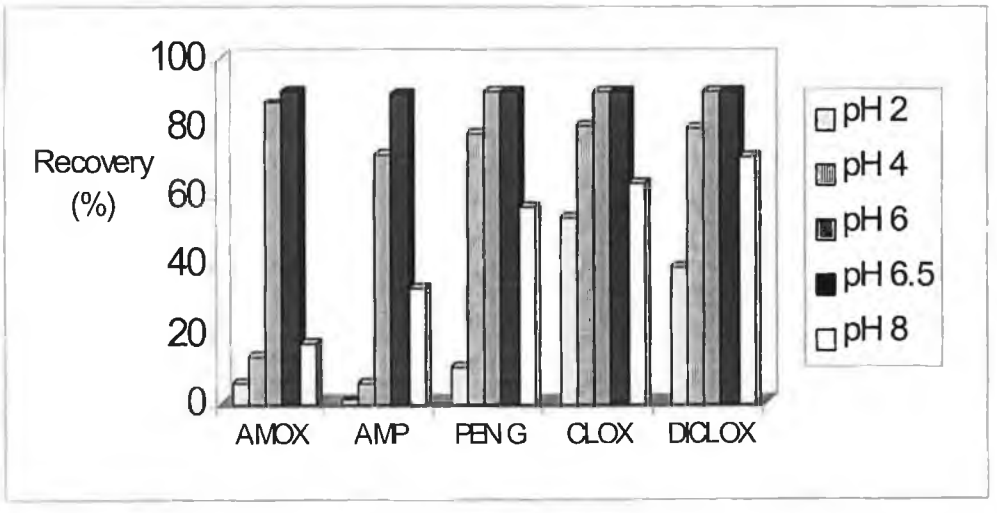

Figure 3.7 *Effect of the pH of the loading buffer solution on the recovery of ß-lactams from the C18 SPE step.*

Conditioning of SPE columns proved important. The column was first washed with methanol to remove any interferences present as a result of the manufacturing process, and to solvate the packing material to ensure the sorbent reacts reproducibly with the analytes. A water wash was employed to prepare the column for loading. The water wash served to prepare the column for the conditioning step, which was composed of loading buffer, and removed any remaining methanol which may have caused buffer precipitation. Following conditioning in loading buffer (2 ml), the extracted residues were applied to the column in this loading buffer. Volumes of 2 ml were employed, which is around three times the volume of the packing material (670 μl). During method development, it was observed that recoveries were sometimes reduced if the SPE columns were allowed to run dry between conditioning and sample loading and between loading and washing.

The effect of buffer volume for dissolving the sample extract was investigated. Figure 3.8 shows that increasing the volume of buffer, up to 6 ml increased recovery, after which no increase was observed. This may be due to greater dilution of the matrix interferences at higher volumes resulting in some interferences being eluted off the column even at the application stage. The effect of varying the concentrations of the two buffer salts was also investigated. The concentration of buffer was seen to be more important for amoxicillin and ampicillin than for the other penicillins. When the concentration of dibasic sodium phosphate ($Na_2HPO_4.2H_2O$) was dropped below 0.07 M, the recovery for these analytes also dropped. This may be explained by the fact that these amphoteric penicillins contain an NH_2 group, and therefore need to be in a buffered environment to assist retention on the C18 SPE column. Increase or decrease in either salt had no effect on penicillin G, cloxacillin and dicloxacillin recovery. Samples were loaded onto the SPE column and allowed to elute at approximately 1 ml min^{-1}. Faster flow rates resulted in poor recovery for amoxicillin and ampicillin.

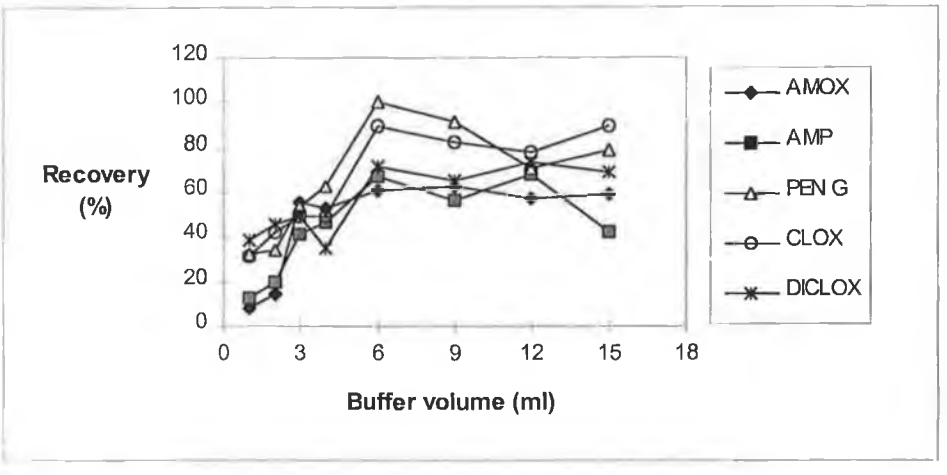

Figure 3.8 *Recovery of ß-lactams from tissue sample extracts spiked at 4 $\mu g\ g^{-1}$ and dissolved in various volumes of buffer prior to SPE.*

To avoid precipitation of the buffer salts that might block the column and inhibit elution of the analytes, a water wash was included following sample application (in buffer) and prior to elution (in acetonitrile). Various volumes of water wash were investigated and 4 ml wash was found to be the most suitable (Figure 3.9). The SPE water wash (4 ml) was also collected and assayed, and was found to contain amoxicillin (26 %). As a result of these findings, a 3 ml water wash was employed, which resulted in better recovery for amoxicillin. Recoveries for penicillin G, cloxacillin and dicloxacillin were marginally better when a 4 ml water wash was employed. Therefore, attempts were made to retain the more polar amoxicillin and ampicillin on the C18 SPE column using sodium pentanesulphonic acid as an ion-pair. However, when the ion-pair was included in the loading solution, recoveries for all analytes, particularly amoxicillin, were relatively reduced, even when an SPE elution volume of 30 ml was employed.

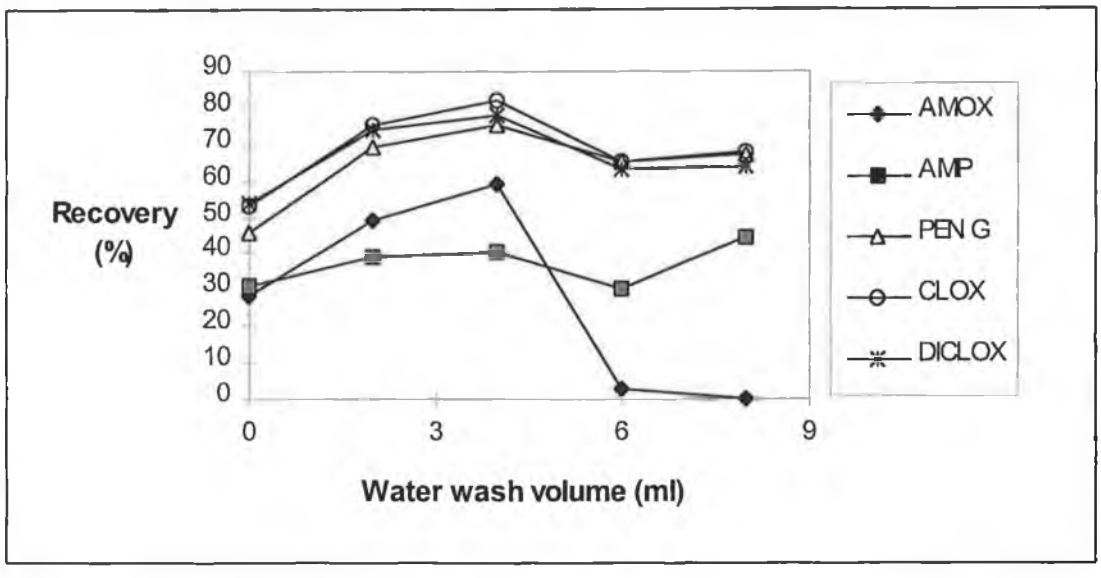

Figure 3.9 *Optimisation of the SPE water wash for the five drug residues isolated from a tissue sample fortified at 4 $\mu g\ g^{-1}$.*

To investigate elution of the penicillins off the C18 SPE, control samples were extracted by MSPD and the extracts were spiked with the five antimicrobials (after MSPD extraction). Various volumes of acetonitrile were used to elute the residues off the SPE column. Table 3.5 shows that 5 ml acetonitrile was sufficient. Reduced recovery was evident when 10 ml acetonitrile was used for elution, probably due to elution of interferences that inhibit the derivatisation reaction. Methanol elution was also investigated and recoveries were significantly reduced, indicating that methanol was capable of eluting the matrix co-extractives that inhibited the derivatisation reaction.

Table 3.5 Effect of SPE elution volume (acetonitrile) on the recovery of the five analytes (spiked at 4 ppm) in the presence of sample matrix.

Elution Volume (ml)	*Recovery (%)*				
	Amox	*Amp*	*Pen G*	*Clox*	*Diclox*
2	19	26	66	55	51
5	68	48	89	99	95
10	62	32	74	73	56

Finally, the acetonitrile elution volume was reduced to 4 ml, allowing faster evaporation with similar recovery. This volume evaporated quickly (20 min) at 40°C. The effect of evaporation at 40°C was investigated by evaporating 4 ml acetonitrile, spiked with the analytes of interest at a standard equivalent to 4 ppm. Penicillin G, cloxacillin and

dicloxacillin exhibited small losses (3-8 %), while losses of 29 % and 9 % were observed for ampicillin and amoxicillin, respectively.

Prior to the SPE step the methanol eluate from the MSPD procedure was centrifuged, the supernatant evaporated and the residue reconstituted in buffer. Centrifugation at 3 speeds was investigated; high (15,000 rpm), medium (7000 rpm) and low (2000 rpm). The high speed centrifugation, at 15,000 rpm, resulted in higher recoveries probably due to removal of some of the proteins that inhibit the derivatisation reaction. Omission of the centrifugation step resulted in blockage of the SPE column, even when SPE columns of 6 cc volume were employed. Following evaporation of the methanol and reconstitution of the extract in buffer prior to SPE, filtering of the extract was required. Where extracts were not filtered, broad peaks were obtained on the HPLC chromatograms, leading to higher variation. This was particularly evident for ampicillin.

Inclusion of 0.5 g C18 material in the MSPD syringe barrel, under the tissue blend was investigated to determine if the sieving effect of the C18 could allow for elimination of the SPE step. However, derivatisation of the penicillins without inclusion of a separate SPE clean-up step was not possible, as the methanol eluted the derivatisation inhibitors off the tissue blend and through the clean C18 material. Various volumes of methanol were investigated to identify a volume capable of eluting the analytes without elution of the derivatisation inhibitors, but this could not be achieved.

3.5.3 Derivatisation reaction

Derivatisation offers many advantages in residue determination, offering the possibility of increased specificity and sensitivity. It often eliminates the need for extensive sample clean-up procedures as detection wavelengths are sometimes beyond the range where matrix co-extractives absorb. Pre-column derivatisation of compounds can render residues separable by methods that are otherwise unsuitable. When UV detection is employed for penicillin determination, greatest sensitivity is normally achieved at wavelengths below 230 nm. At these wavelengths, selectivity is poor, resulting in high matrix interference when residues are determined in tissue samples. Therefore, derivatisation of penicillins is used, in order to form stable derivatives capable of detection at higher wavelengths and exhibiting enhanced absorption properties.

Pre-column derivatisation using BrMmC to form fluorescent derivatives was investigated. This method proved to be unsuitable for the analysis of amphoteric penicillins (amoxicillin and ampicillin). It also necessitated the use of a phase transfer catalyst in the extraction procedure to allow the derivatisation reaction to occur and required the use of dried solvents to obtain clean chromatograms. Pre-column derivatisation using 1-hydroxybenzotriazole was investigated but solubility problems were experienced with the derivatising agent. Derivatisation with triazole was chosen as it was previously used in residue analysis and it was possible to derivatise all the penicillins of interest in this study. It offered sensitivity superior to some of the other methods investigated, such as UV detection at 210 nm, and used inexpensive reagents.

The method used in this study, involving derivatisation with 1,2,4-triazole, is based on the imidazole-catalysed isomerisation of penicillin G described by Hans Bundgaard[69]. In the presence of mercuric chloride, a self-catalysed nucleophilic attack by the imidazole base occurs on the β-lactam ring. The intermediate product formed then reacts with mercuric chloride to form a stable mercuric mercaptide complex. In this study, the imidazole is replaced with 1,2,4-triazole as the base. Reaction rates of penicillins with 1,2,4-triazole and mercuric chloride are faster than those with imidazole[5]. The initial step of the derivatisation reaction involves an acetylation step. Acetic anhydride, pH 9.0, acetylates the side chain amino group in the amphoteric penicillins, ampicillin and amoxicillin, to form N-acetylampicillin and N-acetylamoxicillin, respectively. These compounds then react with 1,2,4-triazole in a similar fashion to the other penicillins. Failure to protect the amino groups of the amphoteric penicillins results in their reaction with 1,2,4-triazole, inhibiting the formation of the mercaptide derivative. The reaction scheme for acetylation of ampicillin is depicted in Figure 3.10. Figure 3.11 shows the reaction mechanism for the β-lactam ring reaction with 1,2,4-triazole.

3.5.3.1 Acetylation/Benzolylation of amphoteric penicillins

The initial step in the derivatisation was the acetylation reaction. In this study, reaction time was set at 5 min, with the reaction proving linear up to this point where it levelled off. Most references allow only a 3 min reaction time. The volume and concentration of acetic anhydride used were 20 µl of 0.02 M solution. Higher volumes resulted in excess reagent peaks and higher concentration did not lead to an increased response. Acetylation,

using acetic anhydride that was prepared more than 48 hours before use, resulted in an interfering peak that eluted at a similar retention time to ampicillin.

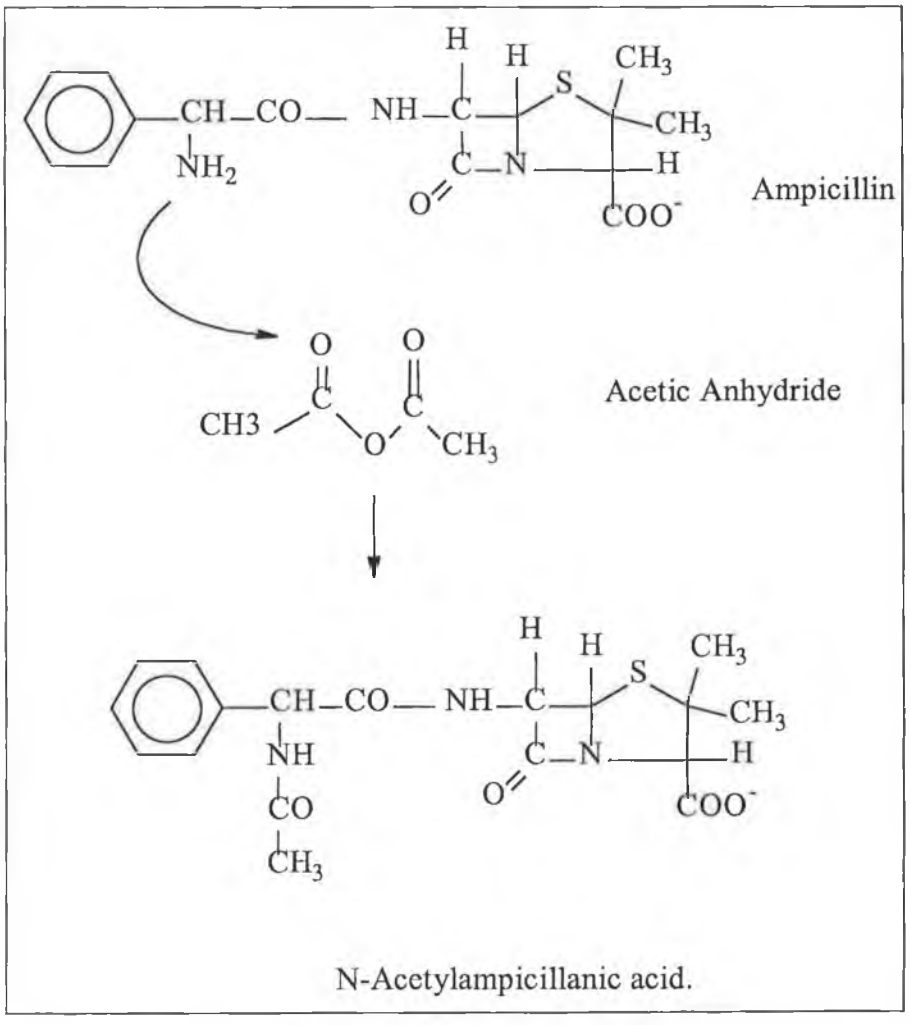

Figure 3.10 *Acetylation of ampicillin*

De-activation of the amino side chain using benzoic anhydride was also investigated. This method was initially described by Tutt and Schwartz[70]. Benzoic anhydride is capable of NH_2 protection by a similar mechanism to acetic anhydride. When dibasic penicillins are acetylated prior to the formation of their mercaptide derivatives, it was found that

acetylamido penicillins still have significant differences in their elution characteristics on a reversed-phase column compared with their monobasic analogues, such that it was necessary to use gradient elution conditions to resolve them. The increased polarity of the acetylamido mercaptide derivatives (compared with the mercaptide derivatives of penicillin G, cloxacillin and dicloxacillin) accounts for the need for gradient elution. However, modification to the chemical derivatisation reaction by using benzoic anhydride enabled resolution under isocratic conditions. Benzoylation confers similar elution characteristics to the benzoylamido mercaptide derivatives of amoxicillin and ampicillin, as the mercaptide derivatives of the monobasic penicillins (penicillin G, cloxacillin and dicloxacillin), allowing isocratic separation. The benzoylamido mercaptide derivatives are less polar and so can elute in a similar time frame to the mercaptide derivatives of penicillin G, cloxacillin and dicloxacillin. A higher acetonitrile content was required in the mobile phase to elute the benzoylamido mercaptide derivatives, compared to the acetylamido derivatives. This is due to the presence of an additional aromatic ring which is retained to a greater extent on the C18 stationary phase. The ring adds a more bulky side-chain, causing it to be delayed on the column. Figure 3.12 depicts the postulated mechanism of reaction for benzoylation. While isocratic elution, following derivatisation with benzoic anhydride, resulted in improved peak shape and decreased variation in chromatography, it proved similar in sensitivity. In this study, derivatisation using benzoic anhydride was rejected when it was observed to produce matrix interferences in the chromatograms at similar retention times of some of the penicillin derivatives.

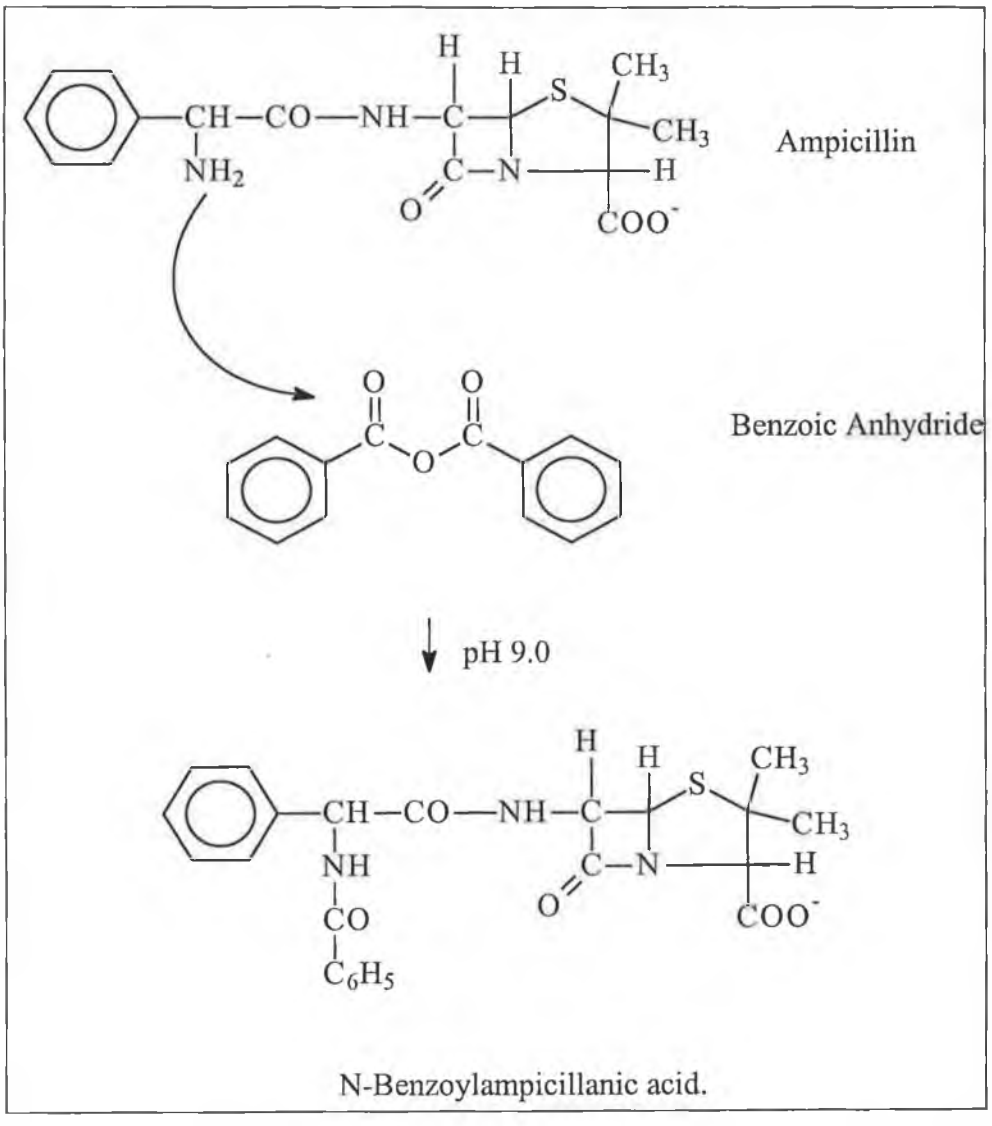

Figure 3.12 *Benzoylation of ampicillin.*

3.5.3.2 Conditions for derivatisation with triazole

The pH of the triazole solution was adjusted to pH 9.0, as basic conditions were necessary to ensure that the triazole molecule was unprotonated so that the reaction with the β-lactam ring (depicted in Figure 3.11) would occur. Also, at an acidic pH, the acetylation reaction would fail to take place as the amino ($-NH_2$) group would become protonated. Mercuric chloride, which stabilises the product formed when the triazole is reacted with

the penicillin, forming penicillanic acid mercury mercaptide, is also known to complex with proteins. This may have been one of the reasons for the problems experienced when attempting to derivatise residues in MSPD extracts. The centrifugation step included after MSPD elution, removed some of the proteins eluted from the MSPD step, however, the supernatant resulting still contained some dissolved proteins. Prior to the introduction of the SPE step, attempts to remove the proteins from the supernatant involved the introduction of acidic pH. However, acidic pH can hydrolyse the β-lactam ring and inhibit the derivatisation reaction. Experiments were carried out to separate the 1,2,4-triazole reaction from the mercuric chloride reaction so that the proteins could be removed before mercuric chloride addition. However, this failed likely due to the instability of the product formed on reaction of the penicillin with 1,2,4-triazole.

The purity of the triazole employed for derivatisation proved important. Triazole of less than 99 % purity resulted in the formation of small interfering peaks which eluted partially resolved from ampicillin and a larger non-retained solvent front caused amoxicillin to elute as a shoulder peak. Derivatisation was carried out at various concentrations of triazole between 1 M and 5 M. Even at high analyte concentrations (20 ppm), the 1 M reagent did not become limiting. Various concentrations of mercury (II) chloride were also investigated. Below 0.005 M, chromatograms showed numerous ghost peaks and reduced peak intensity for amoxicillin and ampicillin. No substantial differences were observed in peak intensity, peak shape or the chromatograms resulting when various mercuric chloride solutions between 0.05 and 0.02 M were employed.

The effect of varying the incubation time for the derivatisation reaction was investigated. Using a penicillin solution of 1 μg ml^{-1}, after 10 min no peaks were apparent for any of the penicillin residues and at 20 min no peaks were evident for ampicillin and amoxicillin. By 30 min all peaks were apparent and responses increased slightly up to 150 min, at which time the reaction appeared to level off. From these experiments, an incubation time of 150 min was selected. Derivatisation was investigated using standards in different media (water, acetonitrile and combinations of these). Standards prepared in water gave the highest peak intensity. The effect of standard diluent was especially significant for ampicillin. When acetonitrile was incorporated in the diluent, the peak shape was broad and tailing. Kubalec *et al.*[71] reported a decrease in the derivatisation reaction speed when the diluent contained 45 % v/v acetonitrile. Therefore, following SPE, samples were reconstituted in water.

Sorensen *et al.*[72] reported the need to employ silanised glassware to avoid irreproducibility in derivatisation, when employing triazole derivatisation for the determination of penicillin residues in milk. On comparing the derivatisation results of standard drug solutions in silanised and non-silanised glassware in this study, no significant difference was observed, so non-silanised glassware was employed similarly to the majority of reported methods. Boison *et al.*[27] reported that it was necessary to maintain the derivatives in the presence of matrix. He claimed the derivatisation of standard drug solutions resulted in high variability due to instability of the derivatives, but the presence of matrix components has a stabilising effect in the derivatives. In this study, the presence of matrix was not seen to result in a more stable derivative. Samples were chromatographed over a period of 12 hours and the derivatives showed similar stability both with and without

matrix. In the method of Boison *et al.*[27], the drug residues were derivatised in a buffer/acetonitrile solution. In this study, the presence of acetonitrile caused poor peak shape and peak splitting for amoxicillin and ampicillin. It was found that the derivatives gave much sharper peak shape when prepared in an aqueous environment. Also, it was desirable to delay exposure of the penicillins to organic solvents, which are reported to cause disruption of the β-lactam ring.

Variation in the derivatisation reaction was assessed by derivatising the same standard solution five times. Table 3.6 shows the variation in the derivatisation of a 1μg ml^{-1} standard solution and the variation resulting when analytes were derivatised in a similar fashion, in the presence of matrix. Slight tailing accounts for the relatively higher variation in the dicloxacillin derivative compared with the others. Variation was observed to be greater in the presence of matrix.

Many authors have used penicillin V as an internal standard, as this synthetic drug is not usually administered as a veterinary drug. Use of penicillin V as an internal standard was investigated to overcome the variation in derivatisation. However, the variation was found not to be sample specific; not all the penicillin residues in one particular sample were observed to be derivatised in a consistent manner. Therefore, an internal standard was not used, as it did not assist in overcoming the inter-sample variation.

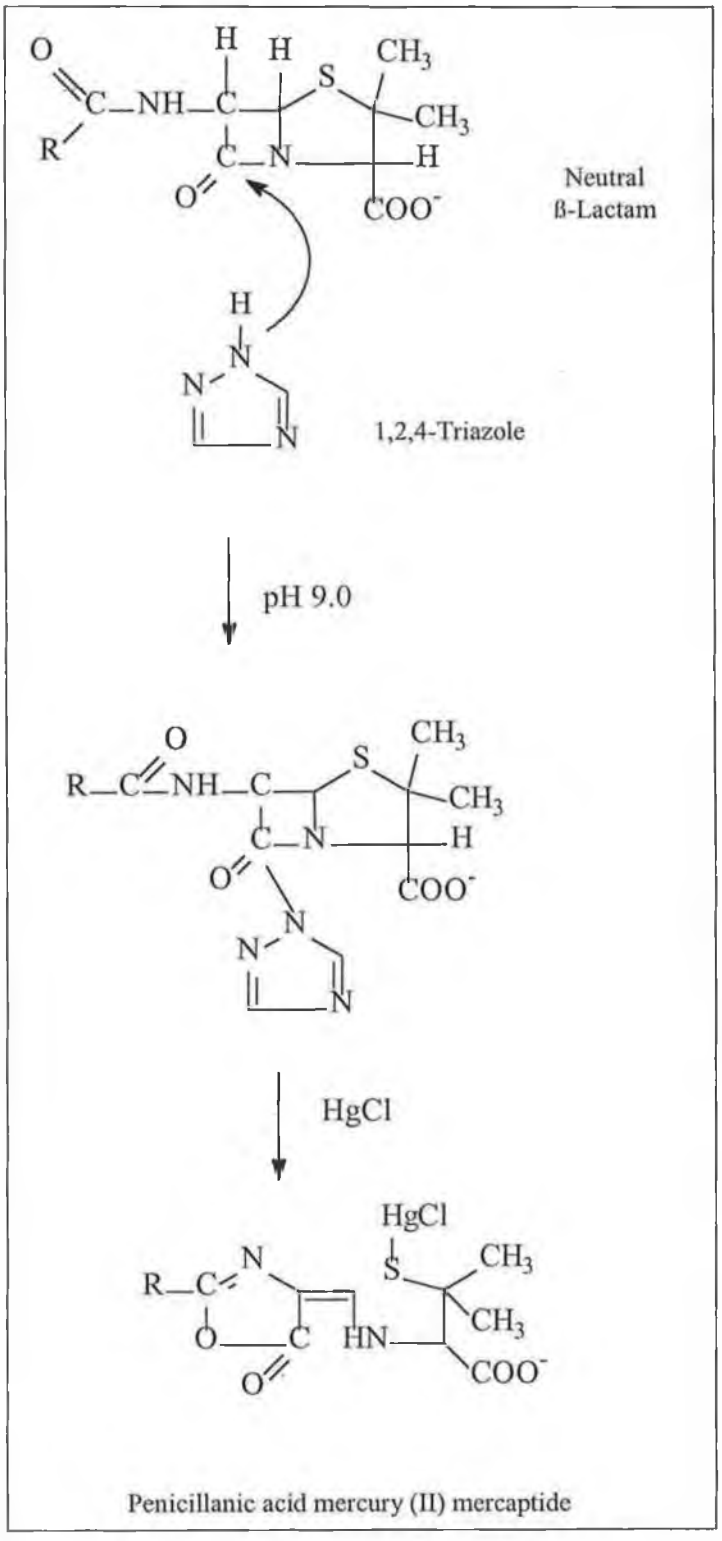

Figure 3.11 *Reaction mechanism for the derivatisation of penicillins using 1,2,4-triazole.*

Table 3.6 Variation in derivatisation of standard penicillins (1μg ml^{-1}, n=5), as a standard solution and in the presence of matrix.

Penicillin	*CV (%) in standard solution*	*CV (%) in matrix*
Amoxicillin	2.4	24.6
Ampicillin	7.8	8.8
Penicillin G	1.8	14.8
Cloxicillin	4.2	4.7
Dicloxicillin	10.2	19.1

3.5.4 Chromatography

Since reversed-phase columns were employed in all previously reported studies, therefore a C18 column was chosen for this work. Gradient elution was necessary to elute the derivatised penicillins in one chromatographic run. Initially, a HPLC method that could include nafcillin was investigated. The derivatised products of nafcillin and cloxacillin could not be separated on a C18 column. Separation could be achieved using a phenyl column, because the aromatic nature of the phenyl packing provides sufficient interaction with the highly conjugated aromatic penicillin derivatives, thereby allowing separation of nafcillin and cloxacillin derivatives. However, this column proved unsuitable for the analysis of tissue extracts. After employing the column for the analysis of MSPD tissue extracts, resolution failed and peak shape deteriorated. The aromatic nature of the column packing renders it more susceptible to degradation and column fouling. Therefore, the

original C18 column was re-employed and nafcillin omitted from further studies. Initial studies investigating various HPLC mobile phases, showed nafcillin and cloxacillin could be separated on a C18 reversed-phase column when an ion-pair (tetrabutylammonium hydrogensulphate) was employed in the mobile phase. This mobile phase was rejected as dicloxacillin eluted very late and the method did not succeed in delaying amoxicillin on the column any further than the current HPLC method employed. Using the mobile phase and gradient system chosen, described in section 3.4, it was necessary to increase the percentage acetonitrile from the initial conditions of 20 % to 40 %, over 25 min, in order to elute the last 4 derivatives off the reversed-phase column. However, maintaining the acetonitrile at 40 % after 25 min resulted in co-elution of the last two derivatives so, after 25 min, their elution was retarded by reverting the gradient back to the initial conditions of 20 % acetonitrile, which was also ideal for preparation of the column for the next sample.

The derivatives were analysed at various wavelengths between 300 and 350 nm. Maximum absorption for amoxicillin, ampicillin and penicillin G derivatives was found to be 325 nm, while cloxacillin and dicloxacillin derivatives showed an absorption maximum at 345 nm. Therefore, at 24 min, the wavelength was switched from 325 to 345 nm to maximise sensitivity for cloxacillin and dicloxacillin. Figure 3.13 shows the separation of the five derivatives of penicillins obtained for a standard solution, for a control muscle sample and for porcine muscle samples fortified at 40 and 200 ppb. A residue free (control) muscle sample is also shown. The change in wavelength is responsible for the inflection in the baseline, observed at 24 min.

The variation in the chromatographic response was assessed by injecting the same standard solution five times, with and without the presence of sample matrix (Table 3.7). As with the variation in the derivatisation, greatest chromatographic variability exists with dicloxacillin and as expected the variation in chromatography is reduced relative to the variation in derivatisation.

Table 3.7 **Chromatographic variation for a 400 ng g^{-1} solution of derivatised penicillins and a solution of derivatised penicillins in the presence of matrix (n=5).**

	Standard solution % CV	in Matrix % CV
Amoxicillin	2.4	2.6
Ampicillin	4.2	2.3
Penicillin G	2.8	3.2
Cloxacillin	2.9	2.6
Dicloxacillin	7.1	4.1

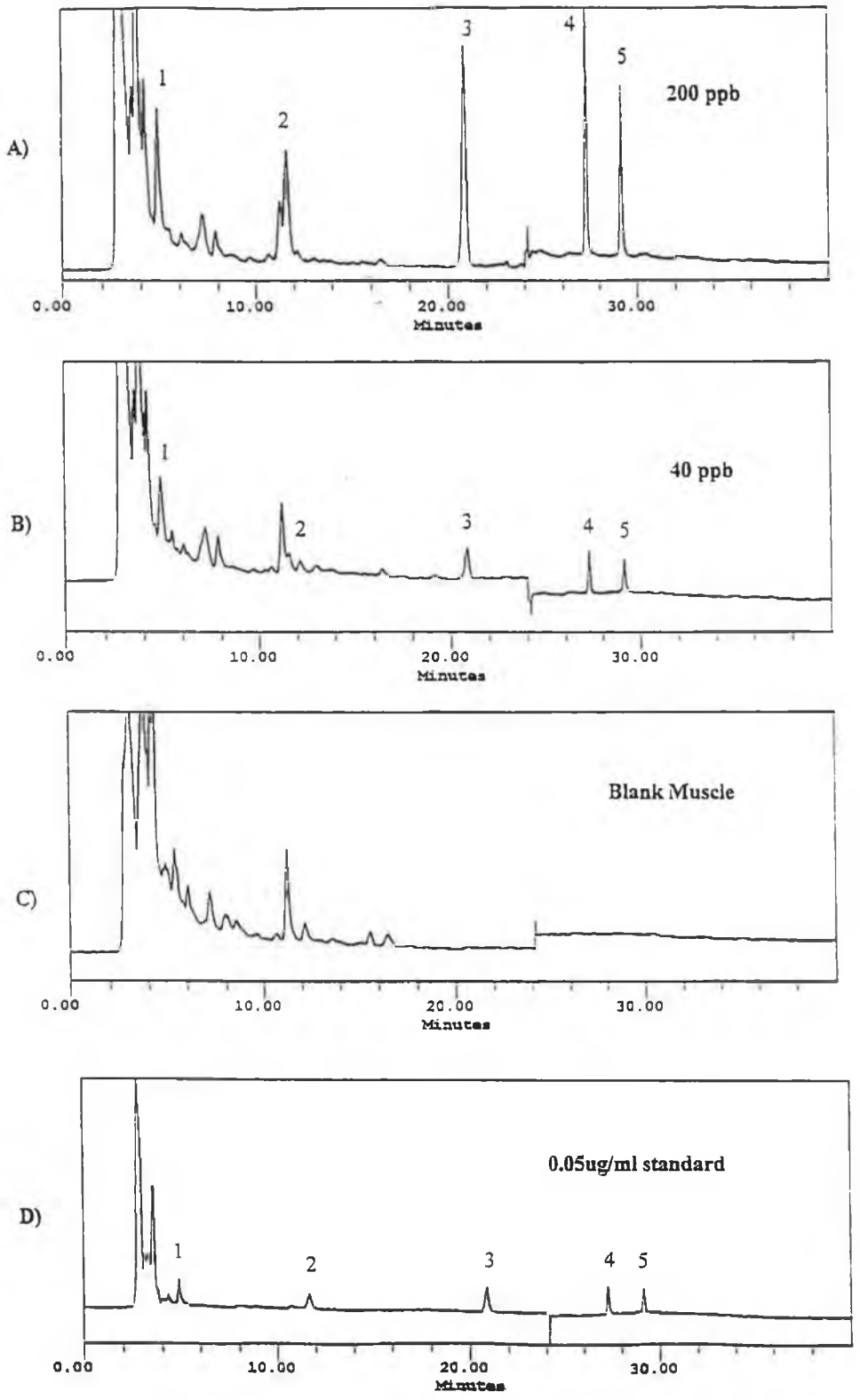

Figure 3.13 *Chromatogram showing derivatised control muscle and muscle samples fortified at 40 and 200 ppb, and a 0.05 μg ml^{-1} standard (equivalent to 40 ppb).*

1) Amoxicillin, 2) Ampicillin, 3) Penicillin G, 4) Cloxacillin and 5) Dicloxacillin

HPLC Conditions: 100 μl injection volume, Waters Symmetry C18 column, 5 μm, 100 Å, 4.6 x 250 mm, Mobile phase and gradient as described in section 3.4, UV detection at 325 nm up to 24 min and 345 nm after 24 min.

3.5.5 Method precision and accuracy

Linearity of the chromatographic method was established between 0 and 0.8 μg ml^{-1}. The highest standard routinely included in this study was equivalent to 800 ppb (MRLs are in edible tissue are 50 for amoxicillin, amoicillin and penicillin G and 300 ppb for cloxicillin and dicloxicillin), therefore it was considered unnecessary to investigate linearity beyond this concentration. The limit of detection was 20 ppb and the limit of determination was 40 ppb for the five analytes in porcine muscle. Method validation was carried out on samples fortified at 40 and 200 ppb.

The within assay (intra) variation and between assay (inter) variation for muscle samples fortified at 40 ppb as shown in Table 3.8. The mean recovery (percentage) and standard deviation are presented. Quantification was not possible for amoxicillin at 40 ppb due to the presence of matrix interference. Ampicillin showed inflated recoveries and higher inter-assay variation as it eluted as a "shoulder" on a peak, caused by matrix interference. For integration purposes, a drop line was forced from the valley between the two peaks to the baseline. Recoveries for cloxacillin and dicloxacillin were low using the method developed. Hou and Poole[73] have reported that the hydrolysis of the β-lactam ring is more difficult to achieve for isoxazoyl penicillins than for the other members of the group. The derivatisation technique employed in this study required hydrolysis of the β-lactam ring. When control tissues were extracted and cleaned-up and extracts fortified prior to derivatisation, recovery for cloxacillin and dicloxacillin were approximately 74 and 68 %, respectively. Therefore, it is likely that in-complete hydrolysis causes low recovery for these two analytes in tissue extracts.

The repeatability and reproducibility of the method for samples fortified at 200 ppb is presented in Table 3.9. At this level, some quantitation of amoxicillin is possible, as it is more easily distinguished from the interfering matrix, due to the higher peak intensity. Variation for intra- and inter-assays is lower at the high level of fortification and recoveries for cloxacillin and dicloxacillin are much improved, relative to that found for samples fortified at 40 pbb (Table 3.8). The improved recovery may be due to the fact that the concentration of the analytes at the high level of fortification greatly out-number the concentration of derivatisation inhibitors, resulting in a higher recovery. At both levels of fortification, intra-assay variation is lower than inter-assay variation. Recoveries for penicillin G are consistently good and variation acceptable.

Table 3.8 Determination of penicillin residues in porcine muscle fortified at 40 ppb, using MSPD with pre-column derivatisation (n=5).

	Mean Recovery (%) ± SD	
Antibiotic	***Intra-assay***	***Inter-assay***
Amoxicillin	NM*	NM*
Ampicillin	120 ± 8.5	130 ± 38
Penicillin G	74 ± 13.1	79 ± 15.1
Cloxacillin	43 ± 14.7	55 ± 19.6
Dicloxacillin	25 ± 9.9	52 ± 25

NM : Not measurable due to matrix interference

Table 3.9 Determination of penicillin residues in porcine muscle fortified at 200 ppb, using MSPD with pre-column derivatisation (n=5).

	Mean Recovery (%) ± SD	
Antibiotic	***Intra-assay***	***Inter-assay***
Amoxicillin	23 ± 18.8	45 ± 16.6
Ampicillin	85 ± 10.5	73 ± 9.4
Penicillin G	81 ± 1.2	76 ± 14.9
Cloxacillin	79 ± 3.4	65 ± 13.1
Dicloxacillin	75 ± 3.5	62 ± 11.9

3.6 CONCLUSION

The study demonstrates the effectiveness of MSPD for the extraction of penicillin drug residues from porcine tissue. Direct UV detection was not possible due to the low UV absorption characteristics of the native penicillins. Therefore, derivatisation was necessary and the technique employed was specific to those compounds containing a penicillin nucleus, resulting in selective enhancement of the signal. MSPD provides a simple, rapid extraction method for the five penicillin residues. The results show that the method was better suited to the analysis of cloxacillin, dicloxacillin and penicillin G, particularly at low levels, due to matrix co-extractives interfering with ampicillin and amoxicillin.

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CHAPTER 4

ANALYSIS OF

AMINOGLYCOSIDE ANTIBIOTIC RESIDUES

IN MILK AND TISSUE USING

PAIRED ION CHROMATOGRAPHY,

ION-EXCHANGE SOLID-PHASE EXTRACTION, AND

IMMUNOAFFINITY CHROMATOGRAPHY.

4.1 INTRODUCTION

Gentamicin, neomycin and kanamycin are the analytes which are the subject of this study. Analytical methods for the determination of gentamicin and neomycin have been reported previously, but few methods are available for the analysis of kanamycin, particularly in biological matrices. None of the published methods are suitable for the analysis of these three antibiotics or present evidence of method validation.

The analysis of aminoglycosides is complicated by the fact that commercial preparations of these veterinary drugs contain various components, which often differ minimally in structure. It has been suggested that the various components may differ in toxicity and there is a trend towards development of chirally pure pharmaceutical preparations. Therefore, it is desirable to have a method capable of monitoring the various components of each veterinary drug preparation. Furthermore, aminoglycosides do not possess any properties that facilitate their direct detection in that they are not inherently fluorescent, they lack chromophores for UV detection, and they are not volatile for GC analysis.

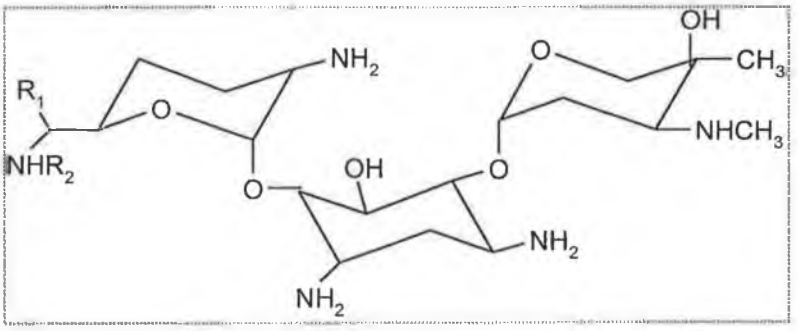
The purpose of this study was to investigate methods of analysis of gentamicin, neomycin and kanamycin in biological matrices. A chromatographic system was developed initially, using ion-pair chromatography with OPA post-column derivatisation. Ion-exchange SPE was employed for the clean-up of the three analytes from milk and the entire method was fully validated. An immunoaffinity

chromatography method was developed for the analysis of gentamicin in milk and tissue and the developed method was also validated.

4.1.1 Classification

Aminoglycosides are antibiotics produced by Streptomyces species (mycins) and Micromonospora species (micins). Structurally these antibiotics consist of amino sugars, attached via a glycosidic bond (sugar-O-R) to a central ring structure called an aminocyclitol. Aminoglycoside antibiotics are divided into 2 main groups, depending on the nature of this central ring; those containing streptidine and those containing 2-deoxystreptamine, with the latter group being the most important. The deoxystreptamine central ring contains amines and hydroxyl groups, whereas the streptidine ring contains more basic guanidine groups (NH-C-(NH_2)).

Deoxystreptamine antibiotics are further classified depending on the substituents attached. The neomycins and paromomycins form one class, where the substituents are at adjacent positions on the molecule, as opposed to the gentamicins and kanamycins where the substituents are isolated on nonadjacent positions of the aminocyclitol moiety. As mentioned in the introduction, aminoglycoside producing micro-organisms produce a mixture of active components; Seidl and Nerad[1] have separated five components of gentamicin designated C_1, C_2, C_{1a}, C_{2a} and C_{2b} (C_{2b} is also known as sagamicin). Of the five components isolated, the first three are major fractions, present in approximately equal amounts, and the remaining two components represent about 4 % of the total composition of gentamicin.



	R_1	R_2	R_3
C_1	CH_3	H	CH_3
C_2	H	H	CH_3
C_{1a}	H	H	H
C_{2a}	CH_3	H	CH_3

Figure 4.1 *Structure of gentamicin and its different forms, C_1, C_2, C_{1a}, C_{2a}.*

4.1.2 Mode of action and consequence of aminoglycoside residues in foods

Aminoglycosides are broad spectrum antibiotics, used in the treatment of disease, since streptomycin was first identified in 1944. They act by inhibiting protein synthesis, thereby preventing the growth of new bacterial cell walls. The 30S ribosomal subunit has multiple reaction sites for aminoglycoside interaction, resulting in inhibition of peptide chain elongation, necessary for the formation of new bacteria.

Aminoglycoside antibiotics are used to treat, control and prevent disease in food producing animals as well as for growth promotion. The presence of residues of these drugs in food is considered a high risk to the consumer. All aminoglycoside antibiotics are potentially toxic, and they are reported as being more toxic than penicillins. In

clinical medicine, blood from patients treated with these antibiotics is carefully monitored, due to the toxic effects of these antibiotics on auditory and nerve function resulting in irreversible damage[2]. Soltes reported that there is a small difference between the effective and toxic concentrations when these drugs are administered for treatment of disease in humans[3]. Also, intravenous administration of neomycin was reported to cause a marked loss of calcium in the urine due to impaired reabsorption in the proximal tubules of the kidneys[4]. However, there are no reports relating such toxic effects to the presence of aminoglycoside residues in foods.

Aminoglycoside antibiotics are most commonly used in veterinary drug medicine in the treatment of infections caused by aerobic gram-negative bacteria, such as mastitis[5]. The most common aminoglycosides used as veterinary drugs include gentamicin, streptomycin, dihydrostreptomycin and neomycin. Kanamycin is also used as a veterinary drug though it has not yet received approval for use in food-producing animals.

4.1.3 General physical/chemical characteristics

Aminoglycosides are soluble in water, possess limited solubility in methanol, and are poorly soluble in other solvents. Aminoglycosides exhibit resistance to heat, acids and bases. Aminoglycoside antibiotics are polar and ionic in nature. They possess very low UV absorption (absorb poorly at 190 nm). Characterisation studies have employed optical rotation. Withdrawal times of 3-63 days (depending on the antibiotic and animal in question) are recommended for the various aminoglycosides to reach a tolerable concentration in food intended for human consumption.

4.2 CHEMICAL METHODS OF ANALYSIS

4.2.1 Introduction

Previously, aminoglycosides have been analysed by non-chromatographic methods including spectrophotometry, colorimetry, polarimetry, polarography and fluorescence. However, these techniques have been used for the analysis of antibiotic levels in fermentation broths and pharmaceutical preparations and not for the analysis of residues in biological tissues and fluids. Current methods for aminoglycoside analysis are primarily based on HPLC. GC methods have been reported, but the requirement for derivatisation has hampered its employment. TLC methods have also been reported, but the sensitivity required for quantification of the analytes in biological matrices has resulted in HPLC becoming the most commonly used method. Most HPLC methods require the use of ion-pair chromatography (IPC) on reversed-phase columns and derivatisation is employed for detection either in pre- or post-column mode. Derivatisation to produce fluorescent products has proven most popular, allowing sensitive detection of analytes.

4.2.2 Spectrophotometry

A spectrophotometric method for the determination of amikacin, neomycin, streptomycin and kanamycin at a minimum concentration of 4 $\mu g\ ml^{-1}$ in pharmaceutical syrups and injections has been described by Zakhari[6]. The method involves nitration of the primary amino groups followed by reduction using cyanoacetamide at 100°C and UV detection at 270 nm. Colorimetric determination of tobramycin, kanamycin, amikacin and gentamicin in pharmaceutical ointments has been described by Ryan[7]. The aminoglycosides were derivatised with 2,4-

dintrofluorobenzene to form a coloured product with an absorbance maximum at 415 nm. However, the derivative exhibited limited stability.

4.2.3 Capillary electrophoresis

Aminoglycoside analysis using capillary electrophoresis has been reported. Flurer and Wolnik[8] separated gentamicin from injectable solutions, into three components using direct detection at 195 nm. The limits of detection were comparable to the more classical microbial methods (1 mg ml^{-1}). Ackermans *et al.*[9] described the analysis of thirteen aminoglycosides using micellar electrokinetic capillary chromatography. Neomycin was determined in a medicinal eardrop solution to test the method. Human plasma has been analysed for amikacin by high performance capillary electrophoresis employing fluorescence detection[10]. The method involves ultrafiltration of plasma prior to derivatisation with 1-methoxycarbonylindolizine-3, 5-dicarboxyaldehyde. Micellar electrokinetic chromatography using SDS was employed for separation of amikacin from the matrix. The authors also compared fluorescence detection of the amikacin derivative with UV detection of the derivative. Using UV detection, resolution was affected by reagent peaks and peak intensity was 1.7 times lower than that obtained by fluorescence detection (which had a limit of detection of 0.5 μg ml^{-1}).

4.2.4 Sensors

Rizk *et al.*[11] have described the fluorimetric determination of aminoglycoside antibiotics using lanthanide ion probe spectroscopy. The technique was applied to the analysis of neomycin, streptomycin, gentamicin, tobramycin, amikacin and kanamycin in pharmaceutical preparations. The method is based on the reaction of aminoglycoside

amino and hydroxyl groups with Eu III ions. The concentration of antibiotic is proportional to the amount of bound ligand, which has fluorescence intensity at 616 nm. Linearity was assessed between 0.2 and 1.0 mg aminoglycoside. The method was tested by analysing veterinary injectables.

Flow injection analysis has been employed in the determination of kanamycin has been described by Alwarthan *et al.*[12]. The authors describe a technique based on the suppression of the chemiluminescence of lucigenin systems when kanamycin is the rate-limiting reagent. The method was applied to the analysis of pharmaceutical preparations. The authors also suggest application of the system to other aminoglycoside antibiotics, but a separation step would have to be included in the assay, as the reaction is not specific.

4.2.5 Immunoassay

A substrate labelled fluorescent immunoassay for amikacin in human serum has been described by Thompson and Burd[13]. The method involves the labelling of amikacin with a fluorogenic substrate for the enzyme β-galactosidase. The enzyme is capable of hydrolysing the labelled analyte to form a fluorescent product. Cross reactivity was low, recovery was high (93-113 %) and inter-assay coefficient of variation was 4.7 % at a level of 5 μg ml^{-1}. Haasnoot *et al.*[14] have recently described the immunochemical detection of aminoglycosides in milk and kidney. Three ELISAs were described for the detection of gentamicin, neomycin and dihydrostreptomycin, with limits of determination of 0.7, 3.6 and 5.1 ng ml^{-1} for gentamicin, neomycin and streptomycin respectively. Monoclonal antibody production and the development of an ELISA for kanamycin, have been described by Watanabe *et al.*[15]. The validated method had a limit

of detection of 0.2 ng ml^{-1} and standard deviation for the analysis of milk, plasma and urine samples fortified at 4 ppb, was in the range 0.1 to 11 %. Brown *et al.*[16] described the extraction of gentamicin residues from tissues, using determination by fluorescence polarisation immunoassay. Sodium hydroxide digestion was compared with TCA precipitation. Tissue homogenisation with sodium hydroxide digestion produced recoveries of 85-94 % with an inter-assay variation of 7-8 %.

4.2.6 Chromatography

As with most methods for antibiotic analysis, chromatographic methods are the most popular for the analysis of aminoglycosides in tissue. Liquid chromatography and thin layer chromatography have been described for analysis of aminoglycoside residues in tissues and biological fluids. Gas chromatographic methods have also been reported, but mainly for the analysis of pure drug solutions.

Thin layer chromatography-bioautography has been reported by Salisbury *et al.*[17] for a variety of different classes of antibiotic residues, including streptomycin, dihydrostreptomycin, neomycin and gentamicin, in beef muscle. Residues were extracted from tissue by liquid-liquid extraction prior to spotting onto TLC plates. The authors reported co-elution of some antibiotics on the silica gel TLC plate so microbiological confirmation was necessary. This made the assay lengthy and time consuming. Limits of detection ranging from 0.33 to 3.3 for the antibiotics of interest were noted. Roets *et al.*[18] separated neomycin into its B and C components using TLC and fluorescence detection after derivatisation with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole. The limit of detection was 0.4 ppm.

Kanamycin and paromomycin determination by gas-liquid chromatography has been reported by Tsuji and Robertson[19]. Aqueous solutions of the antibiotics were silylated and separated on a packed, glass GC column and detected using flame ionisation detection. The method was capable of separating the two peaks of paromomycin and three components of kanamycin. The components were confirmed by mass spectrometric analysis. Neomycin was also analysed using similar derivatisation procedures, but instability of the derivatisation reagents can result in poor linearity and reproducibility.

Liquid chromatography has been described for the analysis of aminoglycoside antibiotics in serum, plasma, blood, urine, liver, kidney and muscle. HPLC separation of these very basic analytes has proved challenging for residue analysts. Reversed-phase chromatography is most commonly employed. Ion-exchange has also been reported for aminoglycoside separation. Fluorescence derivatisation is commonly employed to enhance the sensitivity of the method.

Determination of streptomycin and dihyrostreptomycin in animal tissue has been described[20] using liquid chromatography. Tissue extracts, obtained by acid extraction, were subsequently cleaned up by ion-exchange solid-phase extraction. Ion-pair reagent was added to the eluate prior to on-line sample enrichment and separation on a C8 reversed-phase stainless steel column. Post-column derivatisation, using 1,2-naphthoquinone-4-sulfonic acid was employed, allowing fluorescence detection. Streptomycin and dihydrostreptomycin were partially resolved from each other and recoveries were in the range 46-72 %, from various tissues. The detection limits were 10 ppb for streptomycin and 20 ppb for dihydrostreptomycin.

Four components of gentamicin, extracted from animal tissue and whole milk, were analysed by HPLC[21]. Liquid-liquid extraction was employed followed by ion-exchange solid-phase extraction. The extract was then subject to derivatisation using OPA and separated on a C18 column with isocratic elution. The detection limit for gentamicin in tissue was 1 ppm and 0.6 ppm in milk. Recoveries of greater than 90 % were reported with coefficient of variation no greater than 8.9 %. However, the chromatogram presented by the authors showed the peaks to be broad.

Strong cation-exchange was employed by Seidl and Nerad[1] for the baseline separation of five gentamicin components, with retention times of less than 20 minutes. Two 250 mm columns were required. Peak shapes displayed slight tailing and columns required regeneration after every 500 ml eluent had passed through. Cation-exchange after pre-column derivatisation was employed by Walker and Coates for the analysis of gentamicin, but the components were not separated successfully[22].

Sar *et al.*[23] have described a HPLC method for the analysis of gentamicin in calf tissues using ion-pair reversed-phase chromatography and post-column derivatisation with OPA allowing fluorescence detection. The method did not allow the separation of the gentamicin peaks. Following deproteinisation with trichloroacetic acid, sample extracts were cleaned-up using ion-exchange gel columns. Recoveries ranged from 68 % in kidney to 98 % in liver, for samples fortified at 100 ppb and relative standard deviations were 12.7 and 11.8 respectively. The limit of quantitation was 50 ppb in muscle fat and 100 ppb in liver and kidney. Agarwal[24] has also described a HPLC method for the analysis of gentamicin in animal tissue. The method involves liquid-liquid extraction of muscle tissue, followed by deproteinisation, and solid-phase clean-up on silica and ion-

exchange resin columns. The extract was derivatised by OPA on a silica SPE column. Gradient HPLC was employed to separate components of gentamicin on a reversed-phase column prior to fluorescent detection. Recoveries ranged from 86-108 % and the detection limit was 0.2 ppm.

OPA post-column derivatisation has been employed, allowing fluorescence detection for the determination of neomycin residues extracted from animal tissues[25]. Liquid-liquid extraction was employed followed by heat deproteinisation. The extracts were analysed by reversed-phase HPLC and ion-pair chromatography. The method separates neomycin from streptomycin and dihydrostreptomycin and could also be applied to muscle tissue. The recovery from kidney tissues was 96 %, with 9 % coefficient of variation, for samples fortified at a 1 ppm.

Refractive index detection has been used with ion-pair, reversed-phase, isocratic HPLC separation of eight aminoglycosides[26]. Several ion-pairing systems have been used for the separation of aminoglycosides. The counter ions frequently used are of the alkanesulphonate class, which are very difficult to remove from the sample and can cause deterioration of HPLC columns over time. The authors' report using volatile perfluorocarboxylic acid as the ion-pair agent, allowing separation of eight aminoglycosides when two separate mobile phases were employed. After separation, recovery of pure antibiotic is accomplished by lyophilisation of the mobile phase eluent, so this method may be advantageous if HPLC fractions require further analysis by, for example, MS. The method has not been applied to the analysis of residues in tissues. Refractive index detection has also been employed by Decoster *et al.*[27], for the determination of neomycin components. Ion-exclusion chromatography was employed

for the analysis of neomycin in pharmaceutical preparations and the authors report the use of a resin of a smaller granulometry, which resulted in reduced analysis time. There are no reports on the application of refractive index detection for the analysis of aminoglycoside residues in animal tissues or fluids.

Pulsed electrochemical detection has been reported for the analysis of aminoglycoside antibiotics. The presence of hydroxyl groups presents the possibility of electrochemical detection. Aminoglycosides are anionic at high pH values and are oxidised on a gold electrode. The gentamicin complex has been separated by anion-exchange ion chromatography on a carbohydrate column using gradient elution[28]. The method was applied to the analysis of medicinal injections and allowed the detection of 20 ng gentamicin without derivatisation. Tobramycin has also been detected by pulsed amperometric detection using a gold working electrode[29]. Anion-exchange separation under alkaline conditions was also employed for this hydrophilic analyte.

Lasar-based polarimetric detection has been employed for the analysis of gentamicin in milk[30]. The limit of detection was 7 $\mu g\ ml^{-1}$, well above the MRL (100 ppb). The authors claim to be the first to report a method capable of detecting four gentamicin peaks without employing derivatisation. However, baseline resolution was not achieved and the limit of determination in milk was not addressed.

Derivatisation is common in aminoglycoside analysis. Pre-column derivatisation of gentamicin has been described by Claes *et al.*[31]. Derivatisation with *o*-phthalaldehyde-mercaptoacetic acid reagent allowed UV detection at 350 nm. This derivatisation method required a 15 min incubation step at 60°C. Kanamycin, tobramycin, gentamicin

and siscomicin have also been determined using pre-column derivatisation[32]. The method described is based on the derivatisation of the aminoglycosides with 2,4,6-trinitrobenzenesulphonic acid reagent allowing UV detection at 350 nm. This method also requires a 15 min incubation at 70°C. The stability of the chromophore formed varies from compound to compound, but the addition of acetic acid to the derivative ensured its stability for up to 10 h. The neomycin complex was analysed using pre-column derivatisation with 2-napthalenesulfonyl chloride[33]. Relative standard deviations (RSDs) of between 0.9 and 1.4 % were obtained using this method and linearity was observed between 20-40 μg ml^{-1}. The derivatives were separated on a silica column.

Fluorescein pre-column derivatisation using 9-fluorenylmethyl chloroformate was used for the analysis of neomycin in kidney[34]. Recoveries of 80-120 % were reported from tissue fortified at 0.25-1.0 mg kg^{-1}. Limits of quantitation were 0.125 mg kg^{-1}. The extraction method described is lengthy and peak shape for the neomycin derivative is broad and tailing, when chromatographed on a C4 reversed-phase column.

4.2.7 Mass spectrometric techniques

There are few MS methods reported for aminoglycoside residues in fluids and tissues. No GC-MS method has been reported and few LC-MS methods are available, particularly for analysis of biological matrices. The necessity to use ion-pair reagents for the separation of aminoglycosides has hampered the use of LC-MS as these non-volatile reagents are not compatible with current interfaces. Mass spectrometry of aminoglycoside pure drug standards has been reported. Various ionisation techniques have been employed and with ion-spray proving the most popular. Ion-spray may be

considered suitable for aminoglycosides that are easily ionised, as severe conditions are not necessary[35].

Inchauspe *et al.*[36] used MS detection when investigating the selectivity of perfluorinated ion-pairs for the chromatographic separation of aminoglycosides. Field desorption-MS was employed and the study involved the analysis of pure standards only. Thermospray-MS (TSP-MS) was employed by Getek *et al.*[37] for the analysis of gentamicin by reversed-phase HPLC. Trifluoracetic acid was used as the mobile phase ion-pair, separating gentamicin into 3 peaks (C_{2b}, C_2, and C_1). The authors report that the TSP-MS of gentamicin in the positive ion mode produced low ion intensity for the $[M + H]^+$ ions, but cleavage of the glycosidic bonds resulted in fragments of high intensity. They concluded that the low intensity for the $[M + H]^+$ ions was due to the thermal lability of gentamicin, so it was necessary to monitor the ions produced from the purpurosamine fragment (the part of the molecule that differs from other aminoglycosides) for low analyte concentrations. The limit of detection for the total gentamicin complex was 20 $\mu g\ ml^{-1}$.

4.2.8 Extraction methods

The basic nature of aminoglycoside antibiotics causes them to accumulate in the kidney tissue of the body, where they bind to acidic phospholipids[38]. Therefore, this is the target organ for analysis of aminoglycosides, but many analysts choose to assay biological fluids, such as plasma, serum, milk and urine, as these matrices are easier to assay and the poor absorption of aminoglycosides in the intestinal tract can result in substantial partitioning into biological fluids. The analysis of aminoglycosides in biological matrices requires a deproteinisation step. Proteins can interfere with clean-

up, chromatographic resolution, derivatisation and detection. Organic solvents such as acetonitrile[39] and methanol[40], and acids have been used for extraction and/or deproteinisation. Haasnoot *et al.*[14] used trichloroacetic acid for deproteinisation in the analysis of gentamicin, neomycin and dihydrostreptomycin from kidney samples. Perchloric acid has been employed by Gerhardt *et al.*[41] for the analysis of streptomycin and dihydrostreptomycin in milk. Phosphate buffer, pH 4, has been employed by Shaikh and Jackson[42] for the extraction of neomycin from kidney, with samples being deproteinised by immersion in boiling water. Biological fluids have also been deproteinised using ultrafiltration; Oguri *et al.* used this procedure for the analysis of amikacin in human blood and plasma samples[10]. Following deproteinisation, the extract is usually buffered to an alkaline pH and undergoes further purification.

Brown *et al.*[43] carried out a study to compare sodium hydroxide digestion of tissue with trichloroacetic acid precipitation, with and without homogenisation. Kidney samples were subject to the following treatments; a) homogenisation in phosphate buffered saline (PBS), b) homogenisation in trichloroacetic acid (TCA), c) incubation in sodium hydroxide (NaOH), and d) homogenisation and incubation in sodium hydroxide. The authors concluded that recovery using NaOH digestion (treatment c) was significantly higher than for PBS or TCA treated samples. Recovery using NaOH with or without homogenisation was similar. Ultrasonic probe tissue disruption was compared with a rotary steel probe homogeniser and no significant difference in recovery was found. Gilbert and Kohlhepp[44] also compared homogenisation of kidney tissue with Tris buffer, TCA and NaOH, with heating to facilitate deproteinisation, for the analysis of tobramycin. They found that recovery of tobramycin from kidney fortified at 500 ppb was 32 % using heat deproteinisation in Tris buffer, 89 % using heat deproteinisation in

TCA and 99 % using heat deproteinisation in NaOH. The authors suggest that TCA and NaOH treatment of tissue homogenates results in hydrolysis of anionic tissue components resulting in liberation of the non-hydrolysed cationic aminoglycosides.

Solid-phase extraction is the most popular clean-up method reported, and liquid-liquid partitioning has also been employed. Washing of a tissue extract with methylene chloride to remove the more non-polar interferences has been reported by Salisbury *et al.*[17], in the analysis of muscle, kidney and liver for antibiotics, including aminoglycosides. The residues remained in the aqueous solution, while the non-polar matrix components partitioned into the organic solvent. D'Souza and Ogilvie[45] found liquid-liquid partitioning using acetonitrile/water and methylene chloride sufficient for the analysis of gentamicin in plasma but a C18 SPE step was necessary when the method was applied to the analysis of gentamicin in urine.

Cation-exchange SPE has been employed by Stead and Richards[46] for the analysis of gentamicin in fermentation broth and plasma. Analytes were loaded in phosphate buffer, pH 7.4, and a borate buffer (pH 9.0) wash step was followed by acetonitrile-borate (pH 10.0) elution. The authors explain that a pH of 10.0 was necessary to achieve gentamicin elution and the acetonitrile is thought to assist elution due to improved wetting of the dried solid-phase.

Matrix solid-phase dispersion has been reported by Mc Laughlin and Henion[47] for the extraction of streptomycin, dihydrostreptomycin, hygromycin and spectinomycin from kidney samples. These authors used mass spectrometric detection, so matrix interference was not as significant as with UV or fluorescence detection. Cyanopropyl

sorbent was employed, a hexane wash included and the analytes eluted with 1 ml water followed by 8 ml sulphuric acid. The normal phase sorbent chosen is polar in nature and is suitable for the extraction of aminoglycosides due to the hydrophilic functional groups on the molecules that is the presence of hydroxyl and amino moieties. The hexane wash removed fats and the analytes were eluted from the sorbent/tissue blend using an acidic solution, which protonates the functional groups of the analytes, thereby displacing them off the column.

4.2.9 Summary

To conclude this review, there is a lack of published literature for the determination of some aminoglycosides, such as kanamycin, particularly in biological matrices. The majority of methods for aminoglycoside analysis use time consuming, labour intensive sample preparation techniques. Few methods are capable of determining more than two analytes in one extraction procedure. This study presents the method development and validation for the analysis of gentamicin, neomycin and kanamycin in milk and kidney samples. Ion-exchange SPE and immunoaffinity column chromatography, allow rapid, reliable determination of the three analytes. A wide range of chromatographic features such as column type, system fittings and sample diluent are investigated, in addition to optimisation of the derivatisation technique employed.

4.3 EXPERIMENTAL

4.3.1 Introduction

Methods for the analysis of residues of gentamicin, neomycin and kanamycin in milk and tissue were developed. An ion-pair chromatographic system was developed using heptanesulfonic acid with methanol modifier. Fluorescence detection was achieved by means of OPA post-column derivatisation and derivatisation parameters were optimised. A method was developed for the extraction of the three analytes from milk and clean-up of the extracted residues using ion-exchange SPE. The developed method was validated on the basis of intra- and inter-assay variation in recovery, at five levels of fortification. The analysis of gentamicin residues in milk and tissue was also investigated using immunoaffinity column chromatography. The immunoaffinity chromatography method was validated for both matrices at three levels of fortification, again on the basis of intra- and inter-assays.

4.3.2 Chemicals and Reagents

All solvents were of analytical HPLC grade, unless otherwise stated.

a) Preparation of standards

Gentamicin, neomycin and kanamycin were purchased from Sigma-Aldrich Chemie BV., Zwjindrecht, The Netherlands. Stock solutions of 100 μg ml^{-1} were prepared in deionised water and stored in polyethylene tubes at 4°C for two months.

b) HPLC mobile phase

For ion-pair chromatography, the mobile phase consisted of 70 % buffer/30 % methanol. The buffer contained heptanesulfonic acid sodium salt (0.01 M), sodium

sulphate (0.05 M) and potassium dihydrogen phosphate (0.01 M). The pH was adjusted to 3.5 with ortho-phosphoric acid (98 % v/v) and all chemicals were purchased from Sigma-Aldrich Chemie BV., Zwjindrecht, The Netherlands.

c) Derivatisation reagent

A 0.0075 M derivatisation reagent was prepared by dissolving ortho-phthaldialdehyde (1.0 g) in methanol (50 ml), 500 µl mercaptoethanol was added, before diluting to 1 litre with borate buffer (0.5 M, pH 10). The pH of the borate buffer solution was adjusted using potassium hydroxide (10 M). The derivatising reagent was degassed prior to use and protected from light. It was used for a maximum of twenty-four hours.

d) Ion-exchange

For ion-exchange SPE, Bakerbond carboxylic acid, 3ml, 500 mg columns, purchased from J. T. Baker, Mallincrodt Baker B.V., Deventer, The Netherlands were employed.

20 % aqueous trichloroacetic acid (TCA) was prepared every 3 months.

Ion-exchange dilution buffer: citric acid buffer (0.05 M, pH 10) was prepared weekly, pH was adjusted using aqueous sodium hydroxide solution (50 %).

Ion-exchange washing buffer: citric acid buffer (0.05 M, pH 3.5) was prepared weekly and pH was adjusted using sodium hydroxide solution (50 %). Ion-exchange elution solution: citric acid buffer (0.05 M, pH 2.0) containing sodium sulphate (0.01 M).

e) Immunoaffinity column chromatography solutions

Immunoaffinity dilution buffer: Citric acid buffer (0.05 M, pH 7.0) was prepared weekly and pH was adjusted using aqueous sodium hydroxide solution (50 %). Immunoaffinity elution solution: citric acid buffer (0.10 M) was prepared containing sodium sulphate (0.02 M, pH 2.0). Phosphate buffered saline (PBS) was used for

washing and column conditioning. PBS containing 0.5 % sodium azide was used for column storage.

f) Immunoaffinity column preparation

Two immunoaffinity chromatography columns were prepared, one for gentamicin and one for neomycin clean-up, by covalently binding the respective antibody to these analytes onto a solid support. Polyclonal antibodies were obtained from a rabbit. The support used for the preparation of these columns was agarose, CNBr-activated Sepharose, (available in kit form from Pharmacia Biotech) which binds the antibodies through reaction with their primary amines. Preparation of the columns involved a number of steps, which are briefly outlined below;

1) Gel preparation

The CNBr-activated sepaharose, which is supplied in dried form, was washed with low pH buffer (pH 2-3) to remove additives and activate the reactive groups.

2) Coupling the ligand

The ligand was dissolved in coupling solution (pH 8.3 sodium carbonate buffer containing sodium chloride), while shaking gently overnight. Excess ligand was removed and un-reacted groups were blocked, by washing with mildly alkaline buffer. The gel was then washed with acetate buffer, pH 4, containing sodium chloride, followed by Tris-HCL buffer, pH 8, containing sodium chloride. This washing procedure is repeated three times.

3) Column preparation

The gel is diluted with binding buffer (PBS), to prepare a slurry. The slurry was poured into a plastic syringe barrel, which was fitted with a frit. Once the gel bed has settled, a frit was inserted into the syringe barrel and pushed onto the surface of the gel bed. The

column was flushed with three syringe volumes of buffer, before flushing with PBS, containing sodium azide (0.05 %) to inhibit microbial contamination. The column was then capped and stored at 4°C.

Prior to use the columns were equilibrated to room temperature, before flushing with three column volumes of PBS. The gel was prevented from running dry during this conditioning step or during loading, washing or elution, by the presence of the frit, installed on the surface of the gel. After use, the columns were washed with two column volumes of water, followed by one column volume of PBS, followed by storage, at 4°C, in PBS containing 0.05 % sodium azide.

4.3.3 Instrumentation

Chromatography: A Separations High Precision pump, model 300, (Separations, The Netherlands) was used to deliver the mobile phase at a flow rate of 0.3 ml min^{-1}. The column employed was a glass C8 column, 100 x 3 mm Chromsphere 5, supplied by Chrompack (The Netherlands). Sample volumes of 50 µl were injected using a Gilson autosampler, Model 234 (The Netherlands).

Derivatisation: Post-column derivatisation was used to form fluorescent OPA derivatives which were detected at 340 nm (Ex), 440 nm (Em), using a Separations fluorescent detector, model FP90. The mobile phase post-column was directed into a PEEK mixing tee, where it met with the post-column derivatising reagent, which was pumped at a flow rate of 0.6 ml min^{-1} by a Pharmacia Fine Chemicals pump, model P-500 (Pharmacia Biotech, The Netherlands). The mixing tee effluent was passed through a polypropylene coil of 100 x 0.05 cm before reaching the detector. The system was

allowed to equilibrate for 30 min prior to the first injection and the first injection was not included in validation studies.

4.3.4 Sample Fortification

Standard solutions were prepared prior to analysis, by diluting the aqueous stocks from 100 μg ml^{-1} to 10 μg ml^{-1} (working stock) with water, followed by further dilution to prepare fortification solutions and standards for curve preparation. Samples were fortified by the addition of 50 μl of the standard solution into a measured volume of sample. For milk, the sample was immediately vortexed and left to stand for 15 min. For fortification of tissue samples, 50 μl fortification solution was pipetted into the middle of the tissue, and the sample left to stand at room temperature for 15 min.

4.3.5 Sample Analysis

A) Milk analysis using ion-exchange clean-up

2 ml milk samples were measured into polypropylene tubes and samples were fortified as described in section 4.3.4. 400 μl volumes of aqueous TCA (20 %) were added to the fortified samples, and the samples were vortexed for 30 sec. The tubes were capped and mixed, head-over-head for 30 min. Following centrifugation at 3500 rpm for 20 min at 4°C, the resulting supernatant was immediately decanted off and diluted with 10 ml ion-exchange dilution buffer (see section 4.3.2 d). This solution was then loaded in 3 ml aliquots, onto an SPE column conditioned with 5 ml methanol, followed by 5 ml water, followed by 5 ml dilution buffer. After gravity elution, the column was washed with 2 ml ion-exchange washing buffer (see section 4.3.2 d) and centrifuged at 3500 rpm for

10 min. The analytes were eluted under vacuum with 2 ml elution solution (see section 4.3.2 d). 50 µl of this solution was injected into the chromatographic system.

B) Milk analysis using immunoaffinity chromatography clean-up

A 5 ml milk sample was measured into a polypropylene tube and the sample was fortified as described in section 4.3.4. A 1 ml volume of aqueous TCA (20 %) was added to the fortified sample, and the sample was vortexed for 30 sec. The tube was capped and mixed, head-over-head for 30 min. Following centrifugation at 3500 rpm for 20 min at 4°C, the resulting supernatant was immediately decanted off and diluted with 10 ml immunoaffinity dilution buffer (see section 4.3.2 e). This solution was loaded in 5 ml aliquots, onto the immunoaffinity column conditioned with 5 ml water, followed by 5 ml dilution buffer. After gravity elution, the column was washed with 3 ml PBS. The analytes were eluted under gravity with 2.5 ml immunoaffinity elution solution (see section 4.3.2 e). 50 µl of this solution was injected into the chromatographic system.

C) Kidney analysis using immunoaffinity chromatography clean-up

5 g kidney samples were weighed into 50 ml plastic tubes. The samples were fortified as described in section 4.3.4, 30 ml water was added to each tube and the tubes incubated for 20 min at 80 °C. The samples were centrifuged at 3500 rpm for 20 min and the resulting supernatant decanted off. The immunoaffinity columns were conditioned using 3 x 3 ml distilled water. The supernatant was allowed to flow through the immunoaffinity column under gravity. Following sample loading, the columns were washed with 15 ml PBS, followed by elution of analytes using 3 ml elution solution (see section 4.3.2 e). For tissue sample analysis, during sample loading it was necessary to restrict the flow from the column to approximately 1 ml min^{-1} to

achieve the recoveries presented. Higher flow rates resulted in lower recoveries due to incomplete analyte binding.

4.3.6 Method validation

A) Analysis of gentamicin, neomycin and kanamycin in milk using ion-exchange clean-up

For method validation, samples were fortified with each analyte individually i.e. samples were not fortified with a mixture containing all three analytes. The maximum residue limit set by the EU for the analysis of neomycin in milk is 500 ppb. Samples were fortified at 62.5 ppb (1/8 x MRL), 125 ppb (1/4 x MRL), 250 ppb (1/2 x MRL), 500 ppb (1 x MRL), 1000 ppb (2 x MRL) and 2500 ppb (5 x MRL). Five different samples were fortified at each level and analysed in one assay to assess the intra-assay variation. Inter-assay variation was assessed by the analysis of one sample, fortified at each level, repeated over five separate assays. A standard drug solution of concentration equivalent to that of the fortified sample was also analysed and included in a standard curve from which recovery was calculated. The MRL for gentamicin in milk is 100 ppb and this was assessed similarly (samples were fortified at five levels of fortification; 62.5, 125, 250, 500 and 2500 ppb). Recovery was estimated for the three components of gentamicin (referred to as peak 1, 2 and 3). Kanamycin MRL in milk is 500 ppb and samples were fortified at 100, 200, 500, 750 and 1000 ppb. The lowest level of fortification used for kanamycin studies was higher than the lowest level employed for gentamicin and neomycin due to the presence of slight matrix interference affecting kanamycin, as this was the earliest of the three analytes to elute. The high peak intensity of the kanamycin derivative caused a deviation from linearity at high analyte concentrations, therefore the highest level assayed was 1000 ppb.

B) Analysis of gentamicin in milk using immunoaffinity chromatography

Intra-assay validation was carried out on five milk samples fortified at 50 ppb, five milk samples fortified at 100 ppb and five milk samples fortified a 200 ppb, using the method described in section 4.3.5 (B). Inter-assay validation was also carried out at 50, 100 and 200 ppb, over five separate assays.

C) Analysis of gentamicin in kidney using immunoaffinity chromatography

Intra-assay validation was carried out on three kidney samples fortified at 50 ppb, three kidney samples fortified at 100 ppb and three kidney samples fortified a 200 ppb using the method described in section 4.3.5 (C). These levels are well below the maximum residue limit of 1000 ppb for kidney, set by the EU. Inter-assay validation was also carried out at 50, 100 and 200 ppb, over 3 separate assays.

4.4. RESULTS & DISCUSSION

4.4.1 Introduction

Method development and validation results are discussed in this section. Development of the chromatographic method is presented. Extraction of the analytes from milk with ion-exchange clean-up is discussed. Immunoaffinity chromatography applications are discussed and the results for validation of the method based on ion-exchange and immunoaffinity chromatography are compared.

4.4.2 Chromatography

Chromatographic analysis of aminoglycosides is challenging due to the basic nature of these analytes and the absence of UV chromophores on the molecules. Liquid chromatography of basic compounds may have tailing peaks, which are difficult to quantify and result in lower limits of detection. The primary cause of tailing for basic analytes is the presence of residual silanols on the HPLC column backbone. Despite advances in stationary phase packing, for example, the advent of type B silica, polymer based packing, silica still dominates as the backbone material.

For the analysis of basic compounds, most analysts work at low mobile phase pH in an effort to suppress silanol ionisation. However, basic analytes are positively charged under these conditions, therefore they are highly polar and elute off reversed-phase columns very quickly. Therefore, ion-pair chromatography is commonly employed. This can effectively change the charge on the column and so the polar analytes can be retained longer. This form of chromatography has associated problems: a) the slow

equilibration of the ion-pair reagent between the stationary and mobile phase requires long equilibration times and, therefore use of gradient elution is unsuitable, b) ion-pair systems are more sensitive to temperature changes as temperature affects the equilibrium of the ion-pair between the mobile phase and the column; in this study temperatures above 20°C were observed to result in poor peak shape, c) ion-pair systems often require high concentrations of mobile phase modifiers, increasing the risk of buffer precipitation, and d) there is greater likelihood for varying retention times, due to the long equilibration time required by ion-pair chromatography.

The absence of significant UV chromophores in the aminoglycosides places further demands on the analyst. Typically, derivatisation has been employed, either in pre- or post-column mode. Therefore, the mobile phase must not only be capable of resolving the basic analytes but also be compatible with the derivatisation reagent employed. A chromatographic method employing ion-pair chromatography has potentially poor reproducibility and the inclusion of a derivatisation step serves to increase its potential for high variation.

4.4.2.1 Column choice

The first parameter to be investigated was the choice of column. Despite the fact that one of the aims of the study was to employ derivatisation, UV detection was considered more desirable for this step as it would allow assessment of the native analyte separation by the column prior to derivatisation. However, a UV scan of the three analytes showed no UV absorbance above 190 nm, so UV detection of the native analytes was not possible. Therefore, it was concluded that derivatisation was necessary for detection, and post-column derivatisation proved the most successful.

The first column tested was a Waters C18 μBondapak column (3.9 x 300 mm). The mobile phase employed was a modification of that employed by Caturla and Cusido[48], who used ion-pair chromatography for the separation of kanamycin and amikacin. The mobile phase buffer was composed of heptane sulfonic acid, as the ion-pair, in the presence of sodium sulphate and potassium phosphate. In the initial chromatographic studies a mobile phase of 50 % buffer (containing heptane sulfonic acid as an ion-pair) was used. However, this resulted in co-elution of the derivatives with the solvent front. A mobile phase with 70 % buffer proved better for eluting peaks later, but peak shape still remained broad and tailing. Attempts to improve peak shape by varying mobile phase additives (ion-pair and modifier) proved unsuccessful, so an alternative column of different packing material was investigated.

A Lichrosphere 100 C18 column (5 μm, Merck) was used which had spherical packing particles offering the possibility of increased reproducibility. However, this column eluted all analytes at the same retention time, failed to separate the derivatised components and displayed severe peak tailing. A Lichrosphere 60 RP-Select B column (5 μm, Merck) was also tested. This base-deactivated column was expected to perform better for the analysis of the basic aminoglycosides. Using a mobile phase containing 70 % ion-pair buffer and 30 % methanol, partial separation of the three gentamicin components was achieved. Peak shape was also noted to improve but peaks remained broad. One peak was obtained for kanamycin, and one peak obtained for neomycin, but both peaks were affected by extreme tailing. Variation in mobile phase flow rate and modifier content failed to improve the peak shape and resolution of the gentamicin components.

Glass columns were then investigated. A 10 cm Lichropshpere C8 glass column was tested, but failed to resolve gentamicin into its various components. Kanamycin eluted as a broad tailing peak and neomycin eluted as one peak which had a slight "shoulder". A 10 cm Chromsphere glass C18 column was employed which improved neomycin and kanamycin tailing considerably and resolved gentamicin into three peaks. Figure 4.2 shows the peak shape and resolution for the three analytes on the Lichrosphere RP-Select B column and on the Chromsphere C18 column. To ensure the improvement in chromatography was due to the nature of the packing material, a Chromsphere glass C8 column was tested, for comparison with the Lichrosphere glass C8 column. Both C8 columns resulted in shorter retention than the C18, but the Chromsphere C8 column gave the best resolution for gentamicin, which was resolved as four peaks. The first peak exhibited very low peak intensity and the next two peaks were partially resolved. Neomycin was resolved relatively free from tailing, but it was now resolved as two peaks, one of which exhibited low peak intensity. Kanamycin was resolved as one peak, with good peak shape. When the concentration of analyte was dropped form 10 $\mu g\ ml^{-1}$ to 0.1 $\mu g\ ml^{-1}$, the peaks of low intensity were no longer apparent, therefore, they were not considered in quantitative analysis. The Chromsphere C8 glass column was employed in all further studies. The chromatograms in Figures 4.3-4.6 show peak shape and resolution, in standards and samples fortified with gentamicin, neomycin and kanamycin, using the C8 Chromsphere column. In conclusion, comparing the peak shape and resolution obtained using a Lichrosphere stainless steel column with a Lichrosphere glass column, peak shape was slightly better using the glass column. However, comparing the glass Lichrosphere column with a glass Chromsphere column, both C8 columns of similar dimension, showed the Chromsphere packing to result in much better chromatography. Comparison of the glass C18 Chromsphere with the

shorter chain length (C8), showed the C8 column resulted in shorter retention times but less tailing, particularly for neomycin.

4.4.2.2 Mobile phase

The effect of mobile phase pH on retention and peak shape was investigated. Buffers were prepared at pH 2.5, 3.0 and 4.0. Buffer of pH 2.5 was observed to give the best peak shape and resolution of gentamicin derivatives. As the pH was increased to pH 4.0, the peak area increased, but peaks became broader and resolution decreased. It is likely that the lower pH provides the best chromatographic conditions (due to reduced silanol activity) but the higher mobile phase pH resulted in higher intensities as it is closer to the optimum pH for the derivatisation reaction. A compromise of pH 3.5 was chosen to give both acceptable peak shape and intensity.

It became evident that it was necessary to maintain the column in mobile phase to achieve adequate chromatography. Rinsing of the column removed some of the ion-pair that built up on the column surface. The ion-pair acts by binding to the residual silanols and so rinsing of the column can result in exposure of these silanols to the analytes causing tailing and poor separation. The longer the column is equilibrated in an ion-pair environment, the greater the amount of ion-pair likely to build up until an equilibrium is established, and the greater the charge on the surface of the column. Occasionally, peaks were seen to be broad and poorly resolved. This could be rectified usually by rinsing the column in a gradient manner, starting with a mobile phase of high water content and gradually changing to high methanol content and back to high percentage water.

Many papers report good separation of aminoglycoside antibiotics using volatile acids such as polyflouroacetic acid or triflouroacetic acid. These acids act as ion-pairs, facilitating analyte retention, and their volatile nature renders them suitable for mass spectrometric detection. Non-volatile salts such as the heptane sulfonic acid used in this study are unsuitable for mass spectrometric detection, so the use of a perfluorinated acid was tested so that samples found positive by the post-column method could be confirmed by mass spectrometry. However, attempts to combine a mobile phase containing triflouroacetic acid with post-column derivatisation resulted in a highly coloured solution, which masked the detection of the analytes.

The effect of sodium sulphate in the mobile phase was investigated. Sodium sulphate is thought to compete with the aminoglycosides for the free silanol groups in the column packing material, therefore an increase in the sodium sulphate concentration may result in improved peak shape. A decrease in the concentration of sodium sulphate (from 0.05 M) resulted in late elution of analytes and peaks of low intensity. The delayed retention is likely due to the increased polarity of the sulphate ion relative to the sulphonate used as the ion-pair. Therefore, the sulphate ion is likely competing with the ion-pair for binding to the column and a decrease in sulphate ion concentration allows more ion-pair to bind, resulting in greater retention of the analytes. An increase in the concentration of sodium sulphate above 0.05 M was observed to have no effect, possibly due to the fact that the glass column provides few free silanol groups.

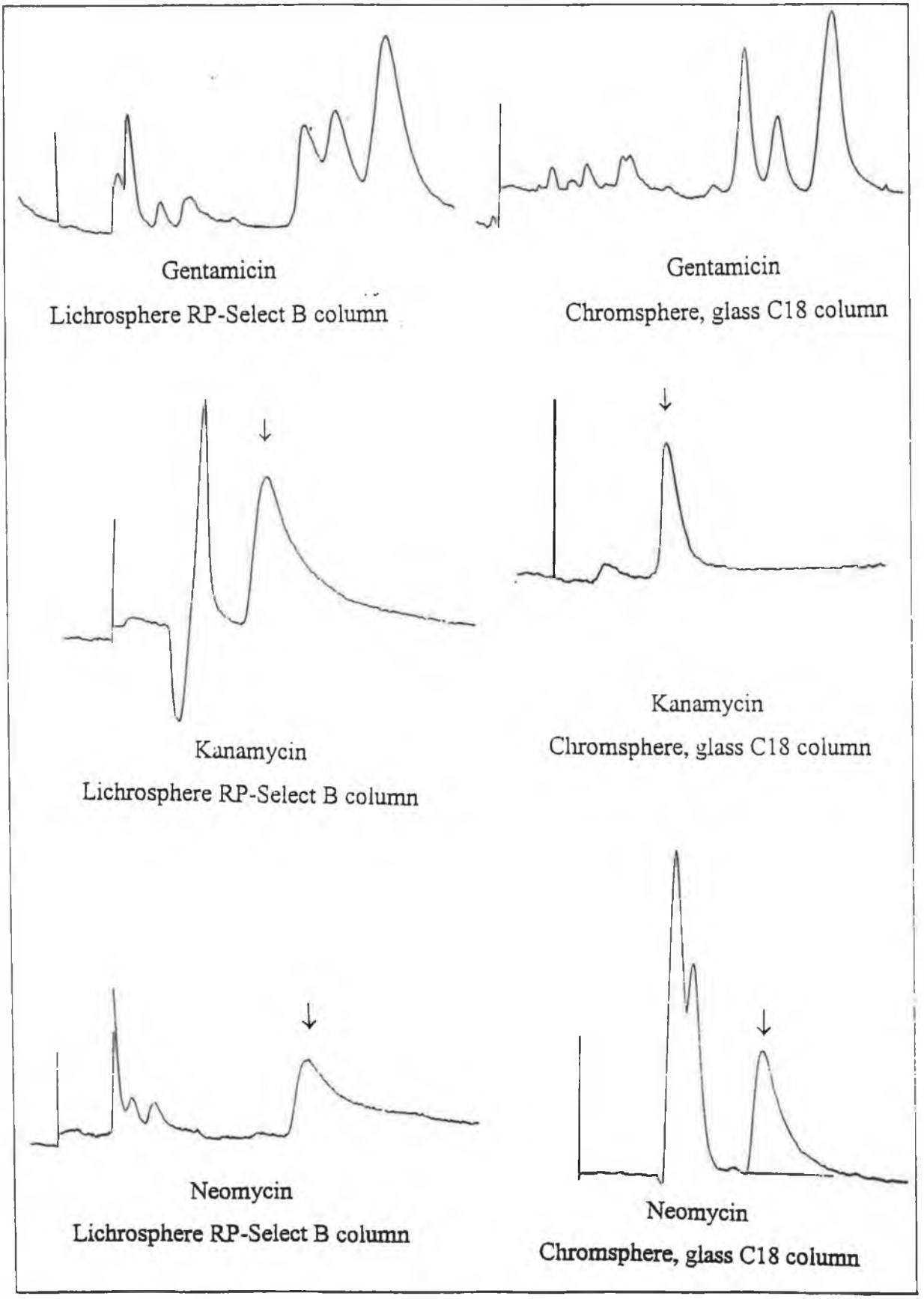


Figure 4.2 *Chromatograms showing peak shapes and resolution on the Lichrosphere RP-Select B column, and the Chromsphere, glass C18 column.*

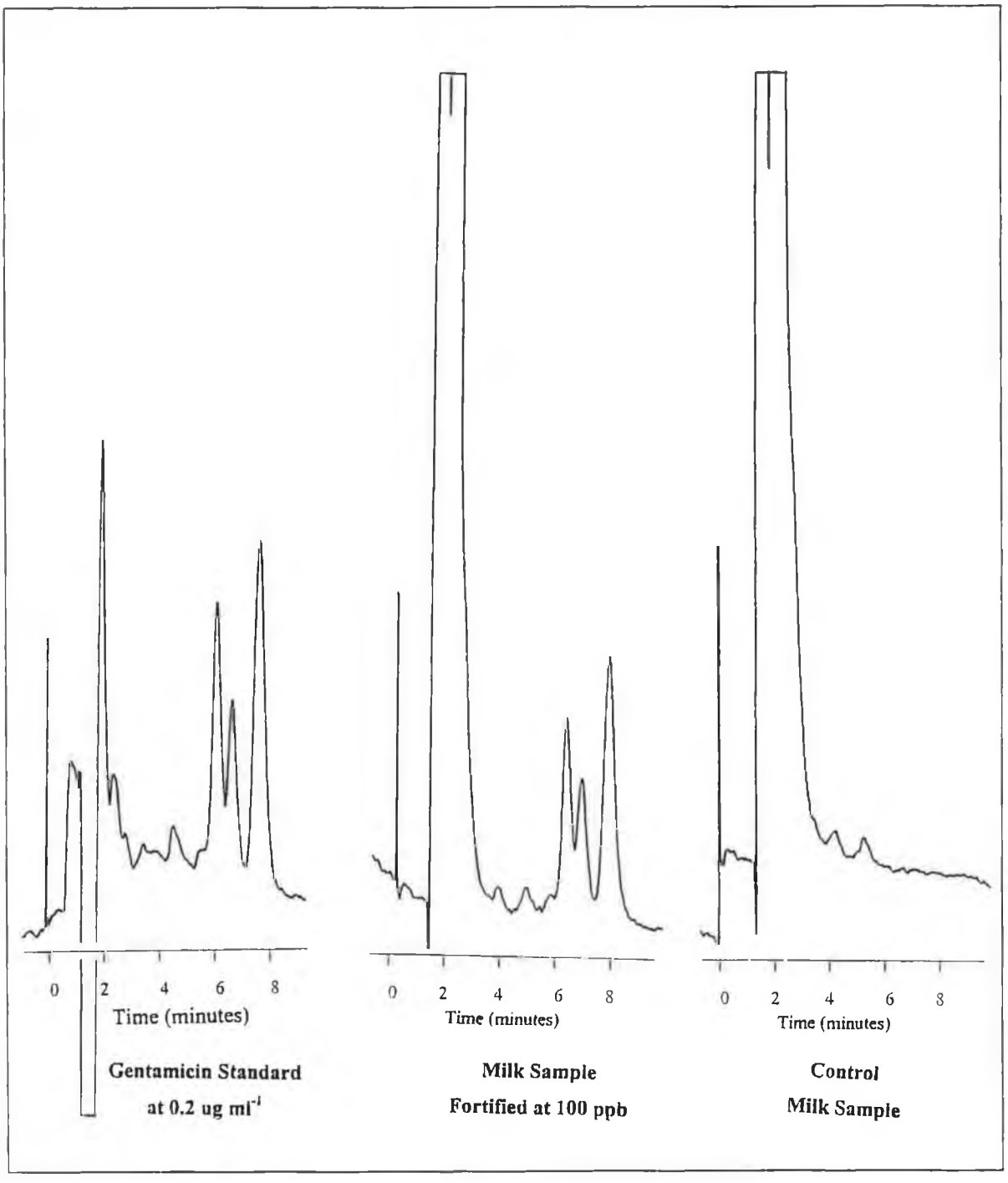


Figure 4.3 *Chromatogram showing a control milk sample and a milk sample fortified at 100 ppb with gentamicin and analysed using ion-exchange SPE.*

HPLC Conditions; Mobile phase: 70 % buffer [heptanesulphonic acid sodium salt (0.01 M), sodium sulphate (0.05 M), potassium dihydrogen phosphate (0.01 M)], 30 % methanol, at 0.3 ml min^{-1}. Injection volume: 50 ul, Post-column reagent: 7.5 mM OPA, containing mercaptoethanol (0.05 %) in borate buffer (0.5 M, pH 10), at a flow rate of 0.6 ml min^{-1}. Fluorescence detection 340 nm (Ex), 440 nm (Em).

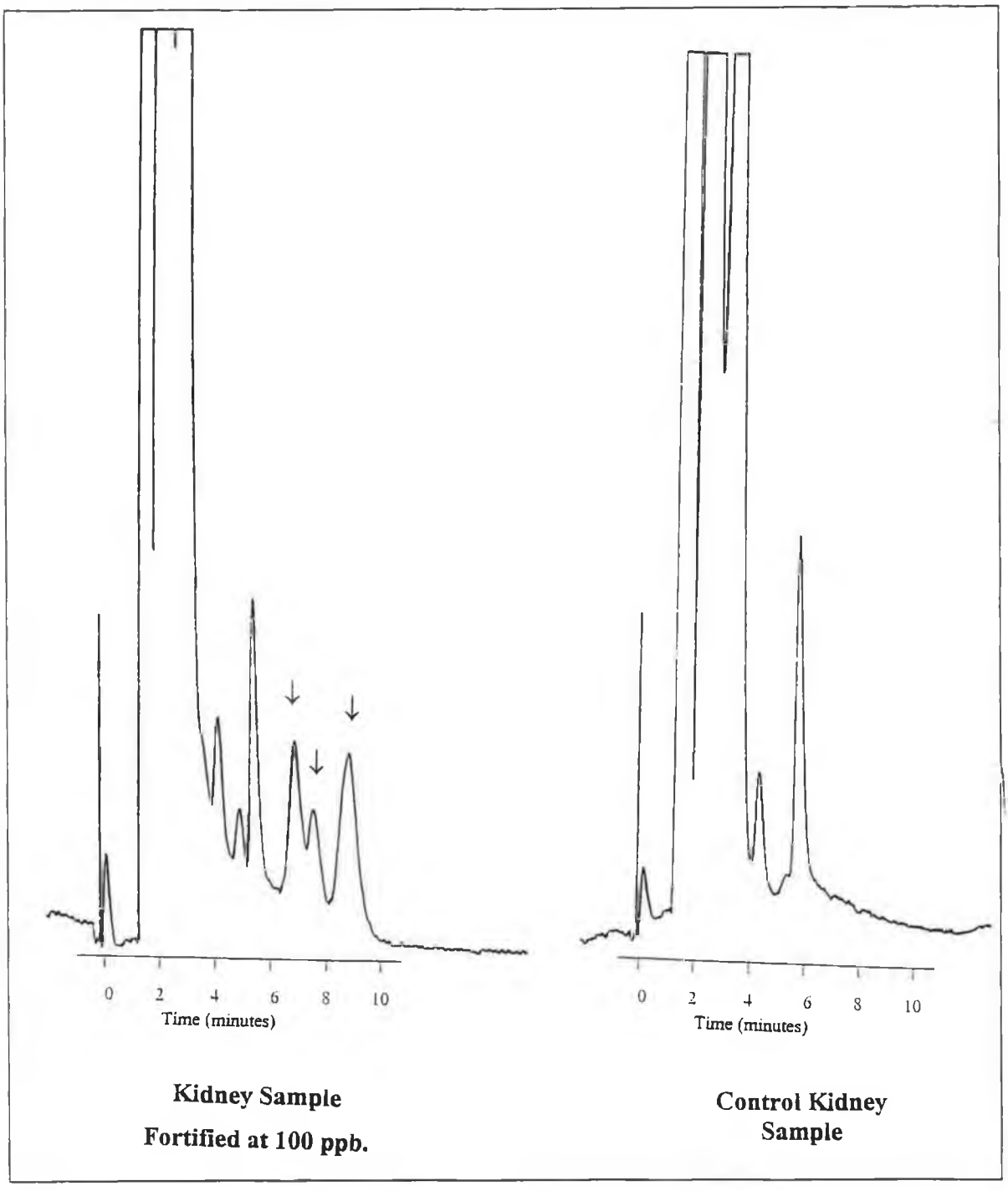


Figure 4.4 *Chromatogram showing a control kidney sample and a kidney sample fortified at 100 ppb with gentamicin and analysed using immunoaffinity chromatography.*

HPLC Conditions; Mobile phase: 70 % buffer [heptanesulphonic acid sodium salt (0.01 M), sodium sulphate (0.05 M), potassium dihydrogen phosphate (0.01 M)], 30 % methanol, at 0.3 ml min^{-1}. Injection volume: 50 ul, Post-column reagent: 7.5 mM OPA, containing mercaptoethanol (0.05 %) in borate buffer (0.5 M, pH 10), at a flow rate of 0.6 ml min^{-1}. Fluorescence detection 340 nm (Ex), 440 nm (Em).

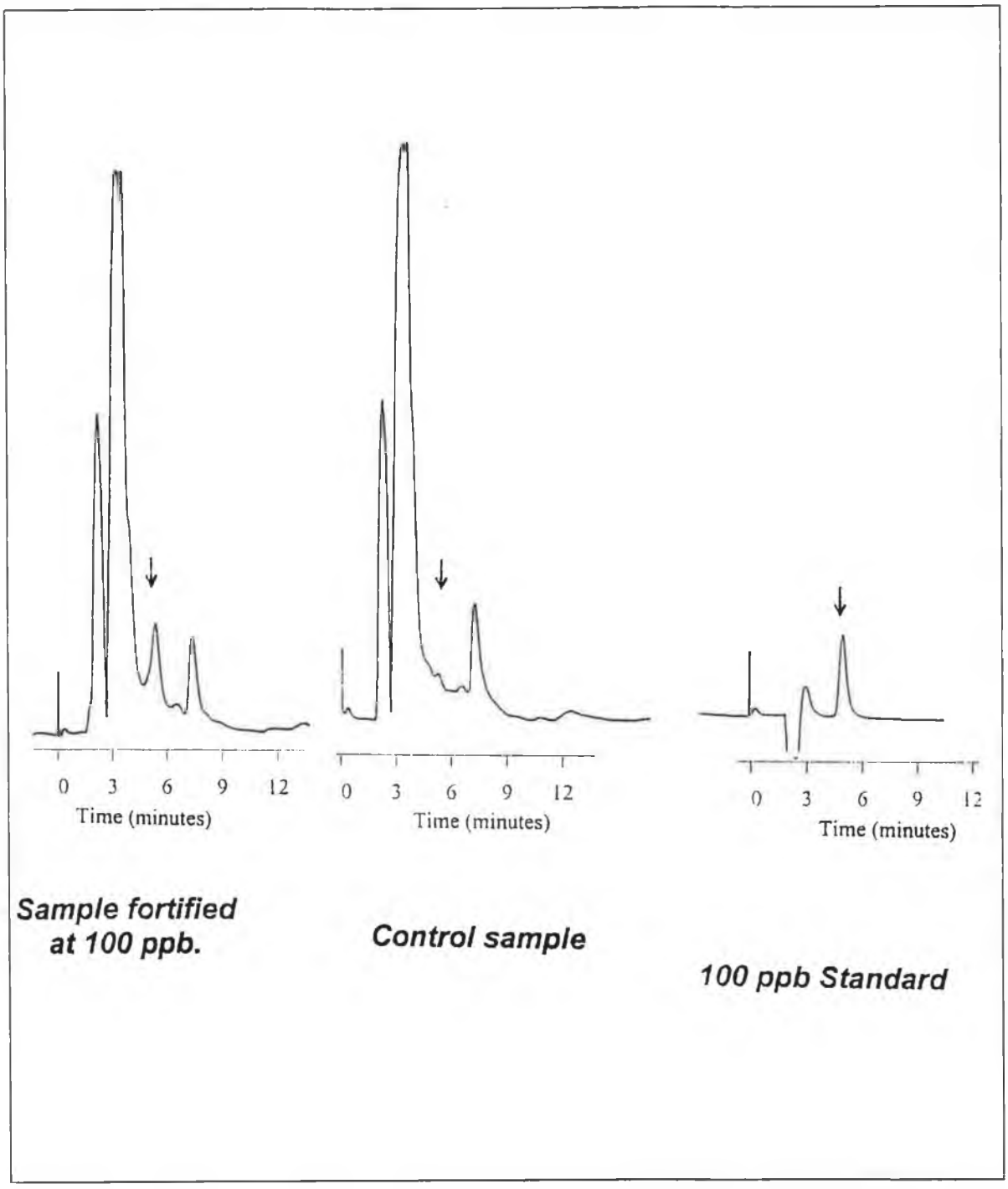


Figure 4.5 *Chromatogram showing a control milk sample and a milk sample fortified at 100 ppb with kanamycin and analysed using ion-exchange SPE.*

HPLC Conditions; Mobile phase: 70 % buffer [heptanesulphonic acid sodium salt (0.01 M), sodium sulphate (0.05 M), potassium dihydrogen phosphate (0.01 M)], 30 % methanol, at 0.3 ml min^{-1}. Injection volume: 50 ul, Post-column reagent: 7.5 mM OPA, containing mercaptoethanol (0.05 %) in borate buffer (0.5 M, pH 10), at a flow rate of 0.6 ml min^{-1}. Fluorescence detection 340 nm (Ex), 440 nm (Em).

4.4.2.3 System fittings

Many authors have reported that aminoglycoside antibiotics absorb to stainless steel. The system employed initially contained a stainless steel injection loop and stainless steel column end-fittings. These were replaced with polyetheretherketone (PEEK) fittings in an effort to improve neomycin tailing. The addition of PEEK fittings was seen to decrease the extent of variation; however neomycin still suffered some tailing. The bar chart in Figure 4.7 illustrates the decrease in variation in peak height when the stainless steel fittings were replaced by PEEK fittings. Variation for gentamicin is relatively high due to the fact that the peaks were not completely baseline resolved.

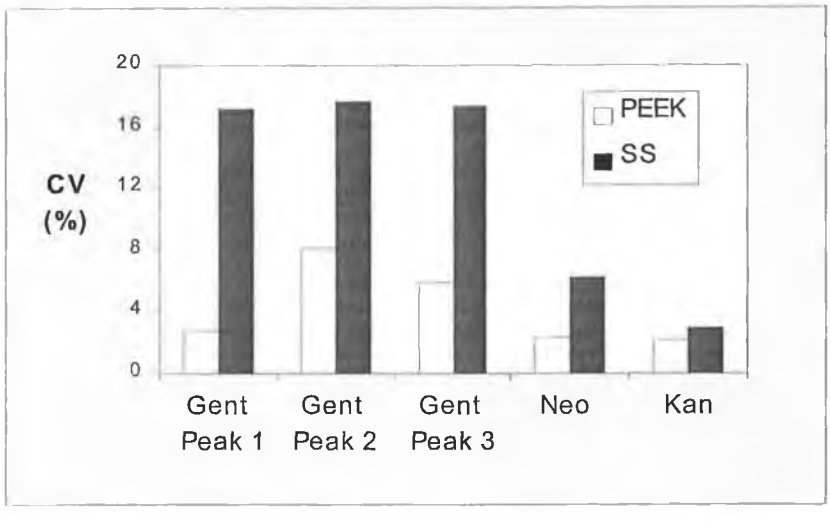


Figure 4.7 *Bar chart demonstrating the variation in peak height for gentamicin at 1 μg ml^{-1}, neomycin at 1 μg ml^{-1} and kanamycin at 0.1 μg ml^{-1} using PEEK and stainless steel (SS) HPLC fittings.*

4.4.2.4 Standard diluent

The composition of the standard diluent was investigated in an effort to improve peak shape. Mobile phase, water and 0.1 M hydrochloric acid were studied. Hydrochloric

acid was used as the compounds all possess very basic properties and liquid chromatography of basic compounds has been traditionally carried out at acidic pH, in an effort to reduce the activity of the residual silanols (by preventing their ionisation). Therefore it was postulated that dissolving the analytes in acid would result in better peak shapes.

When water was used as the standard diluent, carry-over was observed. Carryover was caused by the absorption of the highly basic aminoglycosides to the stainless steel fittings of the HPLC system. When the diluent was changed to 0.1 M hydrochloric acid, the carry-over problem did not occur. However, it was necessary to allow the column a 10 to 15 re-equilibration time between acidic injections. The acidic diluent was capable of stripping the ion-pair from the column, so re-equilibration was necessary. The acidic diluent resulted in taller peaks but neomycin still suffered tailing. Deterioration in peak shape and peak intensity after several injections in acid diluent was evident for gentamicin and neomycin but not for kanamycin.

Therefore, HPLC mobile phase was used as the standard diluent for further investigations. The acidic nature of this diluent, containing ion-pair reagent, prevented the carry-over problem observed when using water but without requiring column re-equilibration which was necessary when using hydrochloric acid. Changing the standard diluent from water to HPLC mobile phase resulted in increased peak intensity, but base-line resolution was not achieved for the gentamicin peaks, when water was used as the diluent.

Having improved chromatography by using PEEK fittings and employing a glass column, sample diluent was again investigated, as peak shapes were now more quantitative. The following diluents, 100 % water, 100 % mobile phase buffer, 70/30 v/v buffer/methanol (which is mobile phase), and 70/30 v/v water/methanol were investigated, with respect to linearity and reproducibility for all three analytes.

Table 4.1 Variation in peak height of multiple injections of the same standard in various diluents, using PEEK fittings.

Diluent	*Coefficient of variation (%), n=5*		
	Neo (2 μg ml^{-1})	**Kan (0.1 μg ml^{-1})**	**Gent (1 μg ml^{-1})**
Water	4.4	7.8	27.8, 13.7, 7.0*
Buffer	0.8	2.1	2.7, 8.2, 5.9*
Mobile phase	4.9	3.0	11.9, 6.2, 2.6*
70/30 water/methanol	7.9	11.9	8.0

* Multiple values for CV for an analyte refer to the different peaks separated

Table 4.1 illustrates the effect of the various diluents on reproducibility.

- When water was used as the diluent, the analytes were retained longer on the column resulting in broad peaks, poor resolution of the three gentamicin peaks, and high variation in peak response.
- When 70/30 water/methanol was used as the diluent, gentamicin resolution was lost and eluted as one peak. Variation was also found to be relatively high for all three analytes.
- Mobile phase showed relatively low variation for most peaks. Gentamicin was resolved as three peaks, however, variation was high for the first peak, possibly

due to the fact that base-line resolution was not achieved between the first and second gentamicin peak. Neomycin and kanamycin eluted as one peak each, with low variation.

- Mobile phase buffer showed the best resolution and the lowest variation for all three analytes and the gentamicin peaks were separated. This may be due to the presence of ion-pair in this sample diluent; some authors have reported the inclusion of ion-pair reagent to sample extracts prior to injection into the chromatographic system to achieve adequate peak shape[49].

Linearity was assessed between 0 and 2 $\mu g\ ml^{-1}$ for gentamicin and neomycin and between 0 and 0.4 $\mu g\ ml^{-1}$ for kanamycin. Using buffer and mobile phase as diluents, R^2 values of 0.99 to 1.0 were obtained for each peak of each analyte, demonstrating the method to be linear in the range studied.

During sample analysis, analytes were eluted off the SPE in citric acid buffer (0.05 M) containing sodium sulphate (0.01 M). This diluent was observed to result in a slight loss of resolution of gentamicin peaks 1 and 2 relative to when mobile phase buffer was used as the diluent. Standards were prepared in citric acid-sodium sulphate buffer containing each of the three mobile phase buffer components separately (heptane sulfonic acid, sodium sulphate and potassium dihydrogen phosphate). The addition of potassium dihydrogen phosphate resulted in a loss of baseline resolution for all gentamicin peaks. The addition of sodium sulphate also resulted in a loss of baseline resolution for the gentamicin peaks, but the addition of the ion-pair reagent (heptane sulfonic acid solution) was seen to restore resolution. However, peak intensity

decreased by approximately 10 %, so unmodified citric acid buffer-sodium sulphate was employed as the standard and sample diluent.

4.4.3 Storage of standards

Stock solutions of 100 μg ml^{-1} of each analyte were prepared in water and stored at 4°C in polypropylene containers. Due to the hydrophilic nature of the aminoglycoside molecules, they are susceptible to adsorption to glass. This theory was confirmed by storing standards in glass and polypropylene and subjecting them to analysis after two weeks storage at 4°C. The peak intensity of the standards stored in glass remained the same for neomycin, but decreased by an average of approximately 10 % for gentamicin and kanamycin.

4.4.4 Derivatisation conditions

From the literature, derivatisation using OPA proved to be most commonly applied to aminoglycoside analysis and also appeared to be the most sensitive. Addition of this non-polar group may also assist retention of the analytes on the column. Pre-column derivatisation was initially investigated, resulting in a highly conjugated product, which could be detected at 350 nm, or by fluorescence detection at 340 nm (Ex) and 440 nm (Em).

The pre-column derivatisation reagent used (OPA in the presence of mercaptoethanol), was similar to that employed by Caturla and Cusido[48]. At an analyte concentration of 20 μg ml^{-1} two peaks were obtained for gentamicin, one peak of low intensity for neomycin, and kanamycin displayed one peak but poor reproducibility. Peak shapes

were notably broad and tailing. The mobile phase composition was changed from 50 % buffer to 70 % buffer which resulted in improved peak shape but caused the derivatives to elute off the column more quickly and so only one gentamicin peak was free from the solvent front. Therefore, post-column derivatisation was investigated. The post-column reaction was optimised in an attempt to maximise sensitivity. The first parameter optimised was the reagent flow rate. The reagent flow rate was varied while the mobile phase flow rate was kept constant, at 0.3 ml min^{-1}. A higher mobile phase flow rate resulted in faster elution and a decrease in the resolution of the gentamicin peaks. Also, at higher mobile phase flow rates, peak intensity was reduced due to greater dilution of the derivatives with mobile phase. Figure 4.8 shows the response of gentamicin peak 3 to increasing the post-column reagent flow rate. Although gentamicin peak 3 was chosen for demonstration purposes, a similar trend was observed for all other gentamicin peaks. A flow rate of 0.9 ml min^{-1} is seen to be optimum at a mobile phase flow rate of 0.3 ml min^{-1}. Increasing the post-column reagent flow rate resulted in increased peak intensity but also in loss of baseline resolution for the gentamicin peaks. A reagent flow rate of 0.6 ml min^{-1} provided the best trade-off between peak intensity and resolution.

The effect of varying the reaction coil length and internal diameter was also investigated in an effort to restore resolution. This experiment was carried out on gentamicin, as this was the only analyte where multiple peaks were required to be resolved. The coil length was first decreased (from 100 cm to 60 cm) resulting in an improvement in the resolution between peaks 1 and 2 of gentamicin, however baseline resolution was still not achieved. While maintaining the coil length at 60 cm, the internal diameter was changed from 0.08 cm to 0.05 cm, which again assisted resolution of peaks 1 and 2.

This internal diameter was maintained and coils of 180 cm and 30 cm length were tested; however, the longer coil resulted in loss of resolution of gentamicin peaks 1 and 2 and the shorter coil resulted in a noisy baseline, due to non-ideal mixing. Therefore, the coil length was maintained at 60 cm and the internal diameter dropped to 0.05 cm. This coil gave the best compromise between baseline noise and gentamicin peak resolution. Gentamicin peaks 1 and 2 were still not completely baseline separated as is evident in the chromatogram in Figure 4.3.

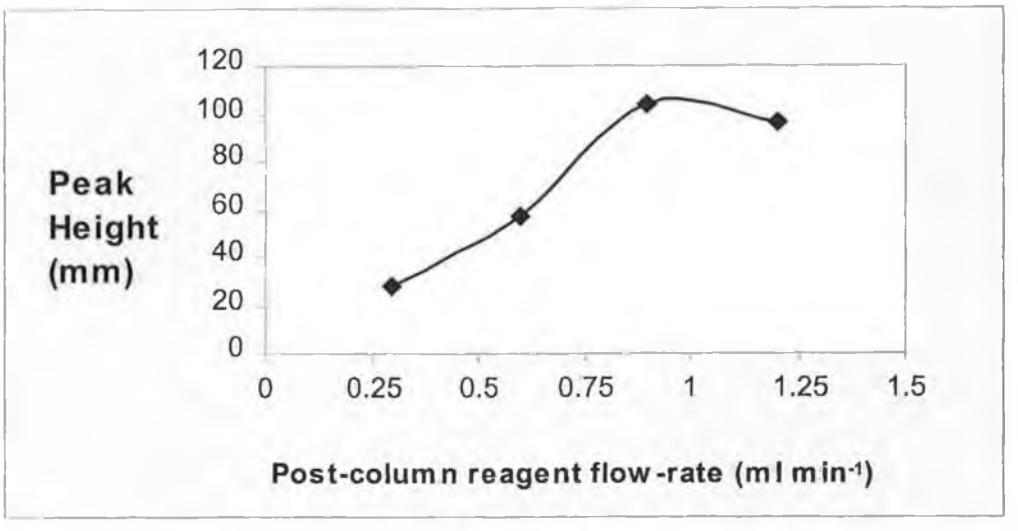


Figure 4.8 *The effect of post-column reagent flow rate on the height of gentamicin peak 3 at a mobile phase flow rate of 0.3 ml* min^{-1}.

Attempts to increase peak separation by increasing the analyte retention time on the column were unsuccessful. Increasing the concentration of ion-pair in the mobile phase resulted in loss of gentamicin resolution and a decrease in peak intensity. Inclusion of EDTA in the mobile phase did not assist analyte resolution and caused ion-pair precipitation during over-night chromatographic runs. Various ratios of organic

modifier to buffer were tested but no improvement was achieved. The nature of the modifier was not found to improve peak resolution.

4.4.4.1 Optimisation of borate buffer pH

The graph in Figure 4.9 shows the results of borate buffer pH investigations. It shows that the pH of the post-column reagent is critical and that optimum pH of borate buffer is 11. The actual pH at which the reaction occurs is due to a combination of the mobile phase pH and the post-column reagent pH. Above and below a pH of 11, the peak intensity is reduced as the reaction is not occurring at its optimum pH of 7-8. Standards of 0.25 μg ml^{-1} were chosen, as this was the concentration equivalent to the MRL (500 ppb) using the method described.

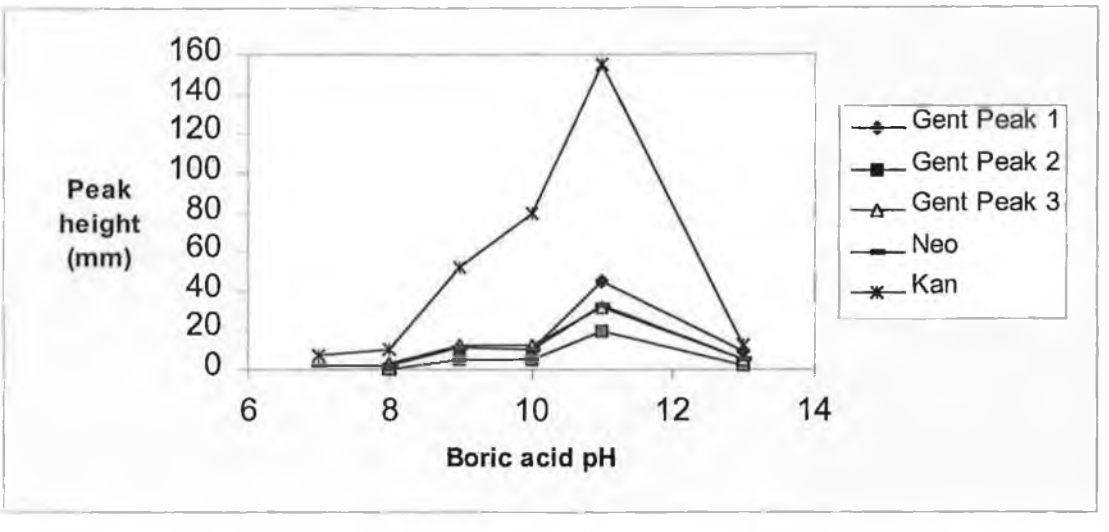


Figure 4.9 *Optimisation of borate buffer pH for derivatisation of gentamicin, neomycin and kanamycin, all at 0.25 μg ml^{-1}.*

4.4.4.2 Optimisation of borate buffer concentration

Borate buffer concentration of 0.5 M was employed in the preparation of the derivatising reagent. From the graph in Figure 4.10, peak intensity was lower, below this concentration. Below 0.5 M, it is likely that the buffering capacity of the solution is not sufficient to maintain the optimum reaction pH in the mixing tee and reaction coil. A slight increase in peak intensity was observed at buffer concentrations beyond 0.5 M, however, higher concentrations, caused intrumental problems.

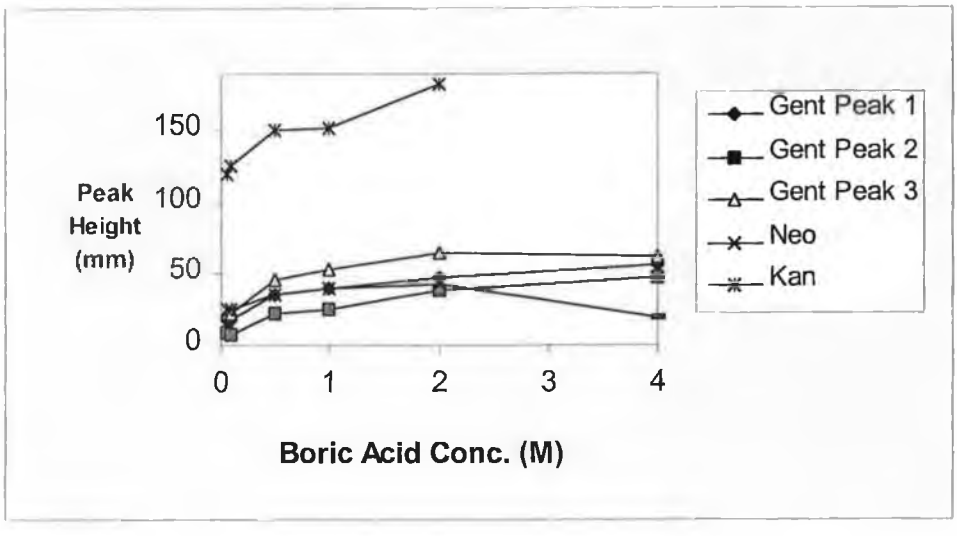


Figure 4.10 *Optimisation of borate buffer concentration for derivatisation of gentamicin, neomycin and kanamycin, all at 0.25 μg ml^{-1}.*

4.4.4.3 Optimisation of OPA concentration

From the graph in Figure 4.11, an OPA solution of 100 mg 100 ml^{-1} results in high peak intensity for a 0.25 μg ml^{-1} analyte solution. There was no significant increase in peak intensity beyond this concentration.

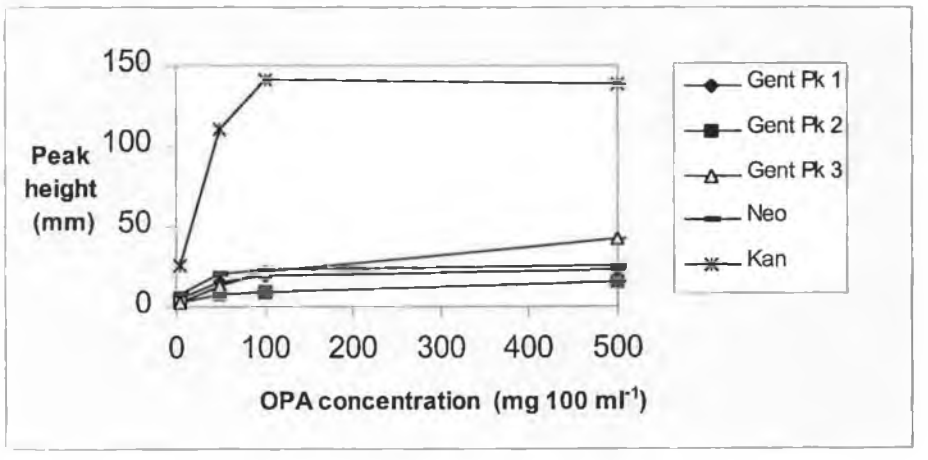


Figure 4.11 *Optimisation of OPA concentration for the derivatisation of gentamicin, neomycin and kanamycin, all at 0.25 μg ml^{-1}.*

4.4.4.4 Mercaptoethanol volume

This is the catalytic agent for the derivatisation reaction, therefore it is important to ensure that this reagent is not limiting the reaction. Using a 100 mg 100 ml^{-1} OPA solution, 25 μl mercaptoethanol was observed to be sufficient for the derivatisation of 0.25 μg ml^{-1} analyte solution. Figure 4.12 below shows the volume of the mercaptoethanol to be one of the less critical parameters.

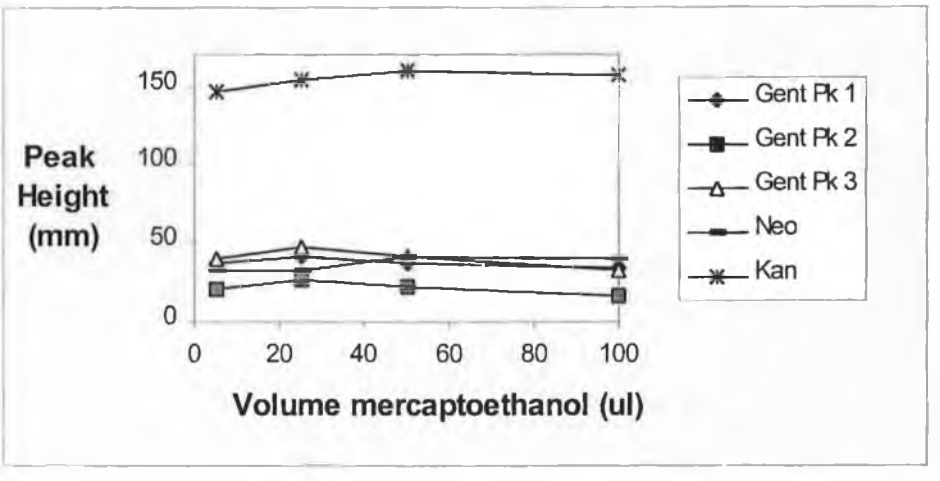


Figure 4.12 *Optimisation of mercaptoethanol volume for derivatisation of gentamicin, neomycin and kanamycin, all at 0.25 μg ml^{-1}.*

4.4.4.5 Two solution post-column derivatisation studies

Derivatisation of aminoglycosides using OPA suffers from instability in both pre-column and post-column mode. The derivatives show limited stability using pre-column derivatisation and the derivatisation reagent shows limited stability using the post-column method. For post-column derivatisation, the reagent is composed of OPA in the presence of mercaptoethanol. The solution is prepared in borate buffer buffer pH 11.0 as this basic pH is necessary to allow the reaction to occur. At this high pH, secondary reactions occur over-time within the reagent itself, thereby reducing its ability to derivatise the analytes. It is postulated that lowering of the pH would reduce these secondary reactions and so remove the problem of instability. However, to allow the derivatisation reaction to occur it would be necessary to increase the post-column mobile phase to a more basic pH. The post-column mobile phase may be made basic by the introduction of sodium hydroxide, after the column and before the introduction of OPA, as in Figure 4.13. In addition to overcoming the instability problem, it was suspected that the two solution system might increase the specificity of the method. In the one solution method, the post-column mobile phase combines with the basic pH and derivatising reagent together, at the same point in time. Using the two-solution method, the post-column mobile phase is made basic firstly and any susceptible substances present were reduced by the sodium hydroxide, before combining with the derivatisation reagent (OPA).

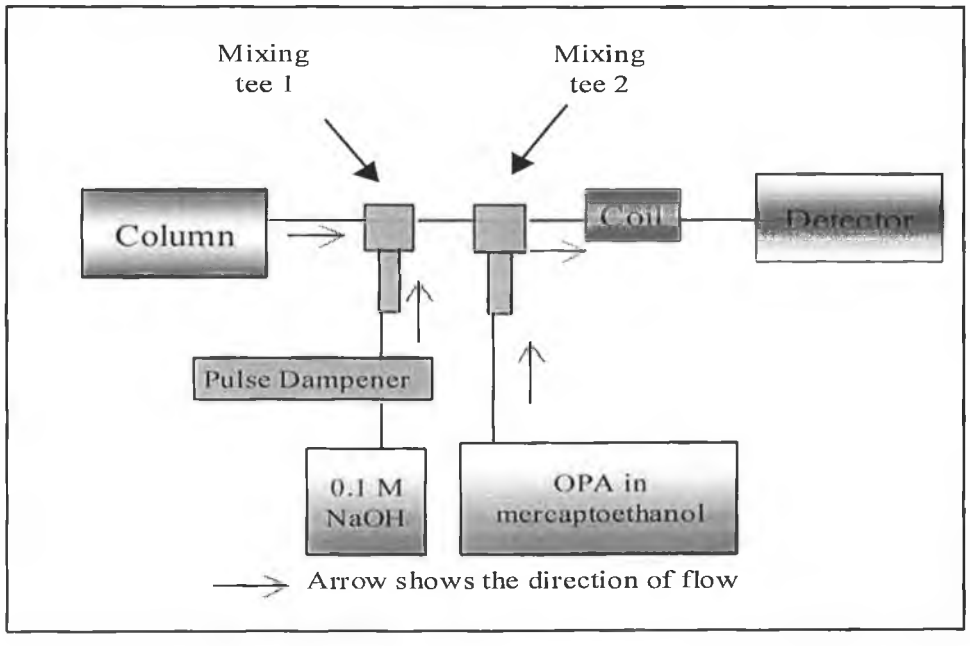


Figure 4.13 *Schematic of two solvent post-column derivatisation system.*

The one solvent and two solvent systems were evaluated with respect to reproducibility, linearity, baseline noise, peak shape and specificity. Various concentrations of OPA were compared. Peak intensity was reduced using the two solvent system and the variation in peak height was generally greater. Peak shape and retention times were similar using both systems. Despite the inclusion of mixing coils before and after mixing tee 2 (see Figure 4.13), baseline noise was greater using the two solvent system, resulting in a less sensitive method. The reagent lifetime of the OPA/mercaptoethanol solution (used in the two solvent method) was increased relative to the OPA/mercaptoethanol/borate buffer solution (used in the one solvent system). With respect to linearity over the range 0.125 to 2.5 $\mu g\ ml^{-1}$, the increased variation of the two solvent system caused R^2 values to be reduced from 0.99 for the one solution system to 0.97 for the two solution system. Fortified samples were also investigated, as it was suspected that the two solvent system might be more specific, with less matrix

co-extractives evident in the chromatogram. However, chromatograms were similar with respect to matrix interferences and the reduced peak intensity of the two solvent system made quantitation more difficult. Therefore, the classical one-solvent post-column derivatisation configuration was employed for further investigations.

4.4.5 Sample extraction

The first step commonly employed in the analysis of antibiotics in complex matrices is a deproteinisation step. The presence of proteins can interfere with clean-up and derivatisation and cause fouling of the analytical column. Sar *et al.*[23] reported the use of trichloroacetic acid (TCA) to deproteinise tissue samples. In this study, TCA (20 %) proved successful in producing a clear supernatant following mixing and centrifugation of milk samples. 30 min shaking in a head-over-head mixing device was found to be necessary to achieve intimate mixing of the TCA with the milk sample; shorter mixing time resulted in a cloudy supernatant following centrifugation, which took longer to flow through the SPE column. Following centrifugation, a firm pellet remained allowing the supernatant to be decanted off. Most milk samples also had a thin fatty layer on the surface of the supernatant, however, this adhered to the plastic tube while the supernatant was decanted off.

4.4.6 Sample clean-up using ion-exchange

Due to the basic nature of the aminoglycosides, it was decided to investigate the use of carboxylic acid columns for their clean-up. These columns possess carboxylic acid (COOH) groups which are capable of trapping analytes possessing positive functional groups, such as the amine groups of the aminoglycosides. To ensure retention of the

analytes on the cation-exchange column it was necessary to dilute the acidic supernatant with a basic buffer. In an acidic environment the acidic groups on the column are fully protonated and so the analytes will pass through unretained. In a basic/neutral environment, the carboxylic acid groups are unprotonated (COO^-) and capable of retaining positively charged species. At pH of 7.0 or above the amino groups of the aminoglycosides exists as positive ions and so are trapped on the activated column. The bar chart in Figure 4.14 shows the effect of citric acid buffer solutions (0.05 M) of various pH values on analyte retention; citric acid buffer pH 10 was found to give maximum retention. The peak height response for gentamicin peak 3 (last eluting peak) is shown, but retention was similar for all gentamicin components.

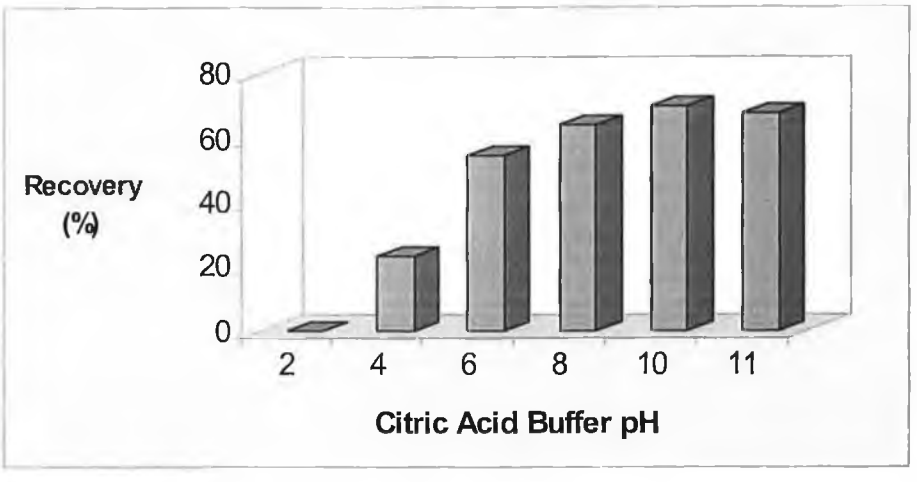


Figure 4.14 *Bar chart of the optimisation of the SPE loading buffer pH for gentamicin peak 3 at 2 μg ml^{-1}.*

Dilution of the acidic supernatant in 5 ml pH 10 buffer resulted a solution of around pH 8, in preparation for SPE. Steps taken to increase the sample extract to pH 10 did not result in a significant increase in recovery. Based on the SPE cation-exchange theory already discussed, the analytes could be removed from the column by an acidic buffer.

HCl (0.1 M) proved capable of eluting the analytes off the column. However, studies on the effect of sample diluent for chromatography (section 4.4.2.4) showed that continuous injection of analytes in HCl (0.1 M) required reconditioning of the column at regular intervals. Citric acid buffer, pH 3.0, was found to elute the analytes off the column, but 4 ml of this solution was required to achieve elution from the SPE column. Inclusion of sodium sulphate with the citric acid buffer resulted in elution of the analytes in 2 ml, resulting in a final solution twice as concentrated. The sodium sulphate provides positive ions capable of competing with and displacing the aminoglycosides off the carboxylic acid column. Sodium sulphate alone was suitable for analyte elution, but it was necessary to adjust the pH to be similar to when citric acid is present. The citric acid-sodium sulphate solution was pH 2 on preparation. It was feared that continuous injection of such an acidic solution onto a reversed-phase column might affect the column so attempts were made to elute the analytes off the SPE in a solution of higher pH or containing an organic modifier. However, recovery was reduced when those solutions were used. Ultimately, continuous injection of the citric acid-sodium sulphate solution did not affect the column performance, so this was employed for SPE elution.

Following loading of the SPE column, a wash step was applied to remove matrix interferences. Initially, a water wash was investigated; however, matrix co-extractives still interfered with the resolution of neomycin and kanamycin and peak 1 of gentamicin. Since elution in citric acid buffer required 4 ml volume, it was considered that a volume of 2 ml might be used to elute matrix components without causing elution of the analytes. Table 4.2 shows the effect of column washing with citric acid buffer of various pH values.

Table 4.2 The effect of citric acid buffer (0.05 M, 2 ml) of various pH values as an SPE wash solution.

Buffer pH	Gentamicin eluted in wash	Neomycin eluted in wash	Kanamycin eluted in wash
Citric Acid Buffer, pH 2.0	✓	✓	✓
Citric Acid Buffer, pH 3.0	✗	✗	✓
Citric Acid Buffer, pH 4.0	✗	✗	✗

Further investigation showed that 2 ml of citric acid buffer, (pH 3.5, 0.05 M) was the most effective wash solution in removing matrix interferences without analyte elution. Increasing the citric acid concentration to 0.10 M resulted in analyte losses in the wash solvent. Applying the sample to the SPE column using 0.05 M citric acid buffer, pH 10.0 as the diluent, washing the column with 0.05 M citric acid buffer pH 3.5 and eluting in citric acid buffer containing sodium sulphate resulted in chromatograms showing no interference with the 3 peaks of gentamicin, neomycin resolved from the matrix and kanamycin eluting close to a small matrix peak. Attempts to remove this interference through sample extraction with sodium hydroxide and acetonitrile, and through varying the nature and concentration of the SPE wash and elution, proved unsuccessful.

4. 4.7 Validation results for the analysis of gentamicin, neomycin and kanamycin in milk, using ion-exchange SPE.

The validation plan for the analysis of gentamicin, neomycin and kanamycin in milk samples, using ion-exchange SPE has been described in section 4.2.6. Intra- and inter-assay results for the determination of gentamicin (three components), neomycin and kanamycin in milk were determined. Recoveries in the range 62-100 % were obtained. The intra-assay results for the determination of gentamicin is shown in Table 4.3. Gentamicin recovery was assessed for the three components and the method proved to be equally capable of recovering all three components. In Table 4.3, variation is seen to be highest at the high level of fortification, 2500 ppb and at the lowest level of fortification, 62.5 ppb.

Table 4.3 Intra-assay variation for the determination of gentamicin residues in milk (2 ml) using ion-exchange SPE, n=5.

	Mean Recovery (%) ± SD		
Fortification Level (ppb)	**Gentamicin Peak 1**	**Gentamicin Peak 2**	**Gentamicin Peak 3**
62.5	110 ± 8.6	72 ± 9.0	96. ± 4.4
125	64 ± 4.3	74 ± 3.6	84 ± 7.7
250	72 ± 2.6	62 ± 3.9	77 ± 8.8
500	78 ± 4.1	80 ± 5.4	82 ± 5.1
1000	87 ± 5.3	96 ± 12.4	97 ± 4.3
2500	72 ± 13.1	99 ± 16.0	99 ± 16.6

Intra-assay results for neomycin presented in Table 4.4, show acceptable recoveries, in the range 69-100 % with relatively low variation. As expected, the highest variation is at the lowest level of fortification, 62.5 ppb. Variation is low at all other levels of fortification and neomycin intra-assay recovery shows less variation than gentamicin intra-assay recovery.

Table 4.4 Intra-assay variation for the determination of neomycin in milk (2 ml), using ion-exchange SPE, n=5.

Fortification Level (ppb)	*Mean recovery (%) ± SD*
62.5	92 ± 9.6
125	100 ± 0.2
250	94 ± 3.5
500	76 ± 1.9
1000	69 ± 1.1
2500	82 ± 5.8

Intra-assay results for kanamycin show the highest recovery and lowest variation for all three analytes. The results presented in Table 4.5 show the highest percentage coefficient of variation to be 6.7 %, and as with neomycin, this value is for the lowest level of fortification. Good peak shape and high peak intensity account for the low variation for kanamycin. Recovery was consistently high at all levels of fortification.

Table 4.5 Intra-assay variation for the determination of kanamycin in milk (2 ml), using ion-exchange SPE, n = 5

Fortification Level (ppb)	*Mean recovery (%) ± SD*
100	88 ± 5.9
200	108 ± 2.4
500	102 ± 1.6
750	95 ± 3.2
1000	101 ± 1.2

Inter-assay results show similar percentage recoveries with higher variation. The bar chart in Figure 4.15 shows the percentage recovery and standard deviation (error bars) for samples fortified at six levels with gentamicin. Recoveries are in the range 60–99 %, similar to the intra-assay results. The variation is seen to be high (standard deviation values between 16 to 19) at the lowest level of fortification, 62.5 ppb. Most other peaks had standard deviation values ranging between 7 and 10. Two exceptions are a standard deviation of 13 % on peak 1 at 2500 ppb and a standard deviation of 14 % on peak 2 at 125 ppb. The method is seen to perform similarly for all three gentamicin peaks as the percentage recovery and standard deviations are similar for all three peaks.

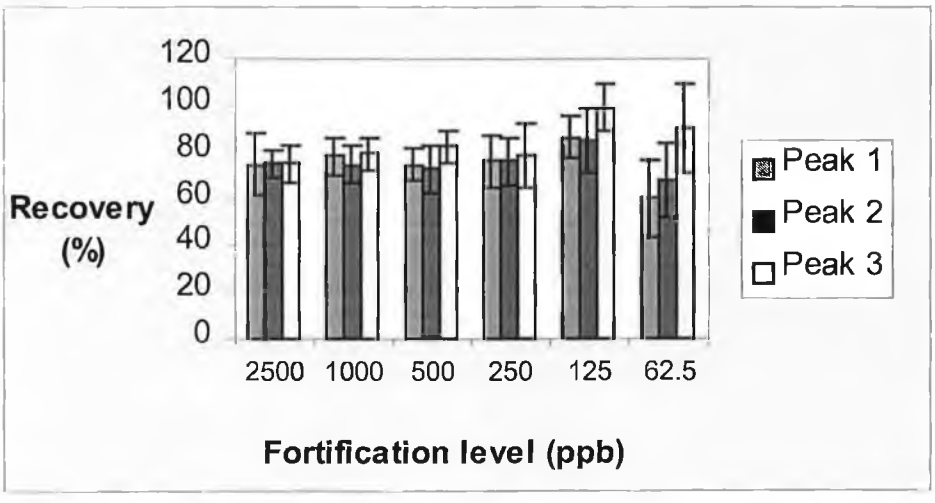


Figure 4.15 *Bar chart demonstrating the between-assay recovery (percentage mean) and standard deviation (error bars) for the three gentamicin components in milk using ion-exchange solid-phase extraction, n=5.*

The bar chart in Figure 4.16 shows the inter-assay percentage recovery and standard deviation (error bars) for samples fortified with neomycin. The percentage recoveries were in the range 64 to 99 %, similar to intra-assay results. Variation was seen to be somewhat lower than gentamicin inter-assay variation, with standard deviation values in the range 8.0 to 11.5 . Recovery was high (99 %) but variation was not elevated at the low level of fortification (standard deviation of 8). Kanamycin inter-assay results, shown in Figure 4.17, show high recoveries (86 to 109 %), similar to the intra-assay results. Variation in the percentage recovery was relatively similar at all levels of fortification (11.6 to 14 .7 standard deviation). As expected, variation was higher for inter-assay results relative to intra-assay results.

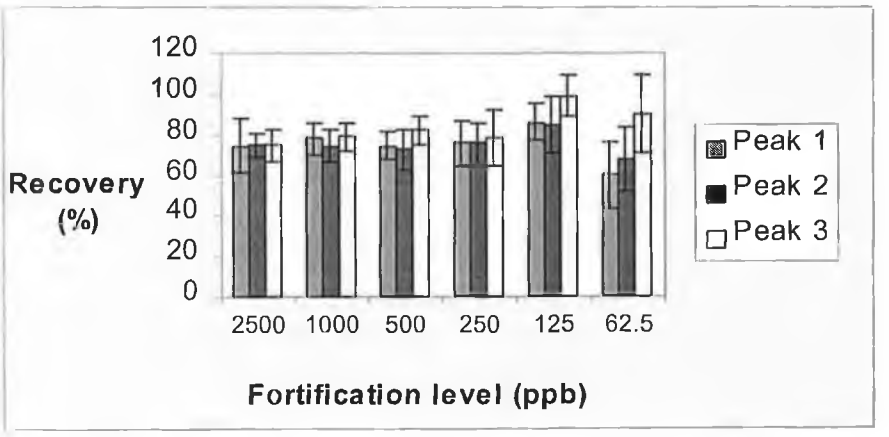


Figure 4.16 *Bar chart demonstrating the between-assay recovery (percentage mean) and standard deviation (error bars) for neomycin in milk using ion-exchange solid-phase extraction, n=5.*

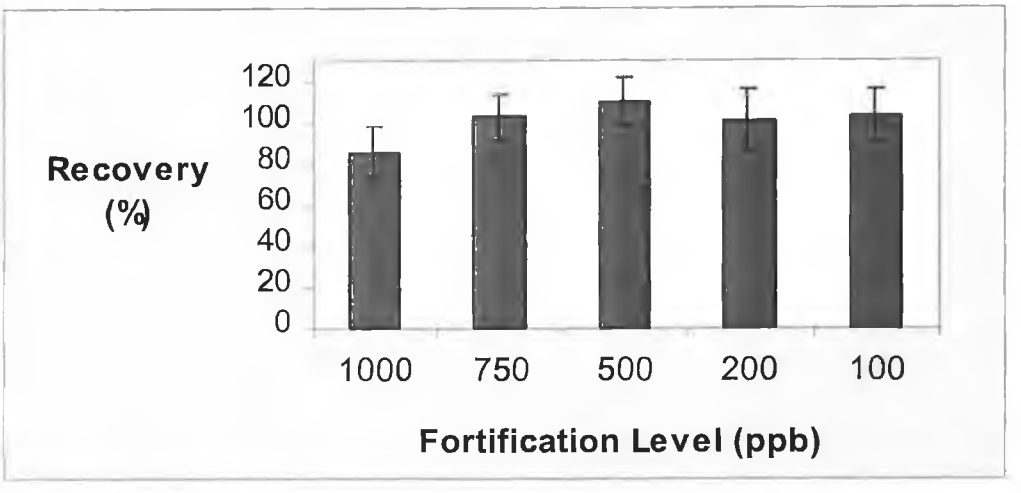


Figure 4.17 *Bar chart demonstrating the between-assay recovery (percentage mean) and standard deviation (error bars) for kanamycin in milk using ion-exchange solid-phase extraction, n=5.*

4.4.8 Immunoaffinity column chromatography

Attempts to analyse the aminoglycosides in porcine muscle, liver and kidney, using the ion-exchange method developed for milk samples failed. The availability of antibodies

to neomycin and gentamicin allowed immunoaffinity column chromatography to be assessed, as a clean-up step.

The aim of this part of the study was to investigate the analysis of neomycin and gentamicin using immunoaffinity column chromatography and apply the developed method to the clean-up of milk and tissue extracts. While the initial studies on binding and elution were carried out using standard drug solutions, it became obvious that the neomycin antibody was of inferior quality, as it possessed low coupling efficiency and low recoveries with high variation when it was employed for sample analysis. Therefore studies in milk and tissue were confined to gentamicin only. The limit of determination for the method was 30 ppb for gentamicin in milk and kidney. The method was validated at 50, 100 and 200 ppb using intra- and inter-assays. The validated method was also applied to the analysis of porcine muscle and liver, and veal muscle. More matrix interference was evident in liver extracts causing gentamicin peak 1 to elute on the matrix front. Also, recoveries were low; in the region of 30 %. This may due to fact that the extraction technique applied was optimised for kidney analysis, and further investigations into sample extraction may enable the application of immunoaffinity clean-up to other tissue extracts. In developing the immunoaffinity method, various optimisation experiments were carried out, which will be discussed in the following sections.

4.4.8.1 Optimisation of the loading solution

The presence of high salt has previously been observed to weaken antibody binding of streptomycin. [Haasnoot, W., Unpublished data] Therefore it was considered necessary to investigate the effects of salt concentration for neomycin and gentamicin binding.

Solutions of varying salt concentration were prepared and used as sample diluents for standard drug solutions such as water, PBS, citric acid buffer of 0.0005 M, 0.005 M, 0.05 M. The column was first conditioned with 6 ml water followed by 6 ml of the loading solution. The results are presented in Figure 4.18. The results for gentamicin peak 3 only are presented, but the results for all gentamicin peaks were similar, as the antibody responded similarly to all gentamicin components.

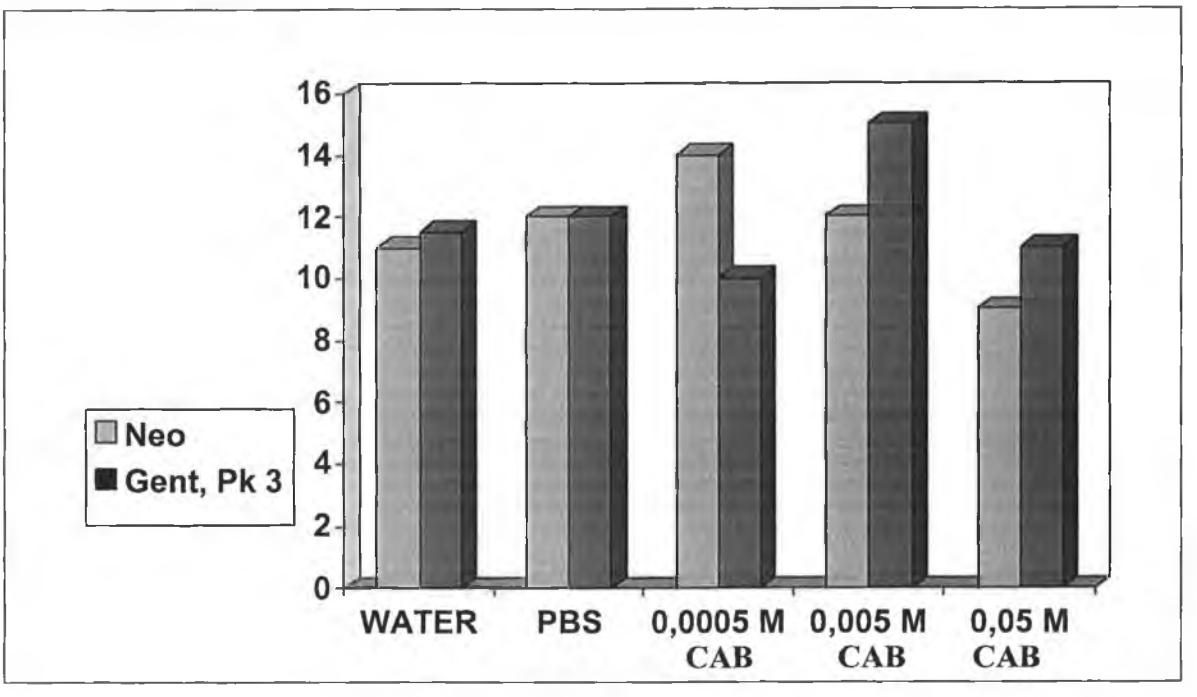


Figure 4.18 *Peak heights resulting from employing solutions of varying salt concentration as immunoaffinity column loading solvents (n=2).*

PBS : Phosphate buffered saline, CAB : Citric acid buffer.

It was evident that the lower the concentration of citric acid buffer, the better the binding affinity for neomycin. This trend was not observed for gentamicin; at the lower gentamicin concentration, binding was reduced. For the analysis of milk samples, the samples were first deproteinised using acid; since the extract was acidic in nature it was unsuitable for application to an immunoaffinity column. It was necessary to dilute the

acidic extract in a solution capable of adjusting the pH to near neutral. Low citric acid buffer concentrations (0.0005 M) were insufficient to achieve pH adjustment in milk samples. Water and PBS proved to be suitable loading solvents, but they were unsuitable for pH adjustment. Therefore, citric acid buffer was necessary and a concentration of 0.005 M was chosen for optimum binding efficiency of gentamicin and neomycin in sample extracts.

The pH of the loading solution was also investigated. Solutions of neomycin and gentamicin were prepared in citric acid buffer, 0.005 M, of varying pH. The solutions were loaded onto a pre-conditioned immunoaffinity column, the eluate collected, the column washed with PBS (3 ml) and eluted with citric acid buffer containing sodium sulphate (4 ml). Table 4.6 shows that retention on the column is increased as pH increases from 2 to 8. Loading analytes in diluent of pH 2 results in incomplete retention on the column for both analytes. Loading at pH 4 results in incomplete retention for neomycin but gentamicin was retained until the elution step. Loading at pH 6 results in some loss of neomycin in the eluate from the loading step and complete retention of gentamicin until the elution step. Finally, loading at pH 8 allows retention of both analytes until the elution step. Therefore, loading gentamicin at a pH of 4 or above ensures retention on the column. However, to ensure complete retention of neomycin on the column, the pH of the loading solution must be above 6. A citric acid buffer solution of 0.005 M, pH 7.0 was finally chosen as the loading solution.

Table 4.6 **Gentamicin and neomycin responses (peak height, mm) found in the loading solution and eluting solution when citric acid buffer of pH 2.0, 4.0, 6.0 and 8.0 were used as loading solutions.**

		Loading solutions			
Antibiotic	**Solution**	**pH 2.0**	**pH 4.0**	**pH 6.0**	**pH 8.0**
Gentamicin	Loading	20	0	0	0
	Eluting	8	21	20	23
Neomycin	Loading	29	13	6	0
	Eluting	6	29	40	41

Experiments were carried out to verify that this solution was capable of adjusting the pH of acidic extracts to achieve analyte retention on the immunoaffinity column. Extracts were diluted in citric acid buffer (0.005 M) of pH 6.0, 7.0, 8.0, 9.0 and 10.0. Results were not significantly different, proving citric acid buffer pH 7.0, 0.005 M to adjust the pH of the extract sufficiently.

4.4.8.2 Optimisation of the washing solution

Initial studies showed that when water was employed as the column wash solution, the gentamicin peaks exhibited poor resolution when chromatographed. This is likely caused by a dilution of the salt concentration in the eluting solution, caused by some of the wash solution (water) still on the immunoaffinity column. Best chromatographic resolution was observed using PBS as the wash solution while use of citric acid buffer provided no significant advantage in removal of matrix or peak resolution. The volume of PBS employed as the wash was varied for the analysis of samples. For milk samples,

increasing the wash solution volume beyond the volume of the column (3 ml) did not assist in reducing the matrix, but all analytes eluted free of matrix interferences using this volume. For kidney extracts, when 3 ml PBS was used as the wash solution gentamicin peak 1 eluted on the descending portion of the matrix peak, and increasing the wash up to 20 ml served to reduce the size of the matrix peak so that gentamicin peak 1 was resolved from matrix interferences.

4.4.8.3 Immunoaffinity column elution

Desorption of the analyte involves cleavage of the antigen-antibody complex. Ideally, the antigen-antibody complex is dissociated and the analyte eluted without damage to the antibody. The binding of the antigen (analyte) to the antibody is described as follows :

$$Ab + Ag \leftrightarrow AbAg \qquad \text{where} \quad K_a = [AbAg]/[Ab]\,[Ag]$$

where K_a is the affinity of the antibody for the antigen and K_a must be reduced to allow analyte elution. This reduction can be achieved using buffers, chaotropic ions or organic modifiers. Buffers of high or low pH are commonly used to elute analytes in immunoaffinity column chromatography. A change in pH can result in a change in the ionic interactions between the antigen-antibody complex. In this study, ion-pairing chromatography was employed together with post-column derivatisation. Section 4.4.2.4 outlines the importance of sample diluent in achieving adequate peak shape and intensity. Citric acid buffer, containing sodium sulphate was previously employed in the elution of the analytes from ion-exchange columns. This solution was of low pH and proved suitable for the chromatographic system employed for quantitation. It also proved capable of eluting the analytes from the immunoaffinity column, as the low pH

together with the sodium sulphate salt causes sufficient changes in the ionic interaction of the antigen antibody complex to elute the analyte.

4.4.8.4 Determination of the coupling efficiency

In immunoaffinity column chromatography, as with SPE, there is a concentration of analyte beyond which the column is incapable of binding any further analyte molecules. This "breakthrough" volume is used to establish the coupling efficiency of the column, that is the maximum concentration of analyte the column can retain. From the previous study, using ion-exchange, protein precipitation of milk samples was achieved using TCA, so the sample extract was acidic. Optimisation of immunoaffinity loading solvent studies show that the analyte will not be effectively retained by the column at low pH, therefore it was necessary to dilute the extract in citric acid buffer, to bring the sample near to pH 7. For this reason, coupling efficiency experiments were carried out using standard drug solutions prepared in this buffer. Fractions were loaded and the eluate collected and analysed. Figure 4.19 shows slight traces of neomycin breakthrough at 2200 ng with complete breakthrough at 2300 ng. The gentamicin column exhibited much higher coupling efficiency with complete breakthrough at 7500 ng. The lower coupling efficiency for the neomycin column reflects the poor quality of the antibody, which became evident when samples were analysed.

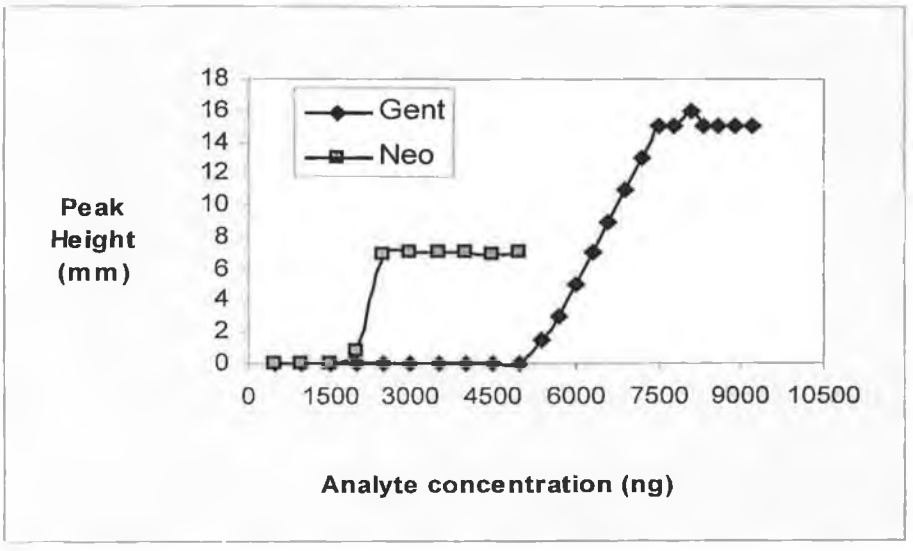


Figure 4.19 *Determination of the coupling efficiency for gentamicin and neomycin immunoaffinity columns. The concentration of analyte in the loading solution, shown on the x-axis, is collected on exit from the column.*

4.4.8.5 Column regeneration and re-usability

The elution conditions developed, should allow the reduction of K_a (allowing release of the analyte) to be a reversible process, so that prolonged use of the column is permitted. After every experiment columns were washed with 20 ml water, followed by 10 ml PBS before being stored at 4°C in PBS containing sodium azide (0.5 %). Between each sample the column was washed with water (3 ml), elution solution (3 ml) and loading solvent (5 ml) in preparation for the next sample. Re-use of columns involves a risk of cross-contamination between samples, but control (blank) samples analysed after samples fortified at 200 ppb, showed no evidence of cross-contamination. For the analysis of milk samples, the same column was employed for up to 30 samples. For kidney samples, column re-usability was permissible when 1 g of kidney was employed, but when 5 g was employed, the column became saturated after 5 samples and the flow through the column became restricted.

4.4.8.6 Specificity of gentamicin antibody

The specificity of the gentamicin antibody was assessed using the following analytes, all at 0.5 μg ml^{-1}: gentamicin, neomycin, kanamycin, streptomycin, dihydrostreptomycin, spectinomyicn, lincomycin, and salinomycin. The analytes were applied onto the column in 1 ml volume, followed by 10 ml PBS wash and 2.5 ml citric acid buffer-sodium sulphate elution. The elution solution was analysed using hydrophilic interaction chromatography-MS (Chapter 5) and no analytes other than gentamicin were detected.

4.4.9 Validation results for the analysis of gentamicin in milk and kidney tissue using immunoaffinity column chromatography

Validation of the immunoaffinity column chromatography method is described in section 4.2.6. Intra-assay results are presented in Table 4.7 for milk samples fortified at three levels with gentamicin, and analysed using immunoaffinity column chromatography. Inter-assay results for milk samples analysed using immunoaffinity chromatography, are presented in Figure 4.20. Recoveries were in the range 63 to 82 %. The bar-chart shows standard deviation values (error bars) were in the range 4.9 to 12.8.

Attempts to analyse tissue samples using the ion-exchange method resulted in excessive matrix co-extractives. However, the application of immunoaffinity chromatography allowed the analysis of gentamicin residues in kidney. A typical chromatogram is shown in Figure 4.4. Intra-assay results are presented in Table 4.8. The intra-assay results show the recovery of gentamicin in kidney (63-78 %) to be reduced relative to milk samples, but recoveries are within the range reported by other authors and validation studies were carried out at limits below most published methods[21,23]. The

standard deviation values were similar to the values obtained for the analysis of milk samples analysed by immunoaffinity column chromatography. The results show that the immunoaffinity column performs similarly for all three gentamicin peaks, and exhibits no bias towards any of the three forms of gentamicin under study.

Table 4.7 Intra-assay variation for the determination of gentamicin in milk using immunoaffinity column chromatography, n=5.

	Mean Recovery (%) ± SD		
Fortification Level (ppb)	***Gentamicin Peak 1***	***Gentamicin Peak 2***	***Gentamicin Peak 3***
50	77 ± 8.4	85 ± 8.5	84 ± 7.5
100	96 ± 7.2	95 ± 13	100 ± 15
200	75 ± 2.3	72 ± 4.9	69 ± 5.3

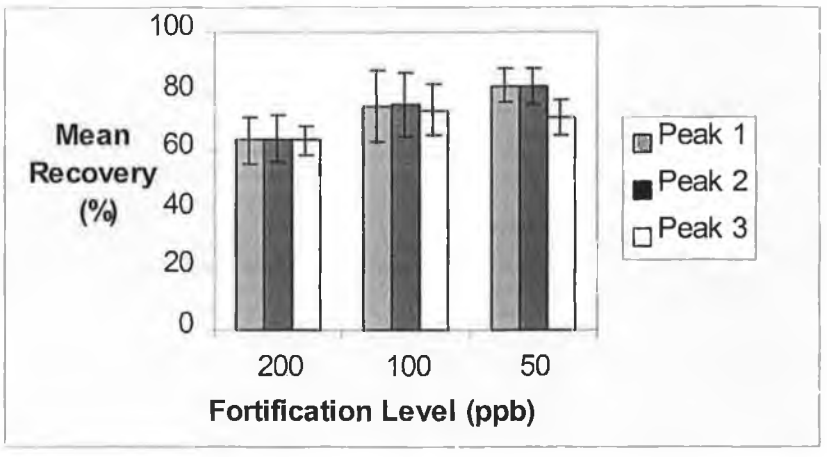


Figure 4.20 *Bar chart demonstrating the between-assay recovery (percentage mean) and standard deviation (error bars) for the three gentamicin components in milk using immunoaffinity chromatography n=5.*

Table 4.8 Intra-assay variation in recovery for the determination of gentamicin in kidney (5 g), using immunoaffinity column chromatography, n=3.

	Mean Recovery (%) ± SD		
Fortification Level (ppb)	***Gentamicin Peak 1***	***Gentamicin Peak 2***	***Gentamicin Peak 3***
50	78 ± 4.2	63 ± 5.9	74 ± 8.9
100	66 ± 13	67 ± 6.3	66 ± 8.1
200	73 ± 6.6	68 ± 9.2	73 ± 5.7

The inter-assay results for the analysis of kidney samples fortified with gentamicin and analysed using immunoaffinity column chromatography, are presented in Figure 4.20. Recoveries were in the range 55 to 79 %, with standard deviation (error bars) in the range 3.8 to 20. 6. The variation for peak 2 was consistently higher, with standard deviation values of 13.8 to 18.8. Since a similar trend in variation was not observed for the analysis of peak 2 in standards or milk samples using immunoaffinity column chromatography, it is unlikely that peak 2 variation is related to the performance of the immunoaffinity column for this form of gentamicin. The high variation may be due to incomplete baseline resolution, as is evident in Figure 4.4. Alternatively this form of gentamicin is not as easily extracted from kidney tissue as peak 1 and 3.

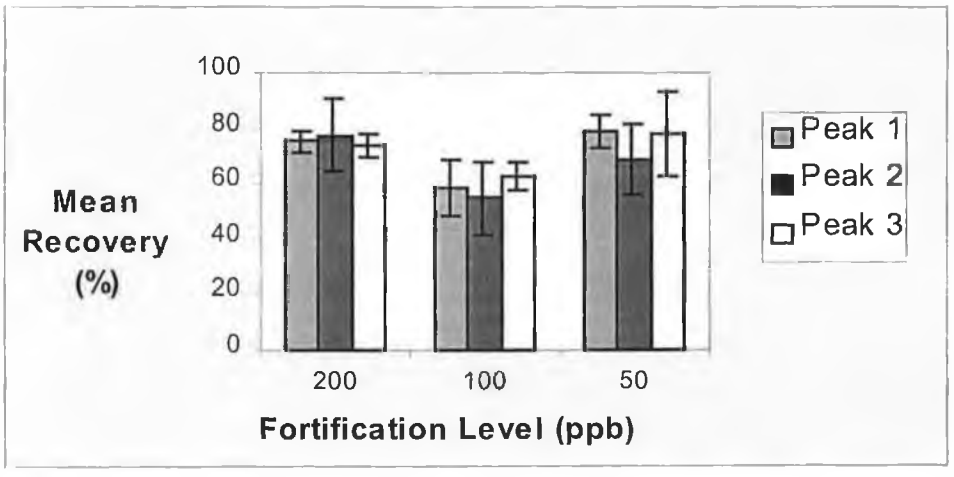


Figure 4.21 *Bar chart demonstrating the between-assay recovery (percentage mean) and standard deviation (error bars) for the three gentamicin components in kidney using immunoaffinity column chromatography, n=3.*

4.4.10 Comparison of ion-exchange SPE with immunoaffinity clean-up

Application of immunaffinity column chromatography clean-up allowed the analysis of gentamcin residues in kidney tissue. For gentamicin residue analysis in milk, the limit of determination achieved using immunoaffinity column chromatography was 50 ppb compared with 62.5 ppb using ion-exchange. Comparison of intra-assay results for milk samples analysed using ion-exchange with those from milk samples analysed by immunoaffinity column chromatography show similar recoveries. Variation in recovery was observed to be slighty higher for samples analysed using immunoaffinity column chromatography; For samples fortified between 50 and 200 ppb, and analysed by immunoaffinity column chromatography, standard deviation values ranging between 2.3 and 15 were obtained. For samples fortified between 65 to 250 ppb, and analysed by ion-exchange, standard deviation values were in the range 2.6 to 9.0. The greater variation may be due to reduced baseline resolution of samples analysed using

immunoaffinity column chromatography, due to the larger sample volume employed for analysis. Comparison of the inter-assay recovery for samples fortified in the range 50 to 200 ppb and analysed using immunoaffinity column chromatography, show recovery was slightly reduced relative to sample fortified in the range 62.5 to 250 ppb and analysed using ion-exchange. For samples analysed using immunoaffinity column chromatography, recoveries were in the range 64 to 82 % with standard deviation values in the range 5 to 13. Using ion-exchange, recoveries for samples fortified between 62.5 and 250 ppb fortification were in the range 60 to 99 % with standard deviation values in the range 9 to 19. Therefore, both ion-exchange SPE and immunoaffinity column chromatography are suitable for the analysis of gentamicin in milk samples, and both methods perform similarly with respect to recovery and variation in recovery. However, lower limits of determination can be achieved using immunoaffinity column chromatography, due to its increased sample capacity.

4.5 CONCLUSION

The results of the study give an insight into the behaviour of the three aminoglycosides, gentamicin, neomycin and kanamycin, under various chromatographic conditions. Chromsphere, C8 packing material was seen to give the best chromatographic resolution and peak shape for these analytes. For ion-pair chromatography, the addition of sodium sulphate in the mobile phase serves to increase retention of these polar analytes on the reversed-phase material and long equilibration times are required, to maintain consistent retention times, using this ion-pair method. To minimise variation, it is necessary to employ inert PEEK fittings, for aminoglycoside HPLC analysis, and to

employ polypropylene containers for analyte storage. The derivatisation conditions for the OPA derivatisation of the three analytes were optimised.

The results show that ion-exchange solid-phase extraction is a suitable clean-up technique for the analysis of gentamicin, neomycin and kanamycin in milk. Immunoaffinity column chromatography is also suitable for the analysis of gentamicin in milk, and for the clean-up of gentamicin residues in kidney tissue, providing a quick, simple method that results in chromatograms free of matrix interference. Both techniques are capable of analysing samples fortified at levels well below MRLs set by the EU.

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CHAPTER 5

THE ANALYSIS OF AMINOGLYCOSIDE ANTIBIOTICS USING HYDROPHILIC INTERACTION CHROMATOGRAPHY WITH MASS SPECTROMETRIC DETECTION.

5.1 INTRODUCTION

Lack of significant UV chromophores necessitates the use of derivatisation techniques for aminoglycoside detection using conventional HPLC detectors. These techniques are not highly specific and they can exhibit non-linearity and increase method variation. Therefore, mass spectrometric detection is of particular interest for the analysis of aminoglycoside antibiotic residues, allowing direct detection of the separated analytes. MS detection is a confirmatory technique, which exhibits specificity, sensitivity, wide applicability and it is quantitative. However, HPLC-MS analysis of aminoglycosides has proven problematic, due to the necessity for ion-pair chromatography. HPLC methods which require the use of non-volatile mobile phase reagents are unsuitable when detection by MS is employed. Ion-pair sulphonate salts are most commonly employed, which are incompatible with MS detection, due to ion suppression, where the mobile phase constituents ionise in preference to the analytes. The aim of this study was to investigate the use of hydrophilic interaction chromatography for the analysis of some aminoglycosides and related compounds.

5.1.1 Direct detection techniques for aminoglycoside analysis

Methods reported for the analysis of aminoglycoside residues have been discussed in Chapter 4, so the discussion here will be limited to direct detection techniques and other MS methods reported. Direct detection techniques used in combination with LC for aminoglycosides include refractive index detection and optical rotation. Refractive index detection has been employed by Inchauspe and Semain[1], following HPLC separation of eight aminoglycosides, but the authors worked at concentrations beyond

that of interest to residue analysts (~ 40 μg/injection). They employed ion-pair chromatography, using volatile perfluorcarboxylic acid as the ion-pair. UV detection at 195 nm has been employed for the detection of streptomycin and dihydrostreptomycin in preparative mode[2]. This wavelength is unsuitable for the analysis of residues in biological samples, due to the presence of co-extracted matrix components, which absorb at this wavelength.

Optical activity allows the use of polarimetry to detect neomycin[3]. Direct detection by pulsed amperometry has also been cited[4]. The presence of amino and hydroxyl groups on the aminoglycoside antibiotics provide electrochemical activity. Statler[5] used it in combination with anion-exchange chromatography, for the analysis of tobramycin in pharmaceutical injectables. To facilitate oxidation of the analyte at the gold electrode surface, it was found necessary to adjust the column eluate to pH 13, by post-column addition of sodium hydroxide. The minimum detection limit was 2 ng (10 ul injected on column). Gentamicin was detected using a carbon electrode[6], and the linearity was assessed between the limit of detection of 16 μg up to 30 μg. The authors comment that concentration of buffer in the mobile phase was crucial to the sensitivity of the method. Pulsed electrochemical detection has been employed for the analysis of neomycin[7] using a polymeric column and ion-pair chromatography, but stability problems were encountered and the method exhibited high variability.

5.1.2 Mass spectrometric detection for aminoglycoside analysis

No GC-MS method has been published for the analysis of aminoglycoside antibiotics and most LC-MS methods are limited to the analysis of pure drug solutions. Aside

from the method reported by McLaughlin and Henion[14], no methods have been published for the analysis of aminoglycosides in tissues. Various moving belt type interfaces were investigated for the analysis of kanamycin, tobramycin and neamine[8]. Chemical ionisation using ammonia was employed and the method was used for molecular weight determination. Time of flight plasma desorption mass spectrometry (TOFPDMS) has been reported for the analysis of eleven aminoglycosides[9]. Positive ion monitoring was employed, resulting in strong molecular ions. The authors also investigated derivatisation of the nine analytes with dinitrophenyl prior to MS detection, as the derivatives proved more readily extractable from an aqueous solution.

Dihydrostreptomycin was determined by continuous flow fast atom bombardment (CF-FAB) using capillary HPLC[10]. Capillary LC offered superior chromatographic efficiency and lower mobile phase flow rates, so that the entire LC effluent could be introduced into the MS. The protonated molecular ion, $(M+H)^+$ of dihydrostreptomycin was detected down to 4.4 ng. Gentamicin analysis has been described by Getek *et al.*[11] using a thermospray (TS) interface and at least three components were identified. Thermospray ionisation was employed with trifluroacetic acid and ammonium acetate in the mobile phase. For aminoglycosides, TS-MS produces low intensity molecular ions $(M + H)^+$, but high intensity fragments. Sakairi and Kambara[12] have detected kanamycin and gentamicin, amongst other analytes, using LC-MS. The gentamicin components were not separated by the LC system, but they have different spectra, while the kanamycin components were separated. Atmospheric pressure chemical ionisation (APCI) was applied and sensitivity was enhanced by employing selective ion monitoring (SIM), where only the specific aminoglycoside ions were recorded. Detection limits between 1 and 50 ng were reported for the various components.

Some authors have reported the use of volatile fluorinated carboxylic acids in the mobile phase, for aminoglycoside separation with MS detection. These acids are volatile and therefore compatible with MS detection, but it has been the experience in our laboratory that, on the one hand, these acids may not provide adequate chromatography at low ion-pair concentrations while, on the other hand, sensitivity was poor when acid concentration was high. Inchauspe *et al.*[13] also reported a decrease in the buffering capacity of perfluorinated carboxylic acids at low concentrations. McLaughlin and Henion[14] reported the use of heptafluorobutyric acid (HFBA) and pentafluoropropionic acid (PFPA) for the separation of streptomycin, dihydrostreptomycin, spectinomycin and hygromycin B in bovine kidney, but confirmation was not possible at the MRL levels (20 ppb for hygromycin B, 100 ppb for spectinomycin and 500 ppb for streptomycin and dihydrostreptomycin) without further pre-concentration. In a later publication[15], these authors employed PFPA as the ion-pair for the analysis of neomycin, gentamicin, streptomycin, dihydrostreptomycin, spectinomycin and hygromycin B in kidney. A pre-concentration step was included, to improve the limit of determination of the assay, thereby allowing detection of streptomycin and dihydrostreptomycin at MRL levels. Ion-spray MS was employed in both studies. It was reported that the retention times of analytes in the presence of matrix varied from those of standard solutions. This was likely due to matrix components competing with the analytes for the ion-pair. These authors also experienced incompatibility problems with sample diluents when using ion-pair chromatography, and have reported column degradation during the analysis of tissue extracts. Therefore, an alternative chromatographic system, without the need for ion-pair reagents is required.

Conboy *et al.*[16] employed an anion membrane suppresser for the MS detection of quaternary ammonium drugs. The authors reported removal of 99.9 % of the ion-pair agent (methanesulfonic acid) from the chromatography eluent, but the technique was not suitable for neutral species or analyte ions having the same charge as the ion-pair, as they could be removed also by the membrane. Ion chromatography systems have been hyphenated to MS, for the analysis of a wide variety of analytes. Milon and Bur[17] used direct liquid introduction (DLI) LC-MS for the analysis of catecholamines using octylsulphonate ion-pair. However, severe sensitivity problems were encountered with large amounts of analyte being required to achieve a reasonable signal, the acidic pH was incompatible with most standard MS fittings and the method required very frequent cleaning of the source.

In this study, the use of hydrophilic interaction chromatography (HILIC) is investigated for the analysis of the basic aminoglycosides. The method offers an LC separation method without the need to employ an ion-pair in the mobile phase. HILIC is described as a variant of normal phase chromatography, therefore elution is expected in the order least to most polar; retention increases as the polarity of the analytes increase.

5.1.3 HILIC mechanism of action

In HILIC, the stationary phase of the HILIC column is hydrophilic in nature and the mobile phase is hydrophobic. The stationary phase used in this study was PolyHydroxyethyl Aspartamide™. This material is extremely polar and is bonded on a silica backbone in a stainless steel column. The mobile phase consists of organic solvent (acetonitrile) in high proportion and, for this study, MS compatible buffer. In

HILIC, it is postulated that there is an immobile liquid phase between the stationary and mobile phase, as depicted in Figure 5.1, which is a result of the affinity of the aqueous content of the mobile phase for the polar stationary phase. Therefore, the stationary phase is polar, the mobile phase mainly organic and an immobile phase sandwiched in between these two phases, which is mainly aqueous. In contrast to normal phase chromatography, which involves the direct adsorption of the analytes onto the stationary phase, separation in HILIC is achieved by partitioning of the analytes between the largely aqueous immobile phase and the mainly organic mobile phase. Analyte retention is proportional to the affinity of the molecule for the immobile phase. Therefore, more polar analytes will partition longer in the more polar immobile phase and so take longer to elute.

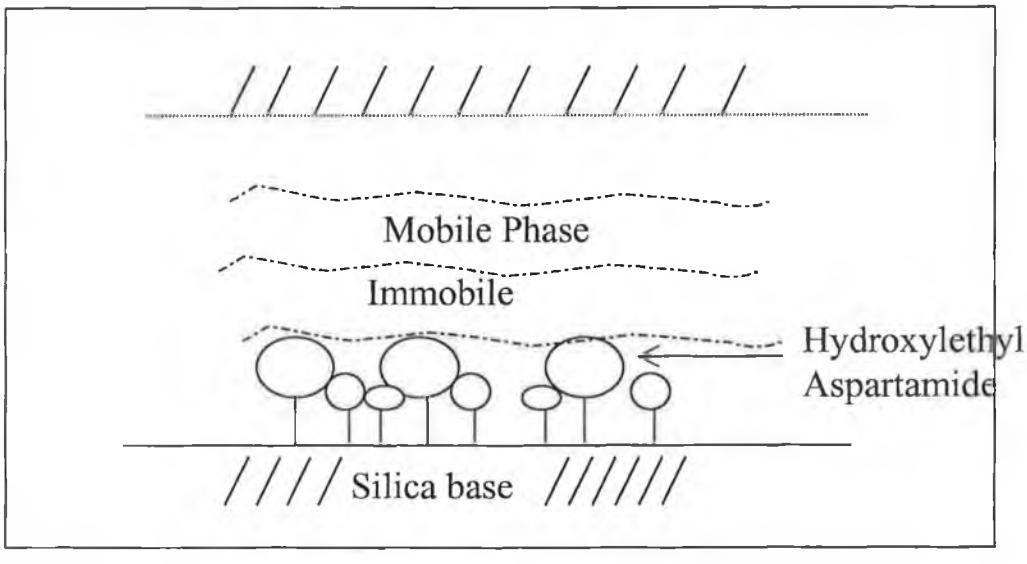


Figure 5.1 *Schematic of the internal structure within a typical HILIC column.*

Alpert[18] described the "contact region concept" to explain retention of peptides in the immobile phase. Analyte molecules orientate themselves so that the most polar end of the structure is in contact with the polar stationary phase (and so in the immobile

aqueous phase), and the least polar end is in contact with the hydrophobic mobile phase. The greater the number of hydrophilic (basic) domains available on the outside of the molecule, the greater its affinity for the immobile phase and therefore the greater the retention. Alpert also stated that the basicity of a peptide promoted retention using HILIC, but to much lesser extent than observed with ion-exchange chromatography. Therefore, the HILIC method may be suitable for multiresidue analysis of analytes of various polarities.

5.1.4 Alternative HILIC applications

This form of chromatography has previously been reported for the analysis of peptides[19]. Extremely hydrophobic or hydrophilic peptides that were difficult to separate by conventional reversed-phase HPLC were separated, with good peak shape and resolution, using HILIC. HILIC has also been employed for the separation of glucose polymers[20] using amperometric detection. Gradient elution allowed the separation of 20 components with low variability (less than 0.4 % RSD). The authors reported similar findings to those obtained in this study, with respect to the effect of buffer and modifier on analyte retention. HILIC was applied to the analysis of a large number of peptides[21] and retention was observed to be in proportion to the basicity of the molecules. For peptide analysis the PolySulfoethyl Aspartamide column is most commonly employed, which is less polar than the packing material used in this study, but possesses charged surface groups. PolyGlycoplex and Polyol-RSiL (a silica phase, to which OH groups have been added) were employed for the analysis of complex carbohydrates[22]. Previously, carbohydrates had been separated on various polar-bonded phase columns, such as silica bound amine[23].

The basic nature of aminoglycosides may render them suitable analytes for separation by HILIC, due to the presence of hydrophilic amino groups and basic hydroxyl groups. Since reversed-phase chromatography is not very suitable for the analysis of aminoglycosides and ion-pair chromatography is not compatible with MS detection, there is a need for an alternative separation method.

5.2 EXPERIMENTAL

5.2.1 Introduction

The aim of the study was to assess the suitability of HILIC for the analysis of aminoglycosides and related antibiotics. The chromatographic behaviour of gentamicin, neomycin and kanamycin were of particular interest, but streptomycin, dihydrostreptomycin, lincomycin, salinomycin and spectinomycin were also investigated. The structures are shown in Figure 5.2 (a). Gradient elution and APCI-MS detection were employed. The effect of acetonitrile, buffer concentration and pH on analyte retention and peak shape was investigated and the effect of packing material pore size was tested. Finally, linearity over the range 0.05–10 μg ml^{-1} was confirmed, and milk and kidney extracts from gentamicin fortified samples were analysed by MS.

5.2.2 Chemicals and Reagents

a) Preparation of standards

All solvents were of analytical HPLC grade, unless otherwise stated. Gentamicin (Gent), Neomycin (Neo), Kanamycin (Kan), Streptomycin (Strep), Dihydrostreptomycin (Dihydro), Lincomycin (Linco), Salinomycin (Sal) and Spectinomycin (Spec) were purchased from Sigma-Aldrich Chemie BV, Zwjindrecht, The Netherlands. Stock solutions of 100 mg ml^{-1} were prepared in milli-Q water unless otherwise stated and stored in polyethylene tubes; these solutions had a shelf-life of two weeks when stored at 4°C.

b) HPLC mobile phases

The organic modifier, acetonitrile, was filtered through a 0.45 μm filter and sparged with helium prior to use. The buffer, ammonium acetate, purchased from Merck (Darmstadt, Germany) was prepared in water, at various concentrations and pH values. The pH was adjusted while continuously stirring, using formic acid. The buffer was also filtered and sparged, using helium, prior to use.

c) Samples (milk and kidney)

Milk and kidney samples, fortified with gentamicin at 100 ppb, were extracted and cleaned-up by a procedure involving immunoaffinity column chromatography, as described in Chapter 4.

5.2.3 Instrumentation

Chromatography: 3 HILIC columns were investigated. The stationary packing was PolyHydroxyethyl A, 5μm, in each case. Further variations are shown in Table 5.1.

Table 5.1 HILIC columns investigated in this study.

Column	*Dimensions*	*Pore Size*
A	200 x 4.6 mm	1000 Å
B	200 x 2.1 mm	100 Å
C	200 x 4.6 mm	500 Å

Column A was a generous gift from PolyLC Inc., (Columbia, MD, USA) and the other columns were purchased from Poly LC. Gradient elution was employed using a quaternary pump (Thermoquest, The Netherlands) to deliver the mobile phase at a flow rate of 0.5 ml min^{-1} through column B, and 1.0 ml min^{-1} through columns A and C. The following gradient was employed, unless otherwise stated:

Step 1: At time = 0, 50 % Acetonitrile, 50 % buffer (ammonium acetate),

Step 2: At time = 5 min, 5 % Acetonitrile, 95 % buffer,

Step 3: At time = 15 min, 5 % Acetonitrile, 95 % buffer,

Step 4: At time = 18 min, 50 % Acetonitrile, 50 % buffer.

Experiments were carried out at room temperature (20°C) and injections (50 µl) were made using a Thermoquest AS3000 autosampler, with cooled sample tray.

Mass spectrometric parameters: The mass spectrometer used was a Thermoquest LCQ (San Jose, CA, USA), equipped with an atmospheric pressure ion source, used in the positive mode. Vaporisation setting: 500°C, Capillary temperature: 180°C, EM voltage: 1500 V, EM gain: 8. The corona discharge needle was maintained at 5 kV with nitrogen sheath nebulisation gas at 70 % of the maximum and auxiliary gas at 20 % of the maximum. The data acquired was processed using the Thermoquest x-calibur™ software. Mass spectrometric parameters were optimised by continuous infusion of a 5 µg ml^{-1} aqueous solution of gentamicin.

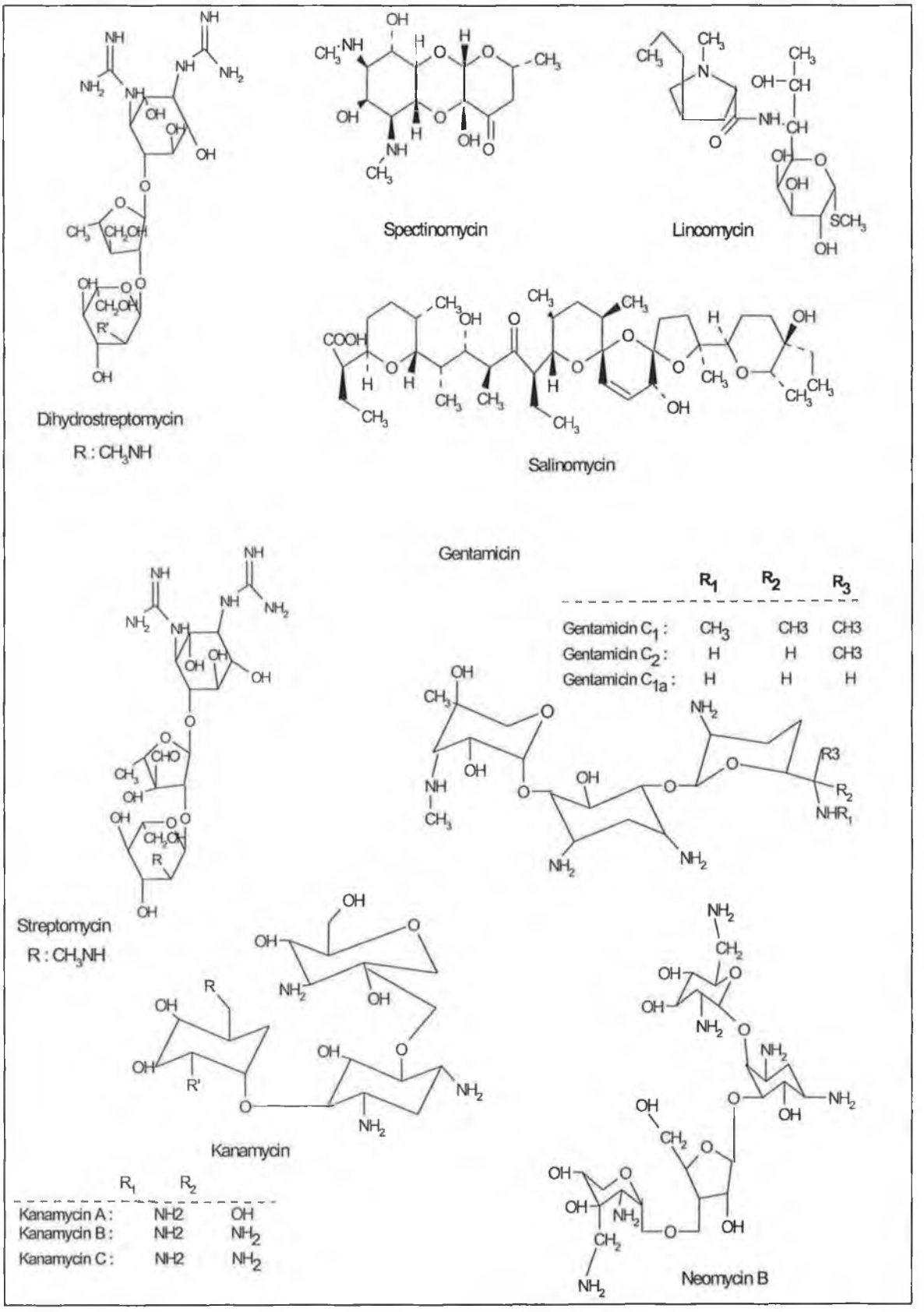


Figure 5.2 (a) *Chemical structures of the analytes under study.*

5.3 RESULTS AND DISCUSSION

5.3.1 Introduction

The aim of the study was to carry out some initial investigations into the applicability of HILIC as a method for chromatographic separation for aminoglycosides and related compounds, and its compatibility with MS detection.

5.3.2 Mass spectrometry

LC-MS offers a combination of separation of analytes and a specific confirmatory detection technique. When interfacing liquid chromatography with MS detection, the interface must be capable of transferring the analyte from the LC column to the MS ion source. The major challenges faced are removal of mobile phase components and dealing with the pressure difference between the LC outlet and the mass analyser. The main problems encountered are 1) limitations on the quantity of analyte which may be introduced into the mass spectrometer, 2) the pumping capacity of the vacuum pump requires reduced LC flow rates, and 3) the mobile phase composition is restricted to volatile reagents. Advances in the instrumentation available have assisted in over-coming problems 1 and 2, but for aminoglycoside analysis, problem 3 remains the main obstacle to successful LC-MS analysis. As discussed in section 5.1, the chromatographic systems commonly employed for aminoglycoside analysis present problems when linked to mass spectrometric detection, due to the necessity for ion-pair reagents in the mobile phase. Ideally, the HPLC eluent should be of low ionic strength and should not contain a high concentration of buffers/ion-pairs, which can compete with ionisation of the analyte, generate high chemical background or precipitate in the source. This has been a limitation especially for aminoglycosides, but with HILIC the use of ion-pair can be avoided, resulting in a potentially more sensitive method.

Since the aim of the study was to investigate the use of HILIC for the MS-compatible separation of aminoglycosides, the detection method was not fully investigated initially. However, some MS parameters were optimised. The most important parameters were temperature of the capillary and vaporiser temperature. Using APCI, the LC effluent is sprayed from a capillary tube and converted into a fine mist by a heated nebuliser. The mist is carried (assisted by a flow of nitrogen) into a region where a corona discharge electrode is installed. Electrons produced by the corona discharge electrode initiate ionisation. The ions and any ion-solvent clusters that may exist are transferred from the atmospheric pressure region into the high vacuum region via a heated capillary. The resulting desolvated ions are introduced into the mass analyser.

Full scan mass spectra were obtained for all analytes and extracted ion chromatograms were constructed from the full scan data. The ions monitored are listed in Table 5.2. Other authors have reported better sensitivity using positive ion monitoring, therefore positive-ion spectra were investigated.

In full scan mode, spectra from sample extracts showed some early eluting matrix co-extractives, but none of these eluted at the same retention times as the analytes of interest. However, the application of MS-MS eradicated the matrix peaks. Using MS-MS, the sample is ionised in the usual fashion, selected ions are induced to fragment and the fragments analysed according to their *m/z* ratios. Therefore, the result is a mass spectrum of the selected ion. The fragment ions resulting from the three gentamicin components are shown in Table 5.3. Figure 5.3 shows the results of HILIC-MS analysis of a kidney extract, fortified with gentamicin at 100 ppb. The three molecular ions were detected and the fragment ions of the C1a ion (Gent 1) are also shown.

Table 5.2 Mass of the molecular ions, $[M + H]^+$ detected for each analyte

Analyte	*m/z*
Gentamicin (C_{1a})	450
Gentamicin (C_2)	464
Gentamicin (C_1)	478
Neomycin	615
Kanamycin	485
Lincomycin	407
Salinomycin	773
Streptomycin	582
Dihydrostreptomycin	584
Spectinomycin	333

Table 5.3 Fragments observed for each component of gentamicin by MS-MS.

Component	$[M+H]^+$	*A*	*B*	*C*	*D*
C1	450	322	163	160	
C2	464	322	163	160	143
C1a	478	322	163	160	

Fragments A and C were monitored in milk and kidney samples fortified at 100 ppb. This level corresponds to the MRL for gentamicin in milk and is well below the MRL for gentamicin in kidney (1000 ppb).

Figure 5.2 (b) shows the postulated origin of each fragment for the three components of gentamicin. Each component had a distinctive molecular ion, which was evident in full scan mode. The *m/z* ratios differ due to varying number of methyl groups (R_2 and R_3). These molecular ions all gave rise to fragment ions A, *m/z* 322, B, *m/z* 163 and C, *m/z* 160. Molecular ion C2 also gave rise to a fragment of *m/z* 143, which differs from C1 and C1a due to the variation in R_2 and R_3. From Figure 5.2 (a), it is evident that fragments A, B and C contain no R groups and so these fragment ions were identical for all components.

5.3.3 Method development for HILIC

Initial studies on the effect of acetonitrile and buffer on chromatography were carried out using column A (1000 Å pore size). This resulted in a chromatographic system that gave good retention and peak shape for the three gentamicin peaks, neomycin, kanamycin, spectinomycin, lincomycin and a split peak for dihydrostreptomycin. Salinomycin and streptomycin were not retained. The developed method was applied to columns B (100 Å pore size) and C (500 Å pore size), thereby allowing the effect of pore size to be investigated. The influence of the mobile phase buffer pH was also investigated and the method applied to milk and kidney sample extracts that contained gentamicin.

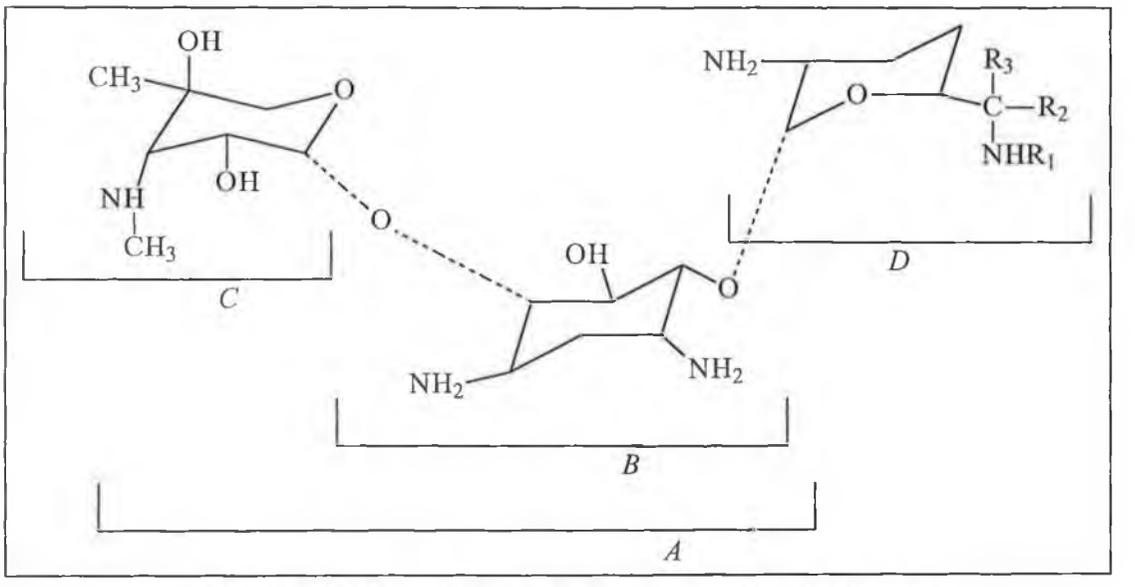


Figure 5.2 (b) *Fragmentation pathway for gentamicin.*

5.3.3.1. The effect of acetonitrile on analyte retention

The effect of acetonitrile on analyte retention was investigated. The graph in Figure 5.4 demonstrates the effect of the acetonitrile content in the initial mobile phase on analyte retention. The composition of the final mobile phase was 5 % acetonitrile /85 % water /10 % buffer (1000 mM). Similarl to that observed by Alpert for the analysis of peptides by HILIC[18], increasing acetonitrile composition of the mobile phase resulted in increased retention for most analytes. Alpert stated that increasing the organic solvent content of the mobile phase increased the selectivity of the sorbent to negatively charged peptide residues. He reported that the effect of the organic solvent content of the mobile phase was to have a major influence on retention of these negatively charged peptides, above 50 % (v/v) acetonitrile. In this study, similar effects were observed when the organic content of the initial mobile phase was increased from 0 to 90 % (v/v). Retention times increased between 20 and 70 % (v/v) acetonitrile content, after which the rate of increase was reduced. Above 40 % (v/v) acetonitrile, most of the analytes

appear to be retained, resolution improved and retention increased for all analytes except salinomycin. Of the analytes studied, salinomycin exhibits little polarity, so it was poorly retained under all conditions. Neomycin, the most polar of the analytes studied, was retained the longest. The three gentamicin components were not completely resolved from each other, but the mass spectrometric detection employed allowed these components to be easily distinguished due to variation in the mass/charge ratio of the molecular ions.

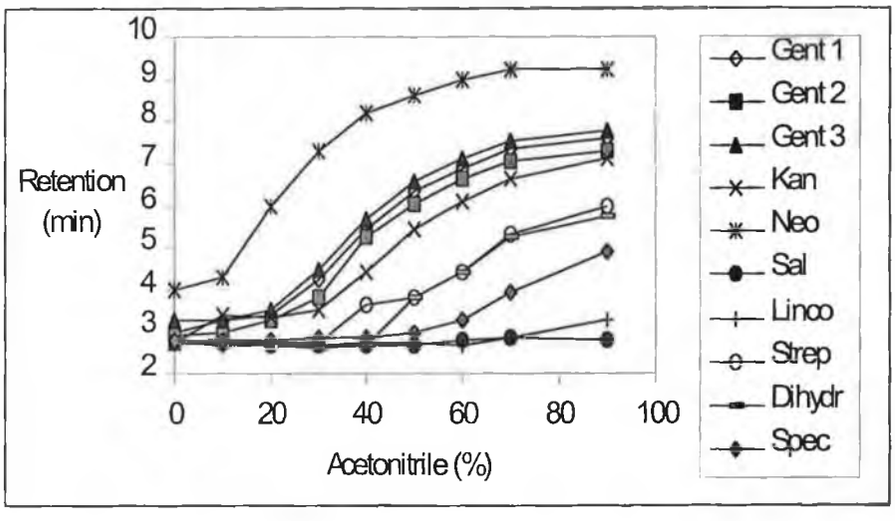


Figure 5.4 *The effect of the acetonitrile content in the initial mobile phase on analyte retention.*

Figure 5.4 depicts the retention times resulting when the percentage acetonitrile was increased while the percentage of buffer was held constant (10 % of 1000 mM), and the remaining percentage was water. Therefore, as the percentage acetonitrile was increased, the water content was decreased (from 90 %, at 0 % acetonitrile to 0 %, at 90 % acetonitrile), and so the molar concentration of buffer in the mobile phase also increased (from 100 to 1000 mM). To establish that the increase in retention time was a result of increasing acetonitrile, and not of increasing the molar concentration of buffer in the aqueous part, or a combination of both, the effect of increasing acetonitrile was

investigated while keeping the buffer concentration of the mobile phase constant (Figure 5.5). The final mobile phase composition employed in this experiment was 5 % acetonitrile/65 % water/30 % buffer (1000 mM). The increase in retention times, with increasing acetonitrile, shown in this graph, supports the view that increasing the volume percent of acetonitrile resulted in increased retention, and any "levelling off" of retention (as observed in Figure 5.4) may be due to high buffer concentration. The increased retention at high levels of acetonitrile may be due to proportionately more water being adsorbed to the polar stationary phase than is present in the flowing mobile phase causing the analytes to partition preferentially into this adsorbed, immobile layer and be retained longer. Nikolov and Reilly[24] stated that the greater the polarity of the organic component of the mobile phase, the smaller the immobile phase, due to competition between both polar components of the mobile phase (organic and aqueous) for the polar adsorbent sites on the sorbent. Acetonitrile was used in preference to methanol, because it is relatively less polar, in order to promote formation of the immobile layer and increased retention of the polar analytes.

Figure 5.6 shows the effect of increasing the percentage volume of acetonitrile on neomycin retention while employing various percentages of 1000 mM buffer in the initial mobile phase. At each percentage buffer, the final mobile phase contained 5 % acetonitrile and similar percentages of buffer to that employed in the initial mobile phase. Neomycin is shown as this is the most polar analyte and the most responsive to changes in chromatographic conditions. Higher buffer content promotes earlier elution and under those conditions the influence of acetonitrile content on retention was not as important.

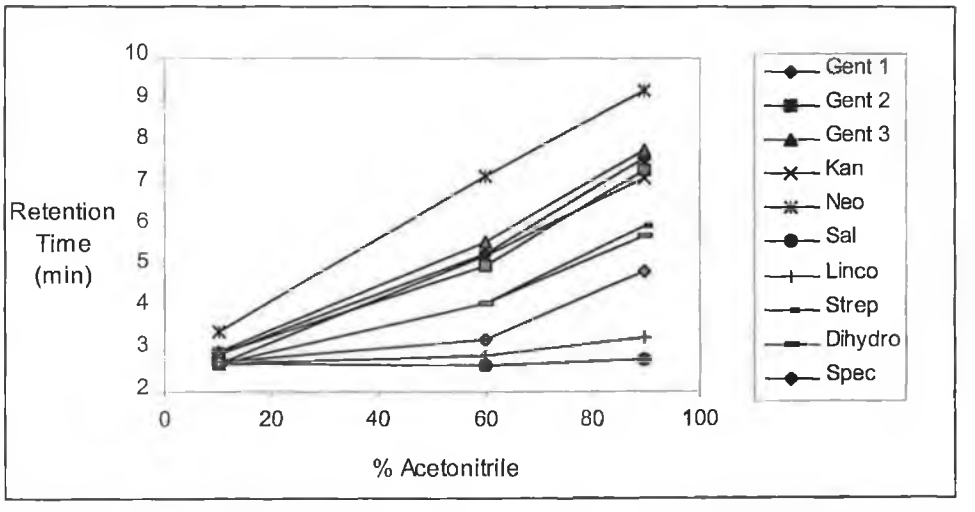


Figure 5.5 *The effect of acetonitrile content in the initial mobile phase on analyte retention, at constant buffer concentration (100 mM) in the aqueous phase.*

The effect of the acetonitrile content in the final mobile phase was also investigated. It was observed that as the percentage acetonitrile increased, analyte retention increased. However, the percentage acetonitrile in the final mobile phase had much less influence on retention time than acetonitrile content in the initial mobile phase.

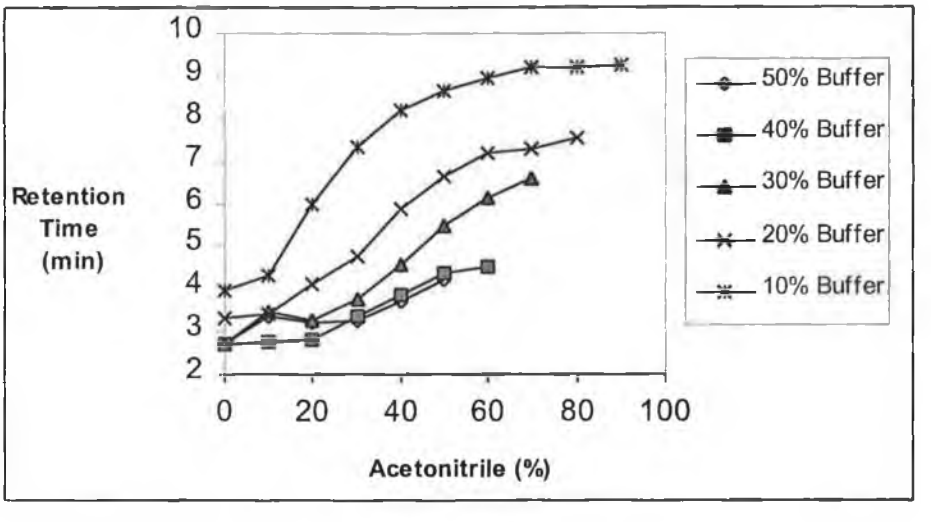


Figure 5.6 *The effect of acetonitrile content in the initial mobile phase on neomycin retention, at various percentages of 1000 mM buffer.*

5.3.3.2 The effect of buffer concentration on analyte retention

Reducing the quantity of buffer in the initial mobile phase promotes retention of the analytes, as shown in Figure 5.7. The results plotted were acquired at constant acetonitrile content (50 %) and using varying contents of ammonium acetate (1000 mM), with water as the remainder, on column A. The final mobile phase contained 5 % acetonitrile and a similar buffer content to that employed in the initial mobile phase. The graph shows that spectinomycin, lincomycin, and salinomycin were not retained, at any content of buffer, under the conditions employed. At the lowest buffer content, longest retention and greater resolution was achieved, but peak shape for all analytes was poor and severe tailing was observed for neomycin. These results indicate that increasing the molarity of the immobile phase serves to decrease the affinity of the analytes for this phase, resulting in reduced retention.

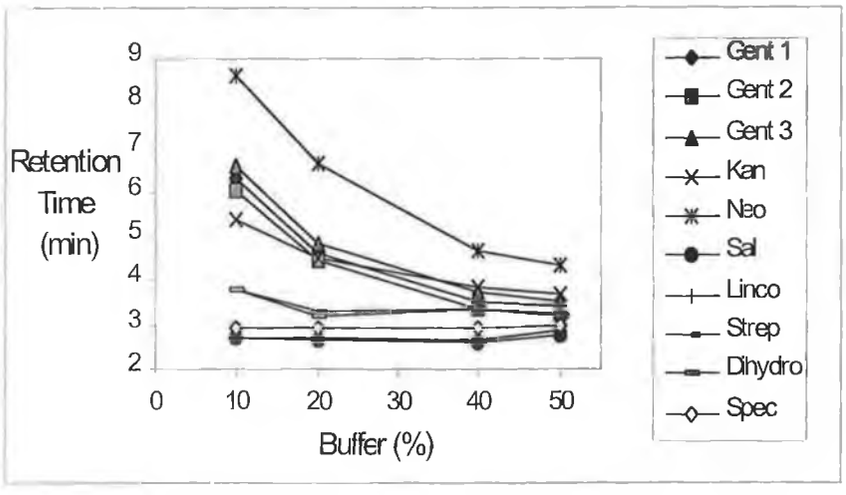


Figure 5.7 *The effect of increasing buffer content in the initial mobile phase on analyte retention (ammonium acetate, 1000 mM).*

Alpert[18] reported a decrease in the retention of basic peptides (arginine and histidine) as the buffer concentration was increased. The graph in Figure 5.8 shows the effect of increasing the buffer content at various contents of acetonitrile, on neomycin retention.

Again, the final mobile phase contained 5 % acetonitrile and a similar buffer content as that employed in the initial mobile phase. In general, retention was seen to decrease with increasing buffer content, but this effect was much less evident at low contents of acetonitrile.

The effect of buffer content in the final mobile phase on analyte retention was also investigated and observed to have a much smaller influence than the buffer content in the initial mobile phase. The lower the buffer content in the final mobile phase the greater the retention of the more polar analytes (neomycin, gentamicin and kanamycin), but no effect was observed on the retention of the less polar analytes (salinomycin, lincomycin and spectinomycin).

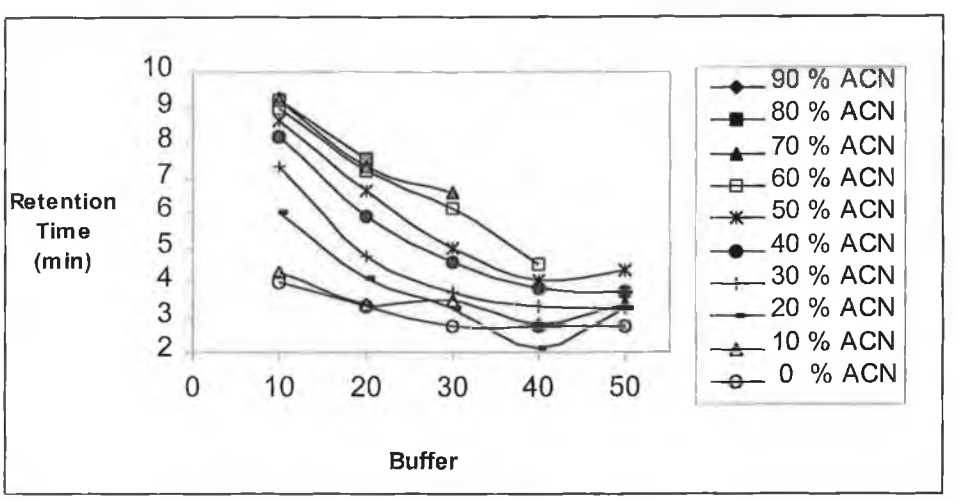


Figure 5.8 *The effect of buffer content in the initial mobile phase on neomycin retention (ammonium acetate, 1000 mM).*

Increasing the buffer content in the mobile phase is likely to cause a reduction in analyte retention as increased buffer would prevent water enrichment of the stationary phase, preventing or limiting the formation of the immobile phase. Reduction of the immobile

phase may also result from competition of the buffer ions for the adsorptive sites on the packing material, and an increase in the polarity of the organic component of the mobile phase, due to the presence of dissolved buffer ions, may contribute to the reduced retention times. In conclusion, increasing the concentration of buffer in the initial mobile phase serves to decrease analyte retention.

5.3.3.3 The effect of buffer pH on analyte retention

Ammonium acetate buffer was investigated at pH values between 3 and 6. The graph in Figure 5.9 shows that as the pH of the mobile phase buffer was increased, retention also increased for most analytes. As the pH increases, the polarity of the mobile phase is increased, therefore the analytes partition more into the immobile, buffer phase and so are retained longer. This is in contrast to the observation by Alpert in the analysis of peptides where retention was inversely proportional to pH[18]. Using buffers at pH 3, 4, and 5, in general, retention was seen to decrease when the buffer concentration was increased from 10 to 100 mM. In this study, pH 5 was the lowest pH at which spectinomycin was retained on the column and increasing the pH to 6 did not result in greater retention, with lincomycin and salinomycin not being retained, even at pH 6. The effect of pH was examined at a number of different buffer concentrations between 10 and 500 mM. In conclusion, increasing the buffer pH served to increase the retention of the analytes.

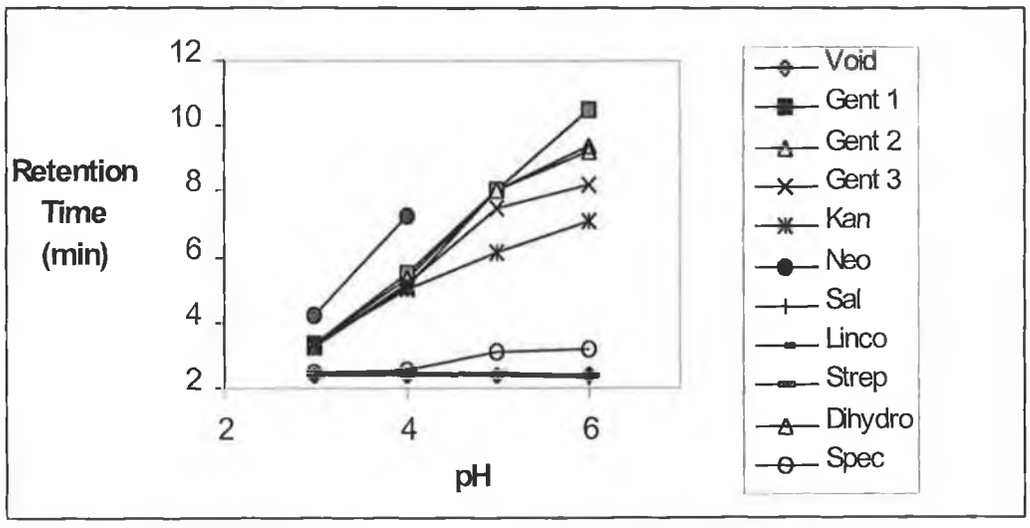


Figure 5.9 *The effect of buffer pH on the retention of analytes, using ammonium acetate, prepared at 100 mM.*

5.3.3.4 Peak shape

So far, discussion has been mainly centered on analyte retention. Retention is a very important parameter in the chromatographic analysis of aminoglycosides, as ion-exchange or ion-pair chromatography is almost always necessary to achieve retention. The peak shape of these basic analytes must also be considered. The effect of acetonitrile and buffer on peak asymmetry were considered. However, peak width was found to be a more suitable parameter to indicate peak shape as very broad peaks could give in good peak asymmetry values, close to 1, where peak tailing did not occur. Figure 5.10 shows how the measurements were made.

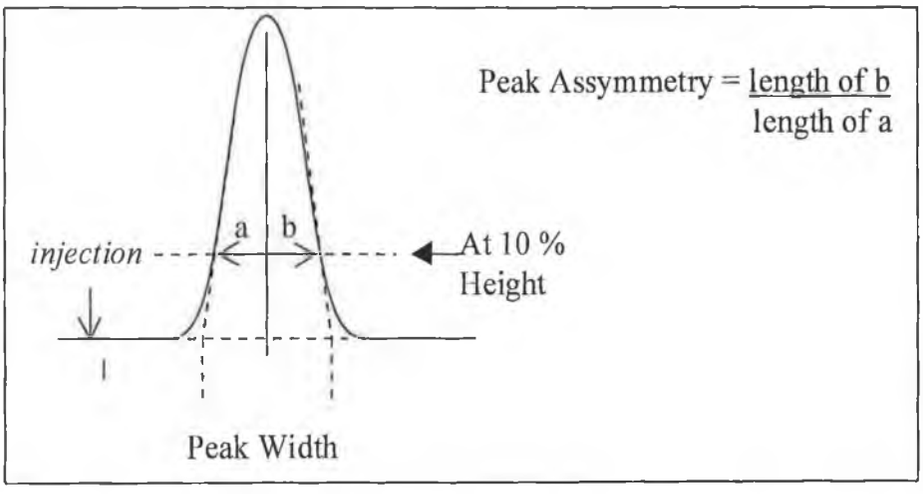


Figure 5.10 *Calculation of peak asymmetry and peak width.*

5.3.3.4.1 ***The effect of acetonitrile and buffer content of the initial mobile phase on peak shape***

When low concentrations of buffer were employed in the mobile phase, peak shape for some analytes was consistently poor, with peaks exhibiting end tailing. The plot in Figure 5.11 shows that as the buffer content of the initial mobile phase was increased, the peak width of neomycin decreased. This effect was evident at all mobile phase contents of acetonitrile. Neomycin, the most polar of the analytes was most susceptible to peak tailing. A similar, but less dramatic effect, was observed for the gentamicin peaks.

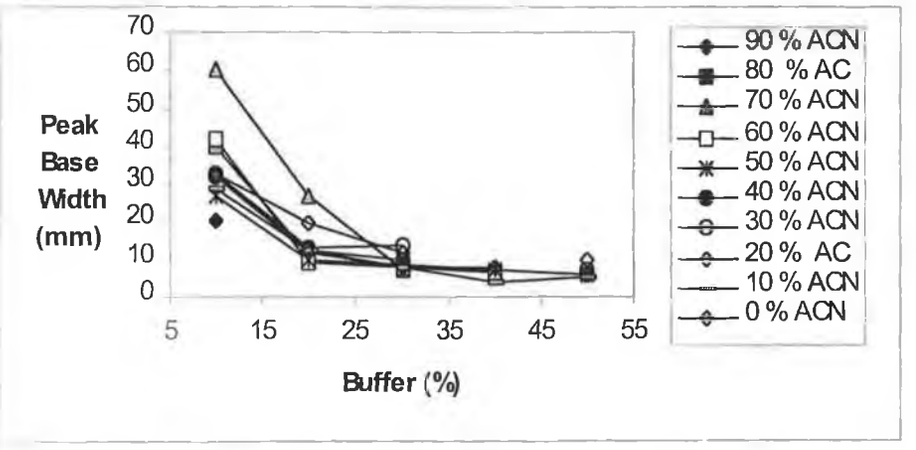


Figure 5.11 *The effect of buffer content of the mobile phase on neomycin peak width at various levels of acetonitrile.*

Figure 5.12 shows the effect of increasing the proportion of acetonitrile, on neomycin peak width, at various percentage contents of buffer in the initial mobile phase. Again, as the buffer content was increased, neomycin peak width decreased and in general, the of acetonitrile content of the initial mobile phase was seen to have little effect on peak width.

In summary, the conclusions on the effects of acetonitrile content, buffer concentration and buffer pH on analyte retention are that retention was promoted by low buffer content (section 5.3.3.2) and high acetonitrile content (section 5.3.3.1), but that high buffer content promotes narrower peaks. Therefore, optimum chromatographic conditions would incorporate high acetonitrile content in the initial mobile phase, to promote retention, and high buffer content to promote good peak shape, without reducing retention dramatically. Buffer pH should be above 3 to promote retention but, above pH 4, neomycin failed to elute within 40 minutes.

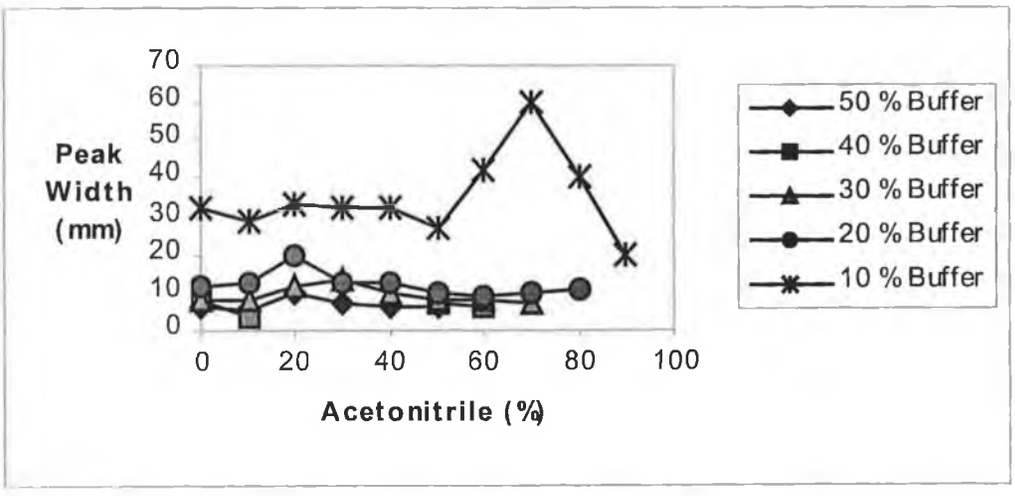


Figure 5.12 *The effect of acetonitrile content on peak width for neomycin at various buffer concentrations.*

5.3.4 Final conditions using column A

To ensure good peak shape and a reasonable retention time for neomycin, a buffer of 250 mM was used, adjusted to pH 4 with formic acid. For adequate retention 80 % acetonitrile was employed in the initial mobile phase. Higher acetonitrile content caused neomycin peak splitting and lower acetonitrile content caused lincomycin to elute as a broad peak and all analytes to have shorter retention times. Inclusion of buffer in the initial mobile phase resulted in lincomycin peak splitting and salinomycin peak broadening. Therefore, the initial mobile phase chosen was 80 % acetonitrile and 20 % water but, because retention times were over long without buffer, over 5 minutes the mobile phase composition was changed linearly to 25 % acetonitrile and 75 % 250 mM, pH 4.0 buffer. Figure 5.13 shows the chromatogram resulting when this mobile phase system was employed, on column A (1000 Å pore size).

5.3.5 Column pore size

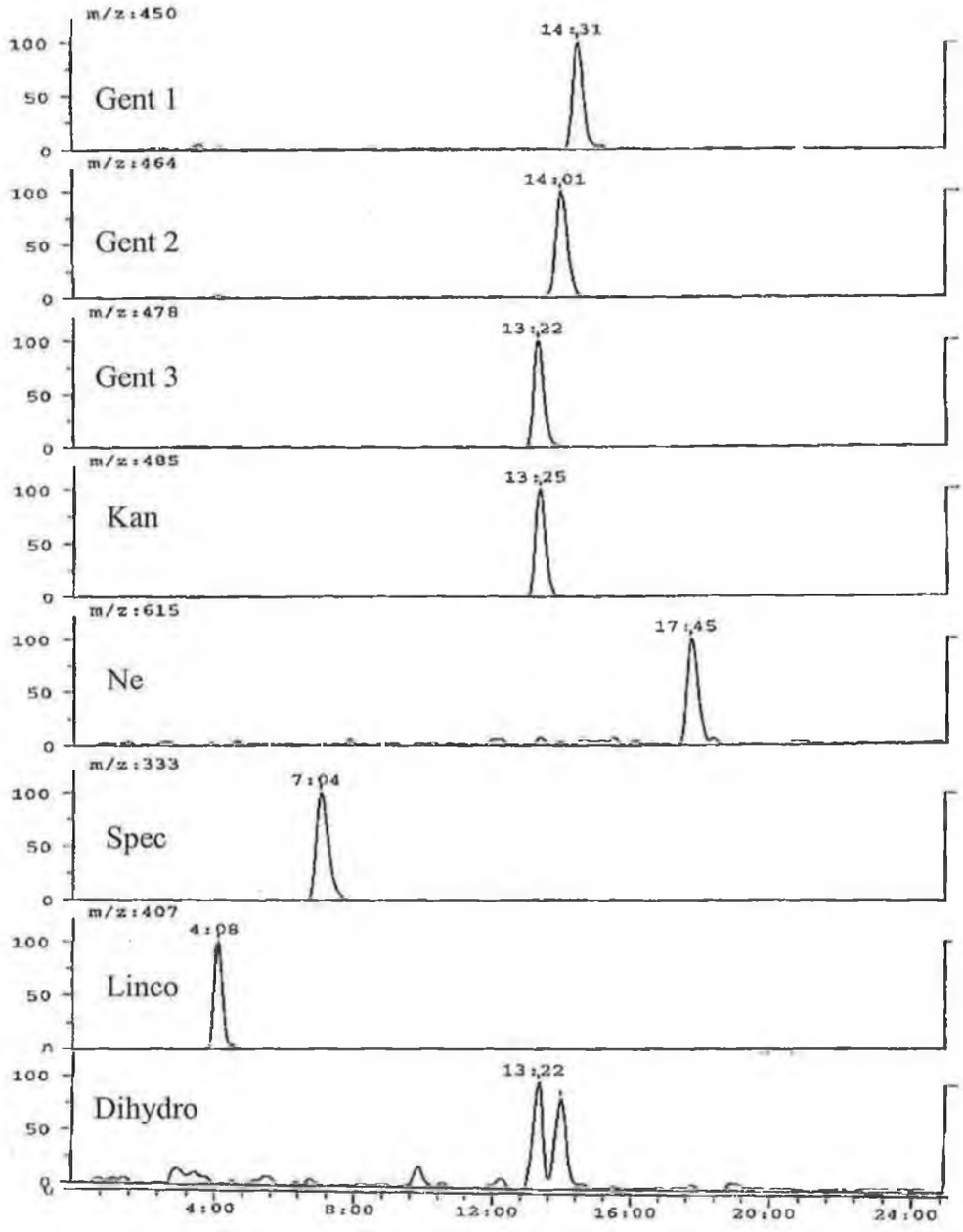
Three columns were investigated: Column A, with pore size 1000 Å, Column B, with pore size 100 Å and column C, with pore size 500 Å. Satisfactory retention and peak shape were first achieved for most analytes on column A, using ammonium acetate prepared at a concentration of 250 mM (see Figure 5.13). Application of these optimised conditions to column B and C did not result in similar chromatography. Using the optimised buffer (250 mM ammonium acetate, pH 4) and column C (500 Å), under a variety of initial and final mobile phase compositions, poor peak shape was observed for all analytes. Tailing was observed for gentamicin and kanamycin and split peaks observed for neomycin. By employing buffer prepared at 500 mM with column C, peak shape for most analytes improved, yet gentamicin peaks were broad and neomycin suffered tailing under a wide range of mobile phase conditions. On column B (100 Å), using the optimum buffer (250 mM ammonium acetate, pH 4), peak shapes for lincomycin, streptomycin, dihydrostreptomycin, spectinomycin and kanamycin were adequate under a wide range of mobile phase conditions. However, retention of the less polar analytes proved more difficult to achieve on the 100 Å column, when compared with the 500 Å column, and peak shape for gentamicin and neomycin was inadequate using the 250 mM buffer. To achieve good peak shape for the more polar analytes with column B, it was necessary to employ ammonium acetate buffer at 500 mM. The best separation achievable with this column is shown in Figure 5.15. While the necessity to use 500 mM buffer (instead of 250 mM) resulted in decreased retention, it was possible to employ 100 % acetonitrile in the initial mobile phase, which helped increase retention and the final mobile phase change was to 5 % acetonitrile, 95 % buffer. Using either column A, B or C, as the buffer in the final mobile phase was decreased below 125 mM, neomycin peak shape deteriorated rapidly, resulting in broad, split peaks.

Alpert observed similar elimination of peak splitting at higher buffer concentration, in the analysis of peptides using HILIC[18].

The effect of pH on peak shape was examined at a number of different buffer concentrations in the range 10-500 mM using columns B and C. It was observed that the higher the pH, the higher the buffer concentration necessary to achieve adequate peak shape. At low buffer concentrations (10, 50 and 100 mM), and pH 5 and 6, most analytes failed to elute within 30 minutes and tailing was observed when elution did occur. All three columns exhibited similar trends with respect to mobile phase composition; as the percentage acetonitrile was increased the retention increased and the acetonitrile content of the initial mobile phase was more influential that its content in the final mobile phase. In general, when using comparable mobile phase compositions, retention times were increased as the pore size decreased.

Figure 5.13: Chromatogram showing the separation achieved for aminoglycosides

HPLC Conditions ; Column : Polyhydroxyethyl Aspartamide™, 200 x 4.6 mm, 5 um, 1000Å.
Mobile phase gradient ; 80 % acetonitrile, 20 % 250 mM NH_4Ac, pH 4 at t=0,
@ 1.0 ml min^{-1}, 25 % acetonitrile, 75 % 250 mM NH_4Ac, pH 4 at t=5 min.
25 % acetonitrile, 75 % 250 mM NH_4Ac, pH 4 at t=15 min.
80 % acetonitrile, 20 % 250 mM NH_4Ac, pH 4 at=18 min.,
Injection volume ; 50 ul of 10 ug ml^{-1}



5.3.6 Investigation of the effect of time taken to change from initial mobile phase conditions to final mobile phase conditions

All experiments, unless otherwise stated, were performed using a gradient which changed linearly between 0 and 5 min, remained until 15 min and changed back to the initial conditions by 18 min as outlined in section 5.2.3. Figure 5.14 shows that retention could be increased and resolution improved by increasing the initial gradient change time beyond 5 min. When the gradient was set to reach final conditions by 7.5 min, tailing resulted for neomycin and a split peak for lincomycin. When the change-time was set to 10 min, spectinomycin was retained well beyond the void volume but eluted as a broad peak, and neomycin exhibited end-tailing. When the change time was set to 15 min, all three gentamicin peaks were resolved from each other, but most other analytes exhibited poor peak shape. Therefore, when a complete multi-residue method is desired, for all the analytes listed, a change time of 5 min is sufficient. However, this time might be varied, to improve resolution, where a method is required for one or some of these analytes.

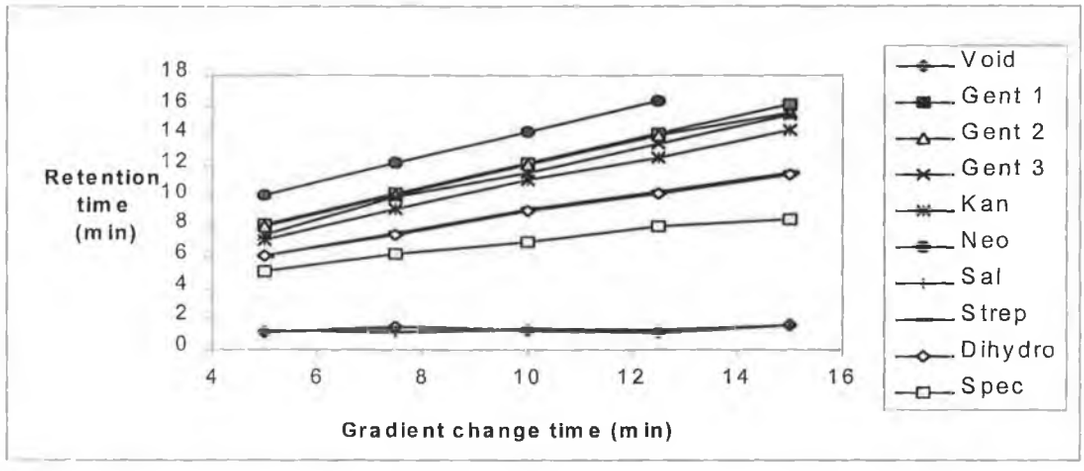


Figure 5.14 *The effect of increasing the time taken to achieve the initial gradient change.*

5.3.7. Chromatography characteristics

Table 5.4. lists the analytes in decreasing order of retention, with neomycin being the most retained and salinomycin retained the least. Also shown are the number of amino groups (NH_2) and number of hydroxyl groups (OH) on each molecule. These groups are shown as they are the functionalities responsible for the hydrophilicity of the molecules and so responsible for partitioning of the analytes within the immobile phase.

Table 5.4 The number of hydroxyl (OH) and amino (NH_2) functionalities on the aminoglycosides.

	Number of OH groups	*Number of NH_2 groups*
Neomycin	7	6
Gentamicin : C1a	3	4
Gentamicin : C2	3	4
Gentamicin : C1	3	3
Kanamycin	6/7	5/4
Streptomycin	8	2
Dihydrostreptomycin	8	2
Spectinomycin	3	0
Lincomycin	4	0
Salinomycin	4	0

The presence of the primary hydroxyl group on neomycin likely causes the increased retention of this analyte, as this functional group conveys high polarity to the molecule. In addition, the molecule contains six amino groups, resulting in a highly basic structure, which is likely to partition in the immobile phase to a relatively high degree.

Kanamycin also contains similar groups, in relatively high numbers, resulting in a polar molecule, which partitions into the immobile phase, resulting in relatively long retention. The gentamicin components are retained possibly due to the presence of three or four amino groups. Amino groups are also likely to be responsible for the retention of streptomycin and dihydrostreptomycin. Spectinomycin, lincomycin and salinomycin display poor retention characteristics due to the lack of any amino groups. Table 5.5 shows the plate number (N) and peak asymmetry (A) values for the analytes, obtained using column A (1000 Å). The high retention achieved resulted in high column efficiency (N) for gentamicin, neomycin and kanamycin. Poor retention resulted in low column efficiency for spectinomycin and lincomycin and the poor retention together with band broadening is responsible for low column efficiency for salinomycin. Peak asymmetry was calculated as depicted in the diagram in Figure 5.10. An ideal gaussian peak shows a value of one. In Table 5.5, the high asymmetry value for salinomycin is due to the end-tailing observed. Values of less than one result for peaks affected by fronting.

Table 5.6 shows the chromatographic properties of separations using optimised conditions for column B (100 Å). Efficiency values (N) are low, relative to those obtained for column A, due to the earlier elution of the analytes and the slightly increased peak width. However, it is worth noting that the efficiency values for salinomycin increased using column B (under the optimised conditions for that column) compared to that obtained for column A. Peak asymmetry values were comparable for gentamicin and kanamycin, using both methods, but using column B, neomycin and

spectinomycin exhibited some end-tailing resulting in elevated peak asymmetry values.

Figure 5.15 shows the separation achieved using column B (100 Å).

Table 5.5 Retention and efficiency data for aminoglycosides on column A (1000 Å)

	Retention (mm)	Peak Width (mm)	Efficiency (N)	Peak Asymmetry (A)
Neo	97.5	5.0	6084	1.56
Gent C1a	80.0	5.0	4096	1.17
Gent C2	77.0	5.0	3795	1.0
Kan	73.5	4.0	5402	1.17
Gent C1	73.0	5.0	3411	1.40
Spec	39.0	6.0	676	1.25
Linco	23.0	4.0	529	0.80
Sal	13.5	7.0	60	4.50

HPLC Conditions : Column A, Mobile phase gradient at 1ml min^{-1} :
At time =0; 80/20 ACN/H_2O,
At time = 5 min; 25/75 ACN/250 mM Ammonium Acetate, pH 4,
At time = 15 min; 25/75 ACN/250 mM Ammonium Acetate, pH 4,,
At time = 18 min; 80/20 ACN/H_2O.

A : Calculated as depicted in Figure 5.10

N : 16 x [(retention time/base width)^2].

Table 5.6 Retention and efficiency data for aminoglycosides on column B (100 Å)

	Retention (mm)	Peak Width (mm)	Efficiency (N)	Peak Asymmetry (A)
Neo	70.0	10.0	784	2.40
Gent C1a	56.0	7.0	1024	1.50
Gent C2	55.0	8.0	756	1.04
Gent C1	54.0	7.0	952	1.50
Kan	50.0	4.0	2500	1.17
Spec	35.5	7.0	412	2.17
Linco	29.0	7.0	275	1.17
Sal	18.5	3.0	608	1.00

Conditions : Column B, Mobile phase gradient at 0.5 ml min^{-1} :
At time =0; 100 % ACN,
At time =5 min; 5/95 ACN/500 mM Ammonium Acetate, pH 4,
At time = 15 min; 5/95 ACN/500 mM Ammonium Acetate, pH 4,
At time = 18 min; 100 % ACN.

A : Calculated as depicted in Figure 5.10

N : 16 x [(retention time/base width)^2].

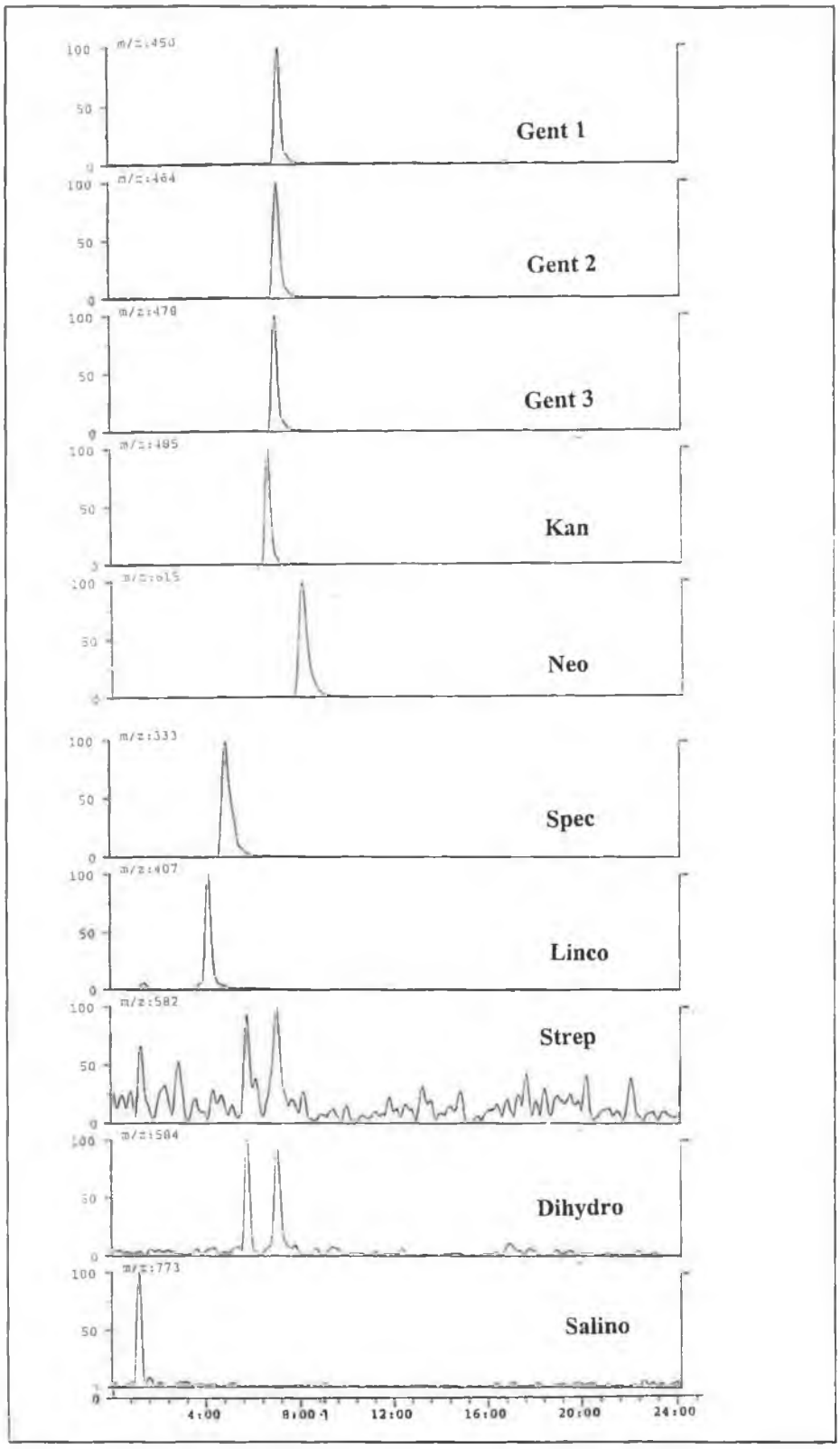


Figure 5.15 *Chromatogram of analyte separation using HILIC Column 100 Å*

HPLC Conditions:	Column : Polyhydroxyethyl Aspartamide™, 200 x 2.1 mm, 5 µm, 100 Å.
Mobile phase gradient;	100 % acetonitrile, at t=0,
@ 0.5 ml min^{-1}	5 % acetonitrile, 95 % 500 mM NH_4Ac, pH 4 at t=5 min
	5 % acetonitrile, 95 % 500 mM NH_4Ac, pH 4 at t=15 min
	100 % acetonitrile, at t=18 min,
Injection volume;	50 µl

5.3.8 Linearity and sample analysis

The conditions described in Table 5.6 were used in conjunction with column B (100 Å), to estimate the linearity of the method and analyse fortified samples. Standards were chromatographed in a number of different diluents, including:

A) 25 % acetonitrile, 75 % water,

B) 75 % acetonitrile, 25 % water,

C) 75 % water, 25 % 500 mM ammonium acetate, pH 4,

D) 75 % acetonitrile, 25 % 500 mM ammonium acetate, pH 4,

When diluent D (acetonitrile and buffer) was employed, the neomycin peak exhibited extreme tailing, streptomycin and dihydrostreptomycin resulted in split peaks and spectinomycin displayed very poor peak shape. Use of diluent C resulted in much improved chromatography. This diluent contained no acetonitrile, which was replaced with water, resulting in a reduction in the molarity of the buffer. Neomycin eluted as a broad peak with slight tailing (the peak was 1 min broad), while all other analytes exhibited acceptable peak shape and retention. Diluent B, comprising mainly of acetonitrile, resulted in poor chromatography for all analytes except salinomycin and lincomycin. Using diluent A, results are comparable with diluent C for gentamicin, neomycin and kanamycin, that is, acceptable peak shape and retention, but spectinomycin, lincomycin and salinomycin all exhibited peak splitting. In conclusion, it appears that the presence of high acetonitrile content in the diluent promotes good peak shape for salinomycin and lincomycin, but results in relatively poor shape for the other aminoglycosides.

Linearity was assessed by injecting 6 standards (50 µl), prepared in diluent C, in the concentration range 0.05 to 10 µg ml^{-1}. R^2 values of 0.999 were obtained, proving that the method gives adequate chromatography over a range of analyte concentrations. Under these conditions, neomycin and spectinomycin peaks exhibited some tailing and streptomycin and dihydrostreptomycin were very poor (low intensity and peak splitting). Gentamicin and neomycin were detectable well above the MRL values shown in Table 5.7; no MRL has yet been set for kanamycin.

Table 5.7 MRLs for gentamicin and neomycin in various matrices.

Matrix	*Gentamicin (ppb)*	*Neomycin (ppb)*
Milk	100	500
Muscle	100	500
Kidney	1000	5000
Liver	200	500
Fat	100	500

Milk samples fortified with gentamicin, neomycin and kanamycin and extracted by ion-exchange SPE, as described in Chapter 4, were analysed by HILIC-MS and by paired-ion chromatography with post-column derivatisation (PIC-PCD). The results are presented in Table 5.8. Recoveries were calculated by comparing peak areas derived from a standard analyte solution of 100 µg ml^{-1} chromatographed alongside the samples and recoveries were found to be similar using both methods.

Table 5.8 Comparison of recovery (%) for milk samples fortified at 100 ppb and analysed by PIC-PCD and HILIC-MS (n=2).

	PIC-PCD	*HILIC-MS*
	Mean Recovery (%)	
Gent Peak 1	68.3	73.3
Gent Peak 2	65.5	76.3
Gent Peak 3	69.0	N
Neo	79.4	84.0
Kan	54.4	55.8

N : Due to incompatibility of the ion-exchange SPE elution diluent with HILIC, this peak was not quantitated.

5.4 CONCLUSION

This preliminary study confirms the applicability of HILIC for the analysis of aminoglycoside drug residues. The suitability of HILIC to mass spectrometric detection has been demonstrated and the method has been tested on milk and kidney sample extracts. The method illustrates a high degree of specificity by providing retention time and structural information for the analytes. The development of an LC-MS system for highly basic analytes is difficult and this is the first report of an LC-MS method for aminoglycosides that does not employ ion-pair or ion-exchange chromatography. This is also the first report on the application of HILIC to veterinary drug residue analysis. The method is capable of detecting residues in tissues well below the MRL and this study shows that some aminoglycoside residues can be detected at levels lower than those reported previously. The studies carried out failed to produce a chromatographic

method capable of separating the basic aminoglycosides gentamicin, neomycin and kanamycin in the same chromatographic run as streptomycin and dihydrostreptomycin. However, further investigations may result in a multi-residue method, capable of achieving this. The results from this study provide a good indication of the factors that affect the chromatography of aminoglycosides, from the most polar (neomycin) to the relatively non-polar (spectinomycin).

This study has shown the important influence of mobile phase composition on analyte retention and peak shape in HILIC separations and has illustrated the importance of optimising the column pore-size for the analytes of interest. The method shows potential for the analysis of highly polar analytes and further studies into the effects of various column packing materials and particle dimensions may allow multi-residue analysis of a large range of highly polar basic analytes, within one chromatographic run. Alternatively, the method could be optimised for the analysis of one specific analyte. A comparison of the chromatograms presented in this study to those presented in Chapter 4, where ion-pair chromatography was employed, shows HILIC offers superior peak shape and MS detection overcomes the problems of matrix co-extractives, in addition to providing a confirmatory method. Also, gentamicin, neomycin and kanamycin can be analysed within one-run, while co-elution prevented single run analysis using ion-pair chromatography. The application of HILIC-MS will allow confirmatory analysis of these analytes with a simple chromatographic method, possessing good chromatographic properties.

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Appendix A

Publications

McGrane, M., O'Keeffe, M., & Smyth, M., sulphonamides, *Irish Journal of Agricultural and Food Research,* 1996, **35,** 192, Analysis of sulphonamides in pork using automated dialysis, (Abstract).

McGrane, M., O'Keeffe, M., & Smyth, M., Multi-residue analysis of penicillin residues in porcine tissue using matrix solid phase dispersion, In *Proceedings of the 3rd International Symposium on Hormone and Veterinary Drug Residue Analysis,* Bruges, Belgium, June 2-5, 1998, 146 (Abstract)

McGrane, M., O'Keeffe, M., & Smyth, M., Multi-residue analysis of penicillin residues in porcine tissue using matrix solid phase dispersion, *Analyst,* 1998, **123**, 2779-2783.

McGrane, M., O'Keeffe, M., & Smyth, M., The analysis of sulphonamide drug residues in porcine muscle using automated dialysis, *Analytical Letters,* 1998, **123**, 2779-2783.

McGrane, M., Keukens, H., O'Keeffe, M., VanRhijn, H., & Smyth, M., A fully validated assay for the analysis of aminoglycoside antibiotics in milk and kidney, In *Proceedings of EuroResidue IV, Conference on Residues of Veterinary Drugs in Food,* Veldhoven, The Netherlands, 8-10 May, 2000, 758.

McGrane, M., Keukens, H., O'Keeffe, M., VanRhijn, H., & Smyth, M., A novel MS compatible method for the analysis of aminoglycoside antibiotics by LC-MS at residue

levels, In *Proceedings of EuroResidue IV, Conference on Residues of Veterinary Drugs in Food,* Veldhoven, The Netherlands, 8-10 May, 2000, 764.