1999-01-01

Chemical Analysis of Veterinary Drug Residues in Salmon Tissue

Patrice Behan
Technological University Dublin

Recommended Citation

Behan, P. (1999). *Chemical analysis of veterinary drug residues in salmon tissue.* Doctoral thesis. Technological University Dublin. doi:10.21427/D7SW3F

Chemical Analysis of Veterinary Drug Residues in Salmon Tissue

A thesis submitted to
Dublin Institute of Technology, Kevin Street
for the degree of PhD

by

Patrice Behan BSc

Supervisors:
Dr. Barry Foley
Chemistry Department
DIT Kevin Street
Dublin 8

Eugene Nixon
Marine Institute
Fisheries Research Centre
Abbotstown Dublin 15

October 1999

Authors Declaration

I certify that this thesis which I now submit for examination for the award of Doctoral Degree (PhD) 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.

This thesis was prepared according to the regulations for postgraduate studies by research of the Dublin Institute of Technology and has not been submitted in whole or in part for an award in any other Institute or University.

The Institute has permission to keep, to lend or to copy this thesis in whole or in part, on condition that any such use of material of the thesis be duly acknowledged.

Signature Patrice Behan
Date 25-09-00

Acknowledgements

I would like to express my sincere gratitude to my supervisors Dr. Barry Foley and Eugene Nixon for their guidance, encouragement and constructive advice during the course of this study.

I am grateful to the Marine Institute, the Irish American Partnership and the DIT Postgraduate Committee for their financial support during this project.

Many thanks to the Marine Institute, Fisheries Research Centre for the opportunity and facilities to carry out this research.

I would like to express my appreciation to the staff of the Fisheries Research Centre, especially the Chemistry staff for samples, use of equipment and constant help throughout this project.

Many thanks to Dr. Kai Bester for the opportunity to visit his laboratory at the University of Hamburg and carry out some research there.
Thanks also to Dr. Ingo Nausch, Dr. Gehard Rimkus and all the staff at the LUVA laboratory in Nuemunster for their help, hospitality and eagerness to share information during a visit there.

Thanks to my editors Eugene Nixon, Barry Foley, Martin Bloxham and Evin McGovern, and also to the FRC and State Laboratory library staff Alice Talbot and Michael O'Gorman.

TABLE OF CONTENTS

Chapter 6: Initial investigations into the use of Size Exclusion Chromatography for the Analysis of Veterinary Drug Residues in Salmon Tissue 244

ABSTRACT

The Irish finfish-farming sector has increased from 20 tonnes in 1980 to 15,440 in 1997. The sales value of the 15,440 tonnes was £37.5 M where exports were worth approximately £30 M of this. This increasing intensity of fish farming has inevitably been paralleled by increases in disease, which has forced the mariculturist to increase the use of chemicals.

A major concern with the use of chemicals in mariculture is residues, which may remain in the fish tissue after harvesting and resulting in exposure to the consumer. Under Council Directive 96/23/EC Member States are required to monitor on a routine basis, veterinary drug residues in animals and animal products. The aim of this monitoring plan is to ascertain that residues, where present are below the maximum residue limit (MRL), and that no residues are present for analytes which do not have an established MRL.

In consistence with this Directive, HPLC methods were optimised and validated according to the criteria set out in Commission Decision 93/256/EEC for the qualitative and quantitative analysis in farmed salmon tissue, of oxytetracycline (OTC), oxolinic acid (OA), sulphadiazine (SD), sulphamethoxazole (SMX) and trimethoprim (TMP). All methods were found to be linear from 50-150% of the respective MRLs, while recoveries were within the required 70-110% range for OTC, OA, SD and SMX. The precision obtained at 100μgkg^{-1} was 2.1% RSD (n=5), 1.5% RSD (n=8), 9.7% RSD (=6) and 8.4% RSD (n=6) for OA, OTC, SD and SMX respectively. The limit of detection (LOD) based on S/N=3, was found to be 10.0μgkg^{-1}, 20.1μgkg^{-1}, 1.0μgkg^{-1} and 2.6μgkg^{-1} respectively, for OA, OTC, SD and SMX. Based on S/N=5, the limit of quantification (LOQ), in tisuue samples was 11.3μgkg^{-1}, 37.6μgkg^{-1}, 1.6μgkg^{-1} and 8.2μgkg^{-1} for OA, OTC, SD and SMX respectively.

In addition to the above chemicals ivermectin and cypermethrin were examined using size exclusion chromatography (SEC) as a possible screening and clean-up technique. A SEC method capeable of screening 16 samples in one overninght run, for residues of SD, SMX and OA in tissue, at 50μgkg^{-1} was developed. Additionally SEC was developed as a cleanup technique to increase sample throughput for ivermectin analysis.

CHAPTER 1: INTRODUCTION

Mariculture inevitably means that marine animals are grown in what are relatively, intensive conditions such as fish farms. The Irish finfish farming sector, consisting primarily of salmon but also including rainbow trout, arctic charr, turbot and eel has grown from 20 tonnes in 1980 [1] to 15,440 in 1997 [2]. The sales value of the 15,440 tonnes was £37.5 M and exports were worth approximately £30 M of this. The industry estimates that over 2,500 people from Malin Head, Co. Donegal to Bantry Bay Co. Cork depend on salmon farming for their livelihoods [1]. The trout industry produced 1250 tonnes in 1997 [2], while 70 tonnes of arctic charr were harvested for the home and export market [2]. Ireland's first land based eel farm using recirculated water technology, was opened in 1997 and has a planned production of 60-80 tonnes per year [2].

The increasing intensity of fish farming combined with the introduction of new species has inevitably been paralleled by increases in disease. The first line of defence against disease outbreaks is the purchase of disease free stock with good husbandry necessary to maintain the stock free of disease. Good husbandry involves the optimisation of culture conditions, which will allow the fish to express natural defences and thus reduce losses. While husbandry is proving to be effective for the control of diseases, situations will arise where the farmer must use other means of control [3].

1.1 Disease groups

Fish diseases are caused by a wide range of infectious organisms including bacteria, fungi, viruses, protozoan and metazoan parasites.

1.1.1 Bacteria

Bacterial pathogens of fish can be divided into (a) primary and (b) secondary pathogens. Primary pathogens may cause serious disease outbreaks and generally cause conditions, which result in significant mortality if left untreated.

Secondary invaders however, are opportunistic in nature and tend to cause problems when the fish are already compromised through reasons such as overcrowding, high waves, temperature etc. Most bacterial infections in the Irish mariculture industry are caused by gram-negative organisms and are readily treated by broad-spectrum antibiotics.

Table 1.1: List of bacteria pathogens, their host, the disease associated with them and the chemical used to treat them [4].

Pathogen	Host	Disease	Chemical
Bacteria			
Yersinia ruckeri	Salmonids	Red mouth	Sulphonamides, oxytetracycline and oxolinic acid.
Vibrio anguillarum	All fish	Vibriosis	Sulphonamides, oxytetracycline and oxolinic acid.
Vibrio salmonicida	Atlantic salmon	Cold water vibriosis	Oxytetracycline
Aeromonas salmonicida	Salmonids	Furunculosis	Sulphonamides, oxytetracycline and oxolinic acid.
Streptococcus spp	All marine fish	Septicaemia	Oxytetracycline
Flexibacter columnaris	All fish	Columnaris disease	Oxytetracycline
Metazoan			
Lepeophtheirus salmonis	Salmonids	Sea lice	Ivermectin, Cypermethrin and Azamethiphos

1.1.2 Fungi

Saprolegnia is the main fungal infection observed in Ireland, and this is readily treated with malachite green. Fungal infections however, can lead to bacterial infections when the opportunistic pathogens invade the site of fungal infection.

1.1.3 Metazoans

In Ireland, the most common type of *ectoparasite* are sea lice, (*Lepeophtheirus salmonis)* [5], which can cause skin erosion and damage which in heavy infestation can result in death. A number of treatments have been proven effective against fish *ectoparasities*, but their use is limited by many environmental factors [4]. The chemicals currently used to treat sea lice in Ireland include ivermectin, cypermethrin and azamethiphos.

1.1.4 Viruses

In Ireland, only two virus isolations from cultured finfish were recorded in the last two years. Unfortunately, viral diseases are not susceptible to control by chemotherapy [4].

1.2 Chemicals used in Mariculture

In mariculture, only a few vaccines have been developed for the control of bacterial diseases of finfish culture. In the case of disease, where vaccines are practical, after good husbandry, these form the ideal second line of defence against destruction of stock by disease. Failing this however, the mariculturist is inevitably forced to employ chemicals.

The use of chemicals in fish culture has a long history, commencing with attempts to control *ectoparisites* with salt baths. The first recorded use of formalin was in 1909 to control *Costia* infections [4]. Other topical "disinfectants" including copper and malachite green were introduced in the 1920's and 1930's and quaternary ammonium compounds in the 1940's [6]. Attempts at therapy of bacterial diseases commenced in the late 1930's and sulphamerazine was introduced in the USA in 1948 [7].

The major chemicals used in mariculture in Ireland are listed in Table 1.2. These chemicals differ in structure and mode of action and fall into different groups. If the compounds are of biological origin, they are termed as antibiotics and antibacterials if they are of synthetic origin [4].

1.2.1 Tetracyclines

Tetracyclines are a group of broad-spectrum antibiotics whose mode of action is mainly bacteriostatic which interfere with bacterial protein synthesis by binding to the bacterial 30S-ribsomal subunit. Effective against a wide range of gram-negative and gram-positive bacteria, the most widely used tetracycline in mariculture is oxytetracycline (OTC), (Figure 1.1).

OTC (4-(Dimethylamino)-1,4,4a,5,5a, 6,11,12a-octahydro-3,5,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide) absorbs in the UV region of 255-390nm and has a molecular weight of 460.44.

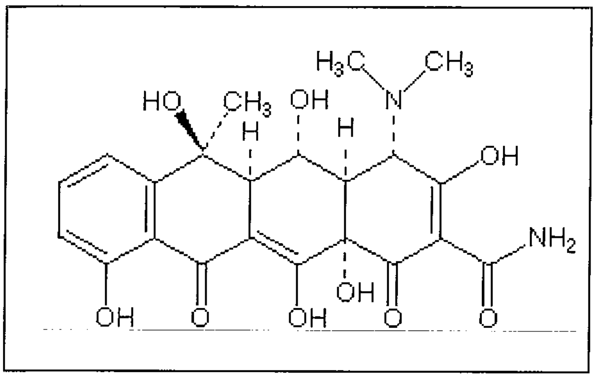


Figure 1.1: Structure of oxytetracycline

OTC is administered to fish in the form of a product called Tetraplex, which consists of 50% OTC [8]. It may be incorporated into feed pellets and fed at a dosage rate of 80mg/kg body weight/day in fresh water and 120mg/kg body weight/day in salt water, over a 10-day treatment period.

The withdrawal period for OTC is 400 degree-days [8], while the EU maximum residue limit (MRL) (Section 1.4), in fish tissue is $100 \mu g kg^{-1}$ [9].

OTC has a low toxicity to fish. After force-feeding fish 200 mg per day for 5 days only mild signs including lethargy in the last day of treatment were reported. Negligible losses of brook trout fry were recorded after 2-hour bath treatment exposures of 100-$500 \mu g g^{-1}$. [3].

The extended use of OTC has led to an increase in the frequency of resistance. When the resistance to OTC develops from a change of bacterial cell membrane permeability, this can result in cross-resistance to other drugs including quinolones [3].

1.2.2 Quinolones

The quinolones are a group of synthetic antimicrobial agents. Oxolinic acid (OA), which has a molecular weight of 261.24, is a member of this group and is effective against gram-negative bacteria (Figure 1.2). OA acts by inhibiting the supercoiling of bacterial DNA by acting against DNA gyrase.

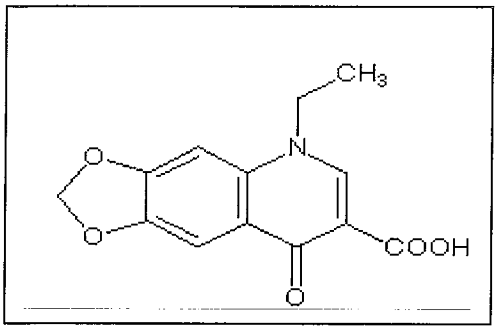


Figure 1.2: Structure of oxolinic acid

In the form of a bath treatment of 30 minutes duration at 10μgg^{-1} of fish tissue, OA is effective against bacterial exoskeleton disease in crustaceans [3]. It can also be administered as an oral dose in the form of feed medications. OA can be administered as Aquinox which consists of 50% OA or Aqualinic which contains 100% OA [8].

The withdrawal period for OA is 500-degree days [8], while at present there is no MRL set for OA in fish tissue in the EU.

The primary route of breakdown of OA in the environment is photolysis, which will not occur in sediments beneath a fish cage [3].

1.2.3 Potentiated sulphonamides

Sulphonamides are a group of antimicrobial agents that act on gram-negative bacteria by blocking folic acid pathways of susceptible microorganisms. Sulphonamides in mariculture are used in combination with a potentiator – trimethoprim (TMP), which blocks a different stage of the folic acid metabolism. Sequential blocking of the pathway can produce synergism, hence the use of potentiated sulphonamides. Two sulphonamides currently used in mariculture in Ireland are sulphadiazine (SD), molecular weight of 250.28, and sulphamethoxazole (SMX) which has a molecular weight of 253.31, while the potentiator is trimethoprim (TMP), molecular weight of 290.32. The structures of these chemicals can be seen in Figure 1.3a-1.3c.

The antibacterials which are currently being used in salmon farming in Ireland are Co-trimoxazole which is a 5:1 mixture of TMP and SMX, and Tribissen which consists of a 40% mixture of SD and TMP in the ratio of 5:1 [8].

For administration, the potentiated sulphonamides are incorporated into feed pellets. Co-trimoxazole is administered in a dose of 30-50mg/kg body weight/day for 5-10 days while Tribissen is administered as an oral medication of 50mg/kg body weight/day for 10 days [3].

The withdrawal period for potentiated sulphonamides varies considerably depending on local veterinary regulations and specific product. For Tribissen the withholding period is 350-degree days in Scotland, while for co-trimoxazole the withholding period is 400-degree days [8].

The EU MRL for the combination of all sulphonamides in fish tissue is $100\mu g kg^{-1}$ while for TMP the MRL is $50\mu g kg^{-1}$ [9].

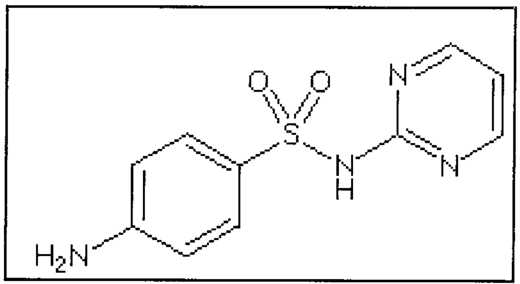


Figure 1.3a: Structure of sulphadiazine

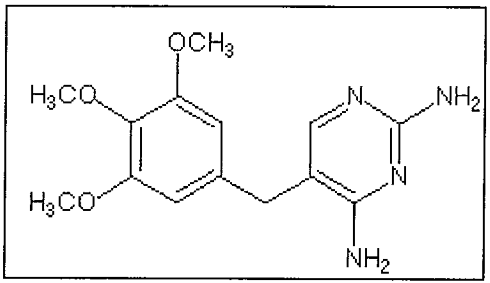


Figure 1.3b: Structure of trimethoprim

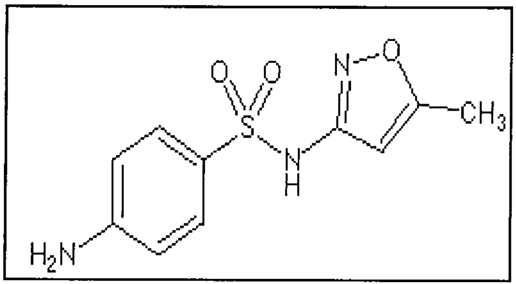


Figure 1.3c: Structure of sulphamethoxazole

1.2.4 Sea Lice control agents

Two chemicals used at present in Ireland, and thus being studied in this work for the control of sea lice on farmed salmon, are ivermectin and cypermethrin.

1.2.4.1 Ivermectin

Ivermectin, (22,23 dihydroivermectin B1), (Figure 1.4), consists of a mixture containing > 80% ivermectin B1a and < 20% ivermectin B1b. It is an insecticidal compound derived from the bacterium *Streptomyces avermitilis* [10].

Ivermectin has been proposed as a chemotherapeutant for the control of farmed salmon infected with sea lice. It is administered as an oral dose on fish feed at typical rates of 0.01-0.05 mg/kg body weight for 3-4 weeks.

No MRL is given for ivermectin in salmon tissue in Commision Regulation (EC) No 508/1999. However, the European Agency for the Evaluation of Medicinal Products (EMEA) recommended a value of 20μgkg^{-1} in deer muscle for inclusion into this regulation [12]. Deer is the only species, which has a MRL for ivermectin in muscle [9].

The withdrawal period for ivermectin from fish tissue is >400 degree days [3].

It has been reported that the concentrations of ivermectin that accumulated in the surface of the under-farm sediments were extremely low. Concentration values reported for a study of an Irish fish farm were 5μgkg^{-1}, while studies of the fauna in these sediments revealed no significant impact on the populations of pollychaete worms, a group thought to be extremely sensitive to ivermectin [11].

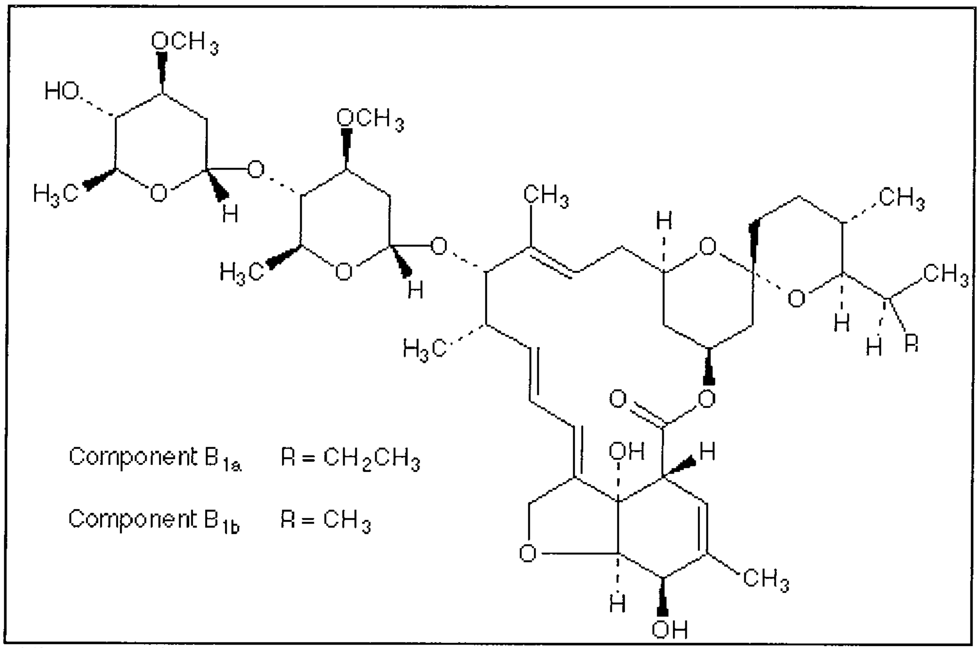


Figure 1.4: Structure of ivermectin

1.2.4.2 Cypermethrin

Cypermethrin is a synthetic pyrethroid insecticide used for the control of sea lice on farmed salmon. It consists of a mixture of 4 *cis*- and 4 *trans*- isomers in a 40:60 ratio for sea lice treatment (Figure 1.5). In Ireland, cypermethrin has a trial licence on certain salmon farms. A MRL of 50μgkg^{-1} has been recommended by the EMEA for salmon tissue and this will, in the future be adopted by the EU [12]. The treatment dose of Excis, which contains 1% cypermethrin, is 5μg per litre of sea water for 1 hour and the withdrawal time for salmon is 72 hours.

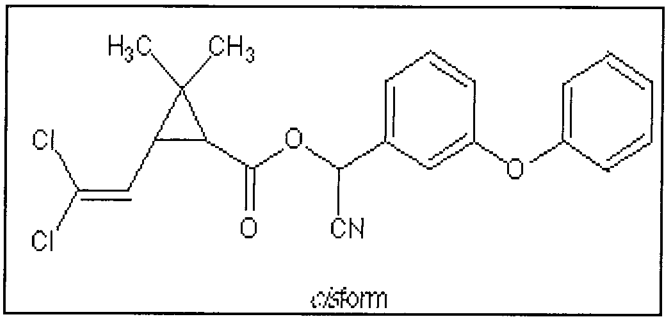


Figure 1.5: Structure of cypermethrin

1.3 Concern over the use of chemicals in mariculture

Many countries engaged in mariculture have few regulatory controls and/or little documentation of the chemicals used by the industry. Even countries that maintain a list of approved chemicals and control their use may not always have information on the quantities actually applied. Ireland is one of those countries, and consequently the need for a national mechanism to compile and maintain records on the types and quantities of chemicals used in mariculture is a high priority [13].

Chemicals employed in mariculture may be of two general types (a) hygiene products for disinfection or for "environmental control", e.g. antifouling agents for fish cages, (b) medical products. The medical products can be divided into chemotherapeutic agents, which act on the invading organism, and pharmacological drugs, which act on the target animal [4].

There are some areas of concern in the use of chemicals in mariculture including persistence in aquatic environments, toxicity to non-target species, stimulation of resistance, and residues of chemicals which may remain in the fish tissue after harvesting and therefore exposed to consumers. An additional subsidiary area of concern involves the potential risks to operators during preparation and application of chemicals [4].

When therapeutants are applied to farmed fish stocks, a significant proportion may not reach the target species but will go directly into the environment. The proportion reaching the environment depends on the route of application of the therapeutants. In the case of an antimicrobial presented in a medicated diet, 30% will reach the environment in the form of uneaten food. This may be consumed by other fish and invertebrates and may or may not be broken down microbiologically, depending on the state of the below-cage environment. Unmetabolised drugs in faeces which are excreted (through kidney, liver or gill) by the farmed fish will fall to the sea bottom and enter the below cage environment with the waste feed. The excretion of a metabolically altered drug by the fish into the marine environment may also have adverse effects on the marine ecosystem. In the case, where fish are treated in the form of a bath treatment almost 100% of the therapeutant goes directly into the environment. Thus, the use of therapeutants and chemical control agents in mariculture forms one of the major factors of the direct environmental impact of the industry [4].

1.4 Regulatory controls to protect the consumer

In order to protect the consumer from residues, which may remain in fish tissue after harvesting, some guidelines have been set up which must be adhered to. These include a drug withdrawal period which is defined as the time interval between the end of drug treatment and slaughter which must be observed in order that the residues of the drug reach levels which present no hazard to the consumer. This level is defined as the maximum residue limit (MRL) which is the maximum permitted level of any veterinary drug residue in any edible tissue of a food animal for human consumption. The MRL is based on the amount of residue considered to have no significant toxicological risk for human health. The MRL is generated by a number of bodies such as the European Union (EU), and more globally within the framework of the FAO/WHO Codex Alimentarius Commission. The FAO/WHO Codex Alimentarius Commission is scientifically advised by the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

The calculation of the MRL is complex, but in brief terms it is calculated from the acceptable daily intake (ADI), which with safety factors is itself based on a No Observable Adverse Effects Level (NOAEL) derived from animal and *in vitro* trials. Therefore, applicants for medicine licenses must provide enough data to enable the ADI to be determined [13]. A list of MRL's for veterinary drug residues in edible animal tissues is available in the Commission Regulation (EC) No. 508/1999 [9], while the relevant MRL's for this project are listed in Table 1.2.

Table 1.2: List of MRL's from Council Regulation (EC) No 508/1999 for salmon tissue. The Limit of Quantification (LOQ) listed refer to the methods optimised in this study for the Marine Institute Fisheries Research Centre Laboratory (MIFRC).

Parent Compound	MRL (μgkg^{-1})	LOQ (μgkg^{-1})
Oxytetracycline	100	38
Oxolinic acid	-	20
Sulphadiazine	100	24
Sulphamethoxazole		24
Trimethoprim	50	Under development
Ivermectin	20 in deer muscle[a]	1
Cypermethrin	50[b]	Under development

a: No MRL is given for ivermectin in salmon tissue. The value listed refers to deer muscle [9].
b: No MRL is given for cypermethrin in salmon tissue. However, the EMEA Committee recommended the inclusion of a 50μgkg^{-1}, MRL for salmon tissue into Comission Regulation (EC) No 508/1999 [14].

Concern for the presence of veterinary drug residues in edible fish tissue is associated with possible effects on consumer health. This can be either in the form of immediate hypersensitivity reactions, such as may occur in people sensitised to β-lactam antibiotics, or a build up of resistance with potential toxicological effects from low levels of antibacterials. The protection of consumers against the risks of ingesting veterinary drug residues is receiving much attention within the EU. Under the Council Directive 96/23/EC member states are required to develop and implement a plan to monitor, on a routine basis, residues in animals and animal products including fish and fish products [15]. Ireland has developed a monitoring programme to ensure that levels of veterinary drug residues in fish tissue are below the recommended MRL where these are set. In the case where no MRL is set for a particular chemical in fish tissue, it is imperative that mariculture products conform to standards no less protective than those already in place for many other areas of animal production.

1.5 Methods of analysis for veterinary drug residues

As a consequence of the concern over the use of chemicals in mariculture, there is a need to monitor the presence of these substances so as to ensure that (1) the wholesomeness of food is not compromised, (2) micro-organisms harmful to man are not developed, (3) the MRL is not exceeded and (4) the regulations are being complied with.

In the past microbiological assays have been more commonly used for the detection of antibiotic residues due to their wide applicability. However, they can be non-specific, they can lack reproducibility, are only semi-quantitative, and in some cases lack adequate sensitivity [8]. These methods are suited to screening for chemicals and groups of chemicals but identification and confirmation methods are required by other chemical techniques.

Physiochemical methods, however, using chromatographic techniques coupled with various detectors do not suffer from these disadvantages. In contrast to the microbiological methods, the chromatographic methods are relatively complex but they do have the potential to identify in a specific manner the chemicals present with considerable sensitivity and accuracy. However, unless the full range of chemicals used is known, they do not provide an assessment of the total chemicals present in the tissue.

1.5.1 Method Validation

The analytical methods developed in this work will, in the future, be used for the routine analysis of veterinary drug residues in farmed finfish. Thus under Council Directive 96/23/EC the methods must be validated according to the criteria set out in Annex 1 of Commission Decision 93/256/EEC [16]. These criteria help to evaluate the performance of the method and, if all the validation criteria are met, it is proven that the method is fit for its intended purpose. The validation criteria include the determination of specificity, injection repeatability, linearity, accuracy, precision, detection limit, quantification limit, range and stability.

Not all the criteria in the Commission Decision 93/256/EEC were applicable to the methods, which were examined in this work e.g. accuracy criteria were reported for certified reference materials (CRM) only and these were not available for the analytes of interest in this work. Therefore, it was necessary to use an alternative source for validation criteria, which was the Codex Committee on Residues of Veterinary Drugs in Foods [17].

Thus, the criteria used to validate the methods examined in this work were where applicable, taken from Commission Decision 93/256/EEC. Otherwise, the Codex criteria were used. The criterion from the combination of the two sources, which was used in this work, is shown below.

1.5.2 Specificity

For chromatographic methods, developing a separation involves demonstrating specificity, which is the ability of the method to accurately measure the analyte response in the presence of all sample components. The response of the analyte in the test mixture, i.e. fish tissue, which contains the analyte and all other sample components, was compared to the response of a standard solution containing only the analyte. Additionally the Diode Array Detector (DAD) was used to obtain the UV spectra of the analyte in the tissue sample and these were compared to that of a standard analyte.

1.5.2.1 Specificity using DAD

The absorption maxima in the spectrum of the analyte in the sample matrix should be at the same wavelength as those of the standard analyte within a typically ±2nm margin. The spectrum of the analyte in the sample matrix above 220nm should not be visually different from the spectrum of the standard analyte.

1.5.3 Injection repeatability

In this study, a minimum of five injections from the same sample vial was made to test the performance of the chromatographic instrument. Once the replicate of injections was carried out, the upper and lower 95% confidence limits were set from the following equation.

$$CL^{95\%} = \overline{X} \pm (S.t_{N-1}^{95\%} / \sqrt{N}) \qquad \text{Equation 1.1}$$

CL = confidence interval.

S = standard deviation.

$t_{N-1}^{95\%}$ = t value read from the table with N-1 degrees of freedom and at a 95% confidence interval.

N = number of injections.

Following this evaluation consideration was given to whether peak height or peak area would be used in the remainder of the evaluation study.

1.5.4 Linearity

A linearity study verifies that the sample solutions are in a concentration range where the analyte response is linearly proportional to concentration. It was determined by examining the test results, obtained by analysis of samples with analyte concentrations across the range of the method. This was achieved by the calculation of the regression line by the method of least squares, of test results versus analyte concentrations. The correlation coefficient of the regression data and the variance of this data provided a mathematical measure of linearity. A correlation coefficient of > 0.999 is generally considered an acceptable fit of the data to the regression line, and the y-axis intercept should be ≤2% of the response obtained for the analyte at the target level [18].

The slopes of the regression line over time are compared by a t-test:

$$t_{calc} = \frac{|b_1 - b_2|}{ESD\sqrt{\frac{1}{\sum(x-\bar{x})_1^2} + \frac{1}{\sum(x-\bar{x})_2^2}}} \qquad \text{Equation 1.2}$$

where:

$$ESD = \sqrt{\frac{df(RSD)_1 + df(RSD)_2}{df_1 + df_2}}$$

$$RSD = \sqrt{\text{Mean Square Residual}}$$

df = (n-2) degrees of freedom

b_1 = the calculated slope on Day 1

b_2 = the calculated slope on Day 2

t_{table} = taken at $(n_1-2) + (n_2-2)$ df

If $t_{calc} < t_{table}$ therefore the slopes are not significantly different at a 5% confidence level

1.5.4.1 Response factor plots

An alternative way of evaluating the linear range involved a plot of response factors against concentration.

$$\text{response factor} = \frac{\text{response - y intercept}}{\text{concentration}} \qquad \text{Equation 1.3}$$

If an equivalent response was obtained at each concentration value, the data points would form a straight line with zero slope. This zero or near zero slope of the response factor plot clearly indicates the linear response range. At the completion of linearity studies, the appropriate linear concentration range for the standards and the injection volume should be set for all subsequent studies.

The confidence interval for the true slope of a regression line is given by [19]:

$$t_{calc} = b \pm \frac{t(ESD)}{\sqrt{\Sigma (x - \bar{x})^2}} \qquad \text{Equation 1.4}$$

where

b = calculated slope

t = taken from the t-table at (n-2) degrees of freedom

ESD = √Mean Square Residual

If the confidence interval contains the value zero then the calculated value of the slope is not significantly different from zero at a 5% confidence level.

To compare the mean response factor values obtained on different days of analysis a t-test was used [20]:

$$t_{calc} = \frac{(\bar{x}_1 - \bar{x}_2)}{s} \sqrt{\frac{1}{n_1} + \frac{1}{n_2}} \qquad \text{Equation 1.5}$$

where

$\bar{x}_1$ = the mean response factor on Day 1

$\bar{x}_2$ = the mean response factor on Day 2

$$s^2 = \frac{(n_1 - 1) \times SD_1^2 + (n_2 - 1) \times SD_2^2}{(n_1 - 1) + (n_2 - 1)}$$

SD_1 = standard deviation for the mean response factors obtained on Day 1
SD_2 = standard deviation for the mean response factors obtained on Day 2
n = the number of determinations
t_{table} = calculated at $(n_1-1) + (n_2 - 1)$ df
If $t_{calc} < t_{table}$ therefore the difference between the means is not significant at a 5% level.

1.5.4.2 y-residual plot

A third method was also used to examine the linearity of the method. This involved calculating the y-residuals and estimating whether or not the sum of these residuals was zero [19]. A y-residual, represents the difference between an experimental value of y and the predicted y-value for the same value of x. The predicted y value, $\hat{y}$, was calculated from the regression line of the standard plot. If a linear calibration plot is appropriate, and if the random errors in the y-values were normally distributed, the residuals, $y - \hat{y}$, themselves should be normally distributed about zero. If the sum of the y-residuals is zero, the data points form a linear calibration plot.

1.5.5 Accuracy

Accuracy is defined as the proximity of a measured value to the true value and it is affected by systematic errors. Systematic error or bias remains constant or varies in a predictable way over a series of measurements. This type of error differs from random error in that it cannot be reduced by making multiple measurements. It can only be corrected for if the exact value of the systematic error and hence the correction factor to apply is known. Random errors are decreased by a factor of √N by repeating the analysis N times, whereas the systematic error may only be eliminated by eliminating its cause.

Commission Decision 93/256/EEC defines the measure of accuracy for routine methods, as the difference between the mean value measured for a reference material and its true value, expressed as a percentage of the true value. However, this Commission Decision also states that if no reference material is available, relevant parameters may be evaluated by analysing fortified sample material. For the analytes of interest to this work, no certified reference materials were available and therefore fortified samples were used for validation purposes.

Therefore for this work accuracy was expressed as the percentage recovery by the assay method, of known, spiked amounts of analyte.

In the absence of CRMs fortified samples, while not ideal, when considering a real sample would contain the incurred analyte, is the best method available for accessing a method. In a recent publication by Cooper et al. [21], it was reported that for OTC analysis in cattle kidney, the recoveries for fortified samples effectively mimicked the recoveries obtained for the incurred kidney samples. While this was not proven by administering radio-labelled OTC to the animals it is a good indication that spiking samples is the best method for determining the accuracy of a method when CRMs are not available. The following is a list of accuracy criteria for fortified tissue samples taken from the Codex guidelines [17].

Concentration	Acceptable range (%)
$\leq 1\mu gkg^{-1}$	50 to 120
$\geq 1\mu gkg^{-1} \leq 10\mu gkg^{-1}$	60 to 120
$\geq 10\mu gkg^{-1} \leq 100\mu gkg^{-1}$	70 to 110
$\geq 100\mu gkg^{-1}$	80 to 110

1.5.6 Precision

The precision limits for analytical methods, expressed as a function of concentration for fortified tissue samples are presented below. These values take into consideration the variety of methods, analytes, matrices and species within the terms of reference of the Codex Committee and that are usually applied to a broad-based residue control programme.

Concentration	% Coefficient of Variation
$\leq 1\mu gkg^{-1}$	35
$\geq 1\mu gkg^{-1} \leq 10\mu gkg^{-1}$	30
$\geq 10\mu gkg^{-1} \leq 100\mu gkg^{-1}$	20
$\geq 100\mu gkg^{-1}$	15

1.5.7 Limit of Detection

The limit of detection (LOD) is described as the concentration, which gives an instrument signal significantly different from the blank or background signal. However, in defining "significantly different" it is important to minimise the probability of reporting false negative and false positive results. A commonly used definition of the LOD which is used for this work describes it as a signal: noise (S/N)= 3 [16].

1.5.8 Limit of Quantification

The limit of quantification (LOQ) is described as lower limit for precise and accurate quantitative measurements, as opposed to qualitative detection. For the purposes of this work the LOQ is defined as the S/N = 5.

1.5.9 Range

The range of an analytical method is the concentration interval over which acceptable accuracy, linearity, and precision are obtained. The method range is considered validated if it satisfies the above criteria when applied to samples containing the analyte at the extremes of the range as well as mid range. To find the dynamic range of the method a plot of standard solutions against concentration was prepared. At the higher concentration levels the line deviates from linearity and the point at which this occurs is taken as the upper limit of the dynamic range. The linear range was taken from the response factor plot (Section 1.5.4.1). A line was drawn through the maximum response factor value and parallel to the x-axis. Additionally a line at 95% of this maximum value was drawn parallel to the x-axis. The concentration values which had response factor values within these limits were deemed to be within the linear range of the method.

1.5.10 Stability

For routine testing in which many samples are analysed each day, it is often necessary to allow for delays such as instrument breakdowns or overnight storage of prepared samples. Therefore, the limit of stability of samples and standards should be tested, and storage conditions identified so that the stability can be improved.

In this work, the stability of prepared tissue samples, was tested by taking a calibration plot of spiked tissue samples and analysing them for three consecutive days using a freshly prepared mobile phase on each of the days. The calibration plot, retention time, response and symmetry were observed on each day and examined to determine the acceptable duration between sample preparation and analysis.

1.5.11 Oxolinic acid

Numerous reports have been published on applications of HPLC to the analysis of OA in trout [59, 60, 61], plasma [62, 45], oyster [63], catfish [64] and salmon tissue [60, 65]. Confirmation methods for OA analysis in fish using ion selective GC-MS has also been reported [66, 67, 68, 69]. The most relevant of these are listed in Table 1.3. Fluorescence detection was used for trout [50, 70], salmon [60, 65], plasma [45], and fish silage [71] while UV detection was used for oyster [63], trout [59, 61], and plasma [62]. The LOQ for trout or salmon tissue using UV detectors was quoted as 50μgkg^{-1} [59] and 200μgkg^{-1} [61]. Since no MRL is available for OA in edible fish tissue, it is difficult to know if these limits are acceptable.

For the HPLC methods using either trout, or salmon tissue as the matrix the HPLC columns used included Nucleosil C18 [59], PLRP-S [60], C18 RP-Silica [65, 70] and Pinkerton ISRP column [61]. Oxalic acid [59, 65, 70], phosphoric acid [60] and potassium dihydrogenphosphate [61] buffers were used in the mobile phase while the organic modifiers included acetonitrile (ACN) [61], methanol (MeOH): ACN [59, 65] and ACN: tetrahydrofuran (THF) [60].

Different cleanup techniques have been reported for OA analysis in tissue samples including solid phase extraction (SPE), for oyster [63] trout [59, 61, 70] chicken [70] and liquid-liquid extraction for trout or salmon [60] catfish [64] and salmon [65]. Different extraction solvents were used in each of the above methods including phosphate buffer [63], hexane: ethyl acetate (1:3) [59], potassium dihydrogen phosphate [61], ACN [70], ACN: ammonia (6:1) [60], ethyl acetate [65] and acetone [64]. The same extraction solvent was not used in any two of the methods reported above, however no specific problems were reported with any of the extraction methods. Of the methods which used SPE, two, reported by Bjorklund *et al.* [61] and Pouliquen *et al.* [63], used the same Bond Elut C18 cartridge. The recoveries quoted were 86.3±3.5% (n=6) at 50μgkg^{-1} [63] and 87.8±2.4% (n=4) at 1000μgkg^{-1} [61]. Baker 10 amino cartridges were used by Iwaki *et al.* and recoveries obtained were 76.1±9.8% (n=4) at 100μgkg^{-1} [59]. Rose *et al.* [70] reported as part of a multiresidue method, an anion exchange resin which gave recoveries of 54±6.2% (n=6) at 10μgkg^{-1}.

Apart from the multiresidue method reported by Rose *et al.* [70], the recoveries obtained in the cited literature are all above 70%, which is acceptable for validation purposes (Section 1.5.5).

Recoveries have been reported at 10μgkg^{-1} for liquid-liquid extraction methods, to be 103±5.7% (n=8) by Steffenak *et al.* [60], 84.5±10.9% (n=5) by Carignan *et al.* [65] and 83.5±7.7% (n=7) by Munns *et al.* [64]. Again as for SPE, liquid-liquid extraction methods all quoted percentage recoveries greater than the required 70%.

Chapter 2 contains additional discussion of these methods with a view to choosing a method for examination in this work.

Table 1.3: Summary of reported analytical methods for oxolinic acid analysis

Author Reference	Column	Detector	Mobile Phase	Clean-up	Accuracy (%)	Range (μgkg^{-1})	L.O.Q. (μgkg^{-1})	Matrix
60	PLRP-S 5µm (150 x 4.6)mm	Fluorescence	ACN:THF:0.002M phosphoric acid (21:15:64)	Liquid extraction	104 ± 6.7	50 - 400	50	Rainbow Trout and salmon
59	Nucleosil C18 5µm (7.5 x 4.6)mm	UV	0.01M Oxalic acid : MEOH : ACN (6:1:3)	Baker 10 amino	76.1 ± 9.8	-	50	Rainbow Trout
70	Silica C18 5µm (150 x 4.6)mm	Fluorescence	[(ACN:MeOH) (3:1)]:0.01M oxalic acid (4:6)	SPE AGMO-1	54 ± 6.2	0-100	10	Trout
63	Lichrospher 100 RP-18E (125 x4.6)mm	Variable wavelength	ACN:0.02M Phosphoric acid	Bond Elut C18	78.3 ± 2.4	50 - 2500	40	Oyster
61	Pinkerton ISRP 5µm (150 x 4.6)mm	Variable wavelength.	ACN:0.1M potassium dihydrogenphosphate (10:90)	Bond Elut C18	77.9 ± 2.4	200-2000	200	Rainbow trout

Table 1.3 contd: Summary of reported analytical methods for oxolinic acid analysis

Author Reference	Column	Detector	Mobile Phase	Clean-up	Accuracy (%)	Range (μgkg^{-1})	L.O.Q. (μgkg^{-1})	Matrix
65	C18 RP-Silica 5µm (150 x 4.6)mm	Fluorescence	ACN:MEOH:0.01M Oxalic acid (30:10:60)	Liquid extraction	84.5 ± 10.9	10-100	10	Tissue
64	RP polystyrene divinyl benzene 5µm (150 x 4.6)mm	UV and Fluorescence	Phosphoric acid: ACN:THF (72:6:12)	Liquid extraction	80.8 ± 6.3	5-80	5	Catfish
62	PLRP-S 5µm (150 x 4.6)mm	UV @ 325nm	ACN:THF: 0.002M phosphoric acid. (21:15:64)	Dialysis and trace enrichment	104 ± 3.1	-	50 (S/N=3)[c]	Plasma
45	Hypersil C8 3µm (150 x 4.6)mm	Fluorescence	0.025M oxalic acid:ACN:MEOH:THF (80:2.5:15:2.5) Gradient	Liquid extraction	94 ± 5.6	50-1000	3 (S/N=3)[c]	Plasma
71	Hypersil C8 3µm (150 x 4.6)mm	Florescence	0.025M oxalic acid:ACN:MEOH:THF (80:2.5:15:2.5) Gradient	Liquid extraction	84 ± 6.0	25-200	10 (S/N=4)[c]	Fish silage

c: S/N - signal to noise ratio

1.5.12 Tetracyclines

In the past, Tetracyclines antibiotics have been analysed by liquid chromatography coupled with an electrochemically pre-treated glassy carbon electrode [22] and chemiluminesence [23]. There are also numerous reports of the applications of HPLC to the analysis of tetracycline antibiotics in sediment [24, 25, 26, 27, 28, 29] and tissues [27, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50]. The methods that are most relevant to the work being examined here are summarised in Table 1.4. Three of these methods use fluorescence detection, two use photodiode array (DAD) while fifteen use UV detection. Based on this UV is the most common detection type for tetracycline analysis. A limit of quantification of 10 μgkg^{-1} was obtained when using a fluorescence detector [31] and a UV detector [38, 40, 46]. Thus, both the UV and fluorescence detectors are capable of detecting OTC at levels below the MRL of $100\mu gkg^{-1}$. The UV was chosen as the detector for this project however, as it could be replaced in the future with a DAD detector and thus the identification confirmed using spectral analysis (Section 1.5.2). Column packings reported in the literature include C18 [32, 34, 36, 38, 49, 50, 51, 52, 53, 54 55] and C8 [31, 33, 46]. A great variety of mobile phases has also been reported. The most commonly used are oxalic acid buffers at acid pH [31, 33, 34, 46, 49, 51, 52], followed by phosphate buffers or phosphoric acid at acid pH (<2.6) [36, 38, 48, 50, 53]. The organic modifiers used in the mobile phase have included MeOH [32], ACN [31, 33, 43, 46, 47, 48, 49, 50, 53], MeOH - ACN [34], ACN – THF [38, 51] and ACN – dimethylformamide (DMF) [36, 52]. DMF was reported as a superior organic modifier in the mobile phase when compared to ACN [54].

Norlander *et al.* [36] reported the use of 1M HCl and 50% aqueous TFA for the extraction of OTC from trout muscle, and the recovery obtained at $50\mu gkg^{-1}$ was 53±2% (n=9). These recovery values do not meet the validation criteria (Section 1.5.5), but were improved in 1992 for salmon tissue, when Iwaki *et al.* [32] substituted 50% aqueous TFA with 24% methanolic TFA. The recoveries for concentration levels of $100\text{-}1000\mu gkg^{-1}$ were 73.6-91.9% (RSD 2.7-4.4%) (n=7) [32]. While these recoveries were an improvement on those reported by Norlander *et al.* [36], no information was given for concentration levels lower than the MRL of $100\mu gkg^{-1}$ for salmon tissue.

Aqueous metaphosphoric acid was also used to extract OTC from salmon tissue and Carignan *et al.* [51] reported that the addition of 2ml DCM to this extraction solution improved the percentage recoveries obtained. At 50μgkg^{-1} the reported recovery was 87.9±2.1% (n=10), but the chromatograms showed large matrix peaks indicating that the tissue sample was not sufficiently clean and thus the column life would be reduced. Bjorklund *et al.* [55] reported the use of McIlvaine buffer in conjunction with a Bond Elut C18 cartridge for the extraction and cleanup of trout tissue samples. The reported recovery at 1000μgkg^{-1} was 87.6±3% (n=8) and the chromatograms showed a clean sample with good resolution of OTC. This same method was adopted for salmon muscle in 1990 by Reimer *et al.* [34] and the recovery obtained was 82±5% (n=6) at 200μgkg^{-1}.

While Table 1.4 contains information on methods cited in the literature for OTC analysis, the most relevant of these are discussed further in Chapter 2.

Table 1.4: Summary of reported analytical methods for oxytetracycline analysis

Author Reference	Column	Detector	Mobile Phase	Clean-up	Accuracy (%)	Range (μgkg^{-1})	L.O.Q. (μgkg^{-1})	Matrix
32	Capcell C18 SG-120 5μm (250 x 4.6)mm	Fluorescence	Buffer :MeOH	Liquid extraction	73.6-91.9 ±2.77-4.44	80-10000	100	Salmon muscle
34	LiChroCART RP-18 7μm (250 x 4)mm	Photodiode array	0.01M oxalic acid:ACN:MEOH (73:17:10)	Alltech C18	82±5	200-1000	80	Salmon muscle
51	Ultrasphere ODS 5μm (150 x 4.6)mm	Variable UV	0.025M oxalic acid:ACN:THF (75:22.5:2.5)	Liquid-liquid extraction	87±2.1	50-1000	50	Salmon muscle
33	Chromspher C8 5μm (200 x 3)mm	Fluorescence	ACN, 0.02M oxalic acid:ACN (90:10) Gradient.	Bond elut cyclohexyl.	75	25-2500	30	Muscle
38	Supelcosil LC-18DB 5μm (150 x 4.6)mm	UV @ 357nm	0.005M phosphate buffer:ACN:THF (81:10:9)	Bondesil C8	89±4.2	10-20	10	Fish muscle
31	Chromspher C8 (100 x 3)mm	Fluorescence	0.01M oxalic acid: ACN (78:22)	Carboxylic acid	73±4	-	10	Trout
55	Spheri ODS 5μm (100 x 4.6)mm	UV @ 355nm	ACN:DMF:0.01M oxalic acid. (22:6:72)	Bond elut C18	87.6±3	200-10000	50	Trout muscle
36	ODS Hypersil 5 μm (250 x 4.6)mm	Variable UV	Phosphate buffer:ACN:DMF (810:190:60)	Waters C18	53-66	50-500	50	Trout muscle

Table 1.4 contd. - Summary of reported analytical methods for oxytetracycline analysis

Author Reference	Column	Detector	Mobile Phase	Clean-up	Accuracy (%)	Range ($\mu g kg^{-1}$)	L.O.Q. ($\mu g kg^{-1}$)	Matrix
43	Hypersil SAS 5μm (200 x 4.6)mm	Variable UV	0.1M citric acid: EDTA buffer in ACN (340:500)	XAD2 resin	72±7.9	-	100	Trout muscle
53	Lichrospher 100 RP-18 5μm (125 x 4.6)mm	Variable UV	ACN:0.02M orthophosphoric acid. (24:76).	Bond-Elut C18, 500mg.	79.8	100-1500	100	Shellfish tissue
44	Wako Gel DMS 10H 10μm (250 x 4)mm	UV@ 370nm	ACN:DMF:0.05M ammonium sulphate (19.5:6.5:74)	Amberlite XAD-2	78.5±5.4	-	50	Yellow tail
42	Novapak Phenyl radical-Pak. 4μm (100 x 8)mm	UV@ 355nm	ACN:0.02M oxalic acid:MEOH (15:80:5)	Sep-Pak C18	89±2.6	25-300	50	Bovine muscle
40	Lichrospher RP-8 5μm (125 x 4)mm	UV@ 355nm	ACN:0.05M oxalic acid (14:86)	Millipore C18	70-80	20-500	10	Catfish muscle
41	ODS 5μm (300 x 4)mm	UV@ 365nm	0.02M oxalic acid:ACN:MEOH (70:27.5:2.5)	MSPD[a] with C18 material	78.9±13.3	50-3200	50	Catfish muscle
37	Spherisorb ODS 2 5μm (250 x 4)mm	Diode array	MEOH:ACN:0.01M oxalic acid (20:35:45)	Separcol C18	86.8±5.4	50-1000	50	Porcine muscle
46	Lichrosorb RP8 (10 x 2.1)mm	UV@ 350nm	0.01M oxalic acid:ACN (1:1)	XAD-2	75±7	10 - 200	10	Animal tissue

Table 1.4 contd. - Summary of reported analytical methods for oxytetracycline analysis

Author Reference	Column	Detector	Mobile Phase	Clean-up	Accuracy (%)	Range (μgkg^{-1})	L.O.Q. (μgkg^{-1})	Matrix
47	PLRP-S 5µm (150 x 4.6)mm	UV@ 360nm	0.01M oxalic acid: ACN (75:25)	SPE SDB I	82±5.6	50-200	20	Porcine tissue
48	PLRP-S 5µm (150 x 4.6)mm	UV@ 350nm	Buffer:ACN/ MeOH/buffer	MCAC[b]	101 ± 4	25-100	25	Salmon
49	Lichrosorb RP-18 (200 x 4.6)mm	Photo-diode-array	MeOH:ACN:Oxalic acid (7:7:26)	Sep-Pak RP-18	86.1 ± 6.2	100-1000	100	Meat
50	Lichrospher 100 RP-18E (125 x 4.6)mm	UV@ 355nm	ACN:0.02M phosphoric acid (6:19)	Bond Elut C18	65.4	40-320	8	Blue mussel
56	Nucleosil C18 5µm (250 x 4.6)mm	Variable UV	ACN:0.02M phosphoric acid (24:76)	Liquid-liquid extraction	73	10-1000	10	Milk
57	Lichrospher RP-8 10µm (15 x 4.6)mm	Variable wavelength	MEOH:ACN:0.01M oxalic acid. (2:3:5)	Bond-Elut C18, 500mg	87.5	-	50	Beef liver
58	Lichrospher 100 RP-18 5µm (15 x 4.6)mm	Variable wavelength	ACN:0.02M orthophosphoric acid. (24:76)	Bond-Elut C18, 500mg	90	50-800	40	Sandy mud
24	ODS Hypersil 3µm (100 x 5)mm	Variable wavelength.	ACN:EDTA buffer (14:86)	Liquid extraction	-	-	-	Sediment
25	ODS RP-18 1.5µm (220 x 4.6)mm	Variable wavelength	DMF:ACN:EDTA buffer (25:10:65)	MSPD	98 ±4.5	-	1200	Sediment

a: MSPD - Matrix Solid Phase Dispersion
b: MCAC - Metal Chelate Affinity Chromatography

1.5.13 Sulphonamides and Trimethoprim

At present two sulphonamides (SD, SMX) are used in the Irish fish farming industry for the treatment of bacterial diseases. These sulphonamides are used in conjunction with a potentiator, TMP, and therefore a method for the simultaneous determination of these three chemicals was desirable. To date only one method exists in the literature for the simultaneous determination of TMP and SD in salmon tissue samples and this was reported by Gentleman *et al.* [72] in 1993. The recoveries recorded in this method do not meet the validation criteria, (63% and 42% for SD and TMP respectively) (Section 1.5.5). The LOD was determined to be 100μgkg^{-1}, which is also unacceptable since the MRL for combined sulphonamides is 100μgkg^{-1} and 50μgkg^{-1} for TMP [9]. In 1998, Andrisano *et al.* [73] reported a HPLC method for the simultaneous determination of SD, SMX and TMP standards. This method however did not examine any matrix or sample preparation procedures.

The sulphonamides [72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88] and TMP [72, 73, 89, 90, 91, 92, 93] methods which are most relevant to the work being carried out at present are summarised in Table 1.5.

These chemicals have been analysed by liquid chromatography with UV detection [71, 72, 75, 76, 77, 78, 79, 80, 89, 90, 94, 95, 86], DAD [73, 84, 85, 87, 93, 96], fluorescence [88] and mass spectrometry [74, 92]. Based on this it is apparent that UV is the most common detection type for sulphonamide analysis. In the literature cited for TMP analysis in trout or salmon tissue only the most recently published methods in 1997 [92, 93] meet the required LOQ of <50μgkg^{-1}, (Table 1.5). An acceptable value of 50μgkg^{-1} was quoted for sulphonamides in trout muscle using UV detection [76] and 5μgkg^{-1} in salmon muscle using post column derivatisation and fluorescence detection [88].

There are numerous reports of the applications of HPLC, to the analysis of sulphonamides in kidney [74], trout muscle [76], pork [78, 87, 95, 96], yellowtail [79], and salmon muscle [72, 84, 88, 94]. Column packings used include C18 [72, 73, 74, 76, 79, 84, 87, 88, 94, 96] and C8 [95]. Various mobile phase buffers have been quoted in the literature including ammonium acetate [74, 84, 87], sodium acetate buffers [73, 95, 96], phosphoric acid [76, 78], acetic acid [88] and oxalic acid [79]. ACN was used as the organic modifier in the majority of the methods listed in Table 1.5. Three methods used the combination of MeOH: ACN [74, 87, 88].

Only C18 column packings have been reported for the analysis of TMP in plasma, pharmaceuticals, bovine muscle, and salmon muscle [72, 89, 90, 91, 92, 93]. The mobile phase buffers reported include ammonium acetate [90], sodium acetate [89], phosphate [72, 93], acetic acid [92] and triethylamine [91]. ACN was reported as the organic modifier in each of these methods (Table 1.5).

Different sample cleanup procedures for sulphonamides were reported in the literature for salmon tissue [84, 72, 88, 94], including liquid-liquid extractions and SPE. Samuelsen [94] reported a simple extraction with an ion-pairing agent, followed by a protein precipitation step for ormethoprim (OMP) and sulphadimethoxine (SDM) analysis in salmon tissue. Recoveries of OMP were reported to be 96.3±7.8% (n=6), at 125μgkg^{-1}, while SDM at the same concentration level were reported to be 84.5±5.5% (n=6). The LOD quoted at the Signal to Noise ratio of 3, (S/N=3) was reported to be 50 and 30μgkg^{-1} for OPM and SDM respectively. No recoveries were quoted at concentration levels closer to these values [94].

In 1992, Reimer *et al.* [84] reported a Matrix Solid Phase Dispersion (MSPD), method for the extraction and cleanup of five sulphonamides including SD from salmon tissue. The sulphonamides were eluted from the C18 MSPD material with DCM and the recoveries were 66±3% (n=8), at 400μgkg^{-1} for SD. This recovery value does not meet the validation criteria of >70% recovery for spiked tissue samples. In addition, no recoveries were quoted at concentration levels nearer the MRL of 100μgkg^{-1}, for combined sulphonamides [84].

Gentleman *et al.* [72] extracted SD and TMP from salmon tissue with ACN and used a Waters C18 sep-pak for the cleanup. The recoveries reported at 100μgkg^{-1}, were 63 and 42% for SD and TMP respectively, (n=5). These recoveries do not meet the validation criteria (Section 1.5.5) and the losses were reported to be due to the lipid constituents in the salmon muscle and the resulting chromatographic interferences [72]. These two methods [72, 84], highlight the need for an effective sample cleanup procedure to remove lipids from the tissue matrix.

Gehring *et al.* [88], reported the extraction and cleanup of fourteen sulphonamides including SD and SMX from salmon tissue. Using a mixture of ACN:MeOH:2% aqueous Acetic Acid (5:10:85), the sulphonamides were extracted. The cleanup involved liquid-liquid partitioning with DCM and hexane. Recoveries at 20μgkg^{-1} were reported to be 89.6±3.4% (n=4), for SD and 89±3.7% (n=4), for SMX. This method was validated according to the EU criteria (Section 1.5.5 and 1.5.6) in terms of accuracy and precision. Recoveries were also reported at 5μgkg^{-1}, which is well below the 100μgkg^{-1} MRL for combined sulphonamides.

However, a similar method was reported by Malisch *et al.* [96] which did not involve the use of the DCM liquid-liquid partitioning step. Thirteen sulphonamides including SD and SMX were extracted from tissue with an aqueous buffer followed by liquid-liquid partitioning with ether: hexane and ethyl acetate. Recoveries from the tissue samples were reported to be 100±13% (n=64), at 100μgkg^{-1} for SD, and 100.7±12.2% (n=9) at 100μgkg^{-1}, for SMX. Malisch *et al.* [96] reported that the percentage recoveries obtained were affected by the length of time the homogenised tissue samples are stored, even at -18°C. Once the tissue sample was homogenised, enzymes were released which act on the tissue causing interfering peaks in the chromatograms and thus lower recoveries. More importantly repeated freezing and thawing of samples resulted in chromatographic problems [96].

An inter-laboratory study involving ten laboratories was carried out on this method and recoveries at 100μgkg^{-1}, were reported to be 89.3±13.3% (n=80) for SD. This method was reported to be validated, and met the criteria of routine methods required by the Commissions Decision 87/410/EEC for both SD and SMX in tissue samples.

Relatively few TMP methods have been reported for biological fluids, pharmaceuticals [89, 90, 91], and biological matrixes [72, 92, 93]. In 1997, Cannavan *et al.* [92] reported the extraction of TMP from tissue with chloroform: acetone followed by a hexane extraction and analysis by liquid chromatographic mass spectrometry (LCMS). Recoveries from bovine tissue were reported to be 79±7.2% (n=5) at 25μgkg^{-1}, which is half the MRL in salmon tissue [9]. This method was validated to the EU criteria [16].

Brandsteterova *et al.* [93], extracted TMP from minced meat with McIlvaine buffer followed by a C18 SPE cleanup step and analysis by DAD detection. Recoveries were reported to be 86 ± 5% at 50μgkg^{-1} and the detection limit was quoted at 280nm to be 15μgkg^{-1}.

Further discussion of the most relevant of these methods can be seen in Chapter 2.

Table 1.5: Summary of reported analytical methods for sulphonamide and trimethoprim analysis

Author Reference	Column	**Detector**	**Mobile Phase**	**Clean-up**	**Accuracy (%)**	**Range (μgkg^{-1})**	**L.O.Q. (μgkg^{-1})**	**Matrix**
84	Supelcosil LC18-DB 5 µm (250 x 4.6) mm	Photodiode array	ACN:0.01M ammonium acetate	C18 Silica SPD	66	60-5000	100	Salmon Muscle
72	Ultrasphere ODS ion-pair 5 µm (250 x 4.6)mm	Variable wavelength	ACN: Phosphate buffer (5:95)	Waters C18	63 and 42	100-1000	100	Salmon muscle
88	Symmetry C18 3.5µm (150 x 4.6)mm	Fluorescence	ACN:MeOH:Acetic Acid Gradient	Liquid extraction	84.6 ± 7.7	5-100	5	Salmon
94	ODS Hypersil 3 µm (150 x 4.6)mm	UV@ 270nm	ACN:Ion Pairing Agent (75:25)	Liquid extraction	88.7 ± 3.1	125-1000	125	Salmon Tissue
76	Wakosil C18 5µm (250 x 4.6)mm	UV@ 272nm	ACN:0.02M phosphoric acid (24:76)	Baker amino	74.7-99.1 (0.9-4.8)	50 - 500	50	Rainbow Trout
96	Spherisorb ODS (2) 5µm (250 x 4.6)mm	Photo-diode array	0.02M sodium acetate:(ACN:H2O) Gradient	Liquid extraction	87.9-109.8	50-10,000	48	Pork
87	Spherisorb ODS (2) 5µm (250 x 4.6)mm	Photo-diode array	0.01M ammonium acetate: (ACN:MeOH) gradient	MSPD C18	85.6 ± 18.5	50-250	50	Pork
78	Varian MCH-10 (300 x 4)mm	UV@ 270nm	0.017M H_3PO_4:ACN (70:30)	MSPD	95.1 ± 15.1	62.8 – 2000	62.5	Pork

Table 1.5: contd. Literature summary for analytical methods for sulphonamide and trimethoprim analysis

Author Reference	Column	Detector	Mobile Phase	Clean-up	Accuracy (%)	Range (μgkg^{-1})	L.O.Q. (μgkg^{-1})	Matrix
95	Li-Chrosorb RP-8 10μm (250 x 4.6) mm.	UV	Sodium Acetate :ACN (82.5:17.5)	XAD-2, XAD-4	85-90 ± 4-10	5-20	5	Pork
80	Novapak 4 μm (100 x 8)mm	UV 190-367nm	0.01M Sodium acetate: ACN Gradient	C18 Silica	77 ± 22	50-100	50	Calf Muscle
86	Spher C8 CP9 8 μm (250 x 4)mm	UV@ 254nm	ACN: Ammonium acetate buffer	Ion Exchange	82	50-500	50	Swine Tissue
79	Intersil ODS 5μm (150 x 4.6)mm	UV@ 265nm	0.005M oxalic acid:ACN (55:45)	Bond Elut C18	88.7 ± 3.7	100-2000	100	Yellow-tail
73	Phenosphere C18 5μm (250 x 4.6)mm	UV@ 254nm	Buffer (pH=6.5) : ACN (80:20)	-	-	-	-	Standard
74	C18 Hypersil, Novapak C18 (150 x 4.6)mm	Mass Spec. Thermospray	Ammonium acetate:MEOH: ACN (85:7:8)	Bond Elut NH_2 and SCX	70.9 ± 14.7	-	-	Kidney
75	Zorbax SB-C8 3.5μm (80 x 4.6)mm	UV @ 267nm and 220nm.	0.1%TFA:ACN pH = 3 (84:16)	Liquid extraction	-	-	-	Plasma
77	Varian MCH-10 10μm (300 x 4)mm	UV@ 270nm	0.05M H_3PO_4:ACN (70:30)	Bulk C18 silica	99.62 ± 5.31	62.5-2000	62.5	Infant formula

Table 1.5: contd. Literature summary of analytical methods for sulphonamide and trimethoprim analysis

Author Reference	Column	Detector	Mobile Phase	Clean-up	Accuracy (%)	Range (μgkg^{-1})	L.O.Q. (μgkg^{-1})	Matrix
85	Spherisorb S5 C8 5µm (250 x 5)mm	Photodiode array	Sodium ethanoate: ethane nitrile	GPC[d]	104.9 ± 4.7	1-100 ppm	1000	Animal feed
72	Ultrasphere ODS ion-pair 5 µm (250 x 4.6)mm	Variable wavelength	ACN: Phosphate buffer (5:95)	Waters C18	63 and 42	100-1000	100	Salmon muscle
92	Partisil 5 ODS 3µm (250 x 4.6)mm	MS	ACN:H_2O:Acetic acid (25:73.7:1.3)	Liquid extraction	79 ± 7.2	4-400	25	Bovine muscle
93	Nova Pak C18 (150 x 3.9)mm	DAD @ 280nm	ACN:5mM phosphate buffer (1:7) with 5mM acid	SPE C18	86 ± 5	15-100	15	Beef Pork Poultry
89	Bondapack C18 150mm	UV@ 254nm	ACN:0.01M sodium acetate (30:70)	Bond Elut C18	82	4 - 40	4	Plasma
90	Partisil C18 10µm (250 x 4.6)mm	UV@ 254nm	ACN:1% ammonium acetate (25:75)	Liquid extraction	99.4 ± 0.87	1-5	2	Tablets
91	Ultracarb ODS 5µm (150 x 4.6)mm	UV@ 254nm	H_2O:0.1% TEA in ACN (80:20)	Liquid extraction	95 ± 5	10-100	20	Plasma

d: GPC – gel permeation chromatography

1.6 References

[1] Marine Institute. (1996). Towards a Marine Policy for Ireland. Proceedings of the Consultative Process. ISBN 0 9529191 0 9.

[2] Bord Iascaigh Mhara (1997). Annual Report. Irish Sea Fisheries Board, Crofton Road, Dun Laoghaire, Co. Dublin, Ireland.

[3] Alderman DJ, Rosenthal H, Smith P, Stewart J, Weston D. (1994). Chemicals used in mariculture. ICES Cooperative Research Report no. 202. ISSN 1017-6195.

[4] Alderman DJ, Michel CM. (1992). Chemotherapy in aquculture today. In Alderman DJ, and Michel CM. Problems of chemotherapy in aquaculture: from theory to reality. Working papers by the Office International des Epizooties, 23-44.

[5] Jackson D, and Minchin D. (1993). Sea lice infestation of farmed salmon in Ireland. Aquaculture and Fisheries Management, **24**, 179-187.

[6] Alderman DJ. (1988). Fisheries chemotherapy, In Roberts RJ. Recent advances in Aquaculture, 3, Croom Helm., London, 1-16.

[7] Gutsell JS. (1948). The value of certain drugs, especially sulfa drugs, in the treatment of furunculosis in brook trout (*Salvelinus fontinalis*). Transactions. of the American Fisheries. Society, **75**, 186-199.

[8] Bacterial Disease Control, Antibiotics and the environment in marine finfish culture: A Review. (1993). A technical report to the marine working group, Scottish Wildlife and Countryside Link.

[9] Commission Regulation (EC) No. 508/1999 of 4 March 1999 amending Annexes I to IV of Council Regulation (EEC) No 2377/90 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin.

[10] Extoxnet: Extension toxicology network from a pesticide information project of cooperative extension offices of Cornell University, Michigan State University, Oregon State University and University of California. Http://ace.orst.edu/cgi-bin/mfs/01/pips/abamect.p541297mfs.

[11] Smith P. Fish farming and our environment. (1998). An international conference. Fish Disease Group National University of Ireland Galway, 178.

[12] EMEA (1998). The European Agency for the Evaluation of Medicinal Products, Veterinary Medicines Evaluation Unit. Committee for veterinary medicinal products, Ivermectin (extension to deer). Summary report (4). EMEA/MRL/487/98-Final.

[13] GESAMP Reports and studies No. 65. (1997). Towards safe and effective use of chemicals in coastal aquaculture. IMO/FAO/UNESCO/IOC/WMO/ WHO/IAEA/UN/UNEP. Joint Group of Experts on Scientific Aspects of Marine Environmental Protection, ISSN 1020-4873, ISBN 92-5-104031-1.

[14] EMEA (1998). The European Agency for the Evaluation of Medicinal Products, Veterinary Medicines Evaluation Unit. Committee for veterinary medicinal products, Cypermethrin (extension to salmonidae). Summary report. EMEA/MRL/436/98-Final.

[15] Council Directive 96/23/EC on measures to monitor certain substances and residues thereof in live animals and animal products.

[16] Annex of Commission Decision 93/256/EEC laying down the methods to be used for detecting residues of substances having a hormonal or thyrostatic action.

[17] Codex Alimentarius, Volume 3, (1994). Section 2: Recommended international code of practice for control of the use of veterinary drugs.

[18] Green JM. (1996). A practical guide to analytical method validation. Analytical Chemistry News and Features, 305-309.

[19] Calcutt R, Boody R. (1993). Statistics for analytical Chemists. 1st edition. Chapman and Hall, ISBN 0 412 23730 X.

[20] Miller JC, Miller JN. (1993). Statistics for analytical Chemistry. 3rd edition. Ellis Horwood PTR Prentice Hall Limited, ISBN 0-13-030990-7.

[21] Cooper AD, Tarbin JA, Farrington WHH, Shearer G. (1998). Effects of extraction and spiking procedures on the determination of incurred residues of oxytetracycline in cattle kidney. Food Additives and Contaminants, **15**, No.6, 645-650.

[22] Hou W, Wang E. (1989). Liquid chromatographic determination of tetracycline antibiotics at an electrochemically pre-treated glassy carbon electrode. Analyst, **114,** 699-702.

[23] Zhang XR, Baeyens WRG, Van den Borre A, Van der Weken G. (1995). Chemiluminescence determination of tetracycline based on their reaction with hydrogen peroxide catalysed by the copper ion. Analyst, **120,** 463-466.

[24] Samuelsen OB. (1992). The fate of antibiotics/chemotherapeutics in marine aquaculture sediments. Chemotherapy in aquaculture: from theory to reality O.I.E., Paris, 162-173.

[25] Smith P, Coyne R, Hiney M, O'Connor B, Kerry J, Cazabon D. (1994). Concentration and persistance of oxytetracycline in sediments under a marine salmon farm. Aquaculture, **123,** 31-42.

[26] Bjorklund H, Bondestam J, Bylund G. (1990). Residues of oxytetracycline in wild fish and sediments from fish farms. Aquaculture, **86,** 359-367.

[27] Bjorklund H, Rabergh CMI, Bylund G. (1991). Residues of oxolinic acid and oxytetracycline in fish and sediments from fish farms. Aquaculture, **97,** 85-96.

[28] Smith P. (1996). Is sediment deposition the dominant fate of oxytetracycline used in marine salmonid farms: a review of available evidence. Aquaculture, **146,** 157-169.

[29] Samuelsen O, Torsvik V, Ervik A. (1992). Long-range changes in oxytetracycline concentration and bacterial resistance towards oxytetracycline in a fish farm sediment after medication. The science of the total environment, **114,** 25-36.

[30] Jacobsen MD. (1989). Withdrawal times of fresh rainbow trout, *Salmo gairdneri* Richardson, after treatment with oxolinic acid , oxytetracycline and trimethoprim. Journal of fish diseases, **12,** 29-36.

[31] Haagsma N, Scherpenisse P. (1993). High performance liquid chromatographic determination of tetracyclines in animal tissue and eggs. Presented at Euroresidues II Veldhoven, NL, 3.5 May 1993.

[32] Iwaki K, Okumura N, Yamazaki M. (1992). Determination of tetracycline antibiotics by reversed phased liquid chromotagraphy with fluoresence detection. Journal of chromatography, **623,** 153-158.

[33] McCracken R, Blanchflower J, Haagen S, Glenn D. (1995). Simultaneous determination of oxytetracycline, teracycline and chlortetracycline in animal tissues using liquid chromatography, post column derivatisation with aluminium and fluoresence detection. Analyst, **120,** 1763-1766.

[34] Reimer G, Young L. (1990). Validation of a method for determination of tetracycline antibiotics in salmon muscle tissue. Journal of the Association of Official Analytical Chemists, **73,** No. 5, 813-817.

[35] Blanchflower J, McCracken R, Rice D. (1989). Determination of chlortetracycline residues in tissues using high performance liquid chromatography with fluoresence detection. Analyst, **114,** 421-423.

[36] Nordlander I, Johnsson H, Osterdahl, Bengtgoran. (1987). Oxytetracycline residues in rainbow trout analysed by a rapid HPLC method. Food additives and contaminants, **4,** No. 3, 291-296.

[37] Sokol J, Matisova E. (1994). Determination of tetracycline antibiotics in animal tissues of food-producing animals by high performance liquid chromatography using solid phase extraction. Journal of chromatography A, **669**, 75-80.

[38] Rogstad A, Hormazabal V, Yndestad M. (1988). Optimization of solid phase extraction of oxytetracycline from fish tissue and its determination by HPLC. Journal of liquid chromatography, **11,** (11), 2337-2347.

[39] Luzana U, Serrini G, Maria V, Moretti V, Luca G, Valfre F. (1994). Effect of temperature and diet composition on residue depletion of oxytetracycline in cultured channel catfish. Analyst, **119,** 2757-2759.

[40] Moretti V, Luzana U, Serrini G, Maria V, Luca G,Valfre F, Albertini A, Bellagamba F. (1994). High performance liquid chromatographic determination of oxytetracycline in channel catfish *(Ictalurus punctatus)* muscle tissue. Analyst, **119,** 2749-2751.

[41] Long A, Hsieh L, Malbrough M, Short C, Barker S. (1990). Matrix solid phase dispersion isolation and liquid chromatographic determination of oxytetracycline in catfish (*Ictalurus punctatus*) muscle tissue. Journal of the Association of Official Analytical Chemists, **73,** No. 6, 864-867.

[42] Mulders E, Van De Lagemaat D. (1989). Determination of residues of tetracycline antibiotics in aminal tissues by high-performance liquid chromatography. Journal of Pharmaceutical and Biomedical Analysis, **7,** No. 12, 1629-1835.

[43] Murray J, McGill A, Hardy R. (1988). Development of a method for the determination of oxytetracycline in trout. Food additives and contaminants, **5**(1), 77-83.

[44] Onji Y, Uno M, Tanigawa K. (1984). Liquid chromatographic determination of tetracycline residues in meat and fish. Journal of the Association of Official Chemists, **67,** No. 6, 1135-1137.

[45] Samuelsen OB. (1990). Simple and rapid method for the determination of flumequine and oxolinic acid in salmon (*Salmo salar*) plasma by high-performance liquid chromatography and fluoresence detection. Journal of Chromatograohy, **530,** 452-457.

[46] Heitzman RJ. (1994). Determination by HPLC of tetracyclines at residue levels in animal tissues. Commission of the European Communities, veterinary drug residues. Residues in food producing animals and their products: Reference materials and methods. Blackwell Science, Second edition. EUR 15127-EN.

[47] Posyniak A, Zmudzki J, Semeniuk S, Niedzielska J, Ellis R. (1998). Determination of tetracycline residues in animal tissues by liquid chromatography. Biomedical Chromatography, **12** (5) 294-299.

[48] Cooper AD, Stubbings GWF, Kelly M, Tarbin JA, Farrington WHH, Shearer G. (1998). Improved method for the online metal chelate affinity chromatography – high-performance liquid chromatographic determination of tetracycline antibiotics in animal products. Journal of Chromatography, A, **812**(1-2), 321-326.

[49] Brandsteterova E, Kubalec P, Bovanova L, Simko P, Bednarikova A, Machackova L. (1997). SPE and MSPD as pre-separation techniques for HPLC of tetracyclines in meat, milk and cheese. Zeitschrift fuer Lebensmittel Untersuchung und Forschung, A. Food Research and Technology, **205**(4), 311-315.

[50] Pouliquen H, Gouelo D, Larhantec M, Pilet N, Pinault L. (1997). Rapid and simple determination of oxolinic acid and oxytetracycline in the shell of the blue mussel (*Mytilus edulis*) by high-performance liquid chromatography. Journal of Chromatography, B:Biomedical Applications, **702**(1-2), 157-162.

[51] Carignan G, Carrier K, Sved S. (1993). Assay of oxytetracycline residues in salmon muscle be liquid chromatography with ultraviolet detection. Journal of AOAC international, **76,** No. 2, 325-328.

[52] Bjorklund H. (1991). Oxytetracycline and oxolinic acid as antibacterials in aquaculture: analysis, pharmacokinetics and environmental impacts. Åbo Akademis fórlag, **5**, No 6, ISBN 951-9498-91-5, ISSN 0001-5105.

[53] Pouliquen H, Keita D, and Pinault L. (1992). Determination of oxytetracycline in marine shellfish (*Crassostrea gigas, Ruditapes philippinarum* and *Scrobicultaria plana*) by high-performance liquid chromatography using solid-phase extraction. Journal of Chromatography, **627**, 287-293.

[54] Knox J, Jurand J. (1979). Mechanism of reversed-phase seperation of tetracyclines by high-performance liquid chromatography. Journal of Chromatography, **186**, 763-782.

[55] Bjorklund H. (1988). Determination of oxytetracycline in fish by high performance liquid chromatography. Journal of Chromatography, **432,** 381-387.

[56] Fletouris D, Psomas J, Botsoglou N. (1990). Trace analysis of oxytetracycline and tetracycline in milk by high performance liquid chromatography. Journal of Agriculture and Food Chemistry, **38,** 1913-1917.

[57] Oka, Hisao, Matsumoto, Hiroshi, and Uno, Keiichi. (1985). Improvement of chemical analysis of antibiotics. VIII. Application of prepacked C18 Cartridge for the analysis of tetracycline residues in animal liver. Journal of Chromatography, **325,** 265-274.

[58] Pouliquen H, Le Bris H, Pinault L. (1994). HPLC Determination of oxolinic acid and oxytetracycline in three types of marine sediments: analytical validation. Quimica Analytica, **13**, Supplement 1, S109-S113.

[59] Ikai Y, Oka H, Kawamura N, Yamada M, Nakazawa H. (1989). Improvement of chemical analysis of antibiotics. XV1. Simple and rapid determination of residual pyridone-carboxylic acid antibacterials in fish using a prepacked amino cartridge. Journal of Chromatography, **477**, 397-406.

[60] Steffenak I, Hormazabel V, Yndestad M. (1991). Rapid assay for the Simultaneous determination of residues of oxolinic acid and flumequine in fish tissues by high-performance liquid chromatography. Journal of liquid chromatography, **14**(1), 61-70.

[61] Bjorklund H. (1990). Analysis of oxolinic acid in fish by high-performance liquid chromatography. Journal of Chromatography. Biomedical Applications, **530**, 75-82.

[62] Agasoster T, Rasmussen K. (1991). Automated analysis of oxolinic acid and flumequine in salmon whole blood and plasma using dialysis combined with trace enrichment as on-line sample preparation for high-performance liquid chromatography. Journal of Chromatography, **564**, 171-179.

[63] Pouliquen H, Pinault L, Le Bris H. (1994). Determination of oxolinic acid in seawater, marine sediment, and Japanese oyster (*Crassostrea Gigas*) by high-performance liquid chromatography. Journal of liquid chromatography, **17**(4), 929-945.

[64] Munns R, Turnipseed S, Peenning A, Roybal E, Holland D, Long A, Plakas S. (1998). Liquid chromatographic determination of flumequine, nalidixic acid, oxolinic acid and piromidic acid residues in catfish (*Ictalurus punctatus*). Journal of AOAC International, **81**, No 4, 825-839.

[65] Carignan G, Larocque L, Sved S. (1991). Assay of Oxolinic acid residues in salmon muscle by liquid chromatography with fluorescence detection: Interlaboratory study. Journal of the Association of Official Analytical Chemists, **74**, No 6, .906-909.

[66] Takatsuki K. (1991). Gas chromatographic-mass spectrometric determination of oxolinic acid in fish using selected ion monitoring. Journal of Chromatography, **538** 259-267.

[67] Takatsuki K. (1992). Gas chromatographic-mass spectrometric determination of oxolinic, nalidixic, and piromidic acid in fish. Journal of AOAC International **75**, No 6, 982-987.

[68] Horie M, Saito K, Nose N, Tera M, Nakazawa H. (1993). Confirmation of residual oxolinic acid, nalidixic acid and piromidic acid in fish by thermospray liquid chromatography-mass spectrometry. Journal of Chromatography, **16**(7), 1463-1472.

[69] Alvarez EJ, Vartanian VH, Brodbelt JS. (1997). Metal complexation reactions of quinolone antibiotics in a quadrupole ion trap. Analytical Chemistry, **69**(6), 1147-1155.

[70] Rose MD, Bygrave J, Stubbings WF. (1998). Extension of mult-residue methodology to include the determination of quinolones in food. The Analyst. **123,** 2789-2796.

[71] Samuelsen OB. (1994). High-performance liquid chromatographic determination of oxolinic acid in fish silage. Journal of Chromatography B, **655**, 311-314.

[72] Gentleman M, Burt H. (1993). High-performance liquid chromatographic determination of sulphadiazine and trimethoprim in Chinook salmon muscle tissue. Journal of Chromatography, **633**, 105-110.

[73] Andrisano V, Makamba H, Bovina E, Cavrini V, Zappoli S. (1998). HPLC Analysis of basic organic compounds in a multi-component ion-interaction system: a mechanistic study. Chromatographia **47**, No.9/10, 493-500.

[74] Porter S. (1994). Confirmation of sulphonamide residues in kidney tissue by Liquid Chromatography-Mass spectrometry. Analyst, **119**, 2793-2756.

[75] Kirkland KM., McCombs DA, Kirkland JJ. (1994). Rapid, high-resolution high-performance liquid chromatographic analysis of antibiotics. Journal of Chromatography A, **660,** 327-337.

[76] Ikai Y, Oka H, Kawamura N, Hayakawa J, Yamada M. (1991). Application of an amino cartridge to the determination of residual sulphonamide antibacterials in meat, fish and egg. Journal of Chromatography, **541,** 393-400.

[77] Long A, Hsieh L, Malbrough M, Short C, Barker S. (1989). A Multiresidue method for the isolation and liquid chromatographic determination of seven sulphonamides in infant formula. Journal of Liquid Chromatography, **12**(9), 1601-1612.

[78] Long A, Hsieh L, Malbrough M, Short C, Barker S. (1990). Multiresidue method for the determination of sulphonamides in pork tissue. Journal of Agricultural and Food Chemistry, **38**, 423-426.

[79] Horie M, Saito K, Hoshino Y, Nose N. (1991). Simultaneous determination of residual synthetic antibacterials in fish by high-performance liquid chromatography. Journal of Liquid Chromatography, **22,** 865-872.

[80] Walker LV, Walsh JR, Webber JJ. (1992). High-performance liquid chromatography of sulphonamides extracted from bovine and porcine muscle by solid-phase dispersion. Journal of Chromatography, **595**, 179-184.

[81] Samuelsen OB, Lunestad BT, Ervik A, Fjelde S. (1994). Stability of antibacterial agents in an artificial marine aquaculture sediment studied under laboratory conditions. Aquaculture **126**, 283-290.

[82] Agarwal V. (1992) A Review. High-performance liquid chromatographic methods for the determination of sulphonamides in tissue, milk and eggs. Journal of Chromatography, **624**, 411-423.

[83] Guggisberg D, Mosser AE, Koch H. (1992) A Review. Methods for the determination of sulphonamides in meat. Journal of Chromatography, **624**, 425-437.

[84] Reimer G, Suarez A. (1992). Liquid chromatographic confirmatory method for five sulphonamides in salmon muscle tissue by matrix solid phase dispersion. Journal of AOAC International, **75,** No 6, 979-981.

[85] Biancotto G, Angeletti R, Piro R. (1996). Detection of veterinary drugs in foodstuffs using gel permeation. Analyst, **121,** 229-232.

[86] Haagsma N, Van De Water C. (1985). Rapid determination of five sulphonamides in swine tissue by high performance liquid chromatography. Journal of Chromatography, **333,** 256-261.

[87] Le Boulaire S, Bauduret JC, François A. (1997). Veterinary drug residues survey in meat: an HPLC method with a matrix solid phase dispersion extraction. Journal of Agricultural and Food Chemistry, **45,** 2134-2142.

[88] Gehring TA, Rushing LG, Thompson HC. (1997). Determination of sulfonamides in edible salmon tissue by liquid chromatography with postcolumn derivatization and fluorescence detection. Journal of AOAC International. **80,** No 4, 751-755.

[89] Svirbely, J. Pesce, A. (1988). Trimethoprim analysis by LC. Journal of liquid chromatography, **11**(5), 1075-1085

[90] Bergh J, Breytenbach J. (1990). High-performance liquid chromatographic analysis of trimethoprim in the pressence of its degradation products. Journal of Chromatography, **513,** 392-396.

[91] Weinfeld R, Macasieb T. (1979). Determination of trimethoprim in biological fluids by high-performance liquid chromatography. Journal of Chromatography, **164**, 73-84.

[92] Cannavan A, Hewitt SA, Floyd SD, Kennedy DG. (1997). Determination of trimethoprim in tissues using liquid chromatography-thermospray mass spectrometry. Analyst **122**(11), 1379-1381.

[93] Brandsteterova E, Kubalec P, Machackova L. (1997). HPLC determination of trimethoprim in meat and milk with an SPE preseparation procedure. Zeitschrift fuer Lebensmittel Untersuchung und Forschung, A. Food Research and Technology, **204**(5), 341-344.

[94] Samuelsen OB. (1994). Simultaneous determination of ormethoprim and sulphadimethoxine in plasma and muscle of Atlantic salmon (*Salmo salar*). Journal of Chromatography B, **660**, 412-417.

[95] Aerts MM, Beek WM. (1988). Monitoring of veterinary drug residues by a combination of continuous flow techniques and column-switching High-Performance Liquid Chromatography. Journal of Chromatography, **435**,97-112.

[96] Malisch R, Bourgeois B, and Lippold R. (1992). Multiresidue analysis of selected chemotherapeutics and antiparasitics. Part 1: Determination of Sulphonamides and their N4-Acetyl-metabolites, chloramphenicol and nicarbazin in meat. Deutsche Lebensmittel-Rundschau, **88**, Jahrg, Heft 7, 205-216.

CHAPTER 2: PROPOSED ANALYTICAL METHODS FOR VETERINARY DRUG RESIDUES

The first step in developing a method of analysis was to establish the degree of resolution, which was required, and the number of components, which need to be resolved. It was also important to know whether quantitative, as well as qualitative data, was to be obtained from the method.

Under Directive 96/23/EC the analytes of interest must be qualitatively and quantitatively analysed in fish tissue samples, and thus resolution was required between the analytes and the matrix components of the tissue sample.

The next step in developing a method was to evaluate the properties of the analyte of interest, such as the chemical structure, the molecular weight and the chemical and physical properties of the components. The structures of the analytes, (oxytetracycline (OTC), oxolinic acid (OA), sulphadiazine, (SD), sulphamethoxazole (SMX), and trimethoprim (TMP)), for which a method of analysis was required can be seen in Figure 1.1 to Figure 1.3.

Once the analyte had been considered in terms of its structure and molecular weight, the question of solubility needed to be addressed. The solubility characteristics of the analytes are important since they must be in solution so that accurate volumes can be injected. In selecting the column packing type to be used for liquid chromatography (LC), consideration of the solubility of the analyte in various solvents to be used is also essential. Table 2.1 shows the molecular weight and the most suitable solvent for each analyte to be analysed.

Table 2.1: Molecular weight and most suitable solvent for the analytes of interest

Analyte	Molecular weight	Most suitable solvent
Oxytetracycline (OTC)	460.44	Methanol
Oxolinic acid (OA)	261.24	Methanol
Sulphadiazine (SD)	250.28	Acetonitrile
Sulphamethoxazole (SMX)	253.31	Acetonitrile
Trimethoprim (TMP)	290.32	Acetonitrile

The similarity in the structures of SD and SMX suggests that both analytes can be analysed by the same or similar analysis method providing adequate resolution can be achieved. TMP, which is used to treat fish in conjunction with either SD or SMX, also has similar properties to both SD and SMX. Thus a method to analyse all three analytes simultaneously was investigated in this work.

While microbiological assays are suited to screening for chemicals and groups of chemicals, identification and confirmation methods are required by other chemical techniques such as chromatography [1].

The preferred method of chromatographic analysis reported in the literature for the analytes of interest is High Performance Liquid Chromatography (HPLC), (Tables 1.3 to 1.5). In contrast to the microbiological methods the liquid chromatographic methods are relatively complex but they do have the potential to identify in a specific manner the antibiotics present with adequate sensitivity and accuracy.

2.1 High Performance Liquid Chromatography

In order to determine the most suitable mode of LC for the analytes of interest, consideration must be given to the analyte structure, molecular weight and solubility. The information in Table 2.1 and the analyte structures (Figure 1.1 to 1.3) were used as the basis for selecting the mode of LC to be used. The molecular weight of all the analytes listed, is less than 2000 and each is soluble in methanol (MeOH) or acetonitrile (ACN). Thus the decision tree, Figure 2.1, indicates that reversed phase high performance liquid chromatography (RPHPLC) is the most appropriate separation technique for the five analytes.

2.1.1 Reverse Phase High Performance Liquid Chromatography (RPHPLC)

The stationary bed in *reversed-phase chromatography* is non-polar (hydrophobic) in nature, while the mobile phase is a polar liquid, such as mixtures of water and MeOH or ACN. In this type of chromatography the more non-polar component will be retained longer on the stationary phase.

When an analyte is first injected onto the LC column, it forms a narrow band at the head of the column. If the equilibrium distributions between the mobile and stationary phases of the analytes present differ, the chromatography will result in a different rate of migration for each analyte. As the mobile phase passes through the column, the initial band separates into individual analyte bands, each of which migrates at a rate governed by the equilibrium distribution of analyte between the mobile phase and the stationary phase. The segmentation of the analytes should be completed before they reach the end of the column and pass as individual bands through the detector cell.

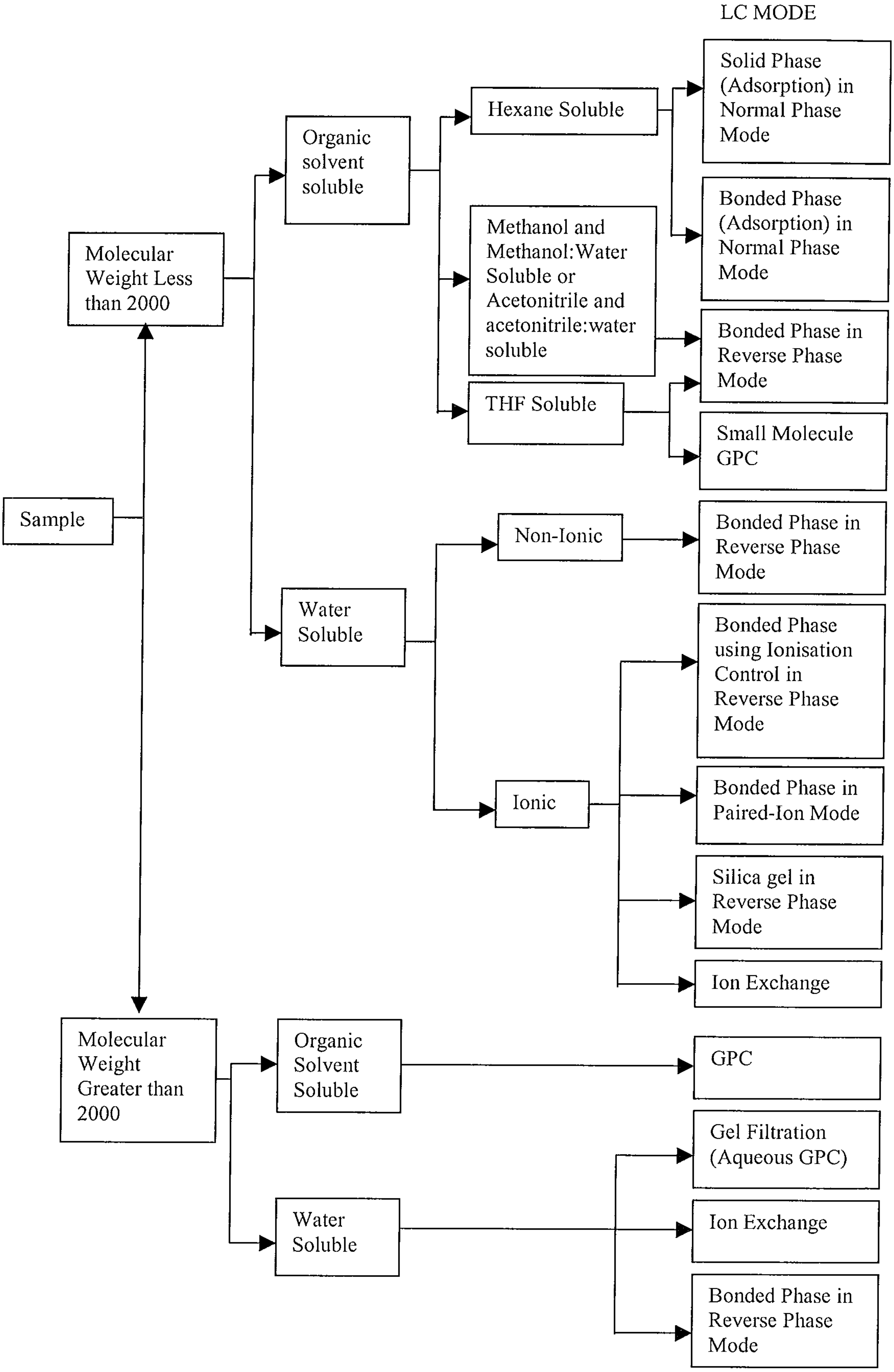


Figure 2.1 Decision tree guide for choosing an LC mode [4]

2.1.2 The separation

In order to obtain an effective separation the following conditions must be met:

- Analyte components must be partially retained on the column
- The analyte components must be segmented from one another
- The analyte components must have relatively narrow bandspreading i.e. sharp peaks
- Analytes must be separated from the matrix components.

Without these essential conditions resulting from the chemical and physical interactions, the separation will not occur within a reasonable time.

The equation, which defines retention for a chromatographic process, is

$$V_R = V_m + KV_S \qquad \text{Equation 2.1}$$

where V_R is retention volume, V_m is mobile phase volume, V_S is the stationary phase volume and K is the equilibrium constant for the concentration distribution between the two phases. [K = (analyte concentration dissolved in the stationary phase)/ (analyte concentration dissolved in the mobile phase)].

2.1.2.1 Capacity factor (k')

The most common factor used to describe retention is the capacity factor, k'. This is a measure of the interaction of an analyte component with a specific stationary phase and solvent combination. The k' term is a measure of the solvent volume required to elute a component (V_1), from the column expressed as multiples of the column void volume (V_0).

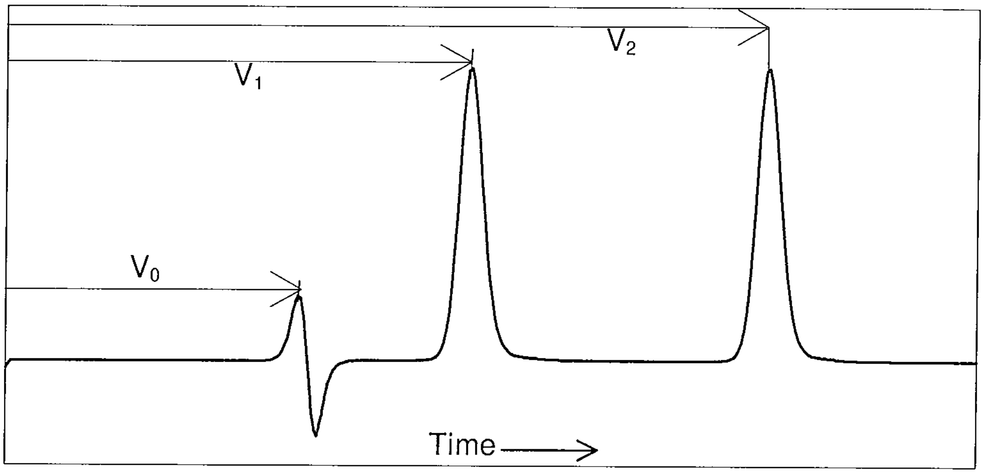


Figure 2.2: Definition of the capacity factor k'. For the first peak, $k'_1 = (V_1 - V_0)/V_0$. For the second peak $k'_2 = (V_2 - V_0)/V_0$.

$$k_1' = \frac{V_1 - V_0}{V_0} \qquad \text{Equation 2.2}$$

To increase retention the analyte must be in an environment where it is preferentially adsorbed by the stationary phase. This is achieved by changing the polarity of the mobile phase. However, too much retention extends analysis time and therefore the optimum value of k' for the best separation is in the range of 2-10.

The capacity factor is influenced by the

- strength of the mobile phase (polarity, hydrogen bonding etc.)
- nature of the stationary phase (surface area, amount of stationary phase and functionality)
- temperature

2.1.2.2 Selectivity

The selectivity of the column is defined as the ability to discriminate between the two components and selectively retard them. When k' values have been determined for each component in the mixture the selectivity, α, can be calculated, as shown in Figure 2.2.

$$\alpha = \frac{k'_2}{k'_1} \qquad \text{Equation 2.3}$$

If the selectivity factor between two components is one, then no separation has been achieved as the peaks coincide. If α = 1.3, the column selectively retards one component 30% more than the other. The larger the α value, the easier the separation is to achieve but a value of 1.1 to 1.4 is typically desired.

Selectivity can be influenced by the

- chemistry of the mobile phase (polarity, hydrogen bonding etc.)
- chemistry of the stationary phase (functionality)
- chemistry of the analyte (e.g. derivatization).

2.1.2.3 Efficiency

The efficiency of the column is a measure of column performance reflecting the amount of peak spreading (dilution) that occurs as the separation takes place. Band broadening is detrimental to the resolution in the separation. This can be reduced by using a column with a higher efficiency. Martin and Synge (Biochem. J., 35, 91, pp1358 (1941)) [2], assumed that a column could be divided into a number of equal sections where an equilibrium distribution of the analytes occurred between the two phases. These equilibrium stages are called theoretical plates. Therefore the separation was modelled like an extraction process, to occur when the mobile phase is transferred to the next stage (or plate), where a new equilibrium is established. For a chromatographic column the number of theoretical plates, N, can be calculated as follows:

$$N = 16(\frac{V_R}{W})^2 \qquad \text{Equation 2.4}$$

where W is the bandwidth.

The efficiency of a packed column is higher when the porous particles are small in size (less than 10 µm). This is due to the shorter distances the analyte molecules must diffuse within the packed bed.

Efficiency can be influenced by the following parameters:

- Flow rate
- Column length
- Average particle size of the packing
- Particle size distribution
- Volume of injection
- Viscosity of injected solution
- Viscosity of the mobile phase (mass transfer term)
- Temperature
- Diameter of the column
- Shape of the particle

2.1.2.4 Resolution

Resolution is a measure of how well two components have been separated. Resolution, R_S, takes into account the separation at peak maxima and the width of the peaks. Components with R_S equal to 1.5 are baseline separated. Resolution results from the physical and chemical interactions that occurs as the analyte passes through the column and therefore can be expressed in terms of the contribution of the individual column characteristics: separation factor (selectivity, α), efficiency (narrowness of peak, N), and capacity factor (k') of the component. The equation, which describes this relationship, is:

$$R = \frac{1}{4}\frac{(\alpha - 1)}{\alpha}\sqrt{N}(\frac{k'}{k'+1}) \qquad \text{Equation 2.5}$$

Of the three factors governing resolution only the selectivity, α, relates to more than one component. This is also the most complex of the three factors and it generally cannot be predicted how changes in the operating conditions will influence this value. The α value needs to be determined by trial and error changes in the mobile phase composition.

The efficiency value, N, improves resolution through a square root factor, and therefore it is most appropriate to start with a column with a relatively high number of theoretical plates. Since N generally varies linearly with column length, the best approach to improve resolution through efficiency is to use a longer column. However, since increasing N, has a square-root effect on improving resolution, a point of diminishing returns is reached where the increase in analysis time or the increase in back pressure does not justify the accompanying increase in theoretical plates. However a variation on this approach to improved efficiency is to reduce the flow rate without changing the column length but this will increase the analysis time and also the peak width.

The third factor which may be altered to improve resolution is the capacity factor, k' of the first component. When k' for the first component is changed so is the k' for the second component, but the effect of both k' values is accounted for in the selectivity factor which was just discussed. Control of k' is obtained by the mobile phase composition. A reduction in the mobile phase strength will increase k' due to the balance in attraction for the analyte being shifted to the stationary phase. If all k' values are too low then the components elute too quickly due to the solvent in the mobile phase being too strong. High k' values reflect long elution times and can be reduced by increasing the solvent strength of the mobile phase. Increasing k', will generally improve resolution but there is a range of k' beyond which the $(\frac{k'}{k'+1})$ term approaches a constant value. The optimum range for k' is 2-5, which means the components should elute between 3 and 6 column volumes.

2.1.2.5 Band Asymmetry

Band tailing or fronting can be measured by the asymmetry factor A_s. The asymmetry factor, which is a measure of peak shape, is defined in Figure 2.3.

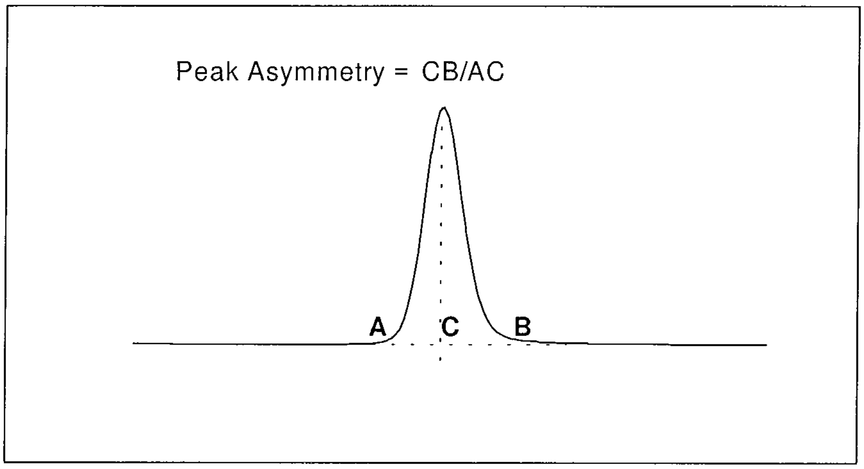


Figure 2.3: Definition of band asymmetry, A_S. [3]

In an ideal case the A_S value would be equal to 1.0 but it is acceptable from 0.8 to 1.2, [3]. For the asymmetry value greater than 1.2, peak tailing is not considered to be acceptable and for the value less than 0.8 peak fronting is not considered acceptable.

2.2 RPHPLC column selection

Since the C18 bonded phase has a wide versatility for retention of most compounds this was chosen as the bonded phase column for the RPHPLC methods. The packing is relatively non-polar and therefore the more polar components elute first from the column. C18 columns have been widely reported in the cited literature, Table 1.3, for OA, Table 1.4 for OTC and Table 1.5 for SD, SMX and TMP. Since it is not clear how to make "the best" bonded-phase column, many types of C18 columns exist on the market. Therefore an understanding must be developed regarding which columns are appropriate for the desired application needs. This information is best gained from experience and from information in the literature. The initial choice of column however, must be made from an application perspective. A comparison should be made between the compounds, which need to be separated, and those separated on the vendor's columns in promotional material. Once the first column is examined, it will be clear from the resolution obtained whether a change in selectivity or efficiency will improve the chromatography. Then using the guidelines above (Section 2.1) which indicate the physical and chemical characteristics which can be altered to change the selectivity and efficiency it is possible to select a more appropriate stationary phase for the separation of the compounds of interest.

The term endcapping was introduced in the late 70's, when it became obvious that some silanols are still accessible after surface modification with long-chain ligands. Trimethylchlorosilane is the most common reagent to cover accessible residual silanols. This molecule is relatively small and it can penetrate to the silica surface and cover "cap" some spotty areas of un-reacted silanols. Generally it leads to the significant improvement of the reversed phase adsorbent properties (less tailing and more predictable retention of polar compounds). Therefore columns with endcapping were considered in this work to keep peak tailing to a minimum.

2.3 Proposed LC conditions for Oxolinic acid

The successful use of fluorescence detection has been reported for the analysis of OA in salmon [5], trout [5, 6] and tissue samples [7]. The use of UV detection has also been successfully reported for the analysis of OA in trout [8, 9] and oyster tissue [10]. Both UV and fluorescence detection methods were compared for catfish tissue and it was found that fluorescence was the best detection method in terms of specificity and sensitivity [11].

However for the purposes of this project an analytical method was required for the identification and confirmation of OA in salmon tissue samples according to Directive 96/23/EC. Since using diode array detection (DAD) would allow for the simultaneous identification, quantification and confirmation of the analyte then this detection method would be ideal. However a DAD detector was not available in the laboratory when this work was being carried out and therefore UV was chosen with a view to adopting the method to a DAD at a later date.

As the organic modifier of the mobile phase for OA analysis in tissue samples, ACN [9, 10], ACN:THF [5, 11], ACN:MeOH [6, 7, 8] and ACN:MeOH:THF [12] have all been reported. While THF has been reported to reduce peak tailing for OA analysis [12], a comparison of ACN, MeOH and THF showed that the most symmetrical peaks were given by ACN [10]. Optimum capacity and resolution factors were also reported for ACN when compared to MeOH for OA analysis in urine samples [13]. Thus ACN was initially examined in this work as the organic component of the mobile phase but if the selectivity needed to be changed during the optimisation of the method then THF would be considered first.

Initially in a RPHPLC method development, the organic component is mixed with water and the ratios changed to examine the effect on retention time and resolution. Often analyte components will have increased ionic interactions with the stationary phase as the concentration of water is increased. These ionic interactions may give rise to a mixed mode of separation (non-polar and ionic attraction), and the result usually can be seen in the form of a peak tail. The undesirable surface interaction is probably due to an electrostatic attraction with residual, unbonded surface hydroxyl groups. In order to change the selectivity to reduce the tailing, attention can be focused on the water in the mobile phase. The addition of a buffer to the water will effectively compete for the electrostatic sites and mask the analytes ionic interactions and therefore reduce any other mixed mechanisms that might be causing the tailing. Pouliquen *et al.* [10] examined different aqueous buffers including disodium hydrogenphosphate, potassium dihydrogenphosphate and dipotassium hydrogenphosphate in order to optimise the chromatographic conditions. All three buffers examined resulted in baseline drift and severe peak tailing. Orthophosphoric acid solution was also examined and in conjunction with the Lichrospher RP-18 column, was reported to produce chromatograms, which were free from interfering peaks, and neither tailing nor baseline drift was evident [10]. Acid was also reported to mask the residual silanol groups on the Hypersil C8 column [12]. Based on this the orthophosphoric acid modifier in the mobile phase was tested in this work to ensure peak tailing was kept to a minimum.

Thus the initial method examined in this work for OA analysis in salmon tissue was a RPHPLC method with UV detection. The column was the Lichrospher RP-18, while the mobile phase consisted of ACN and orthophosphoric acid.

2.4 Proposed LC conditions for Oxytetracycline

OTC is a broad spectrum antibiotic and absorbs in the UV region of 255-390nm with a maximum UV absorbance at 365nm. OTC however, combines with divalent metal ions to give fluorescent chelate compounds, which only show fluorescence under basic conditions [14]. However difficulties in chromatography have been reported when OTC was analysed under basic conditions. These problems were resolved by using an acid based mobile phase, but this resulted in the quenching of the fluorescence [14].

The required sensitivity at the MRL of 100μgkg^{-1} was however obtained for salmon and trout tissue samples with UV detection [15, 16, 17, 18, 19, 20]. Since DAD detection can be used for analyte confirmation and quantification then this was the preferred instrument of choice. However a DAD was not available in the laboratory when this work was been carried out and therefore the UV was chosen with a view to adopting the method to a DAD in the future.

The organic mobile phase components reported in the cited literature for salmon or trout analysis include MeOH [14], ACN [20, 21, 22], ACN: tetrahydrofuran (THF) [16, 17] and ACN: dimethylformamide (DMF) [18, 19]. In theory, when developing a RPHLPC method the most suitable solvent for dissolving the analyte should be examined initially as a mobile phase component. For OTC this solvent was MeOH.

In this study a standard solution of OTC was injected while using 100% MeOH (polarity index: 6.6) as the mobile phase. An injection of an analyte when using 100% of the organic solvent should yield a situation where all the sample components elute as one peak at V_0. When using 100% MeOH as the mobile phase, retention of OTC was obtained and thus a less polar solvent, ACN (polarity index: 6.2), was examined. No retention of the OTC standard peak was found to occur for the mobile phase consisting of 100% ACN and thus this was chosen as the main organic mobile phase component for the analysis. Thus from the literature, the ACN based mobile phases were considered as first choice for the analysis of OTC in salmon tissue.

Therefore the first choice method for OTC analysis in salmon tissue was RPHPLC with UV detection. A C18 column was proposed for examination in conjunction with an ACN based mobile phase.

2.5 Proposed LC Conditions for Sulphadiazine, sulphamethoxazole and trimethoprim

Sulphonamides have been analysed with post column derivatisation for fluorescence detection in salmon tissue [23]. Adequate detection limits below the required MRL of $100\mu gkg^{-1}$ have also been reported for UV detection in finfish or pork tissue samples [24, 25, 26, 27, 28]. Since the analyte identification can be confirmed by DAD detection, which fulfils the requirements of Commission Decision 93/256/EEC, this was chosen as the detection technique for the analysis of SD, SMX and TMP in salmon tissue samples.

Of the literature cited, Table 1.5, most methods used ACN as the organic modifier for the mobile phase. As this was also the most suitable solvent for dissolving SD, SMX and TMP it was chosen as the initial organic solvent component of the mobile phase.

Three methods did use the combination of ACN: MeOH [23, 26, 29], but these were used in a gradient run to separate a wide range of sulphonamides.

Gradient elution can be used to decrease analysis time and is usually used when some components within the sample elute within a reasonable time, (capacity factor value, between 2-10) while others remain on the column much longer. At the beginning of the analysis, the solvent used is appropriate to elute some of the components but is "weak" in terms of its ability to remove other components from the column. As the chromatographic run proceeds, the "strength" of the mobile phase is changed in order to remove all of the analytes from the column, and separate these analytes from one another and from the matrix components in a timely fashion. The solvent composition can vary continuously during the course of the run or can be changed in a step increment.

Thus the method examined initially in this work for the analysis of SD, SMX and TMP in salmon tissue samples was RPHPLC with DAD detection. A C18 column with an ACN based mobile phase was examined initially with a view to using gradient elution if required.

2.6 Sample preparation

For the analysis of veterinary drug residues in salmon tissue samples using HPLC with DAD or UV detection, the tissue sample must be optimally prepared in order to remove the interfering sample components and selectively pre-concentrate the analytes of interest. The interfering sample components must be removed to prevent damage to the HPLC instruments and columns and also to allow for adequate resolution and thus the identification of the analyte of interest. The sample pre-concentration is required to increase the sensitivity of the detector and therefore allow for the quantification of the analyte at the required concentration levels.

The sample preparation and extraction procedures for tissue samples are critical in all residue analysis since they involve the extraction of the analyte of interest, the clean-up of the extracted sample without any loss of analyte and the pre-concentration to allow for detection at the levels of interest. Typical methods for the isolation of veterinary drug residues from tissue samples involve processes such as repeated homogenisation of the tissue in an extraction solvent or a combination of solvents. This is usually centrifuged or filtered followed by a cleanup procedure using solid phase extraction

(SPE) or liquid-liquid extraction. Finally the solvent is evaporated prior to HPLC injection.

2.6.1 Extraction

A critical aspect of residue analysis is the sample extraction step, which requires as far as possible the isolation of all the residues from the biological matrix. If this is not possible, ≥70% should be removed from fortified tissue samples (Section 1.5.5) in a reproducible manner so that an estimate can be made of the remaining un-extracted material and appropriate corrections made. The extraction of analytes from solid samples such as tissue gives rise to more problems than the analysis of liquids. The difficulties encountered can include the binding of the analyte by the matrix or the co-extraction of interfering components along with the analyte of interest. Both of these effects can have profound implications for the validity of the analytical method. The co-extractives can mask the presence of the analyte or give rise to false positive results while the problem of bound analytes with regards to occurrence and degree of binding can very often only be estimated.

When determining an extraction technique, consideration must be given to both the extraction efficiency and also the degree of co-extraction. The technique, which gives the best extraction of the analyte, is not always the method of choice. Soxhlet extraction, which is generally efficient at removing the analyte of interest, also tends to remove many interfering components of the tissue.

Soxhlet extraction is probably regarded as the most exhaustive method of analyte extraction from solid samples available. However due to the long time required and the risk of extracting co-extractives, alternative methods are preferred if applicable. These methods which include blending, sonication and flask-shake methods are quick and easy and are therefore the methods of preference for this study.

2.6.2 Clean-up

In order to achieve the required detection limits for residue analysis the final extract should ideally be completely free from interferences. This separation of the analyte from the co-extracted matrix components, can be the most challenging and demanding stage of the analysis. There are an infinite number of possible co-extractives and some of these may be chemically similar to the analyte. If this is the case then there are two possible routes: to rely upon an end determination technique in which the co-extractives do not interfere or to use separation techniques to isolate the analyte prior to analysis. The former is preferable as with fewer stages in the analysis the associated uncertainty is reduced. In practice however, this type of technique is seldom available and thus extensive cleanup is the only remaining option.

2.6.2.1 Liquid-liquid extraction

Liquid-liquid extraction is the traditional method of dealing with analyte clean up from a liquid sample. This method however, when applied to complex sample matrices such as tissue samples, requires a combination of liquid-liquid extractions to achieve the necessary degree of clean up. Liquid-liquid extraction is relatively easy to perform and requires no specialised equipment. Any pair of immiscible solvents can be used to effect a partition, but the use of the more toxic or environmentally damaging solvents should be avoided if possible. Aqueous samples are immiscible with most organic solvents and thus the partition is easily formed. There is a much narrower choice of extraction solvent when extracting analytes from organic phase extracts. The most widely used combination is hexane/water-modified acetonitrile. These two phases are particularly useful for separating fatty material from analytes of a slightly higher polarity such as the analytes of interest to this work.

2.6.2.2 Solid phase extraction (SPE)

SPE is a sample preparation technique using liquid-solid sorption to remove contaminants or analyte from the sample. SPE cartridges resemble miniature HPLC columns where a pre-separation is carried out on the sample components.

2.6.3 Selection of the appropriate SPE mechanism

There are three common extraction mechanisms used in solid phase extraction: non-polar, polar, and ion exchange. The sorbents within each extraction category exhibit unique properties of retention and selectivity.

The following three factors give the criteria, which should be used to determine which extraction mechanism, would be appropriate for a particular sample.

- The analyte: The important functional groups on the analyte should be identified, as non-polar (alkyl chains, aromatic rings), polar (hydroxyls, amines), or ionic (amines, carboxylic acids) as these can influence the retention on a sorbent.

- The matrix: The matrix from which the analyte is to be extracted will frequently influence which type of extraction to use. If the matrix is aqueous, analytes with non-polar or ionic functional groups can generally be extracted using non-polar or ion-exchange sorbents respectively. If the matrix is oily or is a non-polar solvent, analytes with polar functional groups can generally be extracted using polar sorbents. Also analytes with non-polar functional groups, which are extracted in an aqueous buffer can be analysed using a non-polar SPE cartridge for clean up.

- The objective: SPE cartridges can be used to prepare samples for many types of analysis including liquid chromatography (LC), gas chromatography (GC), UV spectroscopy etc. The final analysis procedure may dictate the need for a certain type of elution solvent. Generally a volatile elution solvent that can be evaporated so as to concentrate the analyte is preferable.

2.6.3.1 Process of SPE

To successfully carry out a SPE procedure, four basic steps are involved:

- conditioning
- loading the sample
- elution of the undesirable material
- elution of the analytes of interest.

2.6.3.2 Conditioning

Conditioning the SPE cartridge has two purposes. Firstly to remove any contamination that may be present in the cartridge due to the packaging and handling processes. Removing the contaminants is essential since they have the potential to elute with and contaminate the analyte. Therefore the first solvent for conditioning should be of equal or stronger eluting strength than the strongest eluting solvent to be used after the sample has been loaded onto the cartridge. Since this is usually the solvent used for eluting the analyte components of interest from the cartridge, it is used as the first conditioning solvent. This ensures that all possible contamination that might elute with the analytes is removed from the cartridge prior to sample introduction.

The second reason for conditioning the cartridge is to wet the packing material and leave it in a state that is compatible with the sample mobile phase. Therefore the second conditioning solvent for the cartridge should ideally be the same solvent used to load the sample. This solvent should also be miscible with the initial conditioning solvent so as to remove all of this solvent prior to the sample introduction.

Adequate conditioning of the cartridge is essential because if there is any of the initial strongest conditioning solvent remaining on the packing or if the packing is dry poor retention, poor recoveries and possibly contaminated analytes may result.

2.6.3.3 Loading the sample

The loading step involves loading the sample onto the packing using, depending on the viscosity of the sample extract, a vacuum or gravity system. The sample solvent is drawn through the cartridge and the analytes of interest are sorbed onto the packing. For the analytes to be retained, the solvent in which they are dissolved must be a weak solvent for the packing type being used. If the solvent is too strong the analytes will not be retained resulting in low recoveries. For reverse phase cartridges the sample should be loaded in water or an aqueous buffer. Using the weakest solvent possible will result in the narrowest bandwidth of analyte on the packing and this is desirable, as it will avoid interferences and reduces the amount of stronger mobile phase necessary for the elution of the analytes from the cartridge.

2.6.3.4 Eluting the undesirable material

After loading the sample, the packing can be rinsed with a mobile phase to wash off any undesirable sample components. A mobile phase that is slightly stronger or the same strength as the sample loading solvent should be used as the rinsing solution. This step removes unwanted sample components which are early eluters from the cartridge. However a rinsing step is not always necessary, as the components of interest are sorbed on to the packing and the undesirable components may be flushed straight through the cartridge to waste while the sample is being loaded.

2.6.3.5 Elution of the components of interest

It is desirable to elute the components of interest in as narrow a band as possible. Using too strong a solvent for this step will result in the co-elution of unnecessary sample components that are more strongly retained than the analytes. A solvent, with the optimal elution strength should keep these matrix components retained on the packing and avoid co-elution with the compounds of interest. It is also important that if the elution solvent is not an appropriate match for the final RPHPLC separation, it must be evaporated and the extracted analytes re-dissolved in another solvent for the HPLC analysis. In this case the elution solvent must be volatile and easily evaporated.

Further discussion of the sample preparation carried out in this work can be seen in Chapter 3 for OA, Chapter 4 for OTC and Chapter 5 for SD, SMX and TMP.

2.7 Gel Permeation Chromatography

Gel permeation chromatography (GPC), also known as size exclusion chromatography (SEC), is a technique used for the separation of macromolecules. It does not rely on chemical differences to effect the separation but separates molecules on the basis of their size relative to the pores in the packing particles. The separation is carried out on columns that are tightly packed with a gel or some porous material and completely filled with solvent. The same solvent is used to dissolve the sample before introducing it onto the column and also for elution. Small sample molecules can diffuse into the pores of the gel, large ones are excluded, and others of intermediate size can penetrate some of the larger pores. The molecules are constantly diffusing back and forth between the pores and the interstices. Solvent pumped through the column flows only in the interstices, sweeping along all sample molecules present there. The molecules in the pores stay behind until they diffuse back out. Larger molecules will pass through the column with no retardation because they cannot enter the gel. Smaller molecules will penetrate the interior to a degree determined by their size.

A species is eluted at a volume exactly equal to the volume available to it in the column. For large completely excluded molecules, the elution volume V_e is equal to the interstitial volume V_o, while for small molecules which can completely penetrate all pores of the gel it is equal to the total liquid volume of the column.

For small molecules:

$$V_e = V_o + V_i \quad \text{Equation 2.6}$$

V_e: elution volume

V_o: interstitial volume

V_i: internal pore volume

For molecules of intermediate size, the elution volume is:

$$V_e = V_o + K_d V_i \qquad \text{Equation 2.7}$$

K_d: the partition coefficient is equal to the ratio of accessible pore volume to total pore volume:

$K_d = V_{iacc}/V_i$

V_{iacc}: the accessible internal pore volume

The elution volume will depend on the gel used. These have a very open, three-dimensional network formed by cross-linking long polymeric chains. Instead of ion exchange sites, most of these gels have polar groups capable of adsorbing the mobile phase. The adsorption causes an opening in the structure, or swelling, leaving the interstices within the gel. Depending on the extent of cross-linking, there will be a critical size (exclusion limit) of a molecule that can just penetrate the interior.

This technique was examined for sample preparation of salmon tissue containing the drug residues of interest and is discussed further in Chapter 6.

2.8 References

[1] Oka H., Nakazawa H., Harada K., MacNeill J. (1995). Chemical analysis for antibiotics used in agriculture. AOAC International, ISBN 0-935584-57-9.

[2] Skoog D., West D., Holler F. (1998). Analytical Chemistry An Introduction. Harcourt Brace Jovanovich College PublishersFifth edition. ISBN 0-03-029924-1

[3] Dolan JW, Synder LR. (1989). Troubleshooting LC systems. The Humana Press Inc. ISBN 0-89603-151-9.

[4] Bidlingmeyer BA. 1992. Practical HPLC methodology and applications. Wiley Interscience Publication. ISBN –471-57246-2.

[5] Steffenak I, Hormazabel V, and Yndestad M. (1991). Rapid assay for the Simultaneous determination of residues of oxolinic acid and flumequine in fish tissues by high-performance liquid chromatography. Journal of liquid chromatography, **14**(1), 61-70.

[6] Rose MD, Bygrave J, Stubbings WF. (1998). Extension of mult-residue methodology to include the determination of quinolones in food. The Analyst, **123**, 2789-2796.

[7] Carignan G, Larocque L, Sved S. (1991). Assay of Oxolinic acid residues in salmon muscle by liquid chromatography with fluorescence detection: Interlaboratory study. Journal of the Associaction of Official Analytical Chemists, **74**, No. 6, 906-909.

[8] Ikai Y, Oka H, Kawamura N, Yamada M, Nakazawa H. (1989). Improvement of chemical analysis of antibiotics. XV1. Simple and rapid determination of residual pyridone-carboxylic acid antibacterials in fish using a prepacked amino cartridge. Journal of Chromatography, **477**, 397-406.

[9] Bjorklund H. (1990). Analysis of oxolinic acid in fish by high-performance liquid chromatography. Journal of Chromatography, Biomedical Applications, **530**, 75-82

[10] Pouliquen H, Pinault L, Le Bris H. (1994). Determination of oxolinic acid in seawater, marine sediment, and Japanese oyster (*Crassostrea Gigas*) by high-performance liquid chromatography. Journal of liquid chromatography, **17**(4), 929-945.

[11] Munns R, Turnipseed S, Peenning A, Roybal E, Holland D, Long A, Plakas S. (1998). Liquid chromatographic determination of flumequine, nalidixic acid, oxolinic acid and piromidic acid residues in catfish (*Ictalurus punctatus*). Journal of AOAC International, **81**, No. 4, 825-839.

[12] Samuelsen OB. (1990). Simple and rapid method for the determination of flumequine and oxolinic acid in salmon (Salmo salar) plasma by high-performance liquid chromatography and fluoresence detection. Journal of chromatograohy, **530,** 452-457.

[13] Duran-Meras I, Galeano-Diaz T, Rodriguez-Caceres MI, Lopez FS. (1997). Determination of the chemotherapeutic quinolonic and cinolonic derivatives in urine by high-performance liquid chromatography with ultra-violet and fluorescence detection in series. Journal of Chromatography-A, **787** (1-2), 119-127.

[14] Iwaki K, Okumura N, Yamazaki M. (1992). Determination of tetracycline antibiotics by reversed phased liquid chromotagraphy with fluoresence detection. Journal of chromatography, **623**, 153-158.

[15] Reimer G, Young L. (1990). Validation of a method for determination of tetracycline antibiotics in salmon muscle tissue. Journalof the Association of Official Analytical Chemists, **73**, No. 5, 813-817.

[16] Carignan G, Carrier K, Sved S. (1993). Assay of oxytetracycline residues in salmon muscle be liquid chromatography with ultraviolet detection. Journal of AOAC international. **76,** No. 2, 325-328.

[17] Rogstad A, Hormazabal V, Yndestad M. (1988). Optimization of solid phase extraction of oxytetracycline from fish tissue and its determination by HPLC. Journal of liquid chromatography, **11**(11), 2337-2347.

[18] Bjorklund H. (1988). Determination of oxytetracycline in fish by high-performance liquid chromatography. Journal of Chromatography, **432** 381-387.

[19] Nordlander I, Johnsson H, Osterdahl, Bengtgoran. (1987). Oxytetracycline residues in rainbow trout analysed by a rapid HPLC method. Food additives and contaminants, **4,** No. 3, 291-296.

[20] Murray J, McGill A, Hardy R. (1988). Development of a method for the determination of oxytetracycline in trout. Food additives and contaminants, **5**(1), 77-83.

[21] McCracken R, Blanchflower J Haagen S, Glenn D. (1995). Simultaneous determination of oxytetracycline, teracycline and chlortetracycline in animal tissues using liquid chromatography, post column derivatisation with aluminium and fluoresence detection. Analyst, **120**, pages1763-1766.

[22] Haagsma N, Scherpenisse P. (1993). High performance liquid chromatographic determination of tetracyclines in animal tissue and eggs. Presented at Euroresidues II Veldhoven, NL, 3.5 May 1993.

[23] Gehring TA, Rushing LG, Thompson HC jr.(1997)). Determination of sulfonamides in edible salmon tissue by liquid chromatography with postcolumn derivatization and fluorescence detection. Journal of AOAC International, **80**, No.4, 751-755.

[24] Ikai Y, Oka H, Kawamura N, Hayakawa J, Yamada M. (1991). Application of an amino cartridge to the determination of residual sulphonamide antibacterials in meat, fish and egg. Journal of Chromatography, **541**, 393-400.

[25] Malisch R, Bourgeois B, and Lippold R. (1992). Multiresidue analysis of selected chemotherapeutics and antiparasitics. Part 1: Determination of sulphonamides and their N4-Acetyl-metabolites, chloramphenicol and nicarbazin in meat. Deutsche Lebensmittel-Rundschau, **88**, Jahrg, Heft 7, 205-216.

[26] Le Boulaire S, Bauduret J-C, Andre F. (1997). Veterinary drug residues survey in meat: An HPLC method with a matrix solid phase dispersion extraction. Journal of Agricultural and Food Chemistry, **45**, 2134-2142.

[27] Long A, Hsieh L, Malbrough M, Short C, Barker S. (1990). Multiresidue method for the determination of sulphonamides in pork tissue. Journal of Agricultural and Food Chemistry, **38**, 423-426.

[28] Aerts MM, Beek WM. (1988). Monitoring of veterinary drug residues by a combination of continuous flow techniques and column-switching High-Performance Liquid Chromatography. Journal of Chromatography, **435,** 97-112.

[29] Porter S. (1994). Confirmation of sulphonamide residues in kidney tissue by Liquid Chromatography-Mass spectrometry. Analyst, **119**, 2753-2756.

CHAPTER 3: ANALYSIS OF OXOLINIC ACID (OA) IN SALMON TISSUE

3.1 Introduction

Oxolinic acid (OA) (Section 1.2.2) is listed as a Group B substance under Directive 96/23/EC. Even though there is no MRL set for OA in edible tissue, farmed salmon under Directive 96/23/EC must be monitored on a routine basis to see if the withdrawal periods are adhered to [1]. Otherwise residues of the drug may remain in the tissue intended for human consumption.

Following the examination of published methods (Section 1.5.11) and a further discussion in Chapter 2, RPHPLC was chosen as the proposed method for OA analysis in salmon tissue. From Section 2.3 the proposed RPHPLC conditions included a Lichrospher RP-18 column, an ACN and orthophosphoric acid mobile phase and detection by UV. Duran-Meras *et al.* [2] reported optimum capacity and resolution factors for ACN when compared to MeOH in the mobile phase. Pouliquen *et al.* [3] compared various phosphate buffers to orthophosphoric acid in the mobile phase and found that baseline drift and peak tailing was kept to a minimum when the acid was used. The proposed conditions are consistent with those reported by Pouliquen *et al.* [3] and thus this method was examined initially in this work for the chromatographic analysis of OA.

Various sample preparation techniques, which are available for the analysis of veterinary drug residues in tissue samples, have been discussed in Section 2.6. However, in order to find the most appropriate method for preparing salmon tissue samples containing OA, a further examination of the literature was necessary.

Different extraction solvents for OA analysis in tissue samples have been reported in the cited literature including phosphate buffer [3], hexane: ethyl acetate [4], ACN [5], ACN: ammonia [6], ethyl acetate [7] and acetone [8]. Since no specific problems were reported for any of the extraction methods above, then any one could be examined for this work. However, the extraction solvent for OA is influenced by the cleanup technique, which follows.

To determine whether to use liquid-liquid extraction or SPE for the cleanup procedure the methods in the cited literature were compared. As discussed in Section 1.5.11, the percentage recoveries obtained were all above the required 70% for both SPE and liquid-liquid extraction methods. The liquid-liquid extraction methods however quoted accuracy and precision values at $10\mu gkg^{-1}$ [6, 7, 8] while the SPE methods quoted values above 50μgkg^{-1} [3, 4, 9]. A comparison was made between SPE and liquid-liquid extraction and it was found that higher and more precise recoveries were obtained for the liquid-liquid extraction method [8]. The SPE cartridges examined were a Baker amino 10 and a Bond Elut C18 while the liquid –liquid extraction involved extraction into acetone, defatting with hexane and extraction of the analytes with $CHCl_3$ [8]. Therefore liquid-liquid extraction was chosen as the method for sample preparation in this work.

From the cited literature two methods were available which involved the extraction and liquid-liquid partition cleanup of OA from fish tissue samples [6, 10]. Samuelsen [10] reported a method which involved the extraction of OA from the tissue sample with McIlvaine buffer/MeOH followed by a subsequent liquid-liquid extraction with the buffer solution and 25ml dichloromethane (DCM). Steffenak *et al.* [6] reported a method, which involved the extraction of OA with ACN/ammonia (NH_3), followed by a protein precipitation step with phosphoric acid and extraction of the analyte with 4ml chloroform ($CHCl_3$). Both methods involved the use of either DCM or $CHCl_3$, but the volume of $CHCl_3$ used was lower than that of DCM. Also cleaner sample extracts were reported by Steffenak *et al.* [6], while interferences in the chromatogram at the retention time corresponding to that of OA was reported by Samuelsen [10]. Thus the proposed method for initial examination, for salmon tissue preparation for OA analysis was based on that reported by Steffenak *et al.* [6].

This chapter presents a description of the method used and the validation study carried out to demonstrate that the method meets the criteria set out under Commission Decision 93/256/EEC to allow it to be used for residue testing (Section 1.5.1).

A study was also carried out to determine if the withdrawal periods were being adhered to for OA in Ireland, by randomly testing farmed salmon from fish farms around the coast.

3.2 Apparatus and materials

3.2.1 Sample preparation

Scalpel and blades
Hi - speed homogeniser: Breda Scientific
Sample jars

3.2.2 Extraction Process

Balance (5 figure)
Centrifuge tubes (10 ml)
Vortex mixer - Fisons
Centrifuge - Sorvall T6000
25 ml glass centrifuge tube
10 ml glass centrifuge tube
Ori block -08-3 Techne + Si3 sample concentrator - nitrogen purge and water bath at 40^{0}C
Disposable glass vials

3.2.3 Chemicals and Reagents

Orthophosphoric acid: Riedel - deHaen 85%
Acetonitrile: Lab Scan - Pestiscan
Tetrahydrofuran: Lab Scan - HPLC
Ammonia: BDH - 35%
Chloroform: Romil - Super purity
Sodium hydroxide pellets: BDH - General purpose
Sodium chloride: Merck - extra pure
Oxolinic acid (O0877), 99% purity: Sigma/Aldrich Ltd.

3.2.4 HPLC System

Pump: Shimadzu LC - 6A

Column oven: Shimadzu CTO - 6A

Column: Lichrospher RP 18 - 5μm Endcapped (125 x 4.6) mm

Guard column: Lichrospher RP-18 Endcapped (7.5 x 4.6) mm
5 μm all guard cartridges.

Auto injector and system controller: Shimadzu SCL - 6B

Detector: UV -Vis spectrophotometric detector-Shimadzu SPD - 6AV

Data handling: Maxima 820 software - Millipore

3.2.5 Column Treatment

The new column was conditioned prior to use by flushing with ACN and water 75:25 v/v (2 hr), 50:50 v/v (2 hr), 40:60 v/v (2 hr) and 30:70 v/v (2 hr). This was followed by overnight conditioning with the mobile phase at a flow rate of 0.2 ml/minute. After each day of operation the column was reconditioned overnight with ACN and water (24:76 v/v) at a flow rate at 0.2 ml/min. This daily reconditioning of the column helped to keep peak tailing and baseline drift to a minimum, and also contributed to extending the column lifetime [11].

3.2.6 Sample storage

All tissue samples were stored in acid followed by methanol rinsed glass sample jars and kept in a freezer at -20°C prior to analysis. Using a hi-speed homogeniser the tissue was homogenised and 3g of this homogenate was used for each analysis.

3.3 Sample Preparation

The procedure as described by Steffenak *et al.* [6], is reproduced here and was followed in this analysis:

Step 1: The homogenised tissue sample, 3g of muscle was weighed into a 10ml centrifuge tube. This was spiked with OA, (300μl of 1.0μgml^{-1} solution, diluted in the mobile phase), and was then extracted with ammonia (NH_3) (1ml), and ACN (5.7ml). The total volume of solution added to the tissue was 7.0mls. The sample was mixed for 1 minute at the whirlimixer, followed by centrifugation for 3 minutes at 3000r.p.m.

Step 2: The supernatant (5ml), from step 1, was transferred by pipette into a glass pyrex test tube (10ml), and 5M sodium chloride (NaCl) (3ml), was added. The sample was vortex mixed for 5 seconds, and centrifuged for 1 minute at 3000r.p.m. which separated the solutions into two distinct layers.

Step 3: The upper ACN layer was discarded, and 85 % orthophosphoric acid (H_3PO_4) (1ml), and chloroform ($CHCL_3$) (4ml) were added to the aqueous NaCl solution. The sample was shaken vigorously for 10-15 seconds followed by centrifugation for approximately 30 seconds at 2500r.p.m.

Step 4: The upper aqueous NaCl layer and any solid particles between the two layers were discarded, or using a glass pasteur pipette, the lower $CHCl_3$ layer was transferred to another glass test tube. The $CHCl_3$ layer was evaporated to dryness at 60°C under a gentle stream of nitrogen. The residue was re-dissolved in the mobile phase (3ml) and then analysed by HPLC.

3.3.1 Modifications made to the sample preparation procedure

Homogenised tissue samples were spiked to known levels with OA, and the above procedure was followed to prepare these spiked tissue samples for analysis. Recoveries from spiked tissue samples at $100\mu gkg^{-1}$ were 45% (RSD 3.5%) (n=3). From this, it is evident that modifications had to be made to the above method in order to improve the recoveries. Therefore each step of the sample preparation procedure was examined individually in detail, starting with the final step and working backwards through the procedure. Any alterations, which were made to the procedure during the examination of these steps, were then incorporated into all other subsequent steps.

3.3.1.1 Step 4: Evaporation of the $CHCl_3$ to dryness

The last step, which involves the evaporation of $CHCl_3$ (4ml), to dryness under a gentle stream of charcoal filtered nitrogen, at 60°C using a dri-block apparatus, was tested and it was found that losses in the recoveries were significant here. In order to resolve this difficulty the temperature at which the $CHCl_3$ was evaporated to dryness was examined. While operating at a temperature of 60°C, 4ml of a standard solution of OA in $CHCl_3$ ($0.075\mu gml^{-1}$) was evaporated to dryness and 3ml of the mobile phase was added to yield a standard solution of 0.1μgml^{-1}. This sample was evaporated under two different conditions. The first of these involved exposing the sample to normal lighting conditions in the laboratory, by using a clear glass pyrex test tube during the evaporation, and in the second case a dark test tube was used which protected the solution from the lighting conditions of the laboratory. The percentage recoveries obtained above were 52% and 53% respectively, which shows that during evaporation the analyte was lost or rendered unavailable for analysis. It also suggests that the lighting conditions of the laboratory was not a significant factor.

These percentage recoveries needed to be improved. To investigate the effect of the temperature at which the evaporation took place, the temperature was reduced from 60°C to 40°C and the same procedure as above was carried out. The percentage recovery obtained when the standard solution was exposed to lighting conditions of the laboratory was 69%, while for the solution kept in the dark was 67%. This is a further indication that light is not a critical parameter.

Although the results were higher than before, an alternative needed to be examined due to the length of time required to evaporate the solution to dryness at 40°C. The Buchi rotary evaporator, R110, was examined as a possible alternative to the nitrogen and dri-block system. When operating this, with a 50ml round bottom flask, and at a temperature >35°C and <60°C it was found that some of the $CHCl_3$ solution tended to "boil" in the flask, and thus moved up inside the condenser and proceeded on to be collected in the waste flask. With the temperature reduced to 30°C or less not all the solution was evaporated and therefore this method of evaporation was deemed unsuitable.

It was noted however, that when the $CHCl_3$ layer was separated from the aqueous sodium chloride solution, it was difficult to eliminate completely the NaCl layer and the aqueous NaCl carried over in the $CHCl_3$ was interfering with the evaporation. This considerably increased the time required to evaporate the solution to dryness. In order to keep this time to a minimum it was decided to take one quarter of the total volume of $CHCl_3$, by using a glass pasteur pipette and removing the $CHCl_3$ from underneath the aqueous layer which gave a cleaner separation. This was evaporated to dryness under nitrogen in a disposable glass vial at 40°C. The final volume of mobile phase, added before analysis, was altered correspondingly, so that 0.75ml was added instead of the previous 3ml. This was vortex mixed for 1 minute followed by analysis by HPLC. The mean percentage recovery obtained from this step, when following the above modified procedure, increased to an acceptable 99%, (RSD 1.2%) (n=3).

Additionally from this study it was concluded that it was not necessary to protect the OA from normal lighting conditions of the laboratory during the analysis.

3.3.1.2 Alterations made to step 2 and step 3:Liquid - liquid extractions

Step 2 involved the liquid-liquid extraction of OA from ACN into an aqueous NaCl layer. For quantification purposes the analyte must be in an organic solvent so the evaporation can take place and the analyte concentrated up to a detectable concentration. Therefore the OA needed to be extracted from the aqueous NaCl layer with 85% H_3PO_4 (2ml) and $CHCl_3$ (8ml), and this solution was treated as described in Section 3.3.1.1.

To check the efficiency of step 2, a second NaCl extraction of the ACN layer was added to the method as follows.

The supernatant from the tissue extraction (5ml ACN) was pipetted into a 25ml glass centrifuge tube and 5M NaCl (3ml) was added. This was mixed for 20 seconds at the whirlimixer, followed by centrifugation for 2 minutes at 1500r.p.m. The upper ACN layer was transferred to a 10ml glass centrifuge tube using a glass pasteur pipette, and the extraction was repeated with a further 3ml of 5M NaCl. After the second centrifugation, the lower aqueous NaCl layer was removed from beneath the ACN layer using the glass pasteur pipette, and transferred to the 25ml test tube which contained the initial NaCl extract. Both aqueous layers were combined in this way, and the ACN layer was discarded.

85% H_3PO_4 (2ml) and $CHCl_3$ (8ml) were added to the 25ml test tube, which contained the combined aqueous NaCl layers. The sample was vortex mixed for 20 seconds, and centrifuged for 3 minutes at 1500r.p.m. which resulted in two distinct layers being formed. The lower $CHCl_3$ layer was transferred to another test tube, using the glass pasteur pipette, while taking care not to remove any of the aqueous solution from the test tube. The upper aqueous layer was re-extracted and again the $CHCl_3$ layer was removed from beneath the aqueous layer, this time taking care not to leave any of the $CHCl_3$ behind. Both $CHCl_3$ extracts were combined and were vortex mixed for 30 seconds followed by centrifugation at 1500r.p.m. for 2 minutes, to separate out any aqueous solution which may have been transferred with the final $CHCl_3$ extract. Using a pipette, and taking care not to take any aqueous solution which may be in the test tube, 4ml of the $CHCl_3$ solution were transferred to a disposable glass vial. This was evaporated to dryness under a gentle stream of nitrogen at 40°C. To this vial mobile phase, (0.75ml) was added and this was vortex mixed for 1 minute, followed by analysis by HPLC.

With the above alterations made to the liquid-liquid extractions, a 98% (RSD 1.5%) (n=3) recovery was obtained from a standard solution, which did not contain any tissue.

3.3.1.3 Step 1:Extraction of the analyte from the fish tissue

The homogenised tissue sample (3g) was transferred quantitatively into a 10ml centrifuge tube. This was spiked with 300µl of a $1.0\mu gml^{-1}$ solution of OA in the mobile phase, which was vortex mixed for 30 seconds to ensure thorough mixing of the OA with the tissue. NH_3 (1ml) and ACN (5.7ml) were added and the sample was mixed for 1 minute at the whirlimixer, followed by centrifugation for 4 minutes at 2500r.p.m.

When the tissue sample was spiked with OA in the mobile phase to a level of $0.1\mu g^{-1}$ the percentage recoveries and the consistency obtained between them were relatively poor, 61±12.6%, (n=3). Therefore the solvent in which the tissue was spiked was changed to ACN, which gave better consistency and higher percentage recoveries.

Recoveries of tissue samples spiked to $100\mu gkg^{-1}$ with OA were 73% (RSD 2.1%) (n=5) (Section 3.7.5) when the sample was prepared according to the procedure with the above adjustments made. However, when a blank tissue sample was spiked immediately after step 1, and just before the liquid - liquid extractions a recovery of 102% (RSD 2.0%) (n=3) was obtained. This indicated that the losses were occurring in the "extraction" of the analyte from the tissue.

Not considered so far, in the extraction of OA from the tissue sample, is the amount of water contained in the 3g of tissue sample, and the dilution effect that this water has on the total volume of supernatant, when NH_3 and ACN solutions have been added to the tissue sample. Investigations into the dilution effects of the water, contained in the tissue, on the percentage recoveries obtained were carried out.

3.3.1.4 The effect of water contained in the tissue on the percentage recoveries

The amount of water contained in the tissue sample (3g) was determined as follows. The tissue sample (3g) was weighed accurately to three decimal places. This sample was dried in the oven at 103-105°C and weighed to a constant weight. The weight difference was taken to be the water content, which at $1gml^{-1}$ equalled the water.

Table 3.1: Wet weight and dry weight of fish tissue, 3g dried in the oven at 103-105°C, to a constant weight.

Sample number	Wet weight / g	Dry weight / g	Water content / g
1	3.015	1.319	1.696
2	2.972	1.379	1.593
3	3.021	1.138	1.883
4	2.985	1.321	1.664
5	3.039	1.176	1.863
Mean	3.006 ± 0.009	1.266 ± 0.08	(1.700 ± 0.07)ml

The volume of water in the fish tissue (3g) was taken as 1.7ml. In the initial step of the cleanup procedure, a total of 7mls were added to tissue sample and 5mls of this was taken and used in the remainder of the procedure. In reality however, it was observed that after 7ml had been added to the tissue and this was homogenised followed by centrifugation, a total of 8.7ml of supernatant could be decanted from the tube containing the tissue sample. This difference in volume was taken as the water content in the tissue sample. When this was considered, it was noted that 5ml of the supernatant was taken from a total of 8.7ml, and this difference was incorporated into the calculations.

The moisture content of a wild salmon is 69.9% while that for a farmed salmon is 71.5% [12]. When these values are compared to the 56.6% moisture content, value obtained above it is apparent that a discrepancy exists between the two sets of data. This can be accounted for by the fact the tissue used to obtain the data in Table 3.1 above was homogenised and frozen prior to the wet weight/dry weight test being carried out. When a tissue sample is homogenised and frozen it loses some of its moisture and thus the lower moisture content value was obtained. It should also be noted that all tissue samples were homogenised and frozen prior to analysis and will therefore have lost some of the moisture content before they are extracted. Therefore, it is necessary, when extracting the tissue with ACN and NH_3, to weigh the supernatant after homogenisation and centrifugation and record this value. The supernatant (5ml) which is used in the remainder of the analysis was also weighed and the calculations for this section of the procedure were carried out on a weight/weight basis. Table 3.2, shows a comparison between the percentage recoveries of OA obtained, from fish tissue (3g), when the water content was taken as zero and as 1.7mls.

It is important that the water content of the sample be determined and the result incorporated into the calculations to account for the dilution effects of the water contained in the sample. As shown in Table 3.2, the water content can account for up to approximately 17% differences in the percentage recoveries obtained.

Table 3.2: Comparison of percentage recoveries obtained with zero water content and 1.7mls water in 3g of fish tissue

Concentration μgkg^{-1}	**% recovery with zero water content in 3g of fish tissue.**	**% recovery with 1.7 mls of water in 3g of fish tissue.**
55.3	64.6 %	82.0 %
99.6	57.7 %	70.6 %
253.3	58.1 %	71.7 %
536.9	62.8 %	77.4 %
974.9	62.8 %	76.9 %

In the individual examination of each step of the cleanup procedure as described by Steffenak *et al.* [3] (Section 1.3.3) changes were deemed necessary to each one. These were recorded and when they were all incorporated into the method, it was found that the percentage recoveries ranged from 70-82%. This is a significant improvement on the 45% recoveries obtained when the unmodified method was examined. The EU guidelines for the validation of routine methods [13], only quote acceptable accuracy ranges for Certified Reference Materials (CRMs), and not spiked tissue samples. No CRMs are available for OA in salmon tissue. Therefore the Codex guidelines were applied to this method for the accuracy test. In the Codex guidelines set out in the establishment of regulatory programmes [14], the recommended acceptable recoveries for spiked tissue samples are 70-110% when the MRLVD (maximum residue limit of veterinary drugs), is 10μgkg^{-1} to 100μgkg^{-1}. Although there is no MRLVD set for OA in fish tissue, the 70-110% recoveries can be taken as a guideline for the accuracy in this study. Therefore the 75.7±6.3% recoveries obtained over the concentration range of 55-975µgkg^{-1}, can be taken as acceptable results. The accuracy and precision are discussed further in the validation study (Section 3.7.6 and 3.7.5).

3.4 Final sample preparation procedure

Muscle (3g) Homogenised

Spike with standard (300µl) in ACN.
Vortex mix for 30 seconds in a 15ml centrifuge tube.

Add NH_3 (1ml).
Add ACN(5.7ml).
Mix for 1 minute at the whirlimixer.
Centrifuge for 4 minutes at 2500r.p.m.

Supernatant (5 ml) of ACN to a 10 ml pyrex tube.

Discard the residue

Add 5 M NaCl (3ml).
Vortex mix for 20 seconds.
Centrifuge for 2 minutes at 1500r.p.m.
Remove the lower aqueous layer to a 25ml pyrex tube.
Repeat the extraction and combine the aqueous layers.

Discard the upper ACN layer

Add 85% H_3PO_4 (2ml) and $CHCl_3$ (8ml).
Vortex mix for 20 seconds
Centrifuge for 3 minutes at 1500r.p.m.
Transfer the lower $CHCL_3$ layer to another 25ml pyrex test tube.
Repeat the extraction of the aqueous layer.

Discard the H_2O

Combine the two $CHCl_3$ layers.
Vortex mix for 30 seconds.
Transfer 4ml of the $CHCl_3$ to a disposable glass vial.
Evaporate to dryness at 40°C.
Dissolve in mobile phase (0.75ml).

HPLC Analysis

3.5 Optimisation of chromatographic conditions for oxolinic acid analysis

As discussed from the literature cited (Section 2.3) RPHPLC was the analytical technique chosen to determine OA in salmon tissue samples. The initial chromatographic conditions examined for the analysis of OA were set out by Pouliquen *et al.* [11]. The column used was a reverse phase C18 column in conjunction with a polar mobile phase.

3.5.1 Chromatographic conditions for oxolinic acid analysis

Detector: UV-VIS @ 262nm.
Detector sensitivity: 0.01a.u.f.s.
Column: Lichrospher RP-18 5μm particles (120 x 4.6) mm.
Oven temperature: 25°C.
Injection volume: 50μl.
Flow rate: 1.0 ml/minute.

3.5.1.1 Standard solutions preparation

A stock standard solution of OA was prepared in aqueous sodium hydroxide (0.03M) at a concentration of 1mgml^{-1}. This solution was shown to be stable for one month when stored at 4°C [11]. This solution was sonicated for 5 minutes to ensure that the entire sample dissolved in the aqueous solution. Working standards were prepared by dilution of the above stock standard in the mobile phase immediately before use. All solutions were stored in the fridge at 4°C prior to use and brought to room temperature prior to injection.

3.5.1.2 Choice of mobile phase

The mobile phase used by Pouliquen *et al.* [11], which was H_3PO_4 (0.02 M), 76%, and ACN, 24%, was examined in this method. The acid was prepared by diluting 1.35ml of 85% pure H_3PO_4 to 1000ml with deionised water. To make 1 litre of mobile phase, 760ml of the acid was combined with 240ml of ACN, and this was filtered using a membrane filter paper. The solution was degassed by sonicating for 15 minutes prior to use, and pumped through the column at a flow rate of 1.0ml/minute for 30 minutes before any samples or standards were injected onto the column. An example of a chromatogram obtained while using this mobile phase can be seen in Figure 3.1. It is evident from this chromatogram that peak tailing is present. This contributed to poor peak symmetry and therefore, modifications were required to reduce the peak tail to a minimum. Peak shape is measured by the asymmetry factor (Section 2.1.2.5), and these values for different ratios of the mobile phase components can be seen in Table 3.3.

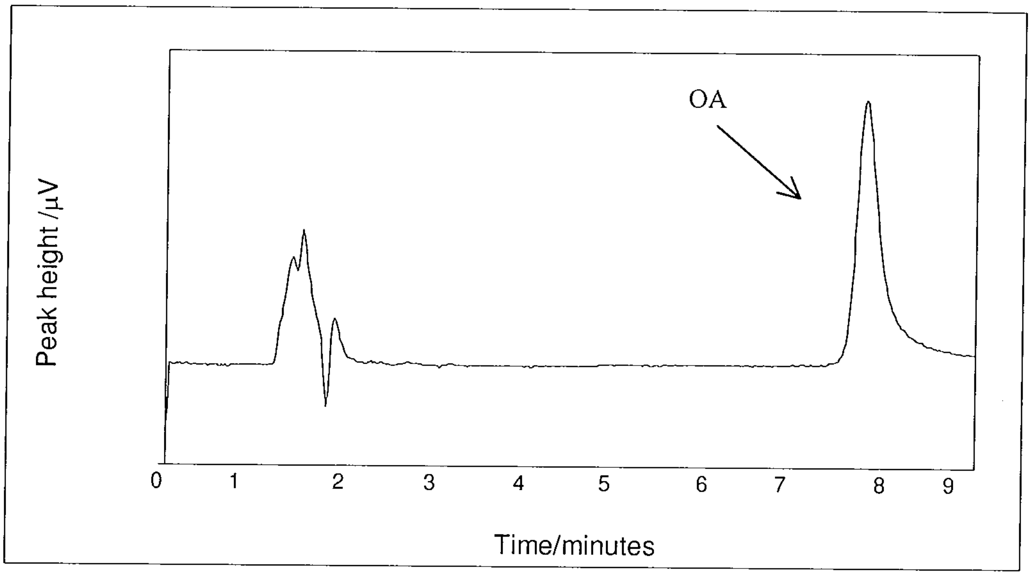


Figure 3.1: Chromatogram of oxolinic acid standard solution 0.1µgml^{-1} diluted in the mobile phase. Injection volume: 50µl. Column: Lichrospher -100 RP18 (120 x 4.6) mm. Oven temperature: 25°C. Mobile phase: H_3PO_4 (0.01M): ACN {76:24}. Flow rate: 1.0 ml/minute. Detector: UV at 262 nm and 0.01 AUFS.

Table 3.3: Asymmetry values for different ratios of the mobile phase components: H_3PO_4 (0.02 M), and ACN. Flow rate: 1.0 ml/minute. The standard solution tested was 0.1µgml^{-1} of oxolinic acid diluted in each of the corresponding mobile phases. Injection volume: 50µl. Column: Lichrospher -100 RP18 (120 x 4.6) mm. Oven temperature: 25°C. Detector: UV at 262 nm and 0.01 AUFS.

0.02M H_3PO_4 : ACN	**pH of mobile phase**	**A_S of analyte peak**	**Retention time (minutes)**
24:76	2.3	2.59	7.67
30:70	2.5	3.17	2.35
40:60	2.55	3.33	1.43
50:50	2.58	0.682	1.81
60:40	2.71	1.36	2.00

The asymmetry values for the above tested mobile phases, which are mostly due to peak tailing, (A_S values over 1.2), were considered unacceptable, and therefore further adjustments to the chromatographic parameters were deemed necessary.

As peak tailing results from a non-ideal equilibrium distribution, between the two phases for the sample components, the difficulty encountered lies with either the mobile or the stationary phase. Therefore, when it became apparent that ACN/acid mixture would not give an acceptable peak shape, when used in conjunction with the Lichrospher-100 RP-18E packing, it was appropriate to change to a different organic/acid mixture, and determine if an improved peak shape could be obtained. A peak tail results from the sample component being retained too strongly by the stationary phase, and therefore it was concluded that a "stronger" (in terms of polarity), mobile phase was required to carry the sample through the column and prevent it being retained by the column packing. A "stronger" solvent, which would be compatible with a bonded non-polar phase packing, was chosen as an alternative to ACN in the mobile phase. Tetrahydrofuran (THF) (polarity index 4.2) was chosen as the organic solvent and examined as a possible replacement for ACN (polarity index 6.2) in the mobile phase. Table 3.4, shows asymmetry values near an acceptable value of 1.2 for this mobile phase, and thus a reduction in peak tailing.

Table 3.4: Asymmetry values for different ratios of the mobile phase components: H_3PO_4 (0.02 M), and THF. Flow rate: 1.0 ml/minute. The standard solution tested was 0.1μgml^{-1} of oxolinic acid diluted in the corresponding mobile phase. Injection volume: 50μl. Column: Lichrospher -100 RP18 (120 x 4.6) mm. Oven temperature: 25°C. Detector: UV at 262 nm and 0.01 AUFS.

0.02M H_3PO_4 : THF	**pH of mobile phase**	**peak width (minutes)**	**A_S of analyte peak**	**Retention time (minutes)**
75:25	2.15	0.425	1.31	4.125
78:22	2.40	0.475	1.19	4.308
79:21	2.34	0.563	1.15	4.717

The mobile phase chosen for the analysis was 78% H_3PO_4 (0.02 M) and 22% THF, with an asymmetry value of 1.19 which fits within the acceptable range. Figure 3.2, shows a chromatogram of a standard solution of OA, which was eluted from the column using H_3PO_4 (0.02 M): THF in the ratio of 78: 22.

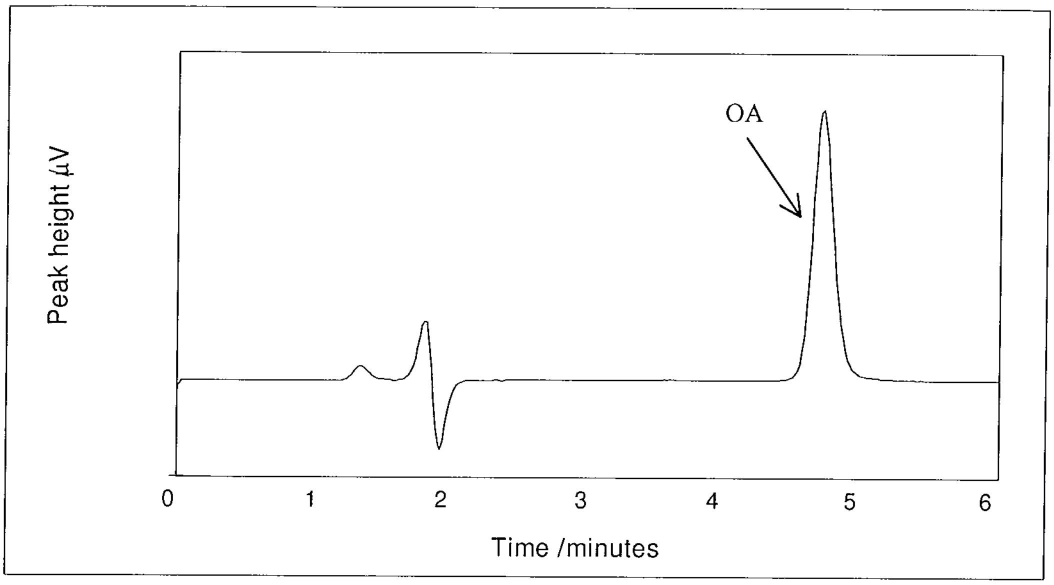


Figure 3.2: Chromatogram of oxolinic acid standard solution 0.1µgml^{-1} diluted in the mobile phase. Injection volume: 50µl. Column: Lichrospher -100 RP18 (120 x 4.6) mm. Oven temperature: 25°C. Mobile phase: H_3PO_4 (0.01M): THF {78:22}. Flow rate: 1.0 ml/minute. Detector: UV at 262 nm and 0.01 AUFS.

When a spiked fish tissue sample was analysed however, the resolution between OA and the nearest eluting matrix peak was very poor, (Figure 3.3), therefore the factors affecting the resolution of the components needed to be improved.

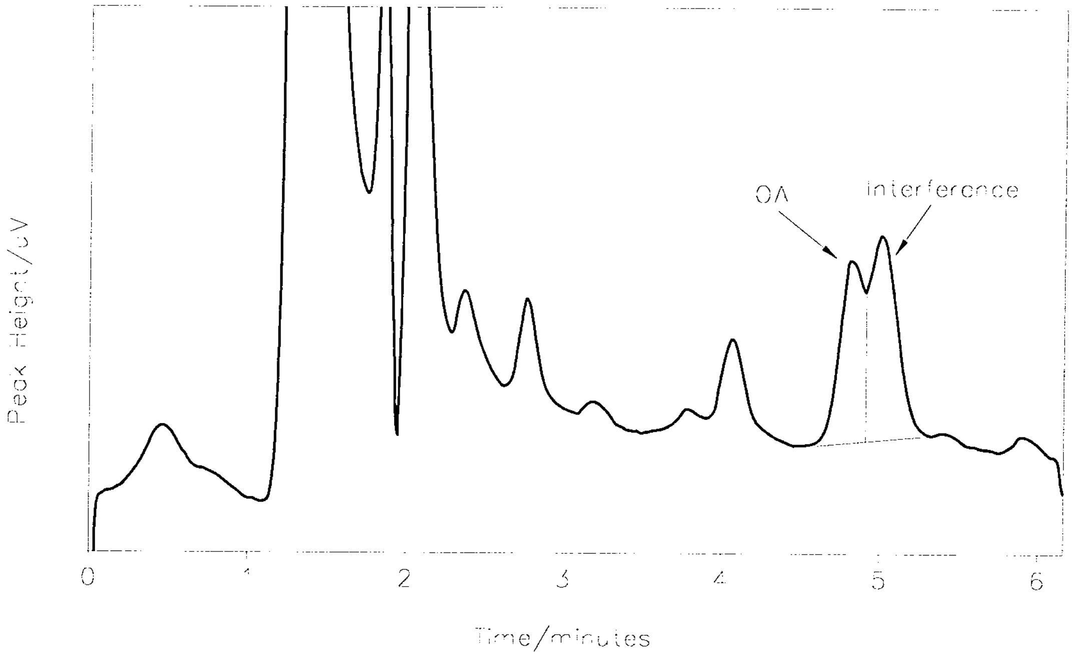


Figure 3.3: Chromatogram of tissue spiked with oxolinic acid 100µgkg^{-1}. Injection volume: 50µl. Column: Lichrospher -100 RP18 (120 x 4.6) mm. Oven temperature: 25°C. Mobile phase: H_3PO_4 (0.01M): THF {78:22}. Flow rate: 1.0 ml/minute. Detector: UV at 262 nm and 0.01 AUFS.

The capacity factor, k′ (Section 2.1.2.1) is a measure of the retardation of the compound in terms of the number of column void volumes it takes to elute the centre of the peak. Ideally, the optimum value of k′ for the best separation is in the range of 2 - 10 [15]. From Figure 3.3, the selectivity factor and the capacity factor for both the analyte and interfering peak was calculated, and finally the resolution, R_S, was estimated (Section 2.1.2.4).

$$\mathbf{k'_1 = (4.90 - 1.35) = 3.55}$$

$$\mathbf{k'_2 = (5.10 - 1.35) = 3.75}$$

$$\boldsymbol{\alpha} = \mathbf{k'_2 / k'_1 = (3.75/3.55) = 1.05}$$

$$\mathbf{N = 16 \{(t_r)^2\}/ (w^2)}$$

k'_1 is the capacity factor for OA.

k'_2 is the capacity factor for the interfering peak.

t_r is the retention time of the analyte peak.

w is the peak width of the analyte peak.

N, the number of theoretical plates was calculated.

$$\mathbf{N = 16\{(4.90)^2\}/(0.50)^2 = 1588}$$

Resolution:

$$\mathbf{R_S = 0.25((1.05-1)/1.05)\sqrt{1588}\{3.55/(3.55+1)\}}$$

$$\mathbf{R_S = 0.38}$$

The resolution obtained was not acceptable and therefore the factors affecting this were examined. Although the capacity factor lies within the acceptable range, the selectivity factor (1.05) was found not to lie within the range acceptable for reasonably well resolved peaks. Therefore the factors affecting the selectivity, and thus the resolution of the sample components, were examined and altered so as to obtain an appropriate R_S value, while also reducing peak tailing to a minimum.

Reverse phase chromatography is characterised by strong interactions between the polar mobile phase and the sample molecules, while interactions between the sample and the non- polar stationary phase are weak. The strong, selective solvent interactions in reverse phase chromatography are largely due to dipole attraction and H-bonding, which means that solvent selectivity can be characterised by, solvent polarity, solvent basicity (proton acceptor), and solvent acidity (proton donor). The preferred solvents for selectivity should differ as much as possible in their polar interactions, therefore a mixture of THF and ACN was chosen to combine with the aqueous acid to improve the selectivity factor and thus resolve the sample components. Figure 3.3 shows the chromatogram when using a mobile phase consisting of H_3PO_4: THF: ACN in the ratio of 78:17:5. From the data shown in Table 3.5, the following values were calculated.

$$\mathbf{k'_1 = 2.33}$$

$$\mathbf{k'_2 = 2.67}$$

$$\boldsymbol{\alpha} = \mathbf{1.15}$$

$$\mathbf{N = 1588}$$

$$\mathbf{R_S = (1/4)\ \{(1.15 - 1)\}\sqrt{1588}\{(2.33)/(1 + 2.33)\}}$$

$$\mathbf{R_S = 1.04}$$

The capacity factors calculated above fit into the optimum range of 2-10 while the selectivity value lies within 1.1 and 1.4 range. With these values acceptable for suitable chromatography the resolution of the two adjacent peaks was calculated and found to have a value of 1.04. Since a value of 1.0 would deem two peaks to be separated for quantification purposes the calculated value of 1.04 is acceptable and therefore the mobile phase for all further analysis was H_3PO_4 (0.02M): THF: ACN in the ratio of 78:17:5.

Table 3.5: Retention time data for oxolinic acid and the nearest eluting peak. Mobile phase H_3PO_4 (0.02 M): THF: ACN (78:17:5). Flow rate: 1.0 ml/minute. The standard solution tested was 0.1μgml^{-1} of oxolinic acid diluted in the mobile phase. Injection volume: 50μl. Column: Lichrospher -100 RP18 (120 x 4.6) mm. Oven temperature: 25°C. Detector: UV at 262 nm and 0.01 AUFS.

	Retention time / minute	Peak start / minute	Peak end minute	Void time of the column (minute)
Oxolinic acid	4.58	4.37	4.88	1.38
nearest peak	5.05	4.88	5.24	1.36

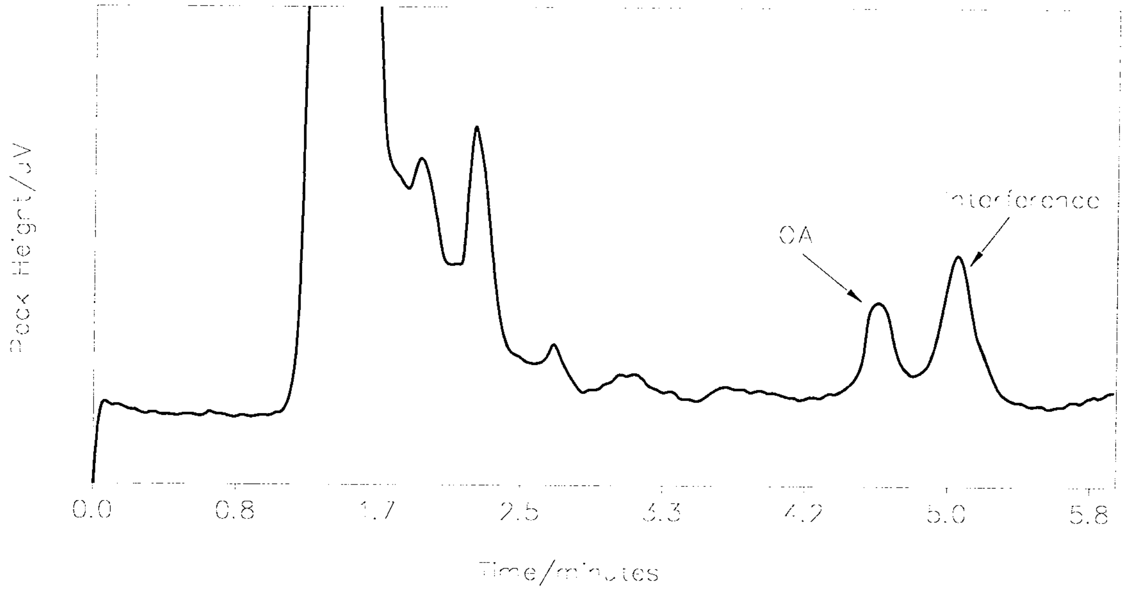


Figure 3.4: Chromatogram of tissue spiked with oxolinic acid 100μgkg^{-1}. Injection volume: 50μl. Column: Lichrospher -100 RP18 5μm (120 x 4.6) mm. Oven temperature: 25°C. Mobile phase: H_3PO_4 (0.01M): THF: ACN {78:17:5}. Flow rate: 1.0 ml/minute. Detector: UV at 262 nm and 0.01 AUFS. $R_S = 1.04$.

Since R_S can be improved by increasing column length (Section 2.1.2.4) a longer (250 x 4.6) mm Lichrospher-100 RP18, 5μm column was examined in the method as opposed to the (120 x 4.6) mm column which was used to date. It was found that the baseline drifted when the conditions described above (Figure 3.4) were applied to this column. Therefore the temperature of the column was increased until a stable baseline was achieved, this occurred at 35°C. When a tissue sample was analysed on this column the resolution obtained between the matrix components and the analyte peak was not acceptable and this is verified in Figure 3.5.

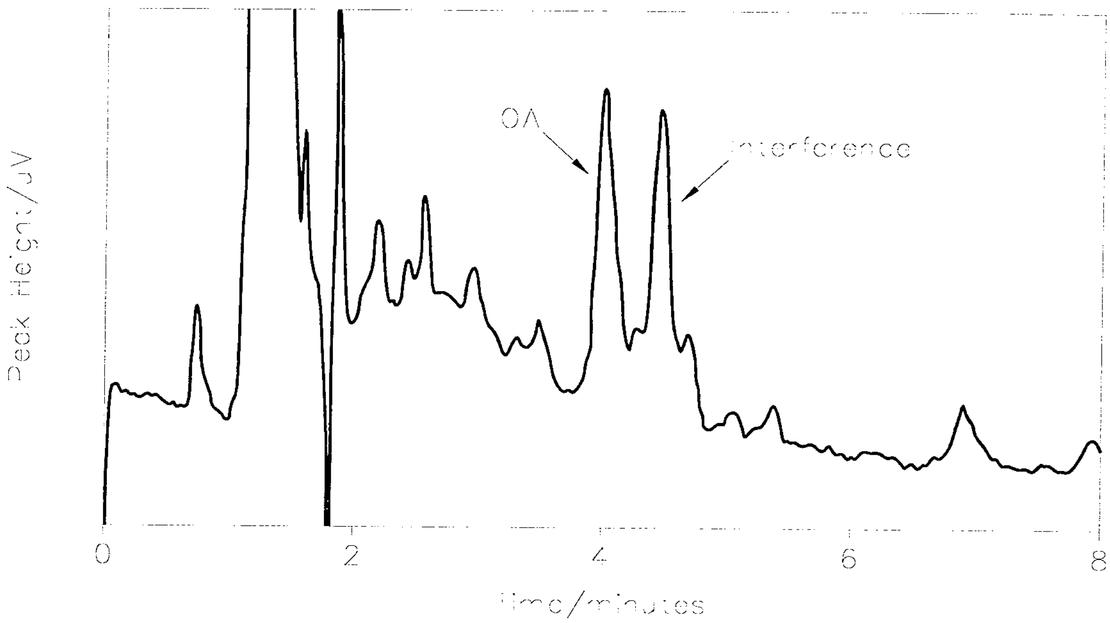


Figure 3.5: Chromatogram of tissue spiked with oxolinic acid 100μgkg^{-1}. Injection volume: 50μl. Column: Lichrospher -100 RP18 5μm (250 x 4.6) mm. Oven temperature: 35°C. Mobile phase: H_3PO_4 (0.01M): THF: ACN {78:17:5}. Flow rate: 2.0 ml/minute. Detector: UV at 262 nm and 0.01 AUFS.

As can be seen in Figure 3.5, the resolution between the matrix and analyte peaks is not acceptable. The OA peak at 4.0 minutes is not sufficiently resolved from the matrix peak beside it. Therefore the mobile phase was examined and the organic phase in this was altered to improve the resolution of the analysis.

The resolution of the analysis can be improved by increasing the "strength" (in terms of polarity) of the organic phase in the mobile phase. Thus the ratios of the components of the mobile phase were kept constant while substituting one organic component to a "stronger" solvent. The ACN in the mobile phase was firstly substituted with ethanol (ETOH), polarity index 5.2, followed by iso-propanol (IPA), polarity index 4.3, and ethyl acetate (EA), polarity index 4.3. The results of this can be seen in Figure 3.6, which shows an overlay of a blank tissue sample on a spiked tissue sample for three different mobile phases. These are 78:17:5 of H_3PO_4 (0.02M): THF: ETOH, H_3PO_4 (0.02M): THF: IPA, and H_3PO_4 (0.02M): THF: EA.

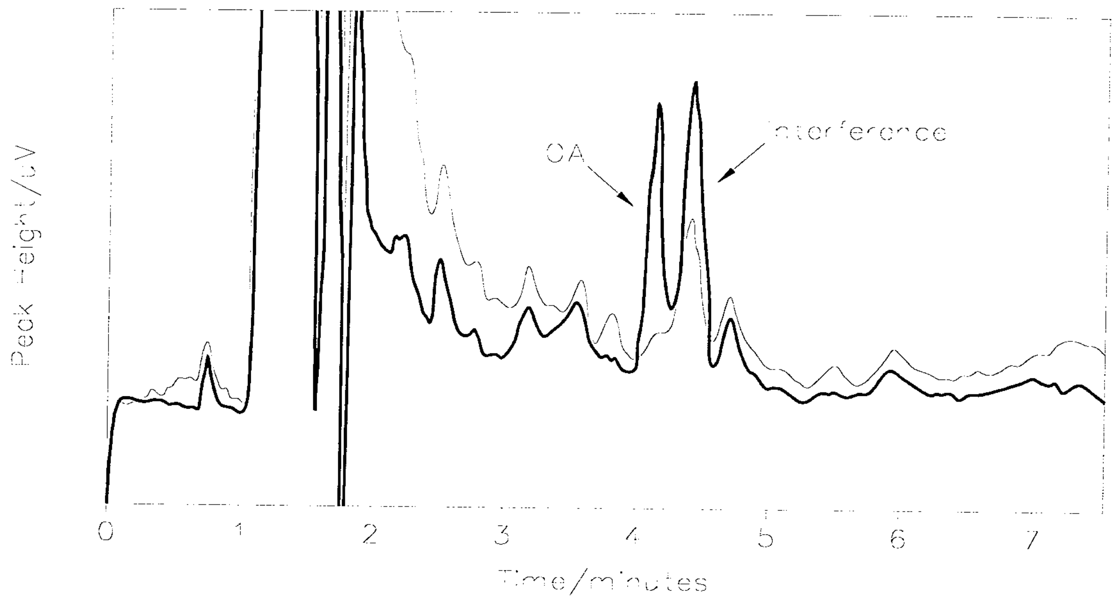


Figure 3.6a: Overlay of a blank tissue on a spiked tissue, 100µgkg^{-1}. Injection volume: 50µl. Column: Lichrospher -100 RP18 5µm (250 x 4.6) mm. Oven temperature: 35°C. Mobile phase: H_3PO_4 (0.01M): THF: ETOH {78:17:5}. Flow rate: 2.0 ml/minute. Detector: UV at 262 nm and 0.01 AUFS.
_______ Spiked ________ Blank

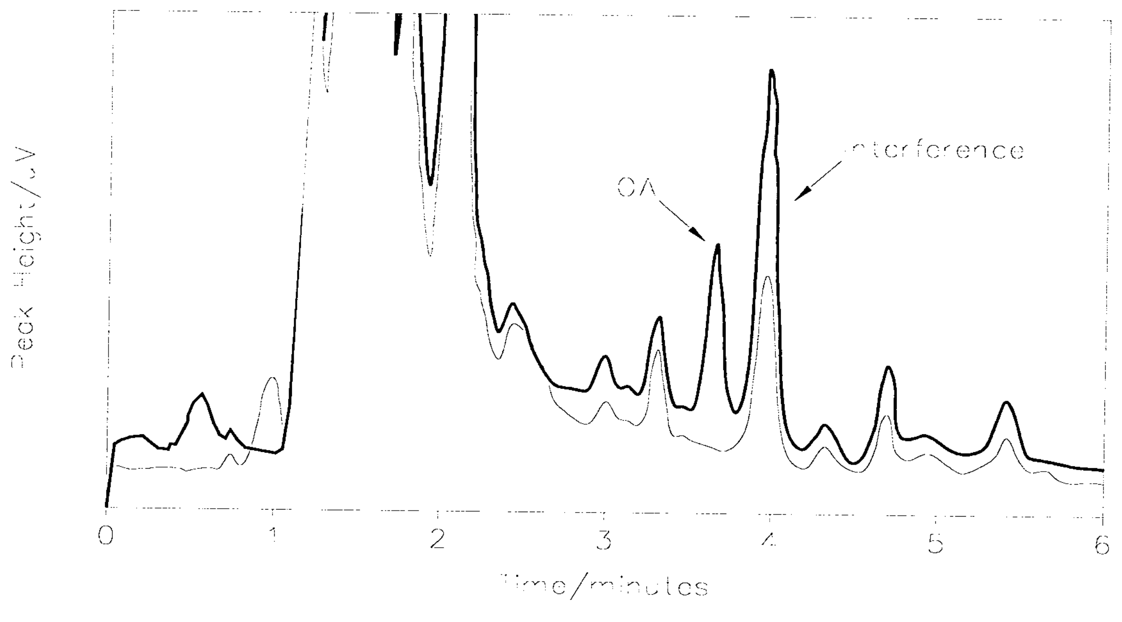
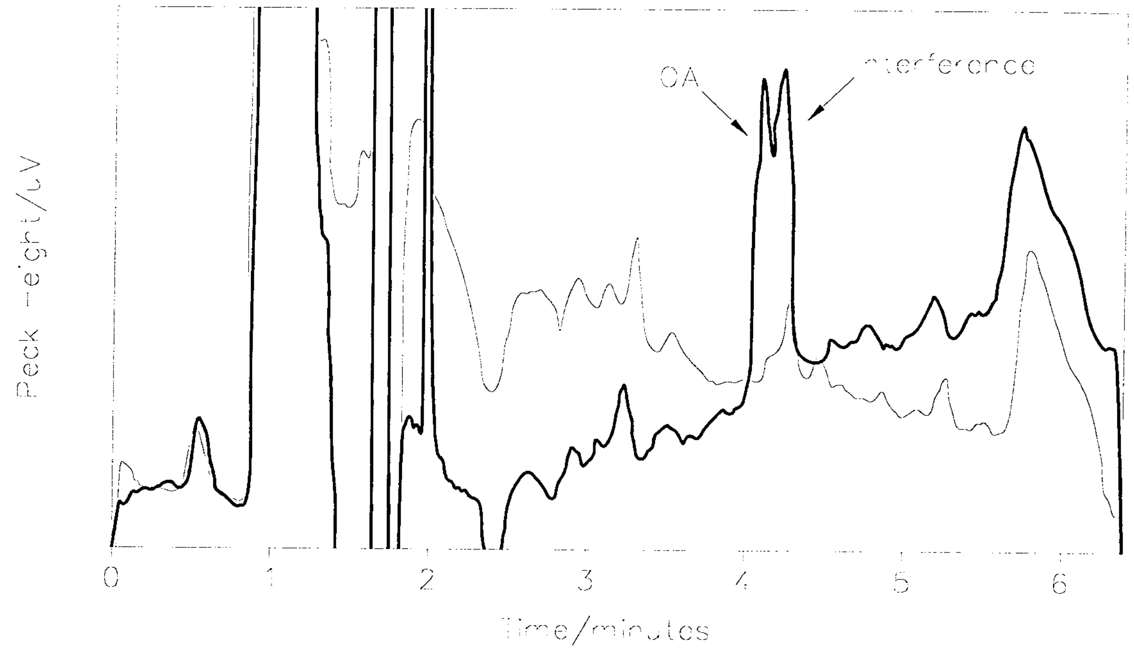


Figure 3.6b: Overlay of a blank tissue on a spiked tissue, 100µgkg^{-1}. Injection volume: 50µl. Column: Lichrospher -100 RP18 5µm (250 x 4.6) mm. Oven temperature: 35°C. Mobile phase: H_3PO_4 (0.01M): THF: IPA {78:17:5}. Flow rate: 2.0 ml/minute. Detector: UV at 262 nm and 0.01 AUFS.
_______ Spiked ________ Blank



μgkg^{-1}. Injection volume: 50μl. Column: Lichrospher -100 RP18 5μm (250 x 4.6) mm. Oven temperature: 35°C. Mobile phase: H_3PO_4 (0.01M): THF: EA {78:17:5}. Flow rate: 2.0 ml/minute. Detector: UV at 262nm and 0.01 AUFS.

_______ Spiked _______ Blank

From Figure 3.6a it can be seen that the matrix components from the blank tissue sample interfere with the analyte peak at 4.2 minutes, when using ETOH in the mobile phase. Therefore by examining the polarity index of solvents, Table 6-4 [15], IPA was seen to be slightly less polar than ETOH and was thus chosen as the next solvent to examine in the mobile phase. A chromatogram of an overlay of the spiked tissue sample on the blank tissue sample can be seen in Figure 3.6b. This shows a clear separation of the matrix components between 3.32 minutes and 3.97 minutes. The analyte peak can then be seen clearly at 3.66 minutes in the chromatogram of the spiked tissue sample. The resolution is visually acceptable and this is confirmed with an R_S value between the analyte peak and the nearest eluting matrix peak calculated to be equal to 1.22. The number of theoretical plates for the new column was found to be 3442, and this shows that this longer 250mm column is more suitable for the analysis of OA than the shorter 120mm column (N=1588). To investigate if the resolution could be improved even further. EA was examined in the mobile phase and the resulting chromatogram consisting of the overlay of the spiked tissue on the blank tissue sample can be seen in Figure 3.6c. This shows that the resolution between the analyte and the nearest eluting matrix peak is unacceptable as both peaks appear as a doublet with virtually no separation being achieved between them.

3.6 Final chromatographic conditions

The final chromatographic conditions are those which were used to obtain the chromatograms in Figure 3.6b. These conditions consist of the following:

Column: Lichrospher RP-18 (250 x 4.6) mm., 5μm particle size.

Guard column: Lichrospher RP-18 Endcapped (7.5 x 4.6) mm
5μm all guard cartridges.

Detector: UV at 262nm and 0.005AUFS.

Mobile phase: H_3PO_4 (0.01 M): THF: IPA in the ratio of 78:17:5.

Flow rate: 2.0 ml/minute.

Temperature: 35°C.

Injection volume: 50μl.

A standard OA peak which was obtained, while using these conditions is shown in Figure 3.7. This shows a peak of acceptable peak width and peak symmetry.

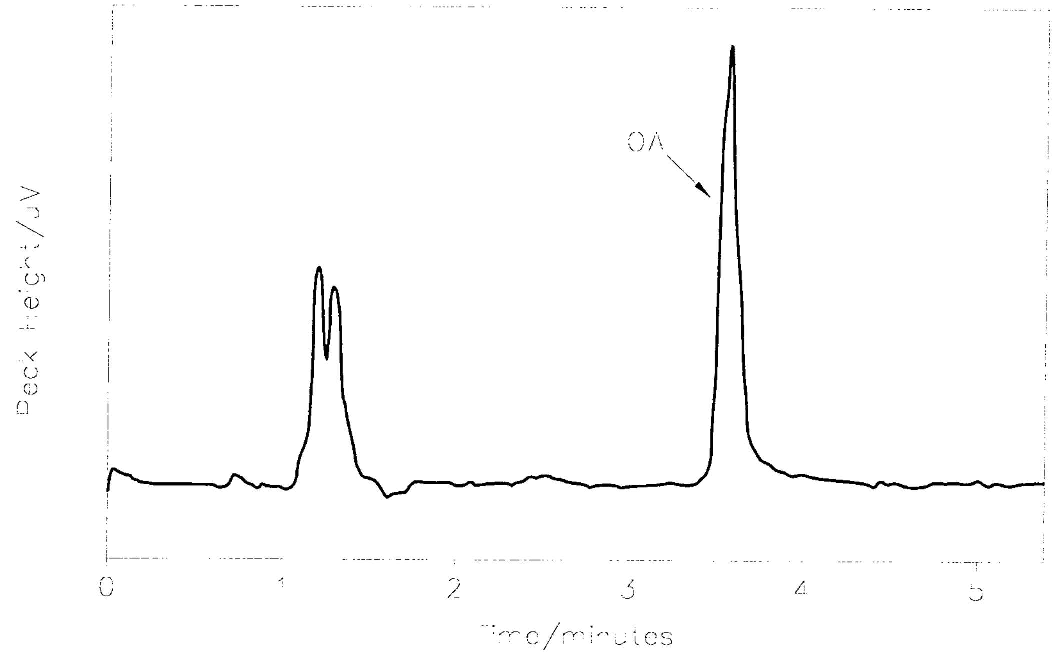


Figure 3.7: Chromatogram of a standard oxolinic acid solution, 0.1μgml^{-1}. Injection volume: 50μl. Column: Lichrospher -100 RP18 5μm (250 x 4.6) mm. Oven temperature: 35°C. Mobile phase: H_3PO_4 (0.01M): THF: IPA {78:17:5}. Flow rate: 2.0 ml/minute. Detector: UV at 262nm and 0.01 AUFS.

3.7 Method Validation

Method validation is a process of proving that an analytical method is acceptable for its intended purpose [13, 16]. Under Council Directive 96/23/EC routine methods must be validated, for the detection and quantification of veterinary drug residues in farmed fish, to the criteria specified in the Commission Decision 93/265/EEC [13]. Similar criteria have been specified by the Codex Committee on Residues of Veterinary Drugs in Foods [14], and these have been discussed in Section 1.5.1. The validation studies were performed during the actual development of the method so that the conditions could be proven to be satisfactory. The analytical conditions used for the validation study are specified in Section 3.6.

3.7.1 Specificity

Specificity is the ability of the method to accurately measure the analyte response in the presence of all the interfering components from the sample matrix.

It was examined in this method by spiking a wild salmon tissue sample, which did not contain any OA (Section 3.10) with a known quantity of analyte. This was analysed, the response was compared to that of a standard solution containing only the analyte, and a percentage recovery was calculated.

To test the robustness of the method a spiked tissue, with the known amount of analyte, was exposed to factors, which might affect the percentage recoveries of OA. These factors included heat and light. The spiked tissue sample was exposed to heat at 50°C, to attempt to reduce the analysis time by evaporating the chloroform to dryness at a temperature nearer that its boiling point (61.2°C). Additionally the sample was exposed to normal lighting conditions in the laboratory for 6 hours. Again these were analysed and compared to the standard solution. The results from these can be seen in Table 3.6.

Table 3.6: Comparison of the analyte standard peak, 0.1μgml^{-1}to a spiked tissue and a spiked tissue exposed to both heat and light

	Retention time (minutes)	Peak width (minutes)	A_S	Percentage recovery (n=3)
Analyte	4.53	0.51	1.06	
Spiked tissue	4.50	0.52	1.07	86.7 %
Heat (50°C)	4.50	0.54	0.83	79.1 %
Light (6 hours)	4.51	0.56	0.92	80.3 %

Consistent retention times and peak widths were obtained for the analyte and spiked tissue samples. The asymmetry values, (Section 2.1.2.5), of the analyte and the spiked tissue showed that an acceptable peak shape was obtained for both, while peak fronting was evident for the spiked tissues which was exposed to both heat and light. Also, a lower recovery of OA was obtained for the samples, which were exposed to heat and light, when compared to the spiked sample which was protected from the heat above 40°C and kept out of direct light. For this reason, the samples were protected from light during the analysis, and temperatures less than 40°C were used when evaporating the solvents to dryness.

As part of the specificity study other analytes which are listed in Directive 96/23/EC for routine residue testing in farmed finfish were tested under the analytical conditions used for OA as described in Section 3.6. These included oxytetracycline, sulphadiazine, sulphamethoxazole and trimethoprim. It was found that none of these analytes interfered in the chromatogram at the retention time of OA under the analytical conditions described in Section 3.6.

The specificity study evaluates the chromatographic conditions for a particular analysis. The study carried out here demonstrated that the conditions used as described in Section 3.6, are suitable for further validation studies.

3.7.2 Injection Repeatability and Linearity

To assess the performance of the chromatographic instrument an injection repeatability test was carried out on a standard solution by analysing five replicates from the same vial. Using a standard calibration plot the concentration of this solution was calculated based on peak height and area (Figures 3.8(A) and 3.9(A) respectively). The mean, standard deviation on the mean, and the percentage relative standard deviation were calculated for both height and area, and the comparison can be seen in Table 3.7.

Table 3.7: Repeatability for a standard solution comparing concentrations obtained based on peak height and peak area.

Vial Number	Concentration (μgml^{-1}) based on Peak Height	Concentration (μgml^{-1}) based on Peak Area
1	0.24	0.24
2	0.25	0.25
3	0.24	0.24
4	0.25	0.25
5	0.25	0.25
Mean	**0.25**	**0.24**
S.D.	**0.0025**	**0.0026**
% RSD	**1.05%**	**1.03%**

Table 3.7 shows %RSD values for repeatable injections to be approximately 1% for both peak height and area. Both indicate that the performance of the instrument is acceptable but to determine whether height or area will be used as the analyte response for further validation studies linearity was examined.

A linearity study checks if the sample solutions are in a concentration range where the analyte response is linearly proportional to concentration. The standard solutions were prepared and analysed by HPLC. A calibration plot based on peak height and area were plotted and examined.

Since the response factor plot is a more useful way of determining the linearity of a method these plots were prepared for both peak height and area. In the ideal situation an equivalent response would be obtained at each concentration, and the data points would form a straight line with a zero slope (Section 1.5.4.1). Table 3.8 shows the response factor for peak height and area of standard solutions while for spiked tissue samples this information can be seen in Table 3.9.

Linearity was further examined by plotting the y-residual against the predicted y-value for both height and area. For an ideal y-residual plot, where the random errors are normally distributed about zero, the slope and intercept of the best fit line would be equal to zero and the sum of the y-residuals would also equal zero [17].

Table 3.8: Response factor data for oxolinic acid standards showing peak height and area values

Concentration μgml^{-1}	Peak height	Response Factor	Peak Area	Response Factor
0	0		0	
0.048	1897	37858	19071	410028
0.098	3959	39549	40880	423104
0.251	9972	39872	103409	419358
0.491	18611	37973	189807	390280
0.989	38576	38961	407335	412894
	Mean	**38842**		**411133**
	SD	**908**		**12749**
	% RSD	**2.3**		**3.1**

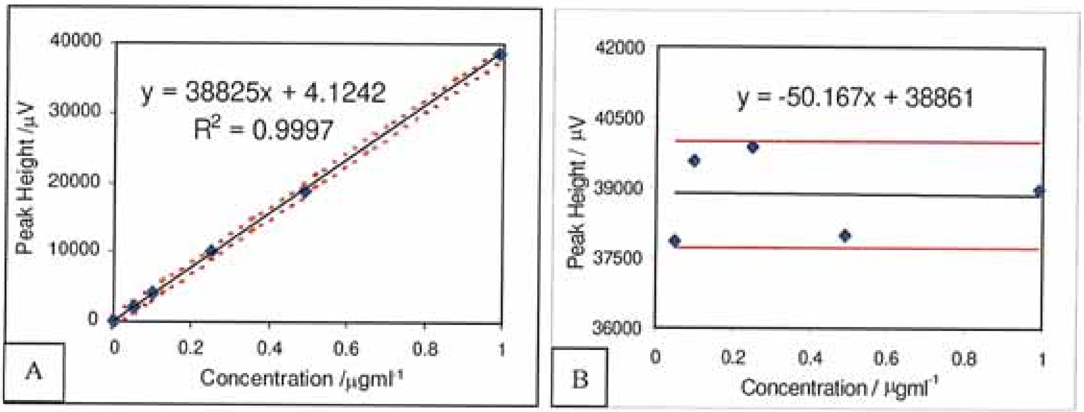


Figure 3.8: Calibration (A) and response factor (B) plots of peak height for oxolinic acid standard solutions showing the best fit lines and the upper and lower 95% confidence limits.

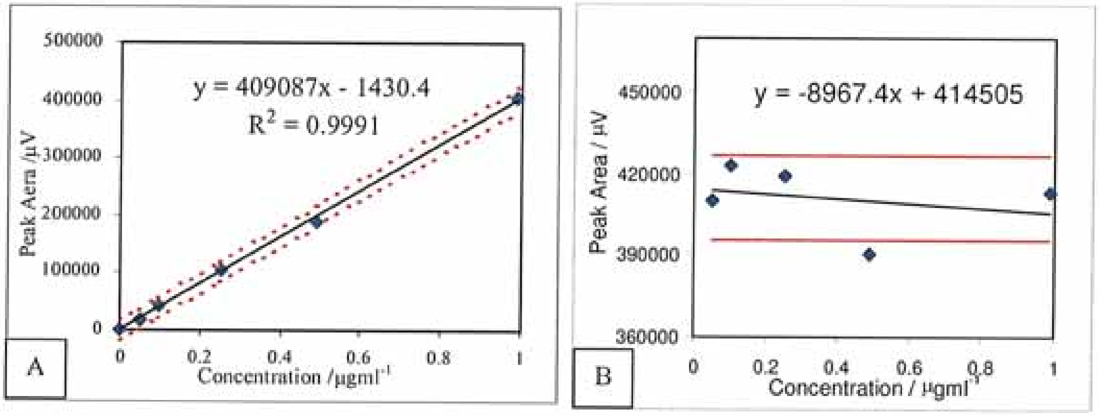


Figure 3.9: Calibration (A) and response factor (B) plots of peak area for oxolinic acid standard solutions showing the best fit lines and the upper and lower 95% confidence limits.

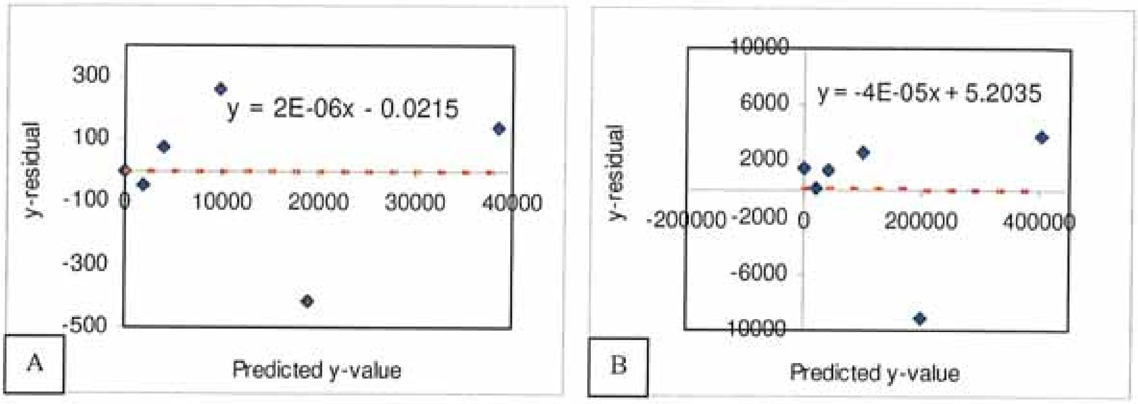


Figure 3.10: y- residual plot for peak height (A) and peak area (B) for oxolinic acid standard solutions.

The calibration plots show a linear response over the concentration range for both peak height and area. The R^2 value was 0.9997 for the peak height while the peak area response shows the R^2 value to be 0.9991. Ideally R^2 for a calibration plot would be equal to 1 indicating a good fit of the data points to the best-fit line for both plots.

The %RSD on the mean response factor values for peak height was 2.3% while the corresponding value for peak area was higher at 3.1%. More importantly however, since an equivalent response should be obtained at each concentration value the scatter of the values obtained about the mean and the slope of the best-fit line was examined. The response factor was plotted against concentration, and the ± 95% confidence limits were included on this plot, Figure 3.8(B) for height and 3.9(B) for area. For peak height the response factor values have a ±5.2% scatter about the mean, while for area this scatter is ±8.0%. If an equivalent response was obtained for each concentration value the data points would form a straight line parallel to the x-axis. Therefore 95% confidence intervals were calculated for the slope (Equation 1.4) to examine if the value obtained was significantly different from zero. The calculated values, which contain the value zero, ranged from (-4.4×10^3 to 4.3×10^3), for peak height and (-6.77×10^4 to 4.98×10^4) for peak area. Using these 95% confidence intervals the slope of the response factor plot was ($-5.02\times10^1 \pm 1.2\%$) and ($-8.97\times10^3 \pm 15.3\%$) for height and area respectively indicating a narrower range was obtained from the peak height data.

The sum of the y-residuals was found to be ($-1\times10^{-4} \pm 2.4\times10^2$) for height and ($1.1\times10^{-11} \pm 4.9\times10^3$) for area at a 95% level of confidence. Additionally in both cases the data points are randomly distributed about the x-axis (Figure 3.10). The 95% confidence intervals were calculated for the slopes and intercepts of these plots and these ranged from (-2.5×10^{-2} to 2.5×10^{-2}) and (-4.55×10^2 to 4.55×10^2) for peak height and (-4.85×10^{-2}

to 4.85x10^{-2}) and (-9.1x10^{3} to 9.1 x10^{3}) for area respectively. From these values the confidence intervals on the slope and intercept was ±0.01% based on peak height and ±0.1% based on peak area. This indicates a narrower spread in the response factor data for peak height when compared to peak area.

To determine the linearity for tissue samples as opposed to standard solutions, spiked tissue samples were analysed, and the relevant plots were examined.

Table 3.9: Response factor data for spiked tissue samples showing peak height and peak area values

Concentration μgkg^{-1}	Peak height	Response Factor	Peak Area	Response Factor
0	0	–	0	–
55.3	1013	20.9	11097	241.3
99.6	1551	17.0	17197	195.2
253.3	3968	16.2	38573	161.2
536.9	9376	17.7	100050	190.5
974.9	17042	17.7	184777	191.8
	Mean	**17.9**		**196.0**
	SD	**1.8**		**28.8**
	% RSD	**10.0%**		**14.7%**

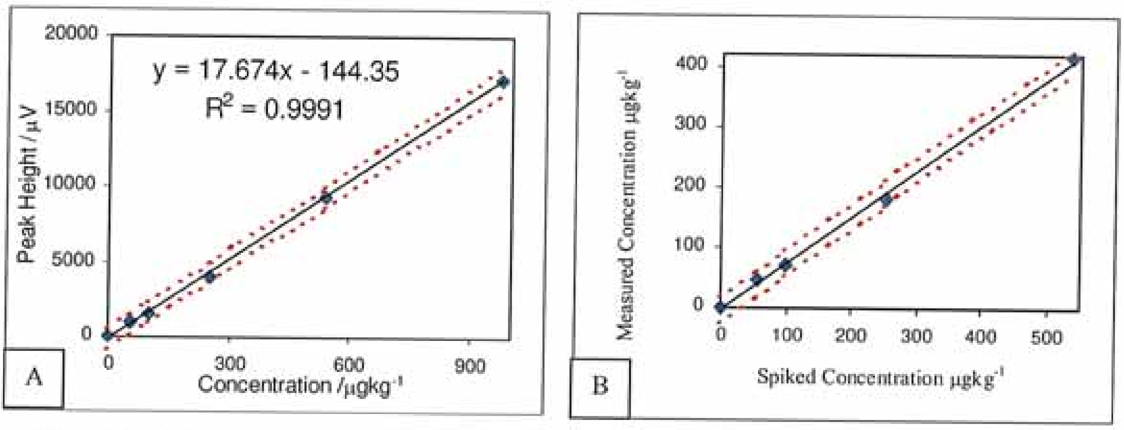


Figure 3.11: Calibration (A) and spiked against measured concentration (B) plots for peak height of tissue samples spiked with oxolinic acid showing the best-fit lines and the upper and lower 95% confidence limits.

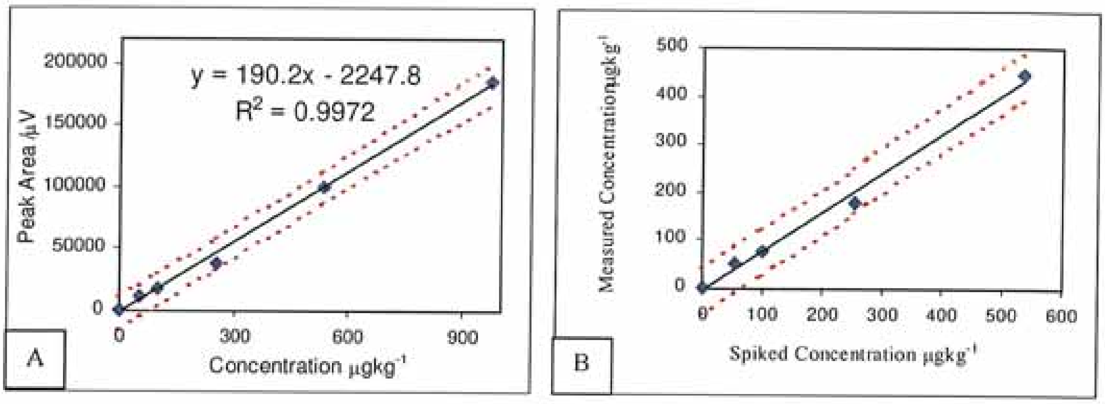


Figure 3.12: Calibration (A) and spiked against measured concentration (B) plots for peak area of tissue samples spiked with oxolinic acids, showing the best fit lines and the upper and lower 95% confidence limits.

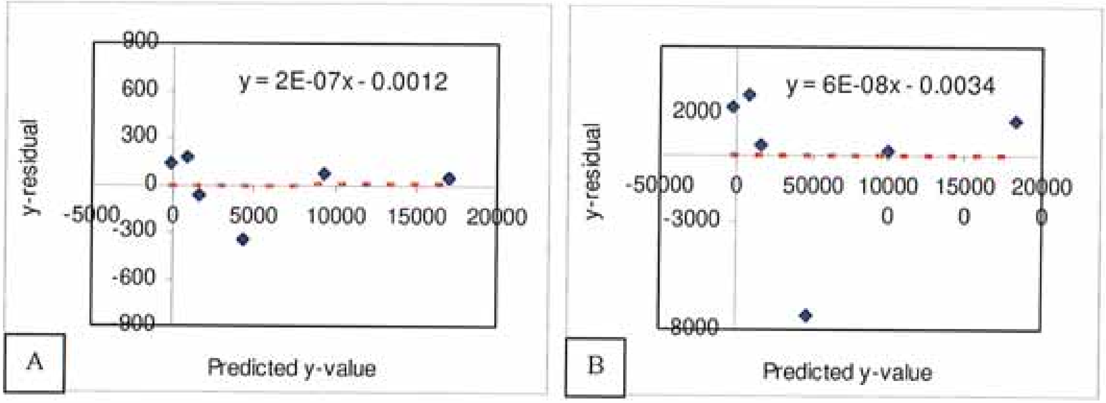


Figure 3.13: y- residual plot for peak height (A) and peak area (B) for tissue samples spiked with oxolinic acid.

The calibration plots show a linear response over the concentration range for both peak height and area. The R^2 value was 0.9991, Figure 3.11(A), for the peak height while the peak area response shows the R^2 value to be 0.9972, Figure 3.12(A). It is evident that the R^2 for height is closer to 1 than that for area indicating that the peak height gives a more linear response that that of peak area.

Spiked concentration was plotted against measured concentration for height and area as shown in Figures 3.11(B) and 3.12(B) respectively. Using the 95% confidence intervals from this plot, the confidence intervals at 250µgkg^{-1} were obtained for both peak height and area. These intervals were 190±20µgkg^{-1} for peak height and 202±47.5µgkg^{-1} for area. When the correction factor for percentage recovery (Section 3.7.4) was applied to these values the measured concentration for 250µgkg^{-1} spiked, was found to lie between 243-284µgkg^{-1} for peak height and 233.5-328.5µgkg^{-1} for peak area. This shows that peak height gives a narrower confidence interval about 250μgkg^{-1} (±10.5%) than peak area (±23.5%), indicating that height should be used for quantification.

The %RSD on the response factor values for peak height was 10.0% while the corresponding value for area was higher at 14.7%. In an ideal situation an equivalent response factor value would be obtained at each concentration, and the data points would form a straight line with a zero slope. The slope of the best-fit response factor line for height ranged from (-9.04×10^{-3} to 7.03×10^{-3}) while for area the range was (-1.55×10^{-1} to 1.09×10^{-1}) at a 95% level of confidence. These confidence intervals indicated a narrower spread in the results obtained for height (-1.01×10^{-1} ±12.5%) when compared to area (-2.32×10^{-2} ±17.5%)

The sum of the y-residuals was found to be ($-1\times10^{-5} \pm 2\times10^{2}$) for height and ($1\times10^{-5} \pm 3.9\times10^{3}$) for area at a 95% level of confidence. The slopes at a 95% level of confidence, of ($2.26\times10^{-7} \pm 4.61\times10^{-2}$) and ($5.87\times10^{-8} \pm 8.35\times10^{-2}$) for height and area respectively, indicate that the data points form a straight line parallel to the x-axis. The near zero intercepts of ($-1.24\times10^{-3} \pm 3.75\times10^{2}$) and ($-3.44\times10^{-3} \pm 7.3\times10^{3}$) for height and area respectively indicate that the points are randomly distributed about the x-axis (Figure 3.13).

From the above study, peak height was chosen as the method of quantification for OA in tissue samples.

3.7.3 Linear Range

To study the linear range of the analysis, standard solutions over a wide concentration range, plus a blank, were prepared and evaluated by using the chromatographic conditions determined in the specificity studies.

From Figure 3.14, the dynamic range of the method can be clearly seen. At the higher concentrations the response deviates from linearity and the point at which this occurs is taken as the upper limit of the dynamic range. The dynamic range of the method extends from 0.01-15μgml^{-1}, which corresponds to 3-5000μgkg^{-1} for tissue samples.

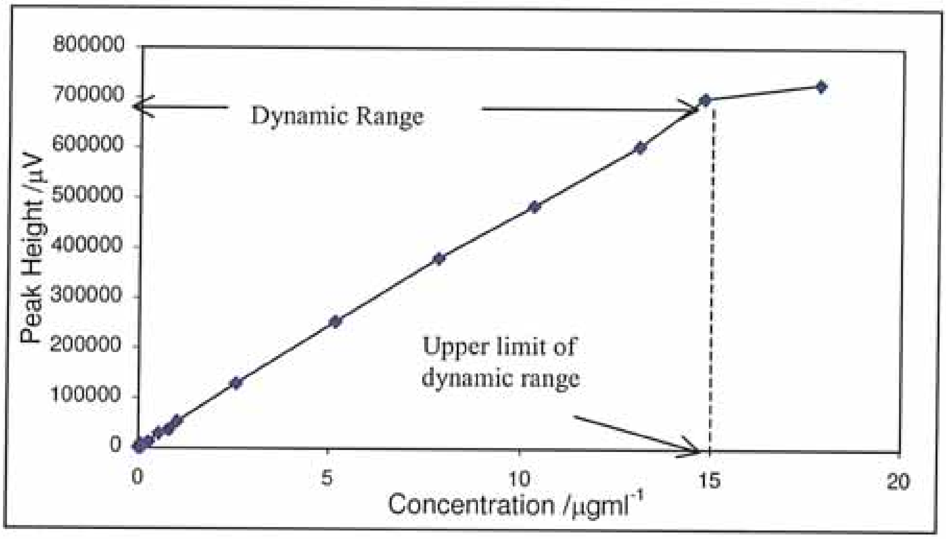


Figure 3.14: Calibration plot of standard solutions of oxolinic acid, diluted in the mobile phase, over a concentration range, which shows the dynamic range of the method. Analytical conditions: Section 3.6.

To find the linear range of the method, a response factor plot (Section 1.5.4.1) was prepared using the data from Figure 3.14. If an equivalent response is obtained for each concentration value, the data points would form a straight line with zero slope. This zero or near zero slope indicated that a linear response was obtained over that concentration range. However, due to the uncertainty of the response factor values at the higher and lower concentrations the linear range was set based on the maximum response factor value obtained (S_{max}) over a defined concentration range. While this is not ideal, the method helps to give a clearer indication of the range of values, which would give a linear calibration plot.

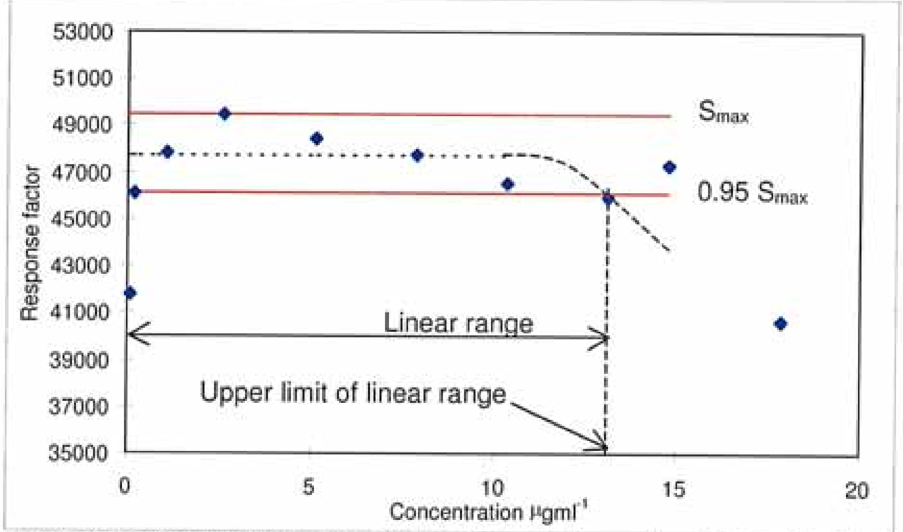


Figure 3.15: Response factor plot of standard solutions of oxolinic acid, diluted in the mobile phase, over a concentration range, which shows the linear range of the method. Analytical conditions: Section 3.6.

The upper line in Figure 3.15 is the maximum response factor value obtained (S_{max}) from the data in Figure 3.14, while the lower line is 95% of this value. All points on the plot must fit within these two lines if the corresponding concentration value is in the acceptable linear range of the method. Thus the linear range was set at the mean response factor value ± 2.5%. The upper limit of linearity is taken as the intersection point of the response factor line and the $0.95S_{max}$ line. From this plot the upper limit of linearity was 13.0μgml^{-1} and the linear range was found to extend from 0.04-13.0μgml^{-1} or 12-3900μgkg^{-1}.

3.7.4 Calculations

Tissue sample weight:	*2.973g*
Concentration of solution used to spike the tissue:	*0.989μgml^{-1} in ACN*
Weight of spike added:	*0.130g*
Density of ACN:	*0.782gml^{-1} at 20°C*

The volume of ACN containing OA, added to the tissue was calculated:

$$(0.130 \div 0.782) \times 1000 = 166\mu l$$

166μl of a 0.989μgml^{-1} solution of OA in ACN was added to the tissue matrix. Therefore the weight of OA added to the tissue was calculated as follows:

$$(166 \times 0.989) \div 1000 = 0.164\mu g \text{ of OA added to the tissue matrix.}$$

Initial weight of tissue was 2.973g, therefore the concentration of OA in the tissue sample is:

$$1000 \times 0.164\mu g \div 2.973g = 55.3\mu g kg^{-1}$$

The height response obtained for this sample was *1013*.

A standard plot for OA standards dissolved in the mobile phase was obtained Figure 3.8(A) and the equation of this plot was:

$$y = 38825x + 4.124 .$$

$$R^2 = 0.9997.$$

The response obtained for the spiked tissue sample was put into the equation of the standard plot:

$$1013 = 38825x + 4.124$$

$$x = 0.026\mu g ml^{-1}$$

A factor which takes into account the volumes, which were used in the sample preparation procedure, was calculated as follows:

Final weight: 0.745g of 0.01M oxalic acid.

Density of 0.01M aqueous acid is 0.983gml^{-1} at 20°C.

Therefore the final volume is:

$$\frac{0.745}{0.983} = 0.757ml$$

4ml of 16mls were evaporated to dryness.

5ml of 7mls were initially taken into the cleanup procedure.

Factor, F:

$$\left(\frac{16}{4}\right) \times \left(\frac{7}{5}\right) \times \left(\frac{0.745}{0.983}\right) = 4.24ml$$

To find the quantity of OA, which was recovered from the salmon tissue sample, the factor, F was multiplied by the concentration found from the equation for the standard plot.

$$0.026\mu g ml^{-1} \text{ x } 4.24ml = 0.108\mu g \text{ recovered}$$

The percentage recovery was then calculated:

$$\left(\frac{0.108}{0.164}\right) \times 100 = 66\% \text{ recovery.}$$

However when the water content of the fish tissue was taken into consideration, Section 3.3.1.4, the factor, F was calculated to be a different value:

$$\left(\frac{16}{4}\right) \times \left(\frac{8.7}{5}\right) \times \left(\frac{0.745}{0.983}\right) = 5.27\,ml$$

The quantity of OA recovered was therefore:

$$0.026\mu g ml^{-1} \times 5.27ml = 0.135\mu g \text{ recovered}$$

The percentage recovery was therefore:

$$\left(\frac{0.135}{0.164}\right) \times 100 = 82.1\% \text{ recovery}$$

3.7.5 Precision

The precision of an analytical method is the amount of scatter in the results obtained from multiple analyses of a homogeneous sample. To be meaningful the precision study must be performed using the exact sample and standard preparation procedures that will be used in the final method. Precision is expressed as the relative standard deviation, which shows the variability as a function of concentration of the analyte in the original sample.

This data was obtained by repeatedly analysing, in one laboratory on one day, aliquots of a homogeneous sample, each of which had been independently prepared according to the method (Section 3.4 and 3.6). Five separate tissue samples were spiked to $100\mu gkg^{-1}$ and each was analysed separately. The recoveries obtained are shown in Table 3.10.

Table 3.10: Repeatability test. Tissue samples spiked to a level of $100\mu gkg^{-1}$

Sample number	**Concentration measured not corrected for percentage recovery (μgkg^{-1})**
1	75.0
2	74.4
3	72.7
4	71.7
5	71.8
Mean	**73.1 μgkg^{-1}**
SD	**1.5**
% RSD	**2.1 %**

The 95% confidence limits were calculated for the measured concentration values and a plot of data points about the mean value showing the upper and lower 95% limits can be seen in Figure 3.16.

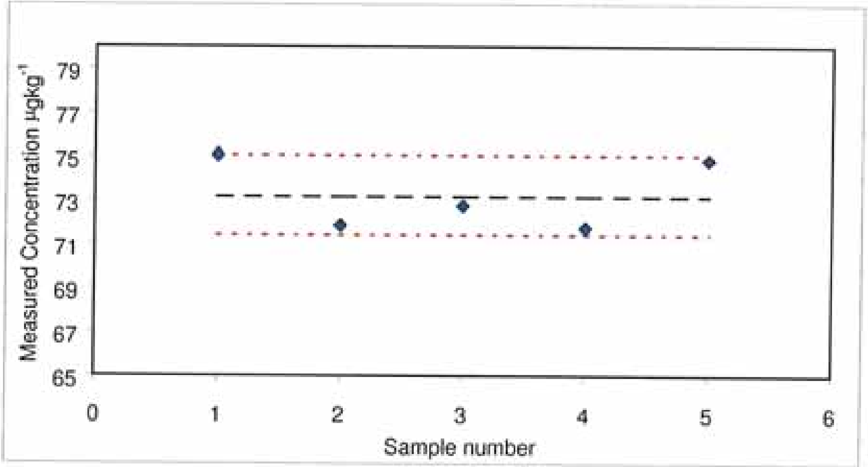


Figure 3.16: Repeatability test for 5 replicates of a tissue sample spiked to 100µgkg^{-1}. The upper and lower dashed lines correspond to the 95% confidence limits while the dashed line in the centre is the mean measured value.

The distribution of the points obtained about the mean value in Figure 3.16 is 73.1±2µgkg^{-1}. The results show that while the percentage recovery of the method is relatively low, the intra-assay precision, at 100µgkg^{-1}, is satisfactory within 95% confidence limits. This is confirmed by the fact that 2.1% RSD value was obtained for the five separate analyses.

The codex guidelines quote acceptable %RSD values of ≤15% for a tissue sample spiked to 100µgkg^{-1}. This is considerably higher than the 2.1% RSD obtained for OA at 100µgkg^{-1}, in this method. It should be noted that the values recommended by the Codex Committee take into consideration the wide variety of methods, analytes and test matrixes and are usually applied in a broad base residue control programme [14]. The EU guidelines 93/256/EEC only quote acceptable precision criteria for CRMs and these do not exist for OA in salmon tissue [13].

From the above precision study it was concluded that, when a sample containing 100µgkg^{-1} is analysed according to the method described (Section 3.4 and 3.6), we can be 95% confident that between 71% and 75% of the total amount of analyte present in the sample will be recovered. The final result can then be corrected for this recovery.

3.7.6 Accuracy

The accuracy of a method is the closeness of a measured value of the sample, to the true value for the sample. Accuracy studies in this method were based on the recovery of known amounts of analyte spiked into the sample matrix (Section 1.5.5). Although high percentage recoveries were preferable, regardless of what average recoveries were observed recovery with low variability was desirable.

To examine the accuracy of the method replicate analyses of calibration plots spiked at five different concentration levels plus a blank were brought through the cleanup procedure and analysed. Precautions were taken to ensure that the spiked sample was homogeneous and that it was in the same or close to the same physical and chemical state as the analyte would be in the unknown sample. This was achieved by taking 3g of a homogeneous tissue sample and spiking it with OA. The spiked tissue was allowed to stand for 30 minutes before the extraction procedure was commenced. The replicates were run in random order, and on different days to subject the method to whatever uncontrolled variables, which may have been operating. The same analyst carried out the analyses on all replicates to eliminate between analyst variations.

Percentage recoveries shown in Table 3.11 were calculated for each analysis.

Table 3.11: Percentage recoveries for tissue samples spiked in triplicate at five different concentration levels within the concentration range of 12-3900μgkg^{-1}

Analysis 1		Analysis 2		Analysis 3	
Concentration (μgkg^{-1})	**Percentage Recovery**	**Concentration (μgkg^{-1})**	**Percentage Recovery**	**Concentration (μgkg^{-1})**	**Percentage Recovery**
0	-	0	-	0	-
55.3	82.0	52.50	72.4	50.2	70.1
99.6	70.6	98.40	70.8	99.6	70.6
253.3	71.7	247.6	70.3	501.1	76.9
536.9	77.4	547.7	79.1	999.4	73.3
974.9	76.9	996.7	77.2		

The percentage recoveries obtained were above 70% at all concentration levels examined (Table 3.11). These values lie within the acceptable range of the suggested accuracy requirements, for spiked tissue samples as set out in the Codex Alimentarius [14] and discussed in Section 1.5.5.

To test the precision of the percentage recoveries obtained in Table 3.11, the upper and lower 95% confidence limits were calculated and the plots for each analysis can be seen in Figure 3.17.

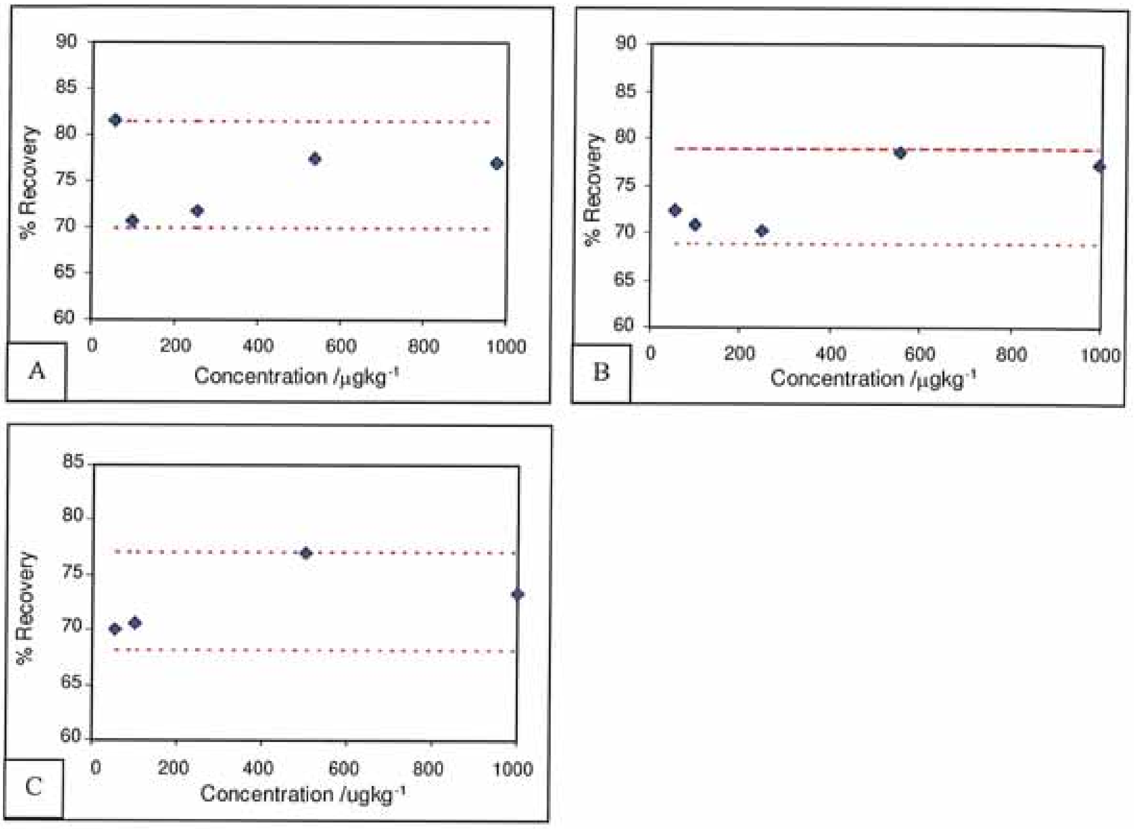


Figure 3.17: Percentage recoveries for Analysis 1 (A), Analysis 2 (B), and Analysis 3 (C), Table 3.11. The upper and lower 95% confidence limits are indicated by (-----)

Using the 95% confidence limits from Figure 3.17, the recoveries obtained for tissue samples spiked over the concentration range of 50-1000μgkg^{-1} were 75.7±5.5%, 73.9±5%, and 72.7±4.5% for analysis 1, 2 and 3 respectively. Therefore at 95% confidence for this method, samples with an OA concentration over the range of 50-1000μgkg^{-1} can be analysed to an accuracy of 74.1% and a precision of 5%. The percentage recovery does not appear to vary significantly over the concentration range studied indicated by the approximate linear response v's concentration plots obtained (Figure 3.18(A) to 3.20(A), Section 3.7.7).

3.7.7 Range and Limits of Detection and Quantification

The range of an analytical method is defined as the concentration interval over which acceptable accuracy, linearity and precision are obtained. The linear range was found to extend from 0.04-13.0μgml^{-1} corresponding to 12-3900μgkg^{-1} (Section 3.6.3). The accuracy and precision over the concentration range of 50-1000μgkg^{-1} was examined and it was found that samples could be analysed to an accuracy of 74.1% and a precision of ±5% at 95% confidence (Section 3.7.6). Therefore in order to evaluate the range of the method the linearity of spiked tissue samples needed to be examined. Triplicate tissue samples spiked at 5 different concentration levels plus a blank, were prepared according to the method described (Section 3.4 and 3.6). Data is shown in Table 3.12 while the peak height plotted against concentration can be seen in Figures 3.18(A) to 3.20(A).

Table 3.12: Response factor data for three separate analyses of spiked tissue samples

Analysis 1		Analysis 2		Analysis 3	
Concentration (μgkg^{-1})	**Response factor**	**Concentration (μgkg^{-1})**	**Response factor**	**Concentration (μgkg^{-1})**	**Response factor**
0	-	0	-	0	-
55.3	20.9	52.50	19.5	50.2	14.3
99.6	17.0	98.40	17.8	99.6	14.6
253.3	16.2	247.6	16.2	501.1	16.2
536.9	17.7	547.7	17.8	999.4	15.4
974.9	17.7	996.7	17.8		
Mean	**17.9**		**17.8**		**15.1**
SD	**1.8**		**1.2**		**0.9**
% RSD	**10.0%**		**6.5%**		**5.6%**

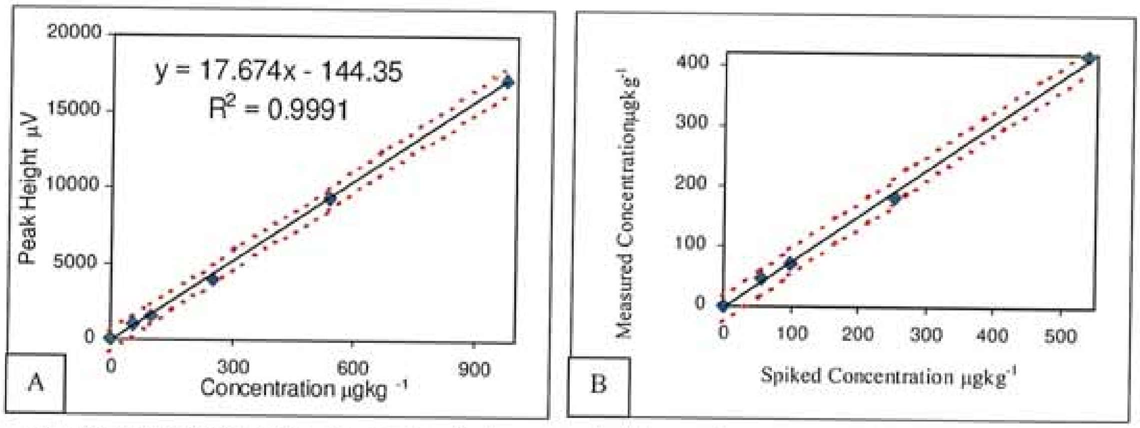


Figure 3.18: Calibration (A) and spiked concentration against measured concentration (B) plots for spiked tissue samples, Analysis 1, showing the best fit and upper and lower 95% confidence lines.

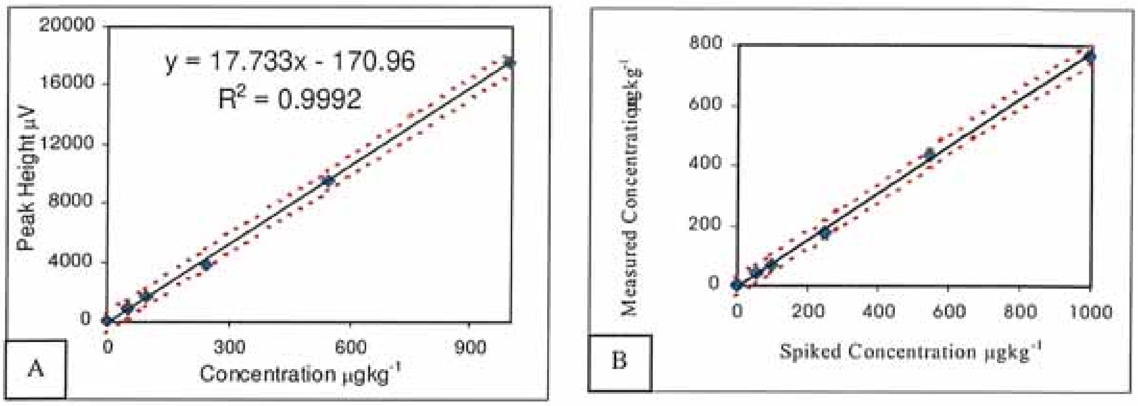


Figure 3.19: Calibration (A) and spiked concentration against measured concentration (B) plots for spiked tissue samples, Analysis 2, showing the best fit and upper and lower 95% confidence lines.

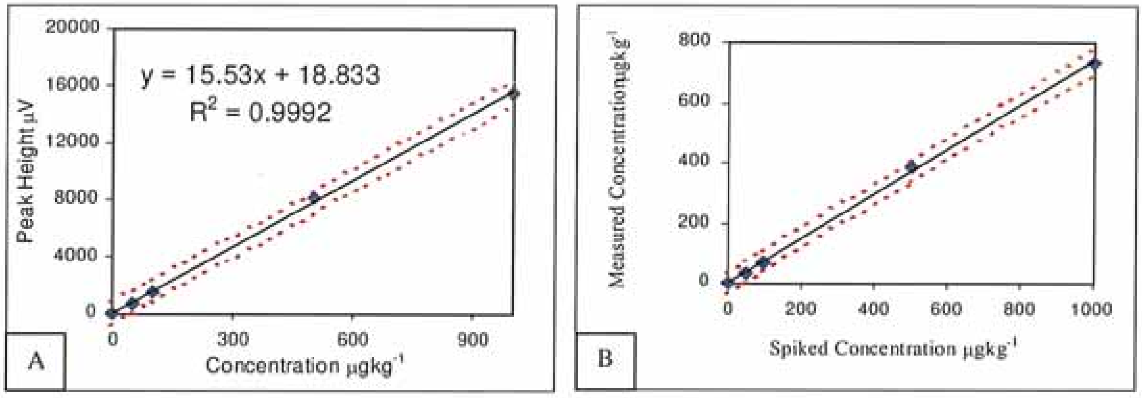


Figure 3.20: Calibration (A) and spiked concentration against measured concentration (B) plots for spiked tissue samples, Analysis 3, showing the best fit and upper and lower 95% confidence lines.

From the calibration plots of peak height against concentration for spiked tissue samples, a straight line with acceptable correlation coefficients of 0.9991, 0.9992 and 0.9993 were obtained for analysis 1, 2 and 3 respectively (Figure 3.18(A), 3.19(A) and 3.20(A)). From the plot of spiked concentration against measured concentration over the range of 50-1000µgkg^{-1}, the 95% confidence intervals around 250µgkg^{-1} were calculated. These were corrected for percentage recovery, and intervals at 95% confidence were found to be 264±20µgkg^{-1}, 264±28µgkg^{-1} and 259±33µgkg^{-1} for analysis 1, 2 and 3 respectively. This indicates that linearity was obtained over the range of 50-1000µgkg^{-1} for spiked tissue samples.

The linearity of the method for spiked tissue samples was further examined using the response factor plots and these can be seen in Figures 3.21(A-C). The 95% confidence limits were calculated and the distribution of the values about the mean were ±8.8%,

±4.5%, and ±6.2% for all analysis 1, 2 and 3 respectively. The near zero slopes of (-1.1×10^{-3} ±12.5%), (-5.4×10^{-4} ±10.3%) and (1.2×10^{-3} ±36.7%) of the response factor plots at a 95% level of confidence, indicate that a linear response was obtained over the concentration range examined for each analysis.

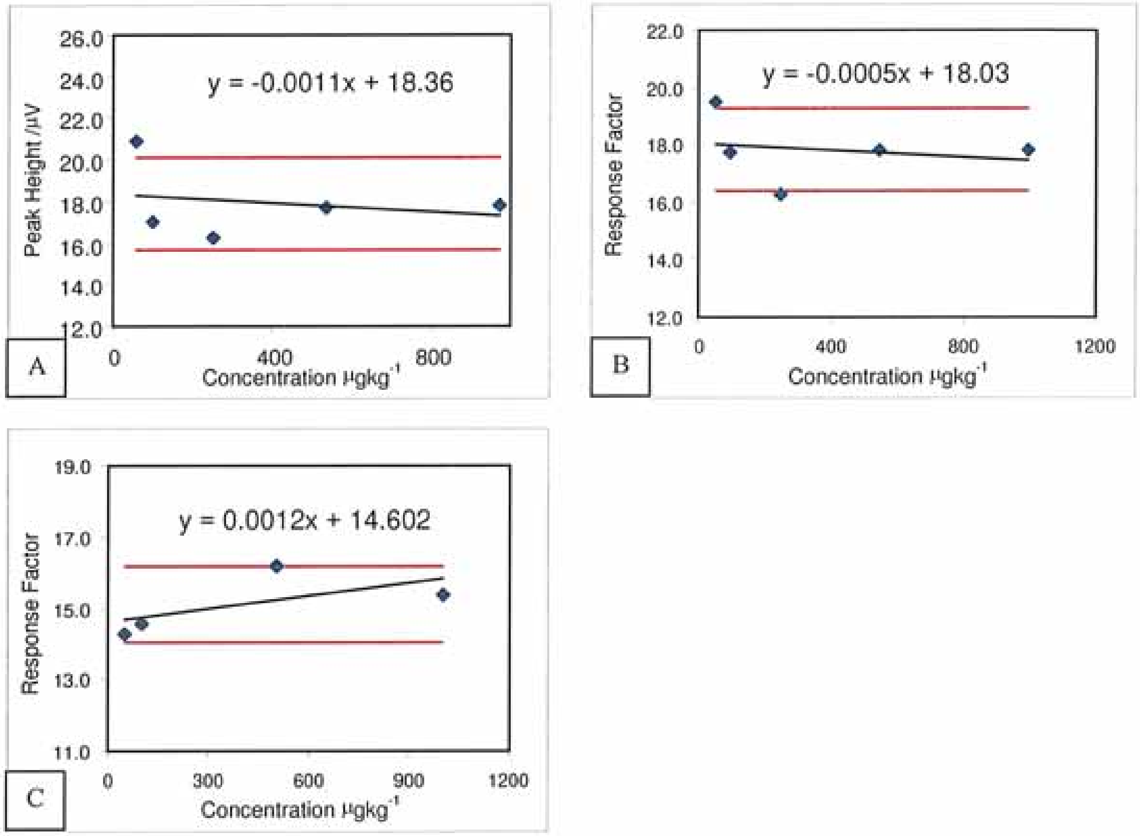


Figure 3.21: Response factor plots for spiked tissue samples over the concentration range of 50-1000µgkg^{-1} for Analysis 1(A), 2(B) and 3(C) respectively.

A third method was used to examine the linearity of the method. This involved calculating the y-residuals for the three separate analyses and the plots can be seen in Figure 3.22(A, B and C) for analysis 1, 2 and 3 respectively.

Table 3.13: y-residual data for 3 separate analyses

Analysis 1		**Analysis 2**		**Analysis 3**	
Height	**y-residual**	**Height**	**y-residual**	**Height**	**y-residual**
0	-4	0	171	0	-19
1013	-48	852	92	736	-62
1551	72	1576	2	1472	-94
3968	262	3842	-371	8127	326
9376	-417	9582	41	15389	-151
17160	135	17568	65		
Sum	**0**	**Sum**	**0**	**Sum**	**0**

The slopes at a 95% level of confidence of the best-fit lines for the y-residual plots, (Figure 3.22(A-C)) of (1.77×10^{-6} ±2.52×10^{-2}), (-1.24×10^{-9} ±4.44×10^{-2}) and (2.15×10^{-9} 5.26×10^{-2}) indicate that the best-fit lines are parallel to the x-axis. (The slopes are not significantly different from zero at a 95% level of confidence). The intercepts of (-2.15×10^{-2} ±4.55×10^{2}), (2.36×10^{-5} ±3.71×10^{2}) and (-1.11×10^{-5} ±4.11×10^{2}), which do not differ significantly from zero indicate that the points are randomly distributed about the x-axis. Additionally the sum of the y-residuals is zero for each analysis (Table 3.13) indicating that the data points form a linear calibration plot over the concentration range examined.

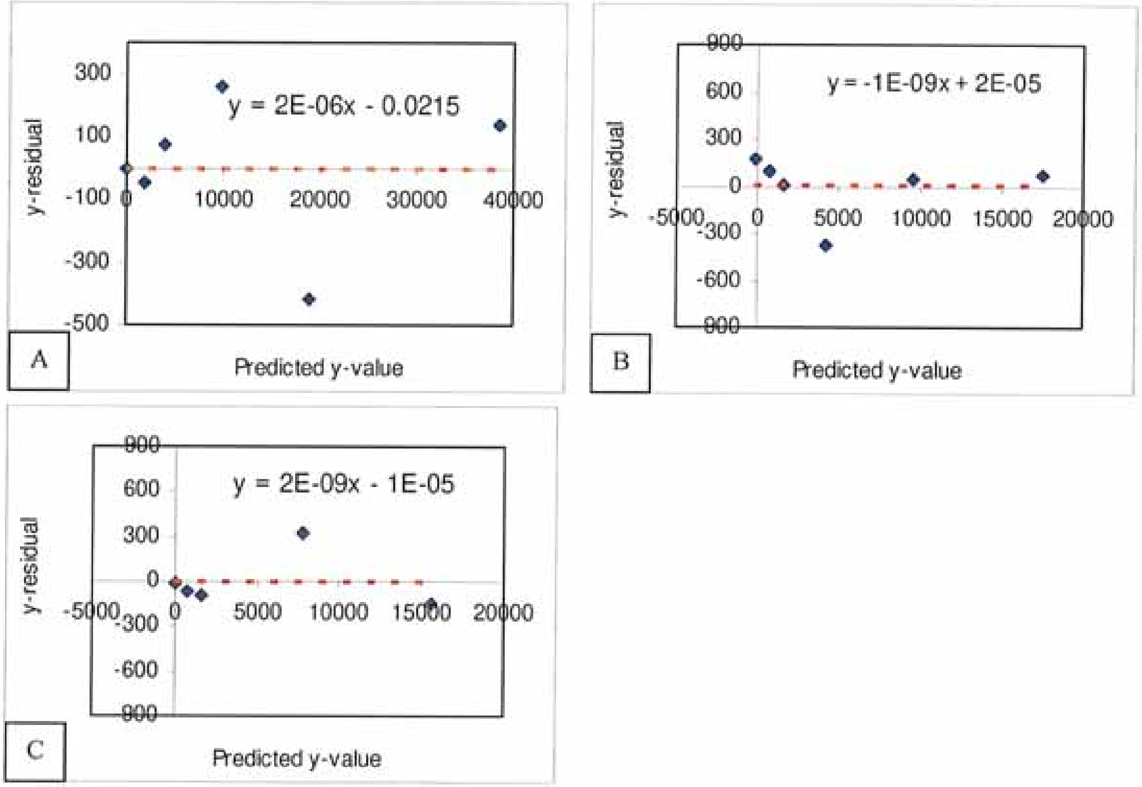


Figure 3.22: y-residual plots for spiked tissue samples for analysis 1 (A), analysis 2(B) and analysis 3(C).

The studies carried out here show that linearity was obtained for spiked tissue samples over the concentration range of 50-1000μgkg^{-1} for three separate analyses carried out independently of each other. However, a lower response was obtained for the samples analysed in analysis 3 when compared to those of analysis 1 and 2. This is indicated by the mean response factor of 15.1 for analysis 3, compared to 17.9 and 17.8 for analysis 1 and 2 respectively (Table 3.12). The lower slope values obtained for the calibration and response factor plots verifies that the response obtained for analysis 3 was lower than that obtained for analysis 1 and 2. This is most probably due to the fact that the three analyses were carried out under separate conditions over a period of three weeks. Slight changes in the mobile phase composition can cause a peak to be either sharper or broader depending on the organic content. Since peak height was used for quantification in this method these changes would have an effect on the response recorded. Standards analysed with the spiked tissue samples in analysis 3 also gave a lower response when compared to those analysed with analysis 1 and 2 indicating that the mobile phase was the most likely cause. This lower response however, did not have a significant effect on the linearity of the method as was shown above.

The limit of detection (LOD) (Section 1.5.7) was estimated by measuring the response obtained for blank tissue samples (n=3) and calculating the LOD based on a S/N = 3. This value was subsituted into the equation of the calibration line to estimate the corresponding concentration value. The equation of the calibration line was $y = 17.67x - 144.35$ and from the information below the LOD was estimated to be 10μgkg^{-1}.

Sample number	**Noise Response**	**3 x noise**	**5 x noise**
1	10	30	50
2	12	36	60
3	11	33	55
Mean	11	33	55
Concentration /μgkg^{-1}		**10**	**11.3**

The limit of quantification (LOQ) (Section 1.5.8) of the method was taken as the S/N = 5. From the data shown this value was estimated to be 11.3μgkg^{-1}. This is the lowest analyte content, which can be quantified as opposed to identified using the above method.

3.7.8 Stability

For routine testing in which many samples are analysed each day, it is often necessary to allow for delays such as instrument breakdowns or overnight analyses. Therefore, the limit of stability of samples, standards and the mobile phase should be tested, and storage conditions identified so that the stability can be improved.

3.7.8.1 Stability of mobile phase

A standard solution of OA was freshly prepared on three consecutive days and analysed on each of these days using the same mobile phase, which was kept in the fume hood of the laboratory for the test period. The concentration of this standard was calculated using a calibration plot of freshly prepared standards on each day of the test. The peak retention time, response and shape were all noted and the observed results can be seen in Table 3.14.

Table 3.14: Stability of the mobile phase over a three-day period

	Retention time (minutes)	**Peak width (minutes)**	**Concentration μgml^{-1}**	**A_S**
Day 1	4.50	0.52	0.99	1.07
Day 2	4.65	0.93	0.98	1.32
Day 3	4.67	0.95	0.97	1.33

Over the three-day period the retention time increased by 0.17 minutes while the peak response decreased by ≈2.0%. The peak shape altered notably with the peak width increasing by 0.43 minutes, and an asymmetry factor of 1.33 showed that a considerable peak tail had developed. The retention and peak width increased while the peak height decreased indicating that some of the organic component of the mobile phase had possibly evaporated off while it was stored in the fume hood over the three days. Therefore it was concluded that the mobile phase should be freshly made up each day prior to the analysis of OA in fish tissue samples.

3.7.8.2 Reproducibility

Reproducibility is the precision relating to measurements made under reproducible conditions, i.e.: same method; different operator; different laboratories; different equipment and a long time period. In this method validation however, it was not feasible to carry out the complete reproducibility study and therefore, a spiked tissue calibration plot was analysed on three consecutive days, using a freshly prepared mobile phase on each of the three days. The calibration and spiked tissue against measured concentration plots are shown in Figures 3.23 to 3.25 for day 1, 2 and 3 respectively.

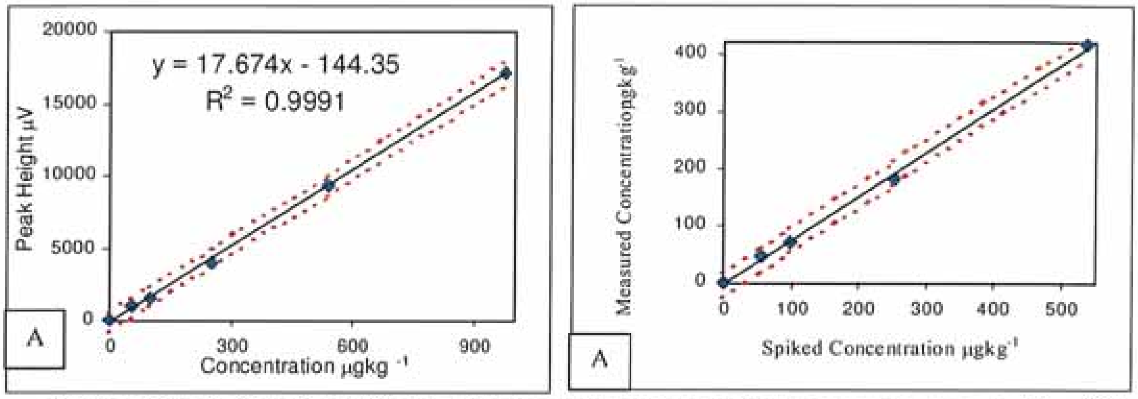


Figure 3.23: Calibration (A) and spiked concentration against measured concentration (B) plots for spiked tissue samples, Day 1, showing the best fit and upper and lower 95% confidence lines.

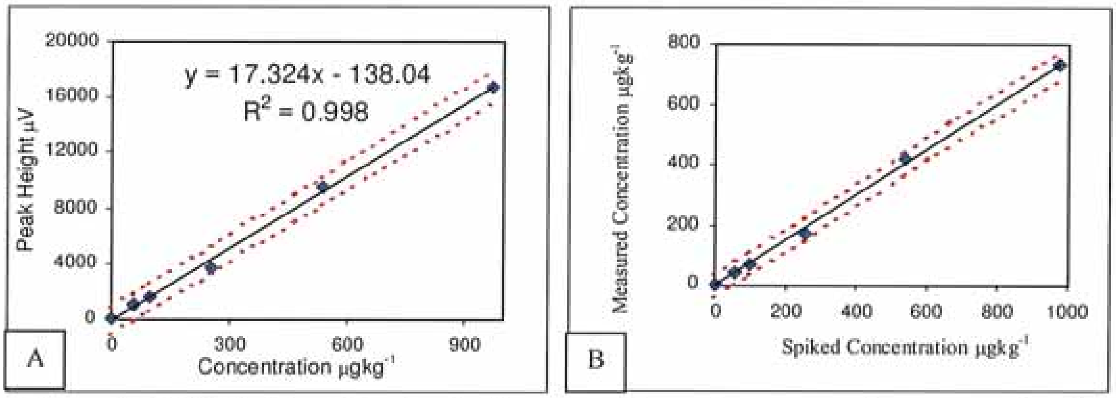


Figure 3.24: Calibration (A) and spiked concentration against measured concentration (B) plots for spiked tissue samples, Day 2, showing the best fit and upper and lower 95% confidence lines.

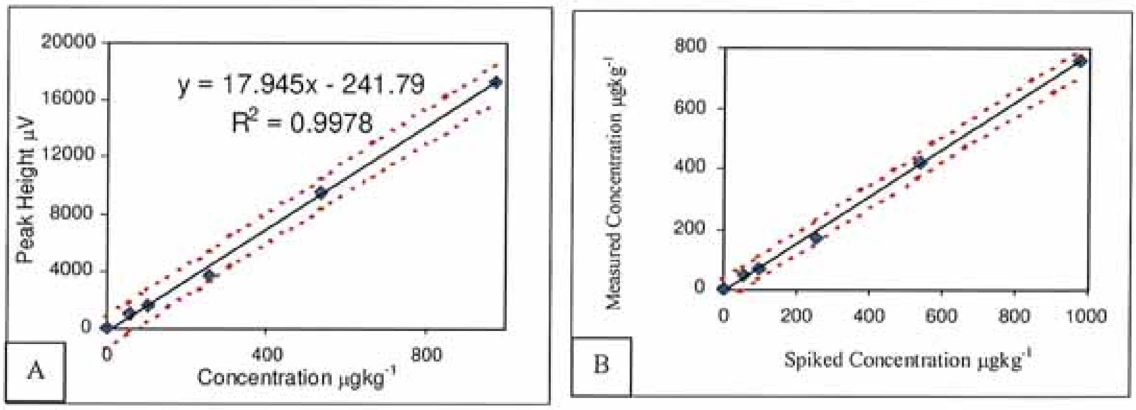


Figure 3.25: Calibration (A) and spiked concentration against measured concentration (B) plots for spiked tissue samples, Day 3, showing the best fit and upper and lower 95% confidence lines.

The R^2 values on the calibration plots decrease from 0.9991 on day 1 to 0.998 and 0.9978 on day 2 and day 3 respectively. Since ideally this value would be equal to 1 then the values obtained indicate that over time there is an increase in the scatter of the points about the line although the response still appears to be essentially linear. To get a better idea if this deviation is significant the 95% confidence intervals about 250μgkg^{-1} were examined using the spiked v's measured concentration plots (Figure 3.23(B), 3.24(B) and 3.25(B). A correction factor for percentage recoveries was applied to these values and the confidence intervals were found to be 250±20μgkg^{-1}, 245±38μgkg^{-1} and 247±38μgkg^{-1} for day 1, 2 and 3 respectively. The confidence intervals increased between days 1 and 2 but remained constant on the third day of analysis.

The measured concentrations were lower than the spiked concentrations (Table 3.15) but these values can be corrected by allowing for the percentage recoveries obtained (Section 3.7.6). Therefore the difference in the responses obtained over the three days appears not to be significant. To confirm this however, the sensitivity of the method was examined by comparing the slopes of the linear regression line on the calibration plots (Equation 1.2). Standards analysed on each of the three days of this test to check the performance of the instrument met the sensitivity criteria indicating that the instrument response was acceptable on each day. Between Day 1 and Day 2 t_{cacl} = 0.0025 while t_{calc} = 0.0038 between Day 1 and Day 3 of analysis. From the t-tables [17], at $(n_1\text{-}2) + (n_2\text{-}2)$ degrees of freedom $t_{critical}$ = 2.45. Since $t_{calc} < t_{critical}$ then the difference in the slopes obtained between Day 1and Day 2, and Day 1 and Day 3 is not significant at a 5% confidence level. Thus the response obtained has not altered significantly over the three days of analysis.

Table 3.15: Measured concentrations of spiked tissue samples over three days of analysis

Spiked Concentration (μgkg^{-1})	Measured Concentration (μgkg^{-1}) not corrected for recovery		
	Day 1	Day 2	Day 3
55.3	45.3	43.8	44.1
99.6	70.3	69.4	67.8
253.3	181.6	171.9	169.3
536.9	415.6	420.3	419.1
974.9	749.7	729.8	758.7

Therefore it can be concluded that the method is linear over the above concentration range, 50-1000μgkg^{-1} for three consecutive days. It was also noted that there was no significant decrease in the peak response over the three days of analysis but the confidence interval widened on the second day of analysis. Therefore the spiked tissue samples are stable for up to three days after the sample preparation is complete.

3.8 Sample Analysis

Thirty-five salmon were obtained randomly from seven major fish farms around the Irish coast. These fish were of marketable size and were intended for human consumption. The tissue samples were homogenised and stored in acid and methanol rinsed glass jars at -30°C prior to analysis. The samples were analysed for OA in edible tissue by RPHPLC.

Results:

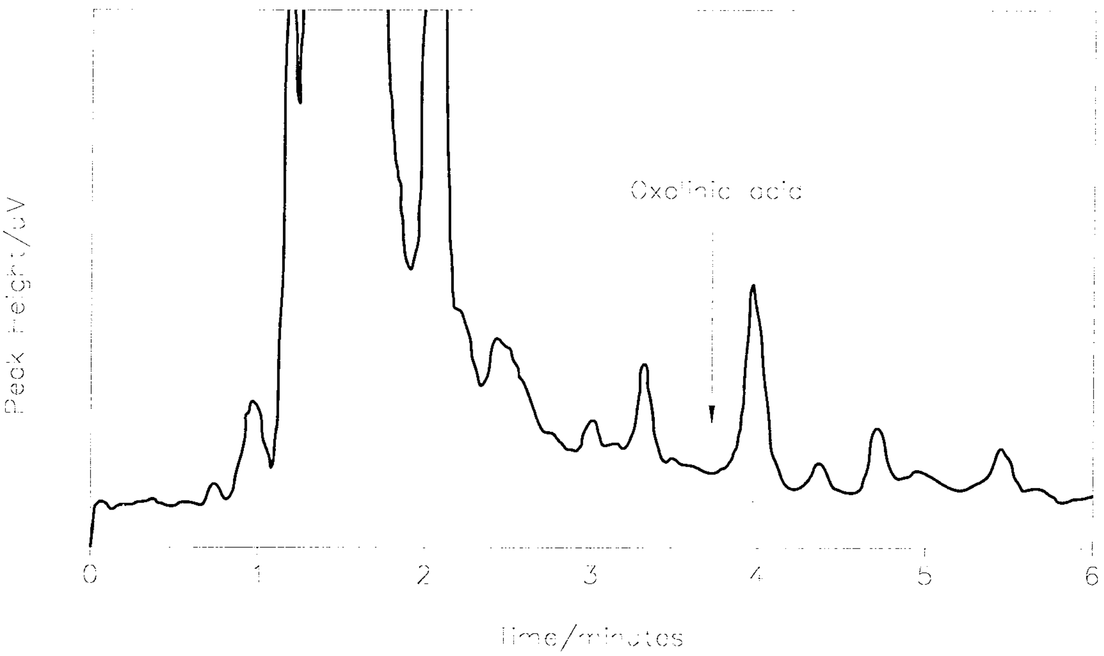


Figure 3.26: Chromatogram of salmon tissue analysed for oxolinic acid. Analytical conditions described in Section 3.6.

The analysis of the thirty-five farmed salmon for OA showed levels of OA below the LOD of of 10μgkg^{-1}. This indicated that the samples, which were obtained randomly, did not contain OA levels, which could be identified using the above method. Subsequent to this work being carried out a MRL of 300μgkg^{-1} was set for OA in salmon. This MRL level and the analysis carried out indicate that the fish tested were safe for human consuption.

3.9 Conclusion

The aim of this work was to develop a method, which could be used on a routine basis for OA analysis in farmed salmon tissue under Directive 96/23/EC. As part of this Directive it is necessary that the method be validated according to the criteria set out in Commission Decision 93/256/EEC. The complete documentation to meet this criterion is lacking in published methods cited for OA analysis in tissue matrixes [3, 4, 5, 6, 7, 8, 9]. However the above method was optimised and validated in this laboratory and from Section 3.7 it is evident that the method meets the specified criteria, and therefore can be used for routine analysis, under Directive 96/23/EC.

The accuracy of the method described is not as good as that quoted in the literature [6] but the precision is improved from 5.7% RSD (n=8), [6] to 2.1% RSD (n=5). The LOQ in this method is 11.3μgkg^{-1} (S/N=5) while in the literature, similar values are quoted [6, 7, 8]. All three of these methods used fluorescence detection for quantification while UV was used in this method. It has been reported that interference from the salmon tissue matrix occurs in the chromatogram at the same retention time as that of OA while using UV detection [8] and therefore fluorescence detection was recommended instead of UV. Since this detection system is more selective than UV the interference did not occur in the chromatogram and thus OA was clearly resolved from any matrix components [8]. The US FDA confirmed the efficiency of that method [8] for determining OA in salmon tissue. However Directive 96/23/EC requires the identity of each compound analysed to be confirmed by a separate detection mode. In HPLC a compound is identified by its retention time using fluorescence or UV detection. To confirm its identity MS has been used in the past for OA [18, 19, 20]. In this laboratory however, this would require the development and validation of a separate MS method for OA. An alternative to the MS method would be to use a DAD detection system where the spectra of the compound in a tissue sample could be obtained and compared to that stored in the library for a standard. If the spectra of the analyte in the matrix matches that stored in the library then the identity of the compound is confirmed. The published methods therefore [3, 5, 6, 7, 8] used fluorescence detection, and avoided the interference from the matrix at the retention time corresponding to that of OA. The method discussed here however involved the optimisation of the chromatography conditions to obtain resolution between the OA and the interferent peak so that the identity could be confirmed by DAD. This resolution was obtained as shown in Figure 3.6b and thus the DAD can be used to confirm identification. In the future the fluorescence and DAD can be coupled in series and fluorescence can be used for quantification, which would result in a lower LOQ, while the identity of the analyte can still be confirmed by DAD.

The method described in this chapter for the determination of OA in salmon tissue involves the use of 16mls of $CHCL_3$. It would be desirable to have a method for this analysis, which did not involve the use of $CHCl_3$ at all. Subsequent to this work Maxwell *et al.* [21], published a paper reporting the successful use of aqueous dialysis, to separate the macromolecules such as proteins from the small molecules such as drugs, followed by trace enrichment for the analysis of OA in chicken tissue. This technique was compared to an AOAC peer verified method, [8], and it was found that the volumes of $CHCl_3$ used were reduced from 125ml [8] to 25ml [21]. While the volume of $CHCl_3$ used was reduced considerably, it was not eliminated altogether. In Chapter 6, size exclusion chromatography is examined as a screening technique for OA in salmon tissue and it will be shown that the method when coupled with a UV detector is capable of detecting residues of OA at $50\mu gkg^{-1}$. This method did not involve the use of any $CHCl_3$ and in the future will be examined further by collecting the eluent from the column and pre-concentrating this for quantification by the HPLC method described in Section 3.6.

3.10 Identification of a blank matrix

Since no certified reference material was available for OA in fish tissue, it was necessary to use blank and spiked tissue samples to check the quality of the data produced in this method development. Therefore it was of extreme importance to ensure that this tissue did not contain any residues of the analytes of interest. A wild salmon was purchased for the analysis. To ensure that this salmon was not a farmed fish it was analysed for canthaxanthin and asthaxanthin.

Asthaxanthin is the main pigment of wild salmon and only smaller amounts of canthaxanthin may be found. Canthaxanthin is a naturally occurring substance found in many algae. It can be produced industrially however, and is used as an additive to fish feed. Therefore a farmed salmon would contain high levels of canthaxanthin and low levels of asthaxanthin, while a wild salmon would show the presence of high levels of asthaxanthin and low levels of canthaxanthin [22].

The method used for the analysis of canthaxanthin and asthaxanthin was HPLC with UV detection [22].

3.10.1 Chemicals used

Diethyl ether - Romil chemicals - Super purity.
n-Hexane - Lab Scan - Pestican
H_3PO_4 - BDH - Analar
Anhydrous Sodium Sulphate - BDH
Chloroform - Romil Chemicals - Super Purity
Canthaxanthin and Asthaxanthin - Roche vitamin and chemical division.

3.10.2 Chromatographic conditions

Mobile Phase: 4.2% Ethanol: 95.8% of 4.5% acetone in n-Hexane
Flow rate: 2 ml/min
Oven temperature: 30°C
Detector: UV 470 nm 0.16 aufs
Column: Hichrom 5u Silica column 24 cm ID 4.6 mm
Run time: 8 min
Retention time: Asthaxanthin: 3-4 minutes
Canthaxanthin: 2-2.5 minutes

3.10.3 Equipment required

3.10.3.1 Sample preparation

Scalpel and blades
Homogeniser - Ultra turrax
250 ml beakers
Balance

3.10.3.2 Extraction process

250 ml beaker
250 ml round bottom flask
100 ml volumetric flask
10 ml Pyrex test tubes
Ori block-Techne + Si3 sample concentrator and water bath at 35°C

Ampoules and vials

3.10.3.3 HPLC system

Shimadzu Sil-6B system controller
Shimadzu Sil-6B auto injector
Shimadzu Cto-6A column oven
Shimadzu SPD-6AV UV-VIS detector
Data was handled by Maxima 820

3.10.4 Column Treatment

The column was conditioned with a solution of 1% v/v H_3PO_4 in ethanol for 4 hours prior to use at 1 ml/min, followed by the mobile phase for 3 hours at 2 ml/min. Standard solutions of canthaxanthin and asthaxanthin in chloroform were injected through the column until the retention times were constant.

3.9.5 Method

The skin was removed from a tissue sample and approximately 20g of flesh were placed in a 250ml beaker. Diethyl ether (50ml) was added to the tissue sample and this was homogenised using the ultra turrax homogeniser, until an obvious colour extract was obtained (approximately 2 minutes). This extraction was repeated twice and the ether extracts were combined in a 250ml round bottom flask. Anhydrous sodium sulphate (2-3) g was added to the combined extracts in the flask and this was mixed for 1-3 minutes. The volume in the flask was then reduced to < 100 ml under reduced pressure at 30-40°C. The extract was decanted into a 100ml volumetric flask and made up to volume. 10ml of the extract were removed and evaporated to approximately 1 ml under nitrogen purge at 30-35°C. To this extract chloroform (1ml), was added and the volume was reduced to 1 ml under nitrogen purge at 30-40°C.

This final extract was then injected, (20μl), and analysed by HPLC.

During the procedure the sample was protected from sunlight and at no stage was the extract evaporated to dryness.

3.10.6 Results

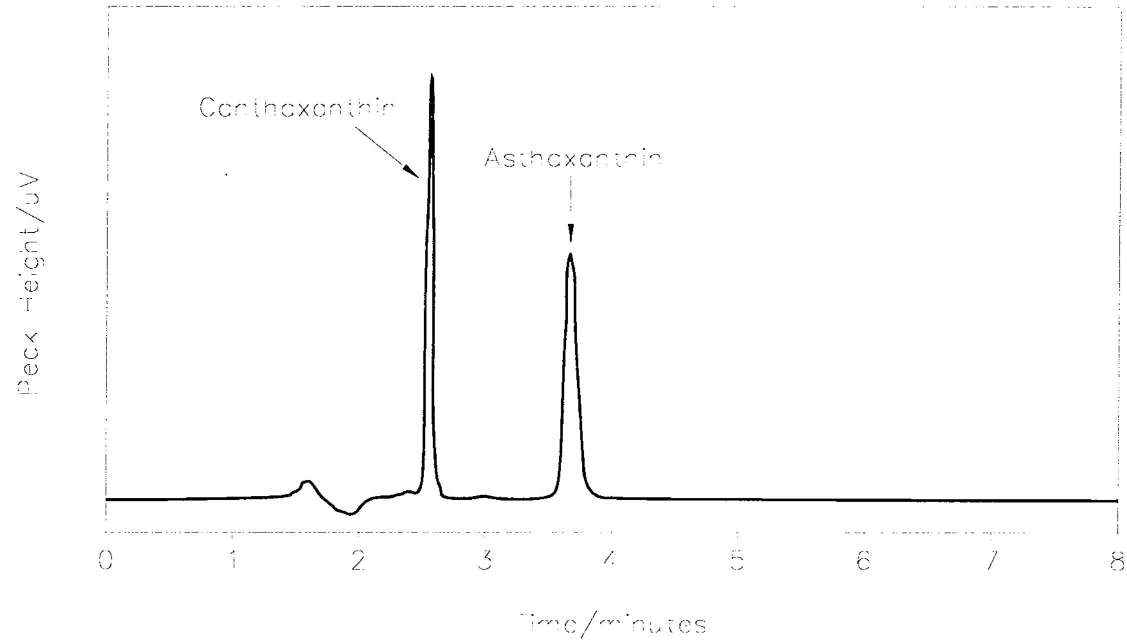


Figure 3.27: Chromatogram of standard solutions of canthaxanthin (2.5 minutes) and asthaxanthin (3.8 minutes). Chromatographic conditions: Section 3.10.2.

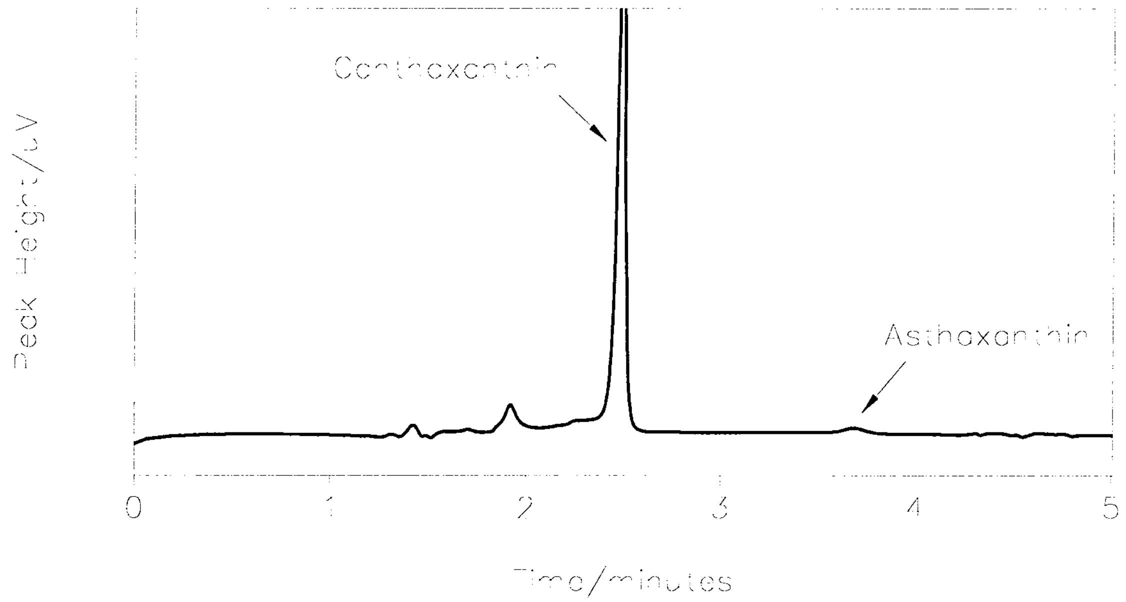


Figure 3.28: Chromatogram of a farmed salmon sample showing the presence of canthaxanthin (2.5 minutes), and a very small quantity of asthaxanthin (3.8 minutes). Chromatographic conditions: Section 3.10.2.

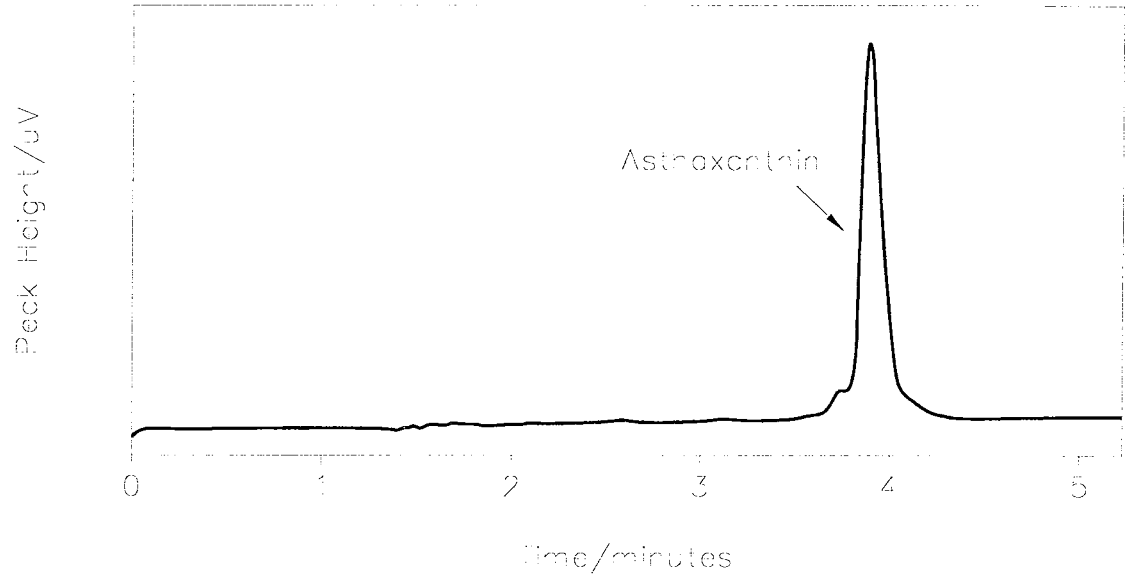


Figure 3.29: Chromatogram of a wild salmon sample showing the presence of asthaxanthin at 3.8 minutes and no canthaxanthin (2.5 minutes). Chromatographic conditions: Section 3.10.2.

Figure 3.29 shows a chromatogram of a salmon with the major component present being asthaxanthin, while Figure 3.28 shows the major component present in the salmon to be canthaxanthin. This indicates that the salmon from Figure 3.29 is not a farmed fish, while the salmon from Figure 3.28, is a farmed fish. However this does not confirm that the salmon from Figure 3.29 is definitely a wild salmon but it is the best method available for distinguishing between the two.

3.11 References

[1] Residue testing plan for Ireland for 1998 drawn up in accordance with Council Directive 96/23/EC on measures to monitor certain substances and residues thereof in animals and animal products. Depatrment of Agriculture and Food, Dublin 2, March 1998.

[2] Duran-Meras I, Galeano-Diaz T, Rodriguez-Caceres MI, Lopez FS. (1997). Determination of the chemotherapeutic quinolonic and cinolonic derivatives in urine by high-performance liquid chromatography with ultra-violet and fluorescence detection in series. Journal of Chromatography, -A. **787**, (1-2), 119-127.

[3] Pouliquen H, Pinault L, Le Bris H. (1994). Determination of oxolinic acid in seawater, marine sediment, and Japanese oyster (Crassostrea Gigas) by high-performance liquid chromatography. Journal of liquid chromatography, **17**(4), 929-945.

[4] Ikai Y, Oka H, Kawamura N, Yamada M, Nakazawa H. (1989). Improvement of chemical analysis of antibiotics. XV1. Simple and rapid determination of residual pyridone-carboxylic acid antibacterials in fish using a prepacked amino cartridge. Journal of Chromatography, **477**, 397-406.

[5] Rose MD, Bygrave J, Stubbings GWF. (1998). Extension of multi-residue methodology to include the determination of quinolones in food. Analyst, **123**(12), 2789-2796.

[6] Steffenak I, Hormazabel V, and Yndestad M. (1991). Rapid assay for the simultaneous determination of residues of oxolinic acid and flumequine in fish tissues by high-performance liquid chromatography. Journal of liquid chromatography, **14**(1), 61-70.

[7] Carignan G, Larocque L, Sved S. (1991). Assay of Oxolinic acid residues in salmon muscle by liquid chromatography with fluorescence detection: Interlaboratory study. Journal of the Association of Official Analytical Chemists, **74,** No. 6, 906-909.

[8] Munns R, Turnipseed S, Peenning A, Roybal E, Holland D, Long A, Plakas S. (1998). Liquid chromatographic determination of flumequine, nalidixic acid, oxolinic acid and piromidic acid residues in catfish (*Ictalurus punctatus*). Journal of AOAC International, **81**, No. 4, 825-839.

[9] Bjorklund H. (1990). Analysis of oxolinic acid in fish by high-performance liquid chromatography. Journal of Chromatography, Biomedical Applications, **530**, 75-82.

[10] Sameulsen O. (1989). Determination of flumequine in fish by high-performance liquid chromatography and fluorescence detection. Journal of Chromatography, **497,** 355-359.

[11] Pouliquen H, Keita D, and Pinault L. (1992). Determination of oxytetracycline in marine shellfish (*Crassostrea gigas, Ruditapes philippinarum* and *Scrobicultaria plana*) by high-performance liquid chromatography using solid-phase extraction. Journal of chromatography, **627**, 287-293.

[12] Lebskaya TK, Dvinin Yu.F, Kuzmina VI. (1991). A comparison of physiological and biochemical indices in wild and farmed Atlantic salmon. International council for the exploration of the sea. C.M. 1991/ M:8 Anadromous and Catadromous Reference F.

[13] Annex of Commission Decision 93/256/EEC laying down the methods to be used for detecting residues of substances having a hormonal or thyrostatic action

[14] Codex Alimentarius Volume 3, (1994). Section 2: Recommended international code of practice for control of the use of veterinary drugs.

[15] Bidlingmeyer BA. 1992. Practical HPLC methodology and applications. Wiley Interscience Publication. ISBN –471-57246-2.

[16] Green JM. (1996). A practical guide to analytical method validation. Analytical Chemistry News and Features, 305-309.

[17] Miller JC, Miller JN. (1993). Statistics for analytical Chemistry. 3rd edition. Ellis Horwood PTR Prentice Hall Limited, ISBN 0-13-030990-7.

[18] Takatsuki K. (1991). Gas chromatographic-mass spectrometric determination of oxolinic acid in fish using selected ion monitoring. Journal of Chromatography, **538**, 259-267.

[19] Takatsuki K. (1992). Gas chromatographic-mass spectrometric determination of oxolinic, nalidixic, and piromidic acid in fish. Journal of AOAC International **75**, No. 6, 982-987.

[20] Horie M, Saito K, Nose N, Tera M, Nakazawa H. (1993). Confirmation of residual oxolinic acid, nalidixic acid and piromidic acid in fish by thermospray liquid chromatography-mass spectrometry. Journal of Chromatography, **16**(7), 1463-1472.

[21] Eng G.Y, Maxwell R.J, Cohen E, Piotrowski E.G, Fiddler W. (1998). Determination of flumequine and oxolinic acid in fortified chicken tissue using on-line dialysis and high-performance liquid chromatography with fluorescence detection. Journal of Chromatography A, **799**, 249-354.

[22] Grethe A.C., Knuthsen P., Skibsted L.H. (1989). Determination of carotenoids in salmonoids. Z. Lebensm Unters Forsch **188**, 413-418.

CHAPTER 4: ANALYSIS OF OXYTETRACYCLINE (OTC) IN SALMON TISSUE

4.1 Introduction

OTC (Section 1.2.1) is a broad-spectrum antibacterial compound commonly used in the prevention and treatment of diseases in farmed salmon. The EU MRL for OTC in fish tissue is 100μgkg^{-1} [1]. Under Council Directive 96/23/EC routine methods are required for the detection and quantification of OTC in salmon tissue, to ensure that withdrawal periods are adhered to, in order to prevent any exposure to the consumer.

Following the examination of published methods (Section 1.5.12) and a further discussion in Chapter 2, a RPHPLC method with UV detection was proposed for analysing OTC in salmon tissue in this work. Since the C18 column is the most commonly used RPHPLC column this was proposed for examination in this method. From Section 2.4, ACN was chosen as the organic component of the mobile phase. For the aqueous phase, water should be examined initially but based on the methods reported in the literature, it is apparent that a buffer must be added to the mobile phase to prevent peak tailing [2, 3]. Thus the analytical conditions as described by Pouliquen *et al.* [2], Knox *et al.* [3] and Bjorklund [4] were deemed suitable for examination in this work.

Sample preparation techniques have been discussed in Section 2.6 but in order to find the most appropriate method for OTC analysis in salmon tissue a further examination of the literature was necessary.

Cooper *et al.* [5] reported that OTC is a polar analyte, which cannot be easily extracted into organic solvents. This was reported to be due to the poor stability of OTC at pH values above 7. However, OTC can pass easily into aqueous solutions at acid pH [5]. The literature reports that it has been extracted from tissue samples with McIlvaine buffer [4, 6], McIlvaine buffer/EDTA [7, 8, 9, 10, 11], hydrochloric acid (HCl) / trifluoroacetic acid (TFA) [12, 13] and metaphosphoric acid with DCM [14]. The acid solutions aid in protein precipitation but since OTC has the ability to chelate metal ions in the tissue the use of EDTA is required to alleviate the OTC-metal ion interactions. During the method development of OTC analysis in tissue samples, the use of McIlvaine buffer for extraction was only moderately successful as reported by Reimer *et al.* [6]. Oka *et al.* [9] reported however, that when EDTA was added to the McIlvaine buffer extraction solution, it was successful in alleviating the OTC-metal ion interactions and thus improved the accuracy of the method. This indicates that the most appropriate extraction solution for OTC in tissue samples was McIlvaine buffer / EDTA solution.

The extraction step of OTC from tissue samples must be repeated to improve the accuracy obtained, and this resulted in relatively large volumes of an aqueous solution. In order to achieve the required detection limits pre-concentration of this aqueous extract was essential. Since OTC is not easily extracted into organic solvents for pre-concentration by means of evaporation, the best option available for both sample cleanup and pre-concentration appears to be SPE as opposed to liquid-liquid extraction.

Using the criteria discussed in Section 2.6.3 the appropriate SPE mechanism for OTC analysis was evaluated. From the structure shown in Figure 1.3, it is evident that OTC has polar hydroxyl functional groups. It is soluble in polar solutions and can be extracted from the tissue matrix into an aqueous buffer. This indicates that a non-polar extraction sorbent would be the most appropriate. In addition since the final analysis procedure for OTC is HPLC, a polar solvent can be used to elute the analyte from the sorbent and this is suitable for non-polar sorbents.

Since C18 is the most common non-polar SPE cartridge, and is widely reported in the literature for OTC analysis in tissue samples [5, 6, 7, 8, 9, 13, 14], it was proposed for examination in this work. The recoveries obtained from C18 cartridges however, have been reported to vary depending on the source of the SPE cartridge [6, 9] and therefore cartridges from various sources will be examined in this work.

Since OTC forms chelate complexes with metal ions and is capable of adsorption on alkyl-bonded reverse-phase columns it can not be eluted from C18 SPE cartridges with either MeOH or ACN [9]. An aqueous Na_2EDTA solution was used to precondition the SPE cartridges to remove any traces of heavy metals and thus prevent OTC-metal ion complexes [6, 9]. Oka *et al.* [9] reported however, that this pre-treatment was not effective for all brands of C18 SPE cartridges. Meinertz *et al.* [10] and Moretti *et al.* [15] reported that silanisation of the cartridge with dimethyl-dichlorosilane (DMDCS) prior to conditioning was successful for removing the metal ions from the cartridge. However, since oxalic acid was found to give satisfactory resolution for RPHPLC analysis of OTC, and separations from a C18 SPE cartridge are based on the same principle, then methanolic oxalic acid was examined and found to be successful as the eluent for OTC from the SPE cartridge [9]. For this work the use of the methanolic oxalic acid solution would be preferred over the use of DMDCS due to the toxic properties of the latter.

Thus from the above analysis sample preparation methods as described by Pouliquen *et al.* [2] and Bjorklund [4] were proposed for initial examination in this work.

The European Union, 93/256/EEC, [16], has laid down criteria for the detection of analytes in test samples for routine monitoring methods as discussed in Section 1.5.1. This chapter presents a description of the method used and the validation study carried out to demonstrate that the method meets the criteria set out under Commission Decision 93/256/EEC to allow it to be used for residue testing.

A study was also carried out to determine if the withdrawal periods were being adhered to for OTC in Ireland, by randomly testing farmed salmon from fish farms around the coast.

4.2 Apparatus and materials

4.2.1 Sample preparation

Scalpel and blades
Hi - speed homogeniser: Breda Scientific
Sample jars

4.2.2 Extraction Process

Balance (5 decimal places)
Centrifuge tubes (15ml)
Vortex mixer - Fisons
Centrifuge - Sorvall T6000
50ml polypropylene centrifuge tube
Varian Bond Elut C18 SPE cartridges 500mg- 3ml
Whatman No. 1 filter papers-30mm diameter
Syringe barrels-5ml
Brown glass 10ml test tubes
Ori block -08-3 Techne + Si3 sample concentrator - nitrogen purge and water bath at 25^0C
Ministart 0.45µm filters
Disposable glass insert vials
Glass vials-4 ml

4.2.3 Chemicals and Reagents

Citric acid monohydrate: GPR-BDH
Oxalic acid dihydrate: BDH
Disodium ethylenediaminetetraacetate dihydrate ($Na_2EDTA.2H_2O$): May and Baker
Disodium hydrogen phosphate dodecahydrate: Merck
Acetonitrile: Lab Scan - Pestiscan
N, N-Dimethylformamide: Riedel-de Haen
Methanol: Lab Scan - HPLC
Oxytetracycline hydrochloride (O5875): 99% purity: Sigma/Aldrich Ltd.

4.2.4 HPLC System

Pump: Shimadzu LC - 6A
Column oven: Shimadzu CTO - 6A
Columns: Spherisorb S5ODS2 5 μm (250 x 4.6) mm
    ODS Hypersil 3 μm (150 x 4.6) mm
    Lichrospher PR18E 5μm (150 x 4.6) mm
Guard column: Sphereclone C18 (30 x 4.6) mm
    5μm all guard columns.
    Hypersil guard cartridges C18 3μm (10 x 3.2) mm
    Lichrospher guard cartridges C18 5μm (30 x 4.6) mm.
Auto injector and system controller: Shimadzu SCL - 6B
Detector: UV -Vis spectrophotometric detector -Shimadzu SPD - 6AV
Data handling: Maxima 820 software - Millipore

4.2.5 Sample storage

All tissue samples were stored in acid followed by MeOH rinsed sample jars and kept in a freezer at -20°C prior to analysis. Using a hi-speed homogeniser the tissue was homogenised and 3g of this homogenate was used for each analysis.

4.3 Chromatographic conditions for OTC analysis

Initially the chromatographic conditions developed for oxolinic acid (OA) analysis were examined to see if OTC could be analysed using the same conditions (Section 3.6). These chromatographic conditions involved the use of the Lichrospher RP-18E column with a mobile phase consisting of H_3PO_4 (0.01M): THF: IPA in the ratio of 78:17:5. A standard solution of OTC diluted in mobile phase to 0.1μgml^{-1} was injected onto the HPLC system. The resultant chromatogram consisted of a peak with retention time of 4.8 minutes, peak width of 0.7 minutes and an asymmetry factor of 0.79. A chromatogram of OTC standard analysed under these conditions is shown in Figure 4.1.

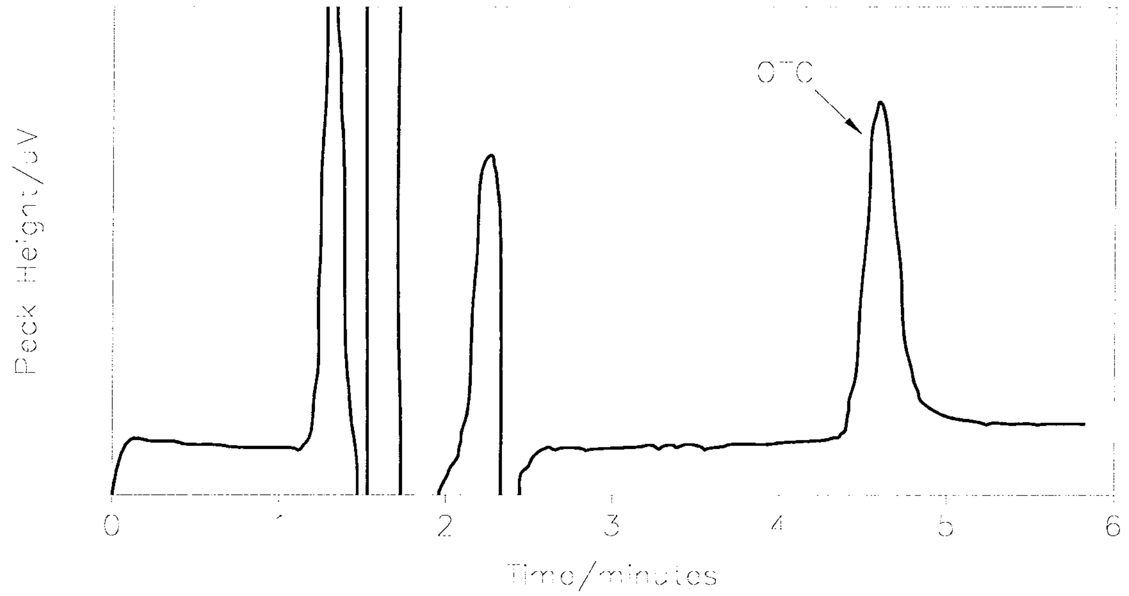


Figure 4.1: Oxytetracycline standard 0.1μgml^{-1}, diluted in the mobile phase. 50μl injected. Column: Lichrospher RP-18E 5μm (250 x 4.6) mm. Temperature: 35°C. Mobile phase: H_3PO_4: THF:ACN (78:17:5). Flow rate: 2ml / minute. Detector: UV at 365nm.

When this chromatogram was examined it was observed that the peak was too broad at 0.7 minutes. In addition the asymmetry factor of 0.79 was outside the acceptable range for good peak symmetry of standard solutions, which ranges from 0.8 to 1.2 [17]. Finally when the response for this standard peak was examined it was found to be too low for a 0.1μgml^{-1} standard solution. Based on the sample intake, this standard is equivalent to 100μgkg^{-1}. It was not possible to increase this value adequately to allow for a limit of quantification of less than 100μgkg^{-1} by adjusting any of the detector parameters. Therefore based on this analysis, OTC could not be adequately determined using the same chromatographic conditions as were used for OA analysis.

4.3.1 Determination of the "strong" solvent for the mobile phase

The theoretical approach for the development of a reversed phase chromatographic separation (Section 2.1) was applied to the OTC method development. The "strong" solvent is defined as one in which the sample is readily soluble. In reverse phase chromatography, this "strong" solvent is used in the mobile phase for separation purposes. The "strength" of a solvent for reverse phase chromatography is determined from its polarity- the lower the polarity index the "stronger" the solvent. As the general guideline in chromatography is to balance the mobile phase "strength" (polarity) with the stationary phase "strength" (polarity) with regards to the samples polarity, it is necessary to find a "strong" solvent for OTC analysis on the Lichrospher C18 column. The "strong" solvent was determined by injecting a standard solution of OTC onto the HPLC system when using different mobile phases. These mobile phases were 100% methanol (MeOH), polarity index 6.6, and 100% acetonitrile (ACN), polarity index 6.2, as OTC is readily soluble in either of these solvents. An injection of the standard when using 100% of the "strong" solvent as the mobile phase should yield a situation where all the sample components elute as one peak at t_0. If there is retention when using 100% of the "strong" solvent, e.g. MeOH, as the mobile phase then a "stronger" solvent such as ACN needs to be examined. OTC was analysed using the two mobile phases above and the results can be seen in Figure 4.2.

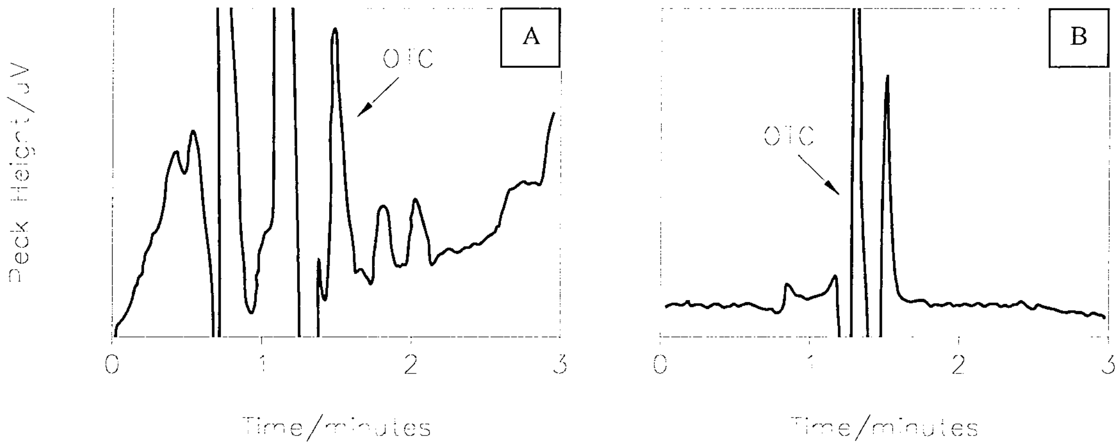


Figure 4.2: Oxytetracycline standard $0.1\mu gml^{-1}$, diluted in the relevant mobile phase and analysed using 100% MeOH (A) and 100% ACN (B) as the mobile phase. Injection volume: 50μl. Column: Lichrospher RP-18E 5μm (250 x 4.6) mm. Temperature: 25°C. Flow rate: 1ml / minute. Detector: UV at 365nm.

Figure 4.2(A) shows the chromatogram obtained when 100% MeOH was used as the mobile phase. As can be seen the standard did not elute as one peak at t_o. There was retention of the OTC beyond the void time of the column and therefore a "stronger" solvent needed to be examined. ACN is the nearest solvent to MeOH with regards to solvent "strength" and therefore this was tested as the mobile phase. Figure 4.2(B) shows the result of this test when the standard diluted in ACN was analysed. With 100% ACN as the mobile phase the OTC eluted at t_o, and therefore this solvent was taken as the "strong" solvent for OTC analysis. Having established the "strong" solvent for OTC a "weak" solvent is required to facilitate the separation. Usually this is water or a weak acid. Following this, different ratios of the solvents in the mobile phase were examined so that retention of the analyte peak could be fine tuned and the appropriate mobile phase, containing the exact blend of the strong and weak solvents determined to achieve the desired resolution.

4.3.2 Mobile phase determination

From the cited literature (Section 1.5.12) Pouliquen *et al.* [2] used a Lichrospher 100 RP-18E column with a mobile phase, which consisted of 0.02M ortho-phosphoric acid and ACN (76:24 v/v). Since ACN was already found to be the "strong" solvent for OTC analysis, the conditions used by Pouliquen *et al.* [2] were taken as a starting point for the determination of suitable chromatographic parameters. Different ratios of the mobile phase components were examined and the results can be seen in Table 4.1.

Table 4.1: Retention times and peak widths for an OTC standard using different ratios of the same mobile phase components. OTC standard, 0.1μgml^{-1}, was diluted in the appropriate mobile phase. Injection volume: 50μl. Column: Lichrospher RP-18E 5μm (250 x 4.6) mm. Temperature: 25°C. Flow rate: 1ml / minute. Detector: UV at 365nm.

ACN : H_3PO_4	Retention Time (minutes)	Peak Width (minutes)
40:60	-	-
30:70	2.40	0.3
24:76	2.45	0.5
4:96	-	-

Figure 4.2(B) shows that there is no retention of OTC when using 100% ACN as the mobile phase. Therefore it was necessary to reduce the "strength" of the mobile phase by combining ACN with 0.02M H_3PO_4. This acid was chosen based on the method by

Pouliquen *et al.* [2]. Therefore the volume of ACN in the mobile phase was reduced while increasing the volume of acid. From Table 4.1 it is evident that no retention is obtained with 40% ACN in conjunction with 60% acid as the mobile phase. A retention time of 2.40 minutes was obtained when using 30% ACN, and 2.45 minutes when using 24% ACN. Based on this it appears that the retention time is increasing with a decrease in the amount of ACN in the mobile phase. When the amount of ACN in the mobile phase was reduced to 4% the retention time was beyond 20 minutes, which was deemed unacceptable. Thus it was evident that the best retention time obtained form the above examination was 2.45 minutes for a mobile phase consisting of 24% ACN and 76% acid. The peak width and symmetry using this mobile phase composition are acceptable, however, the OTC peak was eluting in less than three minutes. This retention time is too short to separate OTC from the co-eluting matrix peaks present in fish tissue. This can be seen more clearly in Figure 4.3, where an overlay of a blank tissue sample with a standard OTC solution, shows that the OTC peak cannot be distinguished from the sample matrix peak.

The blank tissue sample used was tested for canthaxanthin and asthaxanthin (Section 3.10) and found to contain asthaxanthin as the major component. This indicates that the salmon used is not a farmed fish and therefore was not subjected to any veterinary drug treatment. This does not confirm however, that the salmon is definitely a wild salmon, but it is the best method available to determine the difference between the farmed and wild salmon.

It was concluded, therefore, that OTC could not be separated from the matrix peak of the tissue sample under the above conditions and alternative conditions would have to be determined.

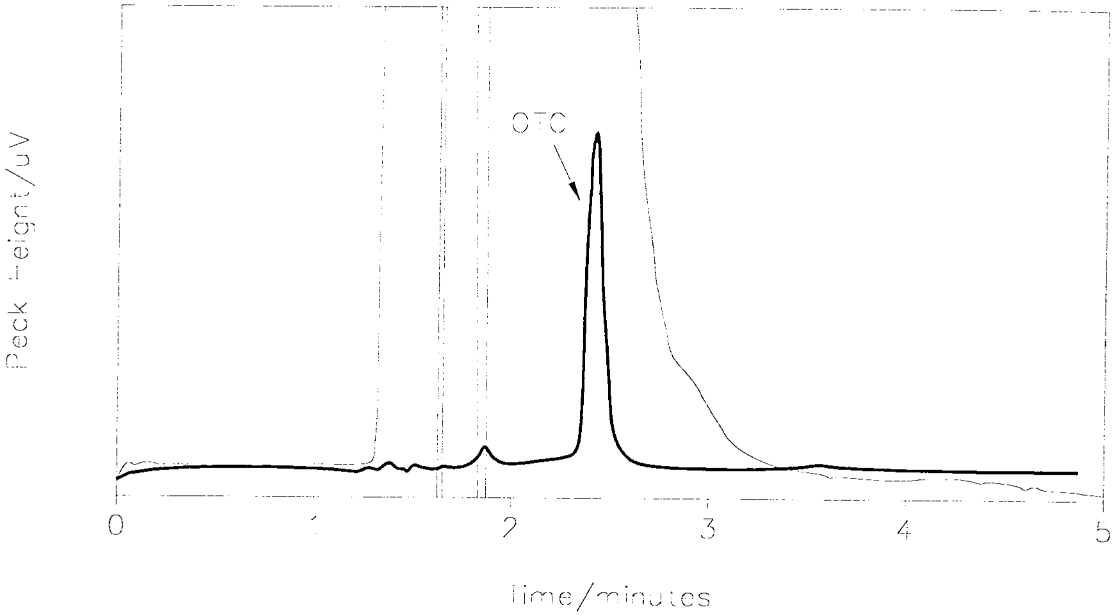


Figure 4.3: Overlay of a standard oxytetracycline solution, 0.1μgml^{-1} diluted in the mobile phase, on a blank tissue sample. 50μl injected. Column: Lichrospher RP-18E 5μm (250 x 4.6) mm. Temperature: 25°C. Mobile phase: 24% ACN and 76% orthophosphoric acid. Flow rate: 1ml/minute. Detector: UV at 365nm.

4.3.3 Determination of chromatographic conditions

The reasoning behind developing a particular mode of LC for the analysis of a particular analyte was explained in Section 2.1. By following the decision tree guide Figure 2.1 a bonded phase column in the reverse phase mode was chosen to examine a separation for OTC. Therefore either the Lichrospher RP-18 or the ODS Hypersil column would be suitable for OTC analysis. Since the stationary phase is a non-polar octadecyl packing and the sample is also non-polar then a polar mobile phase is required for adequate separation. In Figure 4.3 above there appears to be very little retention of OTC by the stationary phase, and thus the sample is more like the mobile phase than the stationary phase in terms of polarity. In order to make the mobile phase less like the sample its polarity needs to be altered. Based on the two methods already examined and a further literature search (Section 2.4) a variation of a method set out by Bjorklund [4], was examined. While using an analytical column which was packed with Spheri 5-μm reversed phased octadecylsilica gel, Bjorklund pumped a mobile phase consisting of ACN, N, N-dimethylformamide (DMF) and 0.01M aqueous oxalic acid (pH 2.1) in the ratio of 22:6:72 through the column at 1ml/minute. When this mobile phase was compared to that used in Section 4.3.2 above it can be seen that both are similar. The mobile phase used in Section 4.3.2 causes little retention of OTC toward the column packing and therefore it needed to be made less polar in order to increase the retention toward the stationary phase. The addition of the DMF in the mobile phase used by Bjorklund [4] has exactly this effect. Since organic solvents are less polar than water then the addition of a second organic solvent to the mobile phase has increased the amount of organic solvent to 28% when compared to 24% in Section 4.3.2, and therefore increasing the strength of the mobile phase slightly. The use of DMF in the mobile phase was reported by Knox *et al.* [3] to be more effective, due to its superiority as an organic modifier, than ACN, with regards to peak symmetry and resolution. Based on this, the combination of ACN and DMF as set out by Bjorklund [4] was examined.

This mobile phase was examined in conjunction with the ODS Hypersil column. Oxalic acid (0.01M) was prepared and different ratios of the mixture of three mobile phase components were tested and the resultant chromatograms were examined. The retention time, peak width and asymmetry factor of the peaks can be seen in Table 4.2.

Table 4.2: Retention times, peak widths and asymmetry factors for different ratios of the mobile phase consisting of oxalic acid (0.01M), acetonitrile and dimethylformamide. Oxytetracycline standard 0.1μgml^{-1}, diluted in the respective mobile phase. 50μl injected. Column: ODS Hypersil 3μm (150 x 4.6) mm. Temperature: 25°C. Flow rate: 1ml/minute. Detector: UV at 365nm.

ACN :DMF : Oxalic acid (0.01M)	Retention time (minutes)	pH of the mobile phase	Asymmetry factor	Peak width (minutes)
27:6:67	6.13	2.6	1.33	0.77
23:10:67	5.95	2.6	1.17	0.51
18:15:67	8.05	2.6	2.1	0.75
18:10:72	7.45	2.5	1.65	0.82
28:10:62	5.68	2.5	2.04	0.59

The first mobile phase tested {27:6:67} (ACN: DMF: Oxalic acid 0.01M), showed a peak with retention time of 6.13 minutes and a peak width of 0.77 minutes. The width of this peak was too broad and the asymmetry factor (Section 2.1.2.5) of 1.33 is outside the acceptable range [17]. Therefore it was necessary to sharpen up the peak and this was attempted by firstly altering the ratio of the two organic components of the mobile phase and attempting to fix the amount of one of these in the final mobile phase. While keeping the amount of acid in the mobile phase constant the amount of DMF was increased by reducing the amount of ACN. This mobile phase {23:10:67} was tested and the result was a peak with retention time of 5.95 minutes, width of 0.51 minutes and an asymmetry factor of 1.17. This peak appears to have acceptable retention time, width, and shape [17], but further tests were carried out to see if the any other improvements could be made. The amount of DMF was increased further to 15% by reducing the ACN to 18%. This resulted in a peak, which had unacceptable peak width and asymmetry. Thus the DMF was kept constant at 10% and the acid increased to 72% by reducing the ACN to 18%. This showed a peak with a considerable peak tail, (asymmetry factor of 1.65), and therefore it was clear that increasing the amount of acid in the mobile phase would not improve the analysis. By decreasing the amount of acid to 62% and increasing the ACN to 28% a peak with an even bigger peak tail was obtained and thus deemed unsatisfactory. Therefore based on the data in Table 4.2 the most appropriate mobile phase for use with the ODS Hypersil column was ACN: DMF: Oxalic acid (0.01M) in the ratio of 23:10:67. The flow rate was increased from 1.0 ml/minute (Table 4.2) to 1.2 ml/minute (Figure 4.4) in an attempt to reduce the analyte peak width. A chromatogram of this at the concentration level of interest can be seen in Figure 4.4.

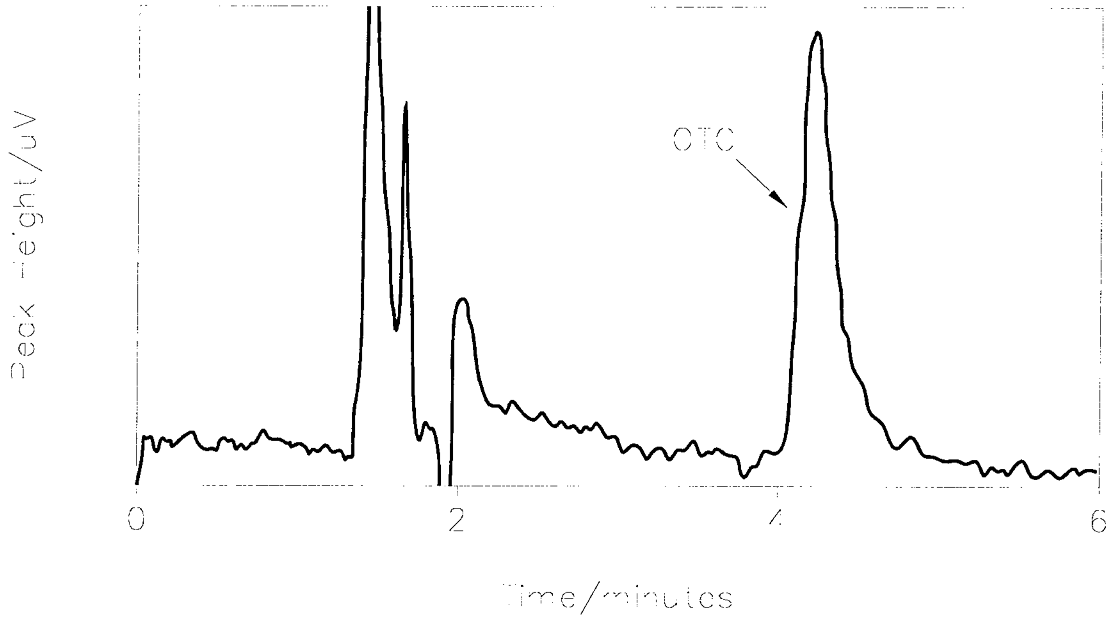


Figure 4.4: Chromatogram of oxytetracycline standard (0.1μgml^{-1} in 0.01M methanolic oxalic acid). Injection volume: 50 μl. Stationary phase: ODS Hypersil 3μm (150 x 4.6) mm. Temperature: 25°C. Mobile phase: ACN: DMF: Oxalic acid {23:10:67}. Flow rate 1.2ml/minute. Detector: UV at 365nm.

It is clear from this chromatogram that improvements can be made to the chromatography conditions. So far the stationary phase and the mobile phase have been examined and the most suitable parameters have been set. Also all the injections made to date were 0.1μgml^{-1} OTC solutions dissolved in MeOH and diluted in 0.01M methanolic oxalic acid. This was used as the sample solvent to date because OTC is readily soluble in MeOH and 0.01M methanolic oxalic acid was the solvent that was used to elute the OTC from the SPE cartridge.

4.3.4 Sample solvent determination

In an ideal situation the sample solvent should be of the same strength or weaker than the strength of the mobile phase. Therefore various sample solvents were examined and the resulting retention time, peak width, asymmetry factor (Section 2.1.2.5) and peak response of these can be seen in Table 4.3.

Table 4.3: Retention time, peak width, asymmetry factor, and response of 0.1μgml^{-1} oxytetracycline diluted in four different solvents. Injection volume: 50 μl. Stationary phase: ODS Hypersil 3μm (150 x 4.6) mm. Temperature: 25°C. Mobile phase: ACN: DMF: Oxalic acid {23:10:67}. Flow rate 1.2ml/minute. Detector: UV at 365nm.

Sample solvent	Retention time (minutes)	Peak width (minutes)	Asymmetry factor	Peak height for 0.1 μgml^{-1}
0.01M Methanolic oxalic acid	4.21	0.50	1.19	1796
Methanol	4.25	0.58	1.19	3417
Mobile phase	4.26	0.52	1.09	4281
0.01M oxalic acid	4.29	0.48	1.12	6775

The peak symmetry and width values of 1.19 and 0.5 minutes are acceptable for good peak shape, when the sample was diluted in 0.01M methanolic oxalic acid. The response for 0.1μgml^{-1} was 1796, and if possible, this needed to be improved to get a suitable limit of the detection for the method. Since the sample solvent should be less than or equal in strength to that of the mobile phase, MeOH was tested as the sample solvent. This resulted in a peak with adequate width (0.58 minutes), and symmetry (1.19), and the response for 0.1μgml^{-1} improved to 3417. The next sample solvent chosen was the mobile phase. The result here was a peak, which again had improved peak width (0.52 minutes), and symmetry (1.09), and the response was 4281 for 0.1μgml^{-1}. The final sample solvent chosen was 0.01M oxalic acid, which had a stronger strength than the mobile phase in terms of polarity. The result in this case was a peak with adequate peak width, (0.45 minutes), and symmetry (1.12), while the response obtained was 6775 for 0.1µgml^{-1}. This response, which was the highest obtained of all four tests, appears to be due to the increased solubility of OTC in aqueous 0.01M oxalic acid when compared to MeOH. Therefore, 0.01M oxalic acid was chosen as the solution to dissolve the sample prior to injection on the HPLC. A chromatogram of a standard sample of OTC diluted in 0.01M oxalic acid can be seen in Figure 4.5.

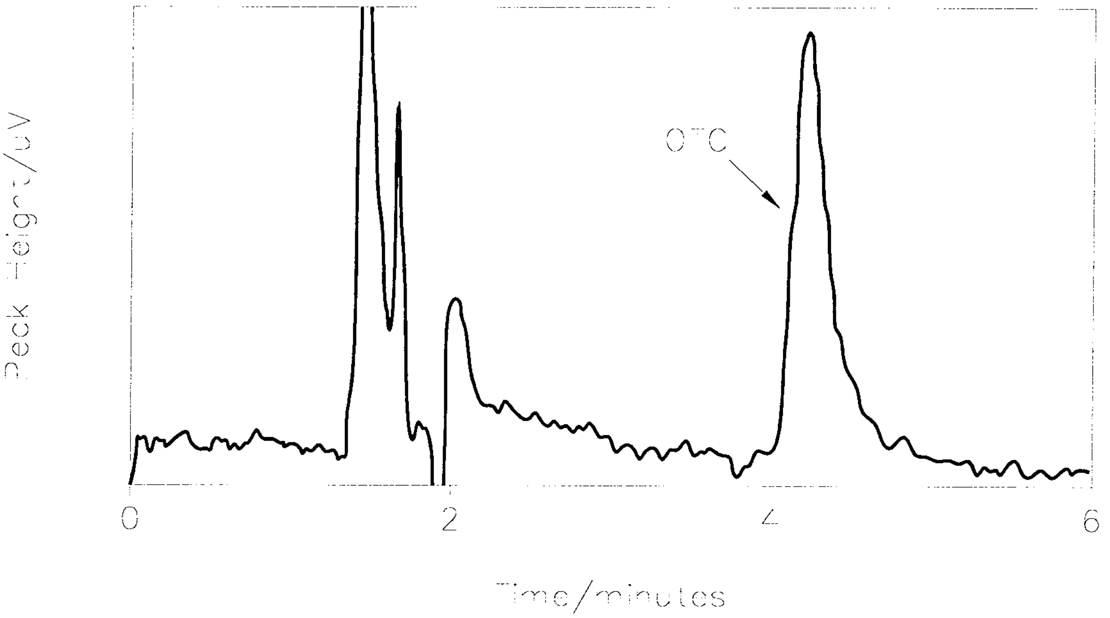


Figure 4.5: Chromatogram of a 0.1µgml^{-1} standard solution of oxytetracycline diluted in 0.01M oxalic acid. Flow rate was 1.0 ml/minute. Injection volume: 50 µl. Stationary phase: ODS Hypersil 3µm (150 x4.6) mm. Temperature: 25°C. Mobile phase: ACN: DMF: Oxalic acid {23:10:67}. Flow rate 1.2ml/minute. Detector: UV at 365nm.

The peak shape and symmetry of a spiked tissue sample (100µgkg^{-1}), dissolved in 0.01M oxalic acid, and in the mobile phase were examined and the resulting chromatograms are shown in Figure 4.6.

From Figure 4.6(A) it can be seen that when the residue of the spiked tissue sample was dissolved in the mobile phase, the resulting chromatogram consisted of a broad shouldered peak. When the residue was dissolved in 0.01M oxalic acid the result was a peak at 4.3 minutes, which showed acceptable peak width (0.45 minutes) and also the asymmetry of 1.12 which is within the acceptable range [17]. From this it is evident that 0.01M oxalic acid was the most suitable sample solvent for the analysis of OTC. Based on these tests it was decided that the for all further analyses the standards and samples would be constituted in 0.01M oxalic acid prior to analysis by HPLC.

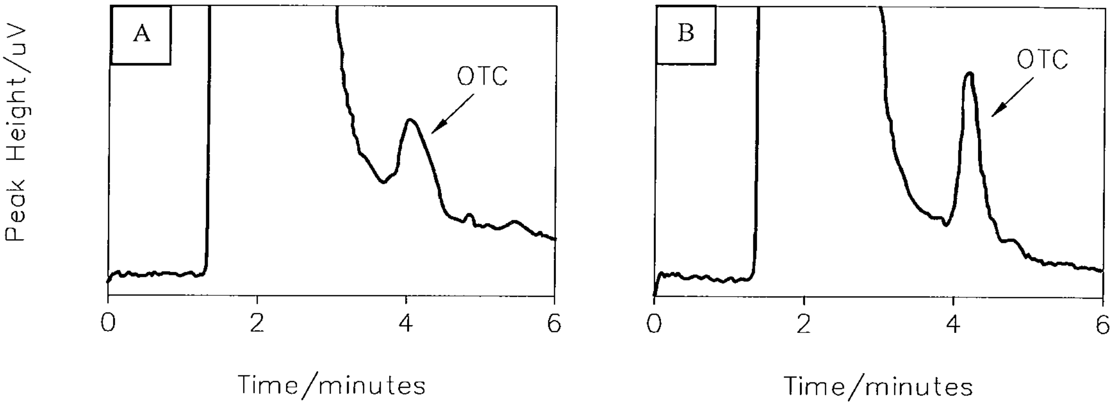


Figure 4.6: Spiked tissue sample of OTC (100μgkg^{-1}) dissolved in the mobile phase (A) and in 0.01M oxalic acid (B). Injection volume: 50μl. Stationary phase: ODS Hypersil 3μm (150 x4.6) mm. Temperature: 25°C. Mobile phase: ACN: DMF: Oxalic acid {23:10:67}. Flow rate 1.2ml/minute. Detector: UV at 365nm.

4.4 Salmon tissue preparation procedure

After a literature search (Section 4.1) a method set out by Pouliquen *et al.* [2], was used to prepare spiked salmon tissue samples for OTC analysis by HPLC. Pouliquen *et al.* analysed shellfish tissues (*Crassostra gigas, Ruditapes philippinarum* and *Scribicularia plana*) and the procedure used was adapted for the determination of OTC residues in salmon tissue. The method as described by Pouliquen *et al.* was as follows:

Step 1: The sample procedure used involved the homogenisation of shellfish tissues using the ultra-turrax homogeniser. A 1g amount of the homogenate was transferred into a polypropylene tube (8ml) and extracted three times with 4,4,2 ml of 0.1M Na_2EDTA McIlvaine buffer (pH 4.0).

Step 2: After centrifugation at 3500 g for 10 minutes at 4 °C, the combined supernatants were filtered through a 90 mm Whatman No. 541 filter-paper.

Step 3: The filtrate was then concentrated by passing through a 3ml Bond Elut solid phase extraction cartridge. Before use, the cartridges were activated with MeOH (4ml) and water (4ml). After the samples had been passed through, OTC was eluted with 2ml of 0.01M methanolic oxalic acid solution and collected in a 3ml flask.

Step 4: The eluate was evaporated to dryness under nitrogen in a water bath at 30°C and reconstituted to 1.0ml in 0.01M oxalic acid. The sample was then centrifuged at 13000g for 10 minutes at 4°C and the supernatant was filtered through an Analypore MC 25 mm, 0.45μm filter. A 50μl aliquot was injected on to the HPLC column.

4.4.1 Response obtained for blank tissue samples

When this clean up procedure was followed for standard OTC solutions it was found that 120% recovery was obtained for a 0.1μgml^{-1} solution while 74% was obtained for a 1.0μgml^{-1} solution. It should also be noted that a blank solution gave a response corresponding to approximately 10% of the response obtained for the 0.1μgml^{-1} solution. This 0.1μgml^{-1} solution was equivalent to the MRL of 100μgkg^{-1} OTC in salmon tissue.

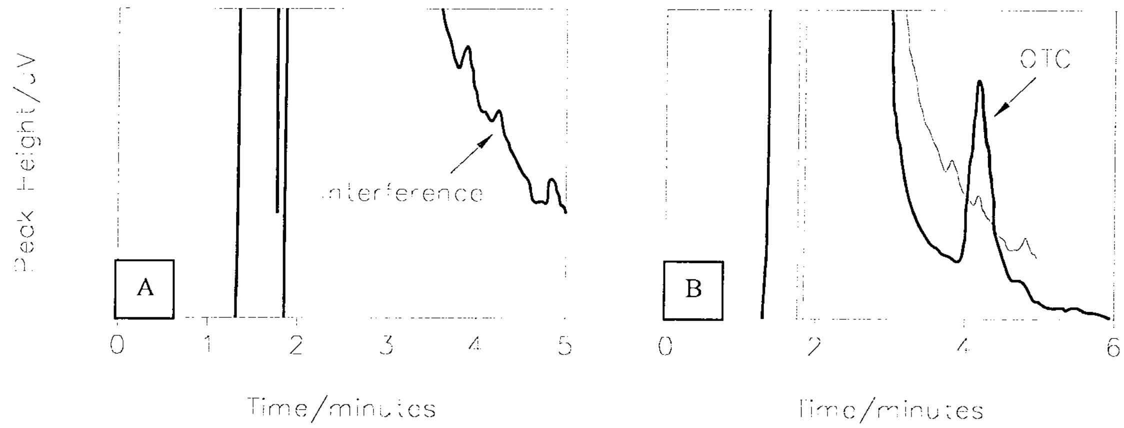


Figure 4.7: Blank tissue sample showing an interference at the R$_T$ of OTC, 4.2 minutes (A) and an overlay of the spiked tissue sample (100μgkg^{-1}) on the blank tissue (B). Analytical conditions: Figure 4.6(B).

When the spiked and blank tissue sample was brought through the above cleanup procedure a response was obtained for the blank tissue sample. A chromatogram, which shows an overlay of a spiked tissue sample on a blank tissue sample, can be seen in Figure 4.7 (B).

It is this response that is causing the inaccuracies of the percentage recoveries and is more noticeable at the lower concentration levels due to the lower response.

Theoretically, it is expected that a lower percentage recovery would be obtained at the lower concentrations since it is more difficult to quantitatively extract and analyse smaller quantities of OTC from tissue samples.

Therefore the sample preparation procedure needed to be examined and alterations made to improve the results obtained. The first step carried out was to analyse a chromatogram of the sample solution containing no standard or tissue sample. This effectively meant that 0.01M oxalic acid was injected onto the HPLC system and the resulting chromatogram was examined, Figure 4.8.

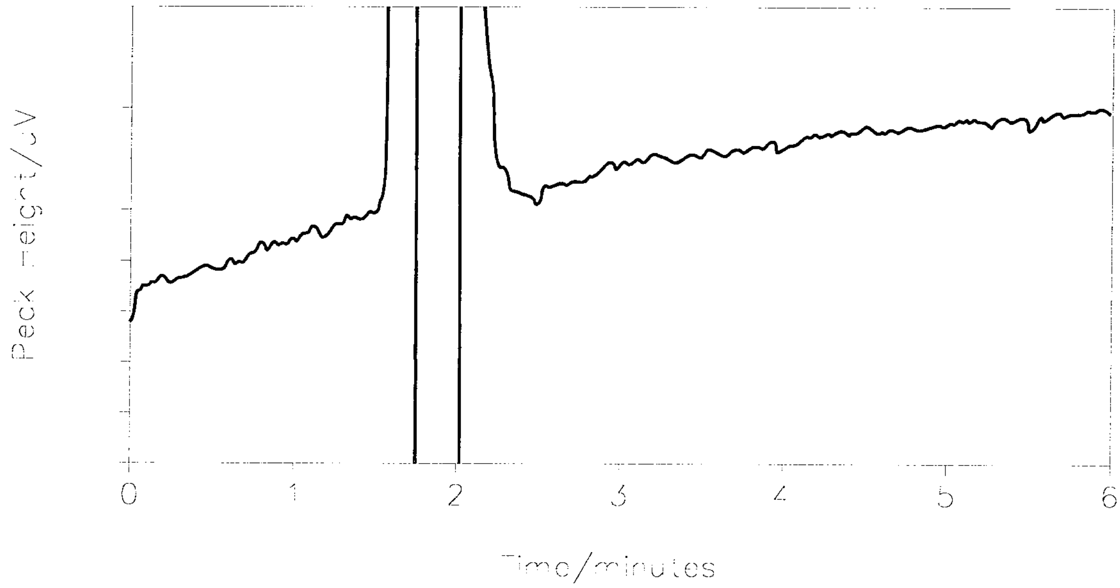


Figure 4.8: Chromatogram of a blank 0.01M oxalic acid solution. Injection volume: 50 μl. Stationary phase: ODS Hypersil 3μm (150 x4.6) mm. Temperature: 25°C. Mobile phase: ACN: DMF: Oxalic acid {23:10:67}. Flow rate 1.2ml/minute. Detector: UV at 365nm. Oxytetracycline and interference peaks at 4.2 minutes.

Figure 4.8 shows that while there is some drift in the baseline, there is no interference at 3.5 - 4.5 minutes of a chromatogram of the 0.01M oxalic acid solution. Therefore, it can be concluded that the response at 4.2 minutes shown in Figure 4.7 for the blank tissue sample is due either to the matrix effects of the sample or it could also be due to interferences from the cleanup procedure.

In order to determine if the response at 4.2 minutes is due to the SPE cartridge, a blank 0.1M Na_2EDTA McIlvaine buffer solution, containing no analyte or tissue was brought through the SPE part of the cleanup procedure as described earlier (Section 4.4). The resulting chromatogram was examined (Figure 4.9).

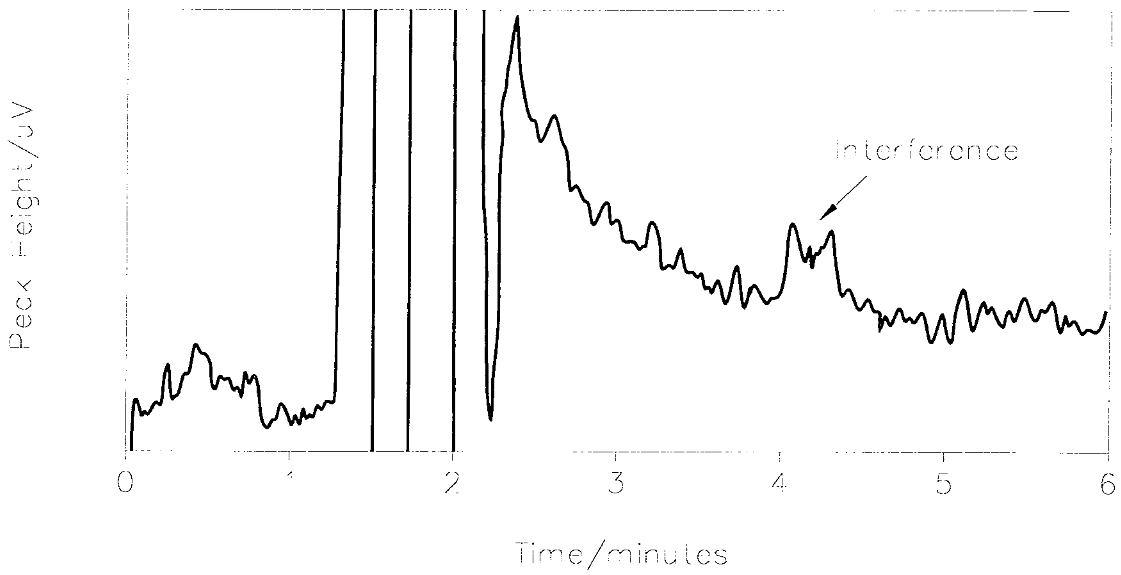


Figure 4.9: Chromatogram of a blank 0.1M Na2EDTA McIlvaine buffer solution, which was brought through the SPE cartridge according to the procedure described (Section 4.4). Sample solvent: 0.01M oxalic acid. Injection volume: 50 µl. Stationary phase: ODS Hypersil 3µm (150 x4.6) mm. Temperature: 25°C. Mobile phase: ACN: DMF: Oxalic acid {23:10:67}. Flow rate 1.2ml/minute. Detector: UV at 365nm.

Figure 4.9 shows that there is a response obtained between 4.0 and 4.4 minutes and this will therefore interfere with the OTC peak, which occurs at 4.2 minutes. It was concluded that the use of the SPE procedure in the analysis needed to be examined.

4.4.2 Separation of the analyte peak and the interfering peak

In an initial attempt to eliminate the interference from the OTC peak in the chromatogram the wavelength of the UV detector was changed and the results of this can be seen in Table 4.4.

Table 4.4: Peak height data for a standard solution of oxytetracycline (0.1µgml^{-1}) and a blank solution for different wavelengths on the UV detector.
Analytical conditions: Sample solvent: 0.01M oxalic acid. Injection volume: 50 µl. Stationary phase: ODS Hypersil 3µm (150 x4.6) mm. Temperature: 25°C. Mobile phase: ACN: DMF: Oxalic acid {23:10:67}. Flow rate 1.2ml/minute.

λ (nm)	350	340	330	365	380
Standard Response	14948	15448	13744	20540	10304
Blank response	315	384	-	650	-

The highest response obtained for the 0.5μgml^{-1} solution of OTC occurs at 365nm (Table 4.4). This wavelength also gave the highest response for the blank solution. It can also be seen that there is no response obtained for the blank solution at 330 and 380nm. However when the response for standard OTC solution at these wavelengths is examined it can be seen that they are considerably lower than the response for the same OTC standard analysed at different wavelengths. Therefore it can be concluded that while the interfering component present in these samples is not as sensitive to detection at these wavelengths, neither is OTC.

Thus the original wavelength of 365nm on the detector was considered the most appropriate. The ratio of the mobile phase components were optimised as shown in Table 4.2, and the most suitable conditions for OTC separation include ACN: DMF: Oxalic acid (0.01M) in the ratio of 23:10:67. Additionally a number of stationary phases were examined in an attempt to improve the chromatography.

4.4.3 Stationary phase examination

A comparison was made between the ODS Hypersil and Spherisorb columns and the findings recorded in Table 4.5. This shows that the Spherisorb column had a larger particle size and was 25cm long as compared to the 15cm long Hypersil column. So that similar retention times and possibly sharper peaks would be obtained the flow rate was increased from 1.2ml/minute on the Hypersil column, to 2ml/minute on the Spherisorb column. The Hypersil column has a pore size of 120 Å, while that of the Spherisorb column is 80Å. It should also be noted that the Spherisorb column has a 12% carbon load on 220m^2/g surface area, while this value is 10% on 170m^2/g surface area for the Hypersil column. Therefore the bonding density is slightly higher on the Hypersil column. The analysis was examined using the Spherisorb S5 ODS (2) HPLC column, (250 x 4.6) mm and the results of this analysis can be seen in Figures 4.10 and 4.11.

Table 4.5: Comparison of the ODS Hypersil and Spherisorb columns

Column	**ODS Hypersil**	**Spherisorb S5 ODS (2)**
Dimensions (mm)	150 x 4.6	250 x 4.6
Particle shape/size (μ)	Spher. 3	Spher. 5
Pore size (Å)	120	80
Pore volume (ml/g)	0.7	0.5
Surface Area (m^2/g)	170	220
Carbon Load (%)	10.0, Monomeric	12.0, Monomeric
End Capping	Yes	Yes

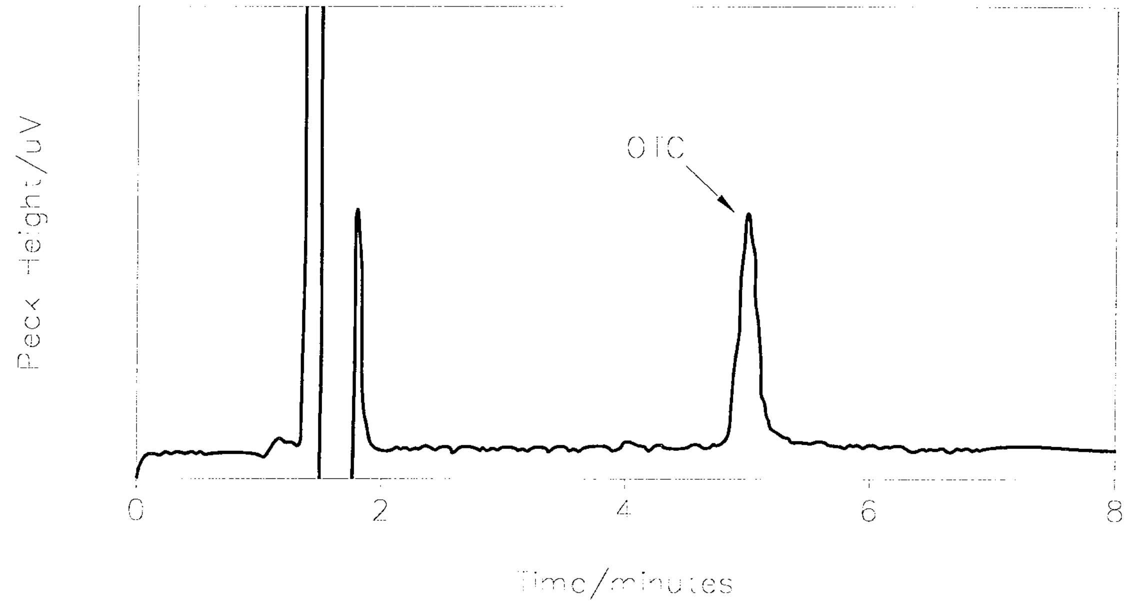


Figure 4.10: Chromatogram of a standard solution ($0.1\mu gml^{-1}$) of oxytetracycline. Sample solvent: 0.01M oxalic acid. Injection volume: 50 μl. Stationary phase: Spherisorb S5 ODS (2) (250 x4.6) mm. Temperature: 25°C. Mobile phase: ACN: DMF: Oxalic acid {23:10:67}. Flow rate 2ml/minute. Detector: UV at 365nm.

Figure 4.10 shows the chromatogram of a standard OTC solution dissolved in 0.01M oxalic acid and analysed using a Spherisorb C18 column with a mobile phase consisting of ACN: DMF: Oxalic acid (0.01M) in the ratio of 23:10:67 at a flow rate of 2 ml/minute. When this chromatogram is visually compared to the chromatogram in Figure 4.5, OTC analysed using an ODS Hypersil column at a flow rate of 1.2 ml/minute the improvement in peak symmetry and baseline noise is evident. Also k′ (Section 2.1.2.1) for Figure 4.10 is 3.16 while in Figure 4.5 this value is 2.23. This will ensure better separation from the tissue matrix components and the analyte peak when analysing spiked tissue sample. This can be seen in Figure 4.11, which shows a spiked tissue sample and an overlay of a blank tissue sample on this spiked tissue.

As can be seen from Figure 4.11(A) there is adequate resolution between the analyte peak and all matrix components to allow for quantification of OTC in fish tissue samples. The overlay of the spiked tissue sample on the blank tissue (Figure 4.11(B)) confirms that the resolution of the analysis is adequate and that there is no interference from the blank tissue at the elution time of the analyte peak.

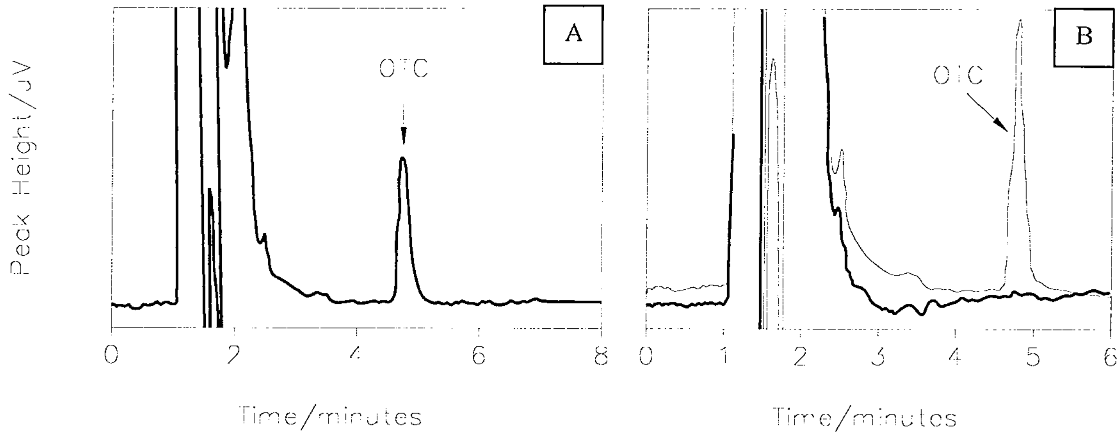


Figure 4.11: Spiked tissue (100μgkg^{-1} OTC) (A) and an overlay of a blank tissue on the spiked tissue sample (B). Chromatographic conditions: Section 4.5.

4.5 Final chromatographic conditions

Therefore the chromatographic conditions developed for OTC analysis were as follows:

Column: Spherisorb S5ODS 2 5μm (250x 4.6) mm.
Guard column: Spherеclone C18 5μm (30 x 4.6) mm.
Detector: UV @ 365nm and 0.002 AUFS.
Oven Temperature: 25°C.
Injection volume: 50 μl in 0.01M oxalic acid.
Flow rate: 2.0 ml/minute.
Pressure: 303 PSI.
Mobile phase: ACN: DMF: Oxalic acid (0.01M) 23:10:67.

4.6 Adjustments made to sample preparation procedure

Using the SPE principles described in Section 2.6.3 the conditioning solvent was changed from MeOH (Section 4.4) to 0.01M methanolic oxalic acid. This was the solvent, which was used to elute the OTC from the SPE cartridge. Water, which was the second conditioning solution reported by Pouliquen *et al.* [2] was also changed in this work. Instead McIlvaine extraction buffer (pH 4.0) was used, since this was the solution which contained the OTC to be loaded on to the SPE cartridge.

4.6.1 Sample spiking solvent

Initially OTC was diluted in 0.01M methanolic oxalic acid and different volumes of this solution were used to spike the tissue sample to different concentration levels. The tissue sample was then extracted with buffer solution and loaded onto a pre-treated SPE cartridge. The same solvent that was used to spike the tissue sample (0.01M methanolic oxalic acid) was used to elute the OTC from the SPE cartridge. However, there was a possibility that this methanolic oxalic acid solution carried some of the OTC through the SPE cartridge to waste while the sample was being loaded on to the SPE cartridge. This may have been the cause of some of the variations in the percentage recoveries obtained and thus the 0.01M methanolic oxalic acid was replaced with MeOH. The MeOH was evaporated to dryness under a gentle stream of nitrogen and the tissue sample (3g) was then weighed into the same centrifuge tube.

4.6.2 Sample filtration procedure

It was found that the Whatman No. 541 filter papers, which were used to filter the combined sample extracts before they were loaded onto the SPE cartridge, were not filtering the samples adequately. This was evident in the fact that the SPE cartridges were becoming blocked, due to the carry-over of particulate matter from the filtering step, when the sample was being loaded. This resulted in very high vacuum pressure being applied to the SPE cartridges and also in some cases forward pressure was required. Thus it became apparent that the filtering method of samples needed to be examined.

The Sartorius Ministart 5μm filters were examined as a possible alternative to the Whatman No. 541 filter papers. While using the Sartorius disposable filters in conjunction with a syringe, each 4ml sample extract was filtered individually using a new disposable filter for each extract. This method of filtration proved unsatisfactory since the filters became blocked even though only 4ml of sample extract were filtered on each. Thus a new approach to filtering the sample extracts was examined.

The tissue was extracted three times with 4, 4 and 2ml of cold extraction buffer (pH 4.0). Each extract was centrifuged at 2700 rpm for 10 minutes. The extract was then decanted from the tube containing the tissue sample to a 50ml polypropylene centrifuge tube. This was carried out for each of the three extracts and these were combined in the 50ml centrifuge tube and vortex mixed for 30 seconds. The combined extracts were again centrifuged for 10 minutes at 2700 rpm. The complete preparation of six samples in a batch took 1½ days and this was an appropriate stage to stop the procedure for overnight storage. Thus the samples were allowed to sit in the fridge overnight at 4°C prior to being loaded on to the SPE cartridge.

Just before the combined extracts were loaded on to the SPE cartridge they were weighed and the weight was recorded to three decimal places. Following this the tubes were again centrifuged for 10 minutes at 2700 rpm. The extracts at this stage contained a white residue at the bottom of the centrifuge tube and care was taken not to disturb this residue.

A reservoir, which consisted of a 5ml syringe barrel, was mounted on top of the SPE cartridge. Into this reservoir was placed a Whatman No. 1 filter paper, 30mm diameter, and this helped to make sure that the combined extracts were thoroughly filtered before they reached the SPE cartridge. The supernatant of the extracts was then loaded on to the preconditioned SPE cartridge. The white residue and some of the liquid extract remained in the centrifuge tube and the weight of this was recorded so the amount of extract, which was loaded on to the SPE cartridge, could be calculated. If at any stage the filter paper in the reservoir became blocked it was removed and replaced with another new filter paper. This proved to be a satisfactory method for ensuring that the SPE cartridges did not get blocked and thus the pressure being applied to the SPE cartridges in the form of either a vacuum pressure or forward pressure was reduced considerably.

4.6.3 Examination of the SPE cartridge wash solution

The large absorbance at the front of the chromatogram in Figure 4.11 is most likely due to proteins or lipids from the tissue sample. In an attempt to reduce this absorbance and protect the column for a longer period of time, a wash step was included in the SPE procedure. This involved passing 30mls of water through the cartridge after the sample had been loaded and prior to the elution step. The result was a cleaner chromatogram, which can be seen in Figure 4.12, and also this wash step had no effect on the percentage recoveries obtained.

Figure: 4.12: Chromatogram of a spiked tissue sample (100µgkg^{-1}), including a wash step in the SPE procedure. Sample solvent: 0.01M oxalic acid. Injection volume: 50 µl. Stationary phase: Spherisorb S5 ODS (2) (250 x4.6) mm. Temperature: 25°C. Mobile phase: ACN: DMF: Oxalic acid {23:10:67}. Flow rate 2ml/minute. Detector: UV at 365nm.

4.6.4 Analysis on various SPE cartridges

While using the mobile and stationary phase described above, (Section 4.5) various different SPE cartridges were examined to prepare the sample for analysis. The results of this test for both blank tissue samples and tissue samples spiked to 100µgkg^{-1} can be seen in Table 4.6.

Table 4.6: Percentage recoveries obtained for spiked tissue samples, (n=3) when analysed using different SPE cartridges. Analytical conditions: Section 4.5.

SPE cartridge	SPE cartridge size (mg)	Concentration (μgkg^{-1})	Peak height	% Recovery
Varian C18-HF	200	blank	-	-
		98.5	999	48
Varian C18-ENV	200	Blank	-	-
		90.0	1497	78
Waters C18	300	blank	-	-
		102.0	1353	66
Varian C18 Bond Elut	500	blank	-	-
		84.0	1485	74
Analytichem Bond Elut C18	300	blank	-	-
		96.0	1492	74

From Table 4.6 it can be seen that the SPE cartridge which gave the best percentage recovery was the Varian C18-ENV. A 78% recovery was obtained when this SPE cartridge was used while 74% was obtained for the Varian C18 Bond Elut SPE cartridge. However it was noted that the C18-ENV SPE cartridge contained 200mg packing while the Bond Elut contained 500mg packing. Based on the data in Table 4.6 the most suitable SPE packing was the Bond Elut C18 since it was not clear if the 200mg packing would be able to cope with concentration greater than $90\mu gkg^{-1}$ without losing any of the analyte to waste. It should be noted at this stage that all the blanks, which were prepared on the different SPE cartridges, did not show any response at the retention time of the analyte peak. This can be attributed to the new method of filtering the extraction buffer and the washing step in the cleanup procedure.

4.6.5 Improvement of the limit of quantification of the method

In order to reduce the limit of quantification of the method to a value lower than the required MRL of $100\mu gkg^{-1}$ a few alterations were made to the method. This involved increasing the initial sample size from 1g to 3g, which resulted in increasing the volumes of extraction buffer used correspondingly. The final sample volume was also reduced from 1ml to 0.5ml of 0.01M oxalic acid. The final method used is described in Section 4.8.

4.7 Examination of the accuracy of the method

Tissue samples were spiked to different concentration levels between 38 and 362μgkg^{-1} ant the percentage recoveries can be seen in Table 4.7.

Table 4.7: Percentage recoveries for spiked tissue samples after the cleanup procedure as described in (Section 4.8)

Concentration (μgkg^{-1})	Percentage Recovery
-	-
38.12	70.7
63.02	77.2
107.13	73.4
131.80	74.5
362.40	75.9

The percentage recoveries obtained all range between 70.7 and 77.2% (Table 4.7). Therefore the sample preparation procedure needed to be examined to identify where the other 20-30% of the analyte was lost. This was carried out by individually examining each of the four steps in the clean up procedure and finding which step was causing the percentage loss. The results of this test can be seen in Table 4.8.

Table 4.8: Percentage recoveries obtained for the individual steps, (n=3), of the sample preparation Procedure as described in (Section 4.8)

Concentration (μgml^{-1})	Step 4: Blowing Down	Step (3+2): SPE	Step 1: Std through full procedure	Spiked tissue: full procedure
blank	-	-	-	-
0.1	109 %	108 %	108 %	70.7 %
1.0	98.5 %	97.5 %	98.5 %	73.4 %

4.7.1 Recoveries from step 4 of the procedure

To test where in the procedure the losses were occurring the last step of the procedure was examined. This involved making up standard solutions of OTC in the solvent used to elute the analyte from the SPE cartridge, i.e. 0.01M methanolic oxalic acid. This solvent, 5mls was then evaporated to dryness and the residue was reconstituted in 0.01M oxalic acid prior to analysis on the HPLC. The percentage recoveries were recorded and these are shown in Table 4.8. For a blank solution no response was obtained, while 109% recovery was obtained for 0.1μgml^{-1} and 98.5% was obtained for 1.0μgml^{-1}. These results indicated that there was no significant loss of analyte occurring in this step of the procedure.

4.7.2 Recoveries from step (2+3) of the procedure i.e. SPE

The next step to be examined was the cleanup involving the SPE cartridge. To examine this step a standard solution of OTC was made up in the extraction buffer (McIlvaine pH 4). This solution was loaded onto the pre-treated SPE cartridge and eluted with 0.01M methanolic oxalic acid (5ml). This was evaporated to dryness under a gentle stream of nitrogen and was then reconstituted in 0.01M oxalic acid (0.5 ml) prior to analysis by HPLC. Again for this step no response was obtained for the blank solution. For the $0.1\mu gml^{-1}$ solution 108% recovery was obtained and 97.5% was obtained for the $1.0\mu gml^{-1}$ solution. This showed that there was no significant loss of analyte in either step 2,3 or 4 of the sample preparation procedure.

4.7.3 Recoveries from step 1 of the procedure i.e. extraction

To examine step 1 of the procedure OTC was made up in MeOH. This was the solution used to spike the tissue sample. The MeOH was evaporated to dryness before the tissue sample was added to the test tube. Therefore in this test the volume of OTC diluted in MeOH required to spike a tissue sample to $0.1\mu gml^{-1}$ and $1.0\mu gml^{-1}$ was evaporated to dryness and the residue reconstituted in 0.01M oxalic acid prior to analysis. This step showed no response obtained for the blank, 108% recovery for $0.1\mu gml^{-1}$ and 98.5% for $1.0\mu gml^{-1}$ solution of OTC. This confirmed that there was no significant losses occurring in the sample preparation procedure and thus the only part left to examine was the extraction of the analyte from the tissue.

When the spiked tissue samples were brought through the sample preparation procedure it was found that 70.7% recovery was obtained for a tissue spiked to $38\mu gkg^{-1}$ and 73.4% recovery was obtained for a tissue spiked to $100\mu gkg^{-1}$. This indicates that the losses in the sample preparation procedure are caused by the fact that all the analyte is not extracted from the tissue sample and in fact the spiked OTC may be coming bound to the tissue. Therefore it is the extraction of OTC from the tissue sample that is causing the losses in percentage recoveries obtained. This is confirmed in some of the literature methods [9, 10, 11], where it is said that the OTC has the ability to chelate metal ions in tissue and is very difficult to remove. Additionally since OTC has the ability to chelate metal ions the recovery could possibly be improved further by using an alternative to the stainless steel Ultra Turrax homogeniser as recommended by Meinertz *et al.* [10].

4.8 Final method procedure

4.8.1 Stock standard preparation procedure

OTC (25mg) was weighed accurately into a 25ml volumetric flask. This was dissolved in MeOH and sonicated for 5 minutes. The volume was made up to 25ml with MeOH and this was stored at 4°C for 1 month. To prepare standards to spike the tissue samples the above stock standard solution was diluted on a weight/weight basis in MeOH to the required concentration levels.

4.8.2 Working standard preparation procedure

The standards used in the calibration plot were prepared by diluting the stock standard solution on a weight/weight basis in 0.01M oxalic acid to the required concentration level.

4.8.3 Preparation of the extraction buffer

McIlvaine buffer (pH 3.0) consisted of 0.1M citric acid-0.2M disodium hydrogen phosphate (79.45:20.55 v/v). 0.1M Na_2EDTA McIlvaine buffer (pH 4.0) [9], was prepared by dissolving 37.224g of $Na_2EDTA.2H_2O$ in water, adjusting the pH to 4.0 with McIlvaine buffer (pH 3.0) and diluting to 1 litre with water.

4.8.4 Sample preparation procedure

Step 1: Spiking the tissue sample.

The tissue sample was spiked by weighing the required amount of OTC (diluted in MeOH) into a 50ml polypropylene tube. The MeOH was evaporated to dryness at room temperature under a gentle stream of nitrogen. The homogeneous salmon tissue, 3g, was weighed accurately into the 50ml tube and the sample was then vortex mixed for 1 minute to ensure an even distribution of the OTC in the tissue sample. The sample was then allowed to sit for 1 hour prior to extraction.

Step 2: Extraction of OTC from the tissue sample.

The OTC was extracted from the salmon tissue with 12, 12, 6 mls of cold McIlvaine buffer, pH 4.0. The buffer was added to the tissue sample, vortex mixed for 30 seconds followed by sonication for 5 minutes. Each extract was then centrifuged for 10 minutes at 2700 rpm and the supernatant was decanted into another 50ml polypropylene tube. The combined extracts were then vortex mixed for 30 seconds to ensure an even distribution of the analyte in the aqueous extract. The combined supernatants were then centrifuged for a further 10 minutes at 2700 rpm and allowed to sit in the fridge at 4°C overnight. Following this the extracts in the 50ml centrifuge tubes were weighed to 3 decimal places and were again centrifuged for 10 minutes at 2700 rpm prior to being loaded on to the SPE cartridge.

Step 3: Solid phase extraction of the analyte.

A 5 ml syringe barrel containing a Whatmann No. 1 filter paper was mounted on to the Varian Bond Elut SPE cartridge. The SPE cartridge was then conditioned with 0.01M methanolic oxalic acid (5mls) followed by McIlvaine buffer pH 4.0 (5mls). As much of the combined sample extract as possible was loaded on to the SPE cartridge using a glass pipette tip and the weight of the remainder of the extract in the centrifuge tube was recorded to three decimal places. The SPE cartridge was washed with water (30mls), and the analyte was eluted from the SPE cartridge with 0.01M methanolic oxalic acid (5mls) and collected in a brown glass test tube.

Step 4: Reconstitution of the residue prior to analysis.

The eluate from step 3 was evaporated to dryness at 25°C under a gentle stream of nitrogen and the residue was redissolved in 0.01M oxalic acid (0.5mls). The residue was then filtered using a Ministart 0.45μm filter. The filtrate was collected into a small insert vial and this was placed in a 4ml vial prior to analysis by RPHPLC.

4.8.5 Sample cleanup procedure

Homogenised fish tissue 3 g

Extract with 0.1 M Na_2EDTA McIlvaine Buffer (pH 4) 12, 12, 6mls

Centrifuge each extract for 10 minutes at 2700 rpm.

Combine the supernatants and vortex mix for 1 minute followed by centrifugation for 10 minutes.

Allow to sit in the fridge overnight.

Condition the Bond Elut SPE cartridge with 0.01M methanolic oxalic acid (5ml) and Buffer pH 4 (5ml).

Pass the sample through the SPE cartridge.

Wash the SPE cartridge with water (30ml)

Elute with 0.01M methanolic oxalic acid (5ml).

Evaporate to dryness at 25°C under nitrogen.

Reconstitute to 0.5ml with the 0.01M oxalic acid.

Analysis by RPHPLC

4.9 Method validation

Method validation is a process of proving that an analytical method is acceptable for its intended purpose [16, 18]. Under Council Directive 96/23/EC routine methods must be validated, for the detection and quantification of veterinary drug residues in farmed fish, to the criteria specified in the Commission Decision 93/265/EEC [16]. Similar criteria have been specified by the Codex Committee on Residues of Veterinary Drugs in Foods [18], and these have been discussed in Section 1.5.1. The validation studies were performed during the actual development of the method so that the conditions could be proven to be satisfactory. The analytical conditions used for the validation study are specified in Section 4.5 while the sample preparation procedure is described in Section 4.8.

4.9.1 Specificity

For chromatographic methods, developing a separation involves demonstrating specificity, which is the ability of the method to accurately measure the analyte response in the presence of all sample components. The response of the analyte in the test mixture, i.e. fish tissue which contains the analyte and all other sample components was compared to the response of a standard solution containing only the analyte. Fish tissue, (3g) was spiked with 0.3μg of OTC to yield a concentration of 100μgkg^{-1} of OTC in the fish tissue. In order for fish tissue to be fit for human consumption the MRL for OTC has been set at 100μgkg^{-1} [1]. This concentration value of 100μgkg^{-1} was chosen to spike the tissue for validation purposes. The spiked tissue was brought through the cleanup procedure (Section 4.8) and analysed under the conditions optimised in Section 4.5. To test the robustness of the method the spiked tissue samples were exposed to stress conditions, which might affect percentage recoveries or peak symmetry. These factors included heat at 40°C and normal lighting conditions in the laboratory for 6 hours. The final eluate from the SPE cartridge in the cleanup procedure was 0.01M methanolic oxalic acid. Since the boiling point of MeOH is 60°C an attempt was made to reduce the analysis time by evaporating this MeOH to dryness at 40°C. The analyte peak was then evaluated for peak purity and resolution from the nearest eluting peak and compared to a standard solution which contained just the analyte peak. Once acceptable resolution was obtained for the analyte peak and potential sample components, the chromatographic parameters were set. These included column type, mobile phase composition, flow rate, oven temperature and detection mode.

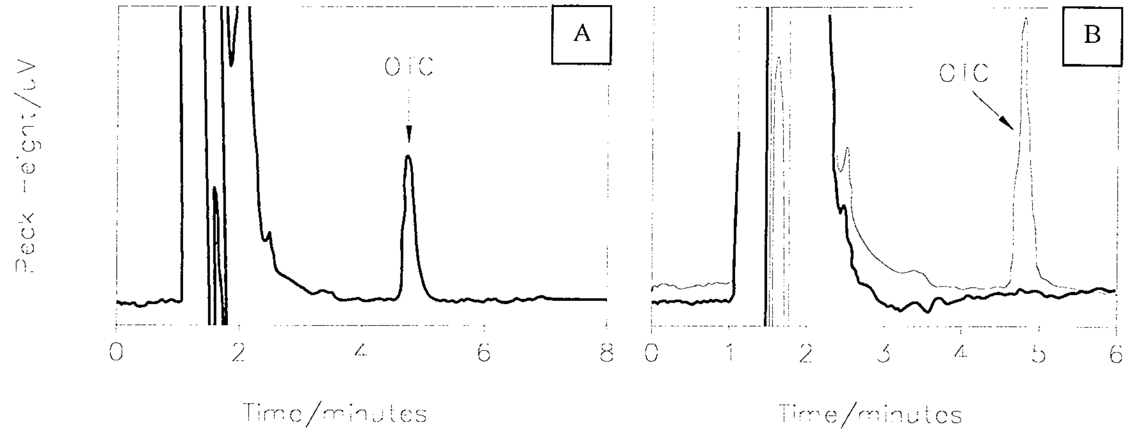


Figure 4.13: Overlay of a blank tissue sample on spiked tissue (100μgkg^{-1}) (B), and a standard solution of oxytetracycline (0.1μgml^{-1}) (A). Analytical conditions: Section 4.5.

Figure 4.13(B) consists of an overlay of a spiked tissue sample on a blank tissue sample. From this it can be seen that there are no matrix interferences from the blank tissue sample between the retention times of 4.0 and 6.0 minutes. It is in this time interval that the analyte peak is observed. This is confirmed by Figure 4.13(A), which shows a chromatogram of a standard solution of OTC at 0.1μgml^{-1}. Since the void time of the column is 1.0 minutes it can be concluded that the analyte peak has baseline chromatographic resolution of greater than the required 1.5 value [20], from all other sample components. As the specificity of the method had been deemed satisfactory the spiked tissue samples were exposed to heat and fluorescent lab lighting, i.e. factors which might alter the peak symmetry and percentage recoveries. Table 4.9 shows the peak retention time, asymmetry (Section 2.1.2.5) and percentage recovery data for these stress tests.

Table 4.9: Comparison of the analyte standard peak to a spiked tissue and a spiked tissue exposed to both heat and light

	Retention time (minutes)	Peak width (minutes)	A_S	Percentage recovery
analyte	4.78	0.55	1.11	
spiked tissue	4.78	0.58	1.16	77.4 %
heat	4.78	0.58	0.85	70.1 %
light	4.78	0.62	0.91	67.2 %

Consistent retention times and peak widths were observed for the analyte and spiked tissue samples. For an ideal peak shape the asymmetry value should be 1.0 but peak asymmetry values of 0.8 to 1.2 are acceptable for quantitative purposes [17]. The asymmetry values of the standard analyte (1.11) and the spiked tissue sample (1.16) are within the range acceptable for good peak symmetry [17]. For the samples exposed to heat and normal lighting conditions in the laboratory the asymmetry values are also within the acceptable range of 0.8 to 1.2. These peaks however, have a tendency to front (asymmetry values less than 1.00) rather than the tendency to tail of the standard analyte and spiked tissue samples (asymmetry values greater than 1.00). Thus even though the peak symmetry is still theoretically acceptable the stress conditions which the samples were exposed to did alter the peak shape. The percentage recovery for the spiked tissue sample exposed to light was 10% lower than the spiked tissue sample which was kept out of direct light. Also, a lower recovery of OTC was obtained for the samples, which were exposed to 40°C in the evaporation step of the procedure when compared to the spiked sample which was evaporated at 25°C. For this reason, the samples were protected from light and temperatures above 25°C during the analysis.

From this specificity evaluation the chromatographic parameters were set and these were used in all further validation studies. The stationary phase consisted of a Spherisorb C18, (250 x 4.6) mm, reverse phase column. A Sphericlone C18 guard column was used to protect the analytical column and help to extend its lifetime. Both of these columns were maintained at 25°C during the analysis. The mobile phase used in the analysis consisted of 23% ACN, 10% DMF and 67% 0.01M oxalic acid at a flow rate of 2.0ml/minute. OTC was detected using a fixed wavelength UV detector at 365nm, while the sensitivity of the detector was set at 0.002 AUFS.

4.9.2 Injection repeatability

In this study eight injections of a standard solution from the same vial were made to test the performance of the chromatographic instrument. Using a standard calibration plot the concentration of this solution was calculated based on peak height and area (Figures 4.14(A) and 4.14(B) respectively). The mean, standard deviation on the mean, and the percentage relative standard deviation were calculated for both height and area, and the comparison can be seen in Table 4.10. Following this evaluation a decision could be made on whether peak height or peak area would be used in the remainder of the study for validation purposes.

Table 4.10: Peak area compared to peak height for the instrument repeatability study

Injection number	**Concentration (μgml^{-1}) based on Peak height**	**Concentration (μgml^{-1}) based on Peak area**
1	0.185	0.189
2	0.187	0.186
3	0.189	0.189
4	0185	0.188
5	0.185	0.187
6	0.185	0.188
7	0.187	0.189
8	0.186	0.188
Mean	**0.186**	**0.188**
Standard Deviation	**0.0014**	**0.001**
%RSD	**0.78%**	**0.60%**

Table 4.10 shows %RSD values for repeatable injections to be approximately 0.7% for peak height and area. Both indicate that the performance of the instrument is acceptable but to determine whether height or area will be used as the analyte response for further validation studies, linearity was examined.

A linearity study checks if the sample solutions are in a concentration range where the analyte response is linearly proportional to concentration. The standard solutions were prepared and analysed by HPLC. A calibration plot based on peak height and area were plotted and examined (Figure 4.14).

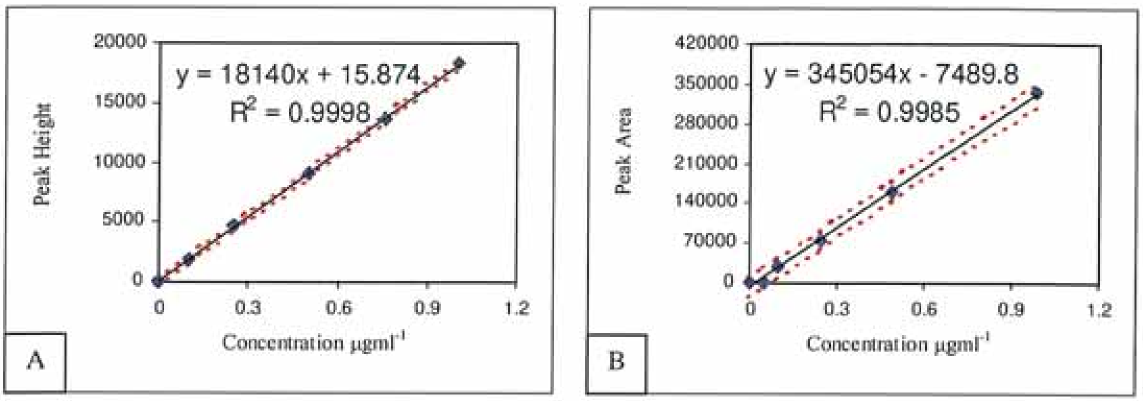


Figure 4.14: Plot of peak height (A) and peak area (B) against concentration of standard solutions. The upper and lower 95% confidence limits are indicated by (----).

These plots show an R^2 value of 0.9998 and 0.9985 for height and area respectively. Since ideally this value should be 1.00, the values obtained indicate that there was a better fit of the data points to the best-fit line for height rather than area. Despite the lower R^2 value for the plot of peak area against concentration, the plot is essentially linear and therefore a further examination involving spiked tissue samples was necessary. Plots of peak height and area against concentration for spiked tissue samples are shown in Figures 4.15 and 4.16 respectively.

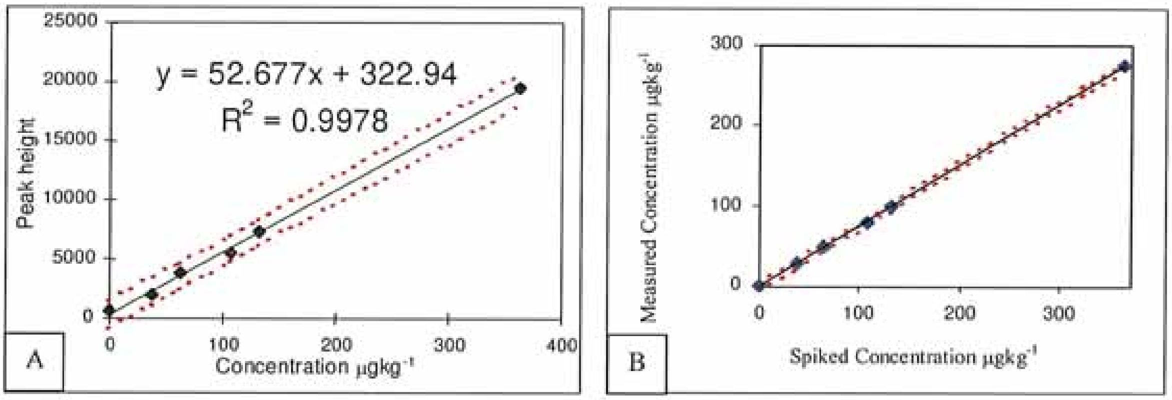


Figure 4.15: Plot of peak height against concentration (A) and spiked concentration against measured concentration (B) for spiked tissue samples. The upper and lower 95% confidence limits are indicated by (----).

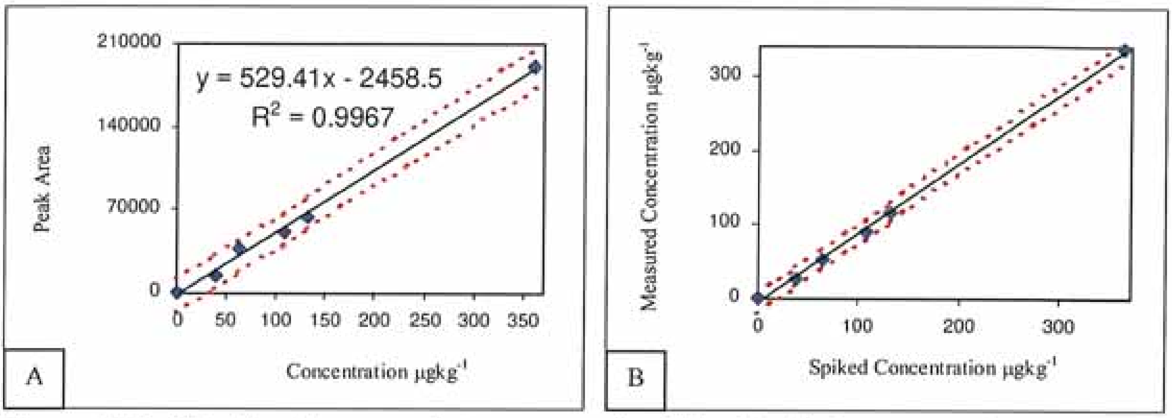


Figure 4.16: Plot of peak area against concentration (A) and spiked concentration against measured concentration (B) for spiked tissue samples. The upper and lower 95% confidence limits are indicated by (----).

The calibration plots for spiked tissue samples show a linear response, all points are within the upper and lower 95% confidence limits, over the concentration range for both peak height and area. The R^2 value was 0.9978 (Figure 4.15(A)) for the peak height while the peak area response shows the R^2 value to be 0.9967 (Figure 4.16(A)). It is evident that the R^2 for height is closer to 1 than that for area indicating that the peak height gives a more linear response than that of peak area.

For both height and area a plot of spiked against measured concentration with 95% confidence intervals included were examined (Figure 4.15(B) and 4.16(B)). From these plots, the confidence intervals at the MRL of 100µgkg^{-1} were obtained for both peak height and area. The values obtained were corrected for percentage recovery (Section 4.9.4) and the intervals were 97±7µgkg^{-1} for peak height and 111±13µgkg^{-1} for area. Based on this it was evident that peak height gave a narrower confidence interval about 100µgkg^{-1} when compared to that of peak area. Therefore peak height should be used as the analyte response for quantification.

4.9.3 Linearity

A linearity study checks if the sample solutions are in a concentration range where the analyte response is linearly proportional to concentration. Standard solutions of OTC were prepared and analysed by HPLC. In order to find a concentration range for which linearity, accuracy, and precision studies will be examined the linear range of the method must first be determined. A plot of the concentration of the standard solutions against peak height measurements can be seen in Figure 4.17. From this plot it is clear that at the higher concentrations the line deviates from linearity and this is most likely due to detector saturation. The point at which this occurs (12.6μgml^{-1}) is taken as the upper limit of the dynamic range of the method. The lowest concentration value on this plot (0.0055μgml^{-1}) was taken as the lower limit of the dynamic range. The dynamic range of the method extends from 0.005-12.6μgml^{-1}, which corresponds to 1.8-4200μgkg^{-1}.

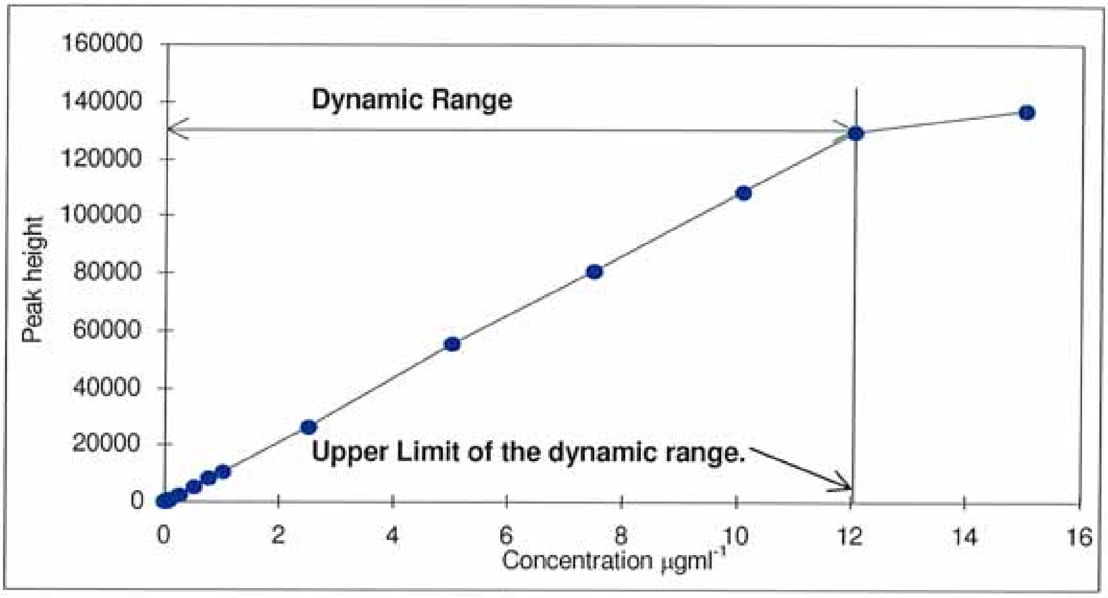


Figure 4.17: A plot of concentration (μgml^{-1}), against peak height shows the dynamic range of the method.

The linear range of the method was estimated by mathematical treatment, of the peak heights obtained by analysis of standard solutions with analyte concentrations across the dynamic range of the method. The treatment is normally a calculation of the regression line by the method of least squares, of peak heights versus analyte concentrations. The correlation coefficient of the regression data and the variance of this data provides a mathematical measure of linearity. A correlation coefficient of > 0.999 is generally considered as evidence of acceptable fit of the data to the regression line. Although it is a very practical way of evaluating linearity data, by itself it can be misleading and should not be used without a visual examination of the response versus concentration plot. An alternative and more realistic way of evaluating the data is to plot response factors against concentration (Section 1.5.4.1).

If an equivalent response is obtained at each concentration value, the data points would form a straight line with zero slope. This zero or near zero slope of the response factor plot indicates that a linear response is obtained over that concentration range. At the completion of linearity studies, the appropriate linear concentration range for the standards should be set for all subsequent studies.

To find the linear range of the standards the response factors from the plot in Figure 4.17 were calculated and these were plotted against concentration. This plot, shown in Figure 4.18 was examined and the linear range determined.

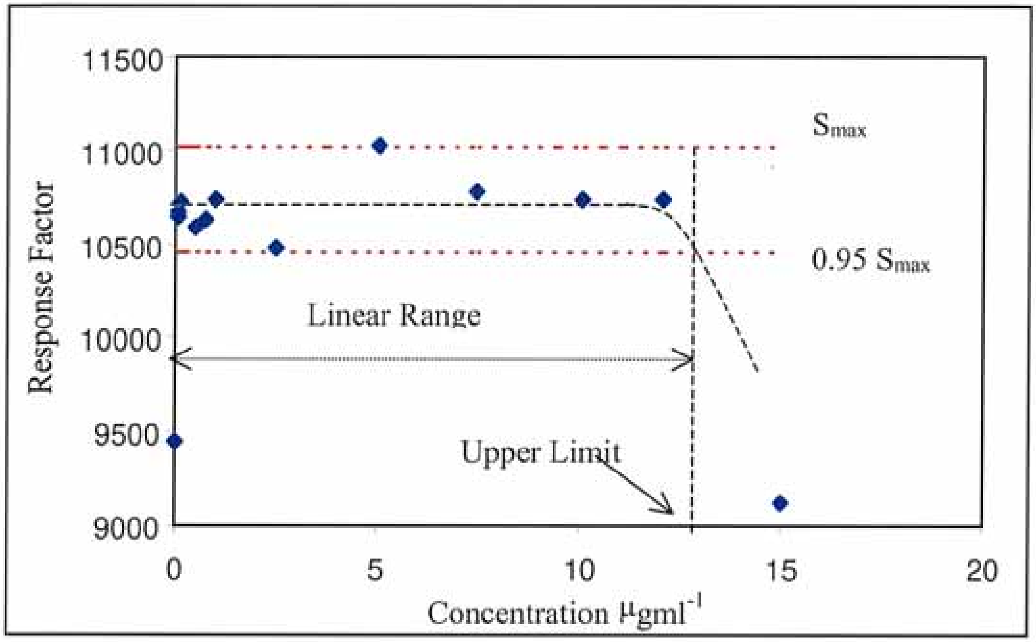


Figure 4.18: Plot of response factor against concentration (μgml^{-1}). S_{max} is the maximum response factor value while 0.95 S_{max} is 95% of this maximum value.

In practice an equivalent response was not obtained for each concentration value. Therefore the linear range was determined by estimating a range of response factor values which fall within the mean ±2.5%. Due to the uncertainty of the response factor values at the higher and lower concentrations the linear range was set based on the maximum response factor value obtained (S_{max}). While this method is not ideal, when combined with the results from the calibration and y-residual plots, it helps to define the linear range of the method.

The upper dashed line in Figure 4.18 represents the maximum response factor (S_{max}) and the lower dashed line is 95% of this value. In order to determine the linear range of the method all the points on this plot must lie within these limits. Thus any concentration value which gave a response factor value within ±2.5% of the mean response factor value was within the linear range of the method. However it is clear that the points for the lower and higher concentrations are outside the lower limit and therefore these concentration values are not in the linear range of the method. These points are omitted from the plot and the result can be seen in Figure 4.19.

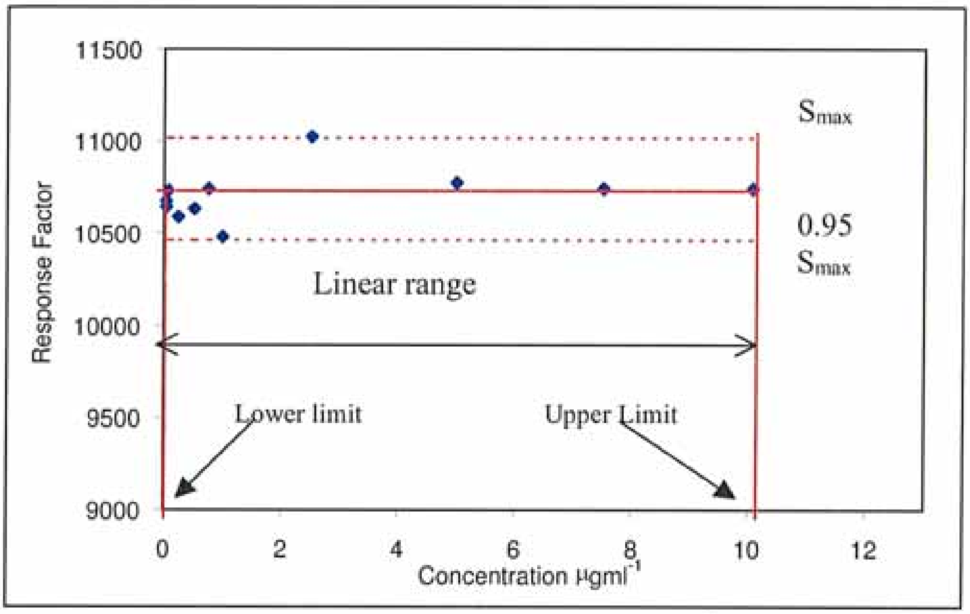


Figure 4.19: Plot of response factor against concentration (µgml^{-1}) showing the linear range of the method.

The linear range of the method can be taken from Figure 4.19, 0.05µgml^{-1} to 10.07µgml^{-1}, which corresponds to 18-3600µgkg^{-1} based on a 3g tissue sample. The red dashed line represents the mean response factor value. The upper limit of the linear range is taken from the point where this mean response factor value line crosses the 0.95S$_{max}$ line. The upper limit therefore is taken as 10µgml^{-1}. A plot of standard solutions of OTC against single peak height measurements was plotted over the defined linear range, Figure 4.20.

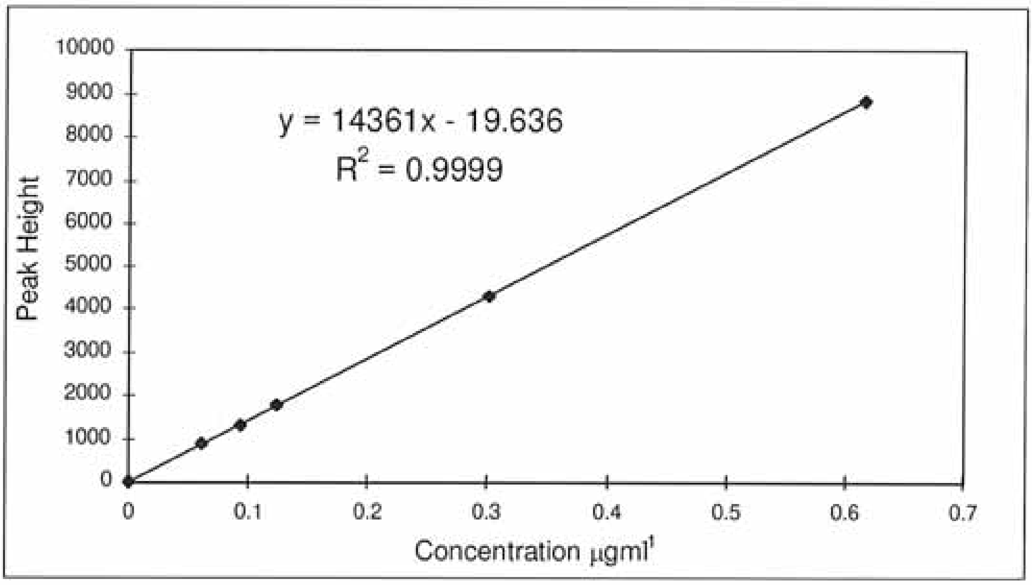


Figure 4.20: Calibration plot of oxytetracycline standards (µgml^{-1}) over the linear range defined

From this plot it is evident that there is a good fit of the data points to the least squares line and this is confirmed by a value of 0.9999 for the coefficient of variation. The y-intercept of the plot is -19.636. The response for the concentration of most interest on the plot i.e. $0.1\mu gml^{-1}$, is 1416. Therefore the y-intercept is 1.4% of the $0.1\mu gml^{-1}$ response and this value $\leq$ the required 2% difference which is set out in the guidelines for testing the linearity of the method [21].

4.9.4 Calculations

1.065μg of OTC was added to 2.938g of tissue. This yielded a $362.4\mu gkg^{-1}$ sample. The peak height obtained for this sample was *19507.*

From a plot of OTC standards against peak height a straight line was obtained (Figure 4.20)

The equation of this line was: $y = 14361x - 19.366.$

When the height for the sample was substituted into this equation a concentration value corresponding to $1.359\mu gml^{-1}$ was obtained.

$$\text{i.e. } 19507 = 14361x - 19.366$$
$$x = 1.359\mu gml^{-1}$$

30.003g of extraction buffer was used to extract the OTC from the tissue sample. However only *25.231g* of this buffer was added to the SPE cartridge and therefore only this portion of the sample was analysed. Thus the concentration value obtained from standard equation needs to be adjusted to allow for this fact.

i.e. the factor used to adjust the concentration value was:

$$\frac{30.003}{25.231} = 1.189$$

The final weight in which the residue was dissolved was *0.498g* of oxalic acid. The density of this solution is $0.996gml^{-1}$, therefore 0.498g corresponds to *0.499ml.*

The total factor is therefore:

$$1.189 \times 0.499 = 0.593\text{ml}$$

The total number of µg recovered is therefore the value obtained from the equation multiplied by the factor calculated above.

$$1.359\mu\text{gml}^{-1} \times 0.593\text{ml} = 0806\mu\text{g recovered}$$

The total amount of OTC recovered was: *0.806µg.*

The total amount of OTC added to the tissue was: *1.065µg.*

The percentage recovery was:

$$(\mu\text{g recovered} / \mu\text{g added}) \times 100$$

$$\text{i.e. } (0.806/1.065) \times 100 = 75.7\%$$

4.9.5 Precision

The precision of a method is the amount of scatter in the results obtained from multiple analyses of a homogeneous sample. To be meaningful the precision studies were performed using the exact sample and standard preparation procedures that were used in the final method. The first type of precision study is the instrument precision or injection repeatability, which was dealt with in Section 4.9.2. The second type of precision study is the repeatability or intra-assay precision. Intra-assay precision data was obtained by repeatedly analysing, in one laboratory on one day, aliquots of a homogeneous sample, each of which was independently prepared according to the method procedure (Section 4.8). From these precision studies the sample preparation procedure for the final method was set.

In order to carry out the intra-assay precision study, replicate samples were prepared containing the MRL of OTC ($100\mu\text{gkg}^{-1}$). Fish tissues samples (3g), were spiked with $100\mu\text{gkg}^{-1}$ of analyte and each sample was independently prepared according to the sample procedure. These samples were analysed by HPLC and measured concentration values recorded.

Table 4.11: Results from 8 replicate tissue samples spiked to 100μgkg^{-1}

Sample number	Measured Concentration not corrected for recovery (μgkg^{-1})
1	77.5
2	77.4
3	79.2
4	75.9
5	75.9
6	78.9
7	77.6
8	77.2
Mean	**77.4 μgkg^{-1}**
Standard deviation	**1.19**
% RSD	**1.5%**
Upper 95% CL	**78.5 μgkg^{-1}**
Lower 95% CL	**75.9 μgkg^{-1}**

The 95% confidence limits for these results were calculated and a plot of the data points about the mean value and the upper and lower confidence limits is shown in Figure 4.21.

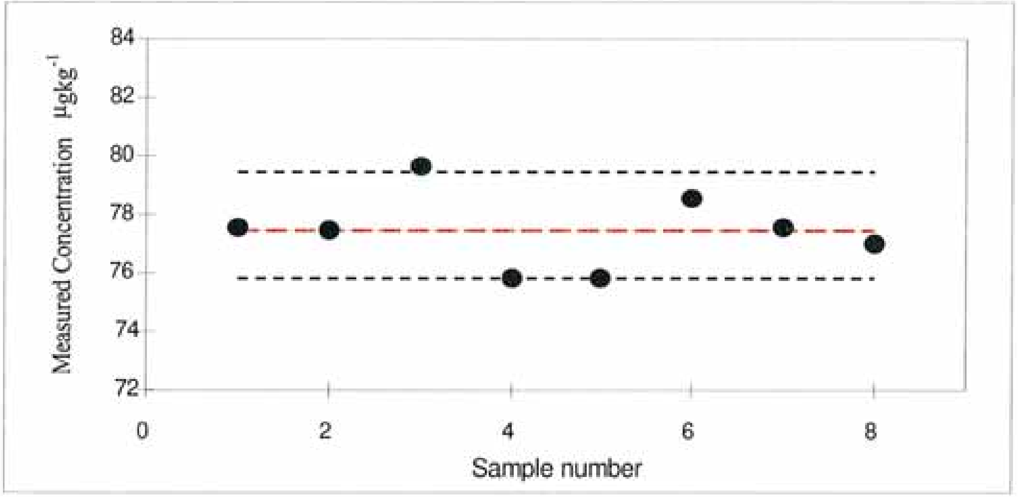


Figure 4.21: Intra-assay precision study showing concentration measured plotted against sample number for 8 replicates of tissue spiked to 100μgkg^{-1}. The upper and lower dashed lines correspond to the 95% confidence limits, while the red line in the centre is the line corresponding to the mean value recorded.

This plot shows the distribution of the data points around a mean value of 77.4μgkg^{-1}, i.e. the centre red dashed line in Figure 4.21. The plot also shows that when a sample is analysed using the described method, between 76 and 78.5% of the total sample present is recovered at a 95% confidence level. The result obtained for an unknown sample can be corrected for this recovery.

4.9.6 Accuracy

Accuracy is defined as the proximity of a measured value to the true value and it is affected by systematic errors. Systematic error or bias remains constant or varies in a predictable way over a series of measurements. This type of error differs from random error in that it cannot be reduced by making multiple measurements. It can however, be corrected for if detected but the correction would not be exact since there would inevitably be some uncertainty about the exact value of the systematic error. Accuracy is expressed as the % recovery by the assay method, of known spiked amounts of analyte.

Precautions were taken to ensure that the spiked sample was homogeneous and that it was in the same or close to the same physical and chemical state as the analyte would be in the unknown sample. This was achieved by taking 3g of a homogeneous tissue sample and spiking it with OTC. The spiked tissue was allowed to stand for 30 minutes before the extraction procedure was commenced. The replicates were run in random order, and on different days to subject the method to whatever uncontrolled variables which may have been operating. The same analyst carried out the analysis on all replicates to eliminate between analyst variations. When all the analyses were carried out, the data was then evaluated. The precision of the data obtained, which involves the repeatability study, can be seen in Section 4.9.5.

The percentage recoveries for the three analyses above are in the range of 70.7% to 79.5% (Table 4.12). The acceptable range for a method with a MRL of $\leq 100\mu g kg^{-1}$ is 70-110% [18]. Therefore even though the method is not very accurate the recoveries obtained fit within the range as set out in the Codex guidelines for control of the use of veterinary drugs. To test the precision of the percentage recovery values, over the concentration range examined, the upper and lower 95% confidence limits in analysis 1, 2, and 3 were calculated using Equation 1.1. The percentage recoveries and the 95% upper and lower confidence limits were plotted against the sample number and these can be seen in Figure 4.22.

Table 4.12: Percentage recoveries for triplicate spiked tissue samples analysed over the concentration range set in Section 4.9.3

Analysis 1		Analysis 2		Analysis 3	
Concentration (μgkg^{-1})	Percentage recovery	Concentration (μgkg^{-1})	Percentage recovery	Concentration (μgkg^{-1})	Percentage recovery
-	-	-	-	-	-
38.12	70.7	36.8	74.4	70.90	74.4
63.02	77.2	69.06	76.9	105.18	78.8
107.13	73.4	103.62	75.7	144.95	79.5
131.80	74.5	128.49	77.4	367.42	75.6
362.40	75.9	334.77	74.0	598.95	74.2
Mean	**74.2**		**75.7**		**76.5**
Standard deviation	**2.76**		**1.49**		**2.48**
% RSD	**3.71**		**1.97**		**3.24**
Upper 95% CL	**77.6**		**77.6**		**79.6**
Lower 95% CL	**70.0**		**73.8**		**73.4**

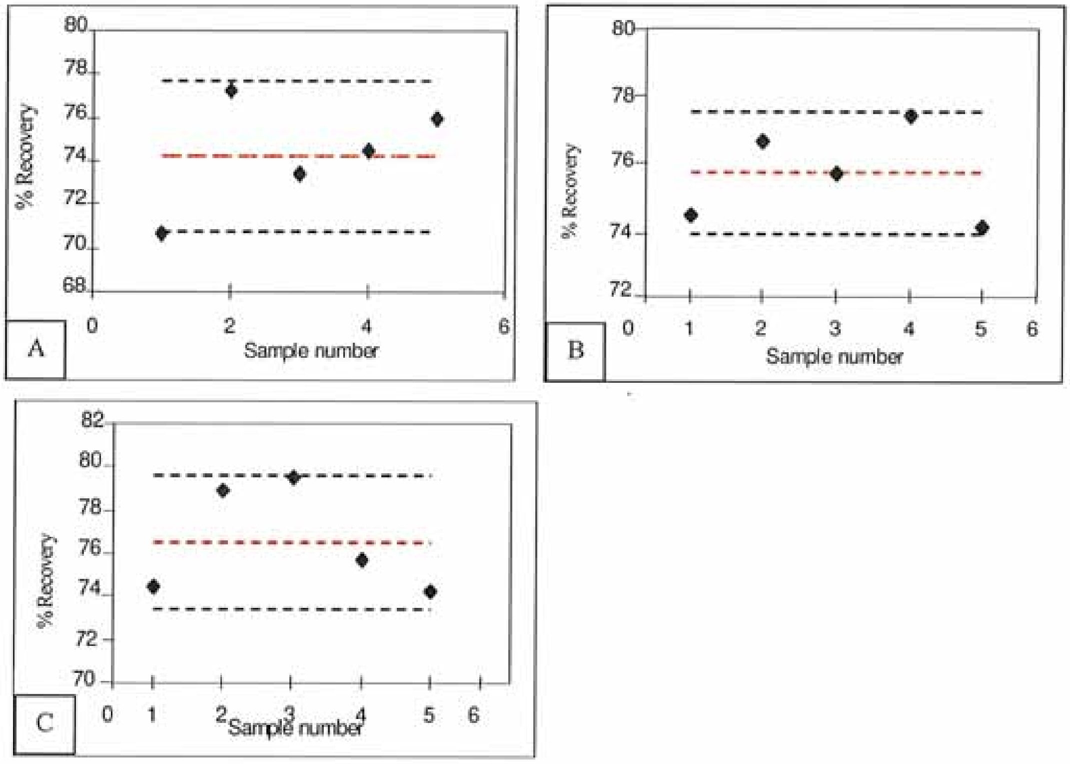


Figure 4.22: Plot of percentage recovery against sample number for Analysis 1(A), 2(B), and 3(C). Table 4.12.

The plots show a scatter of 74.2±3.8%, 75.7±1.9% and 74.2±3.1% for analysis 1, 2 and 3 respectively for samples analysed over the concentration range of 36-600μgkg^{-1}. Therefore at 95% confidence for this method, samples with an OTC concentration over the range of 36-600μgkg^{-1} can be analysed to an accuracy of 74.7% and a precision of 2.9%. The percentage recovery does not appear to vary significantly over the concentration range studied indicated by the approximate linear response v's concentration plots obtained (Figure 4.23(A) to 4.25(A), Section 4.9.7).
Directive 93/256/EEC only gives validation criteria for certified reference materials and not spiked tissue samples, which is the ideal situation. However, since no CRM exists for OTC in salmon tissue the spiked tissue samples must be used. It is worth noting that it has been reported in the literature that spike recoveries effectively mimic the recovery of the incurred analyte. However this was not proven as it was beyond the scope of the work published in the paper [5]. The authors did show however, that while the recoveries differed for extraction methods, 71±4% (n=10) for McIlvaine buffer and 90±6% (n=5) for ethyl acetate, for fortified tissue, similar results were obtained for incurred tissue for both methods when the results were corrected for percentage recovery. The results obtained were 1280μgkg^{-1} ±7%(n=10) for McIlvaine buffer and 1170μgkg^{-1} ±7%(n=5) for ethyl acetate. This gives an indication that the results from fortified and incurred tissue samples are comparable for OTC analysis.

4.9.7 Range and Limits of Detection and Quantification

The range of the analytical method was determined on the basis that the concentration value of most interest in the method is the MRL of 100μgkg^{-1}. The range of an analytical method is defined as the concentration interval over which acceptable accuracy, linearity and precision are obtained. The range was determined using data from the linearity, accuracy and precision studies, which were carried out earlier. The linear range was found to extend from 0.05-12.6μgml^{-1} corresponding to 18-4200μgkg^{-1} based on a 3g tissue sample, and this was deemed acceptable for the required analysis. The accuracy of the method was shown to be within 20-30% of the "true" value for three separate analyses, which were prepared and analysed independently of each other. Therefore in order to find the range of the method, the only remaining factor to be evaluated is the linearity of spiked tissue samples. This study will also show that the accuracy and precision are constant over the concentration range examined.

The calibration plots for the spiked tissue samples were prepared by spiking (5 x 3g) of tissue with known levels of OTC. The spiked tissue samples plus a blank were extracted and analysed according to the method procedure (Section 4.8). The calibration plot was examined to determine if a straight line with correlation coefficient of ≥ 0.999 was obtained. A response factor plot was examined and once acceptable linearity was obtained the range and the limit of quantification of the method were determined. The calibration plots and the response factor plots for each of the three analyses can be seen in Figures 4.23-4.25 for analysis 1-3 respectively.

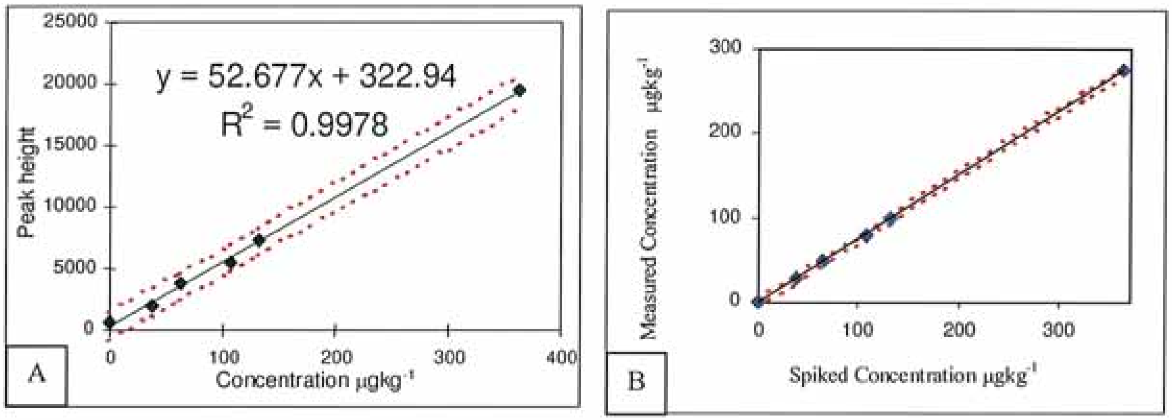


Figure 4.23: Oxytetracycline calibration plot (A) and spiked against measured concentration plot (B) for Analysis 1 of spiked tissue samples. The dashed lines represent the upper and lower 95% confidence limits.

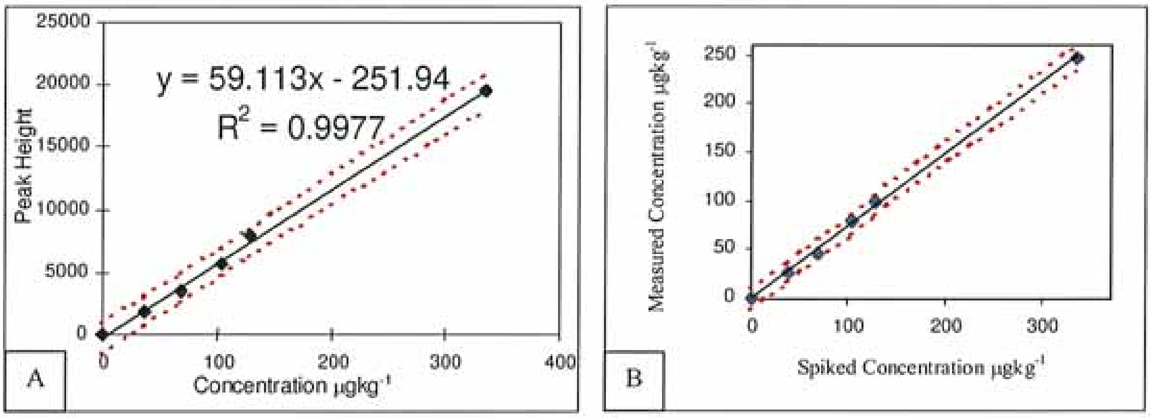


Figure 4.24: Oxytetracycline calibration plot (A) and spiked against measured concentration plot (B) for Analysis 2 of spiked tissue samples. The dashed lines represent the upper and lower 95% confidence limits.

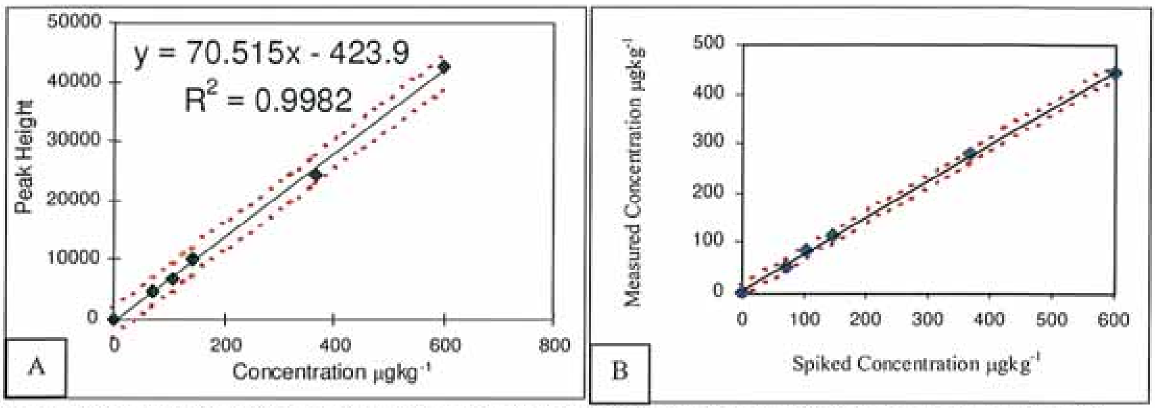


Figure 4.25: Oxytetracycline calibration plot (A) and spiked against measured concentration plot (B) for Analysis 3 of spiked tissue samples. The dashed lines represent the upper and lower 95% confidence limits.

On first examination of the linearity of the calibration plots, straight lines with correlation coefficients of 0.9978, 0.9977 and 0.9982 were obtained for analysis 1,2 and 3 respectively as can be seen from Figures 4.23(A), 4.24(A), and 4.25(A). Using the 95% confidence intervals from the plots of spiked concentration against measured concentration the confidence interval at the MRL was calculated for each analysis. These were corrected for percentage recovery (Section 4.9.4) and the intervals were found to be 97±7µgkg^{-1}, 95.5±10µgkg^{-1}, and 99±11µgkg^{-1} for analysis 1, 2 and 3 respectively. This indicates that linearity was obtained over the range of 36-600µgkg^{-1} for spiked tissue samples.

The linearity of the method for spiked tissue samples was further examined using the response factor plots and these can be seen in Figures 4.26(A-C). The 95% confidence limits were calculated and included in these plots.

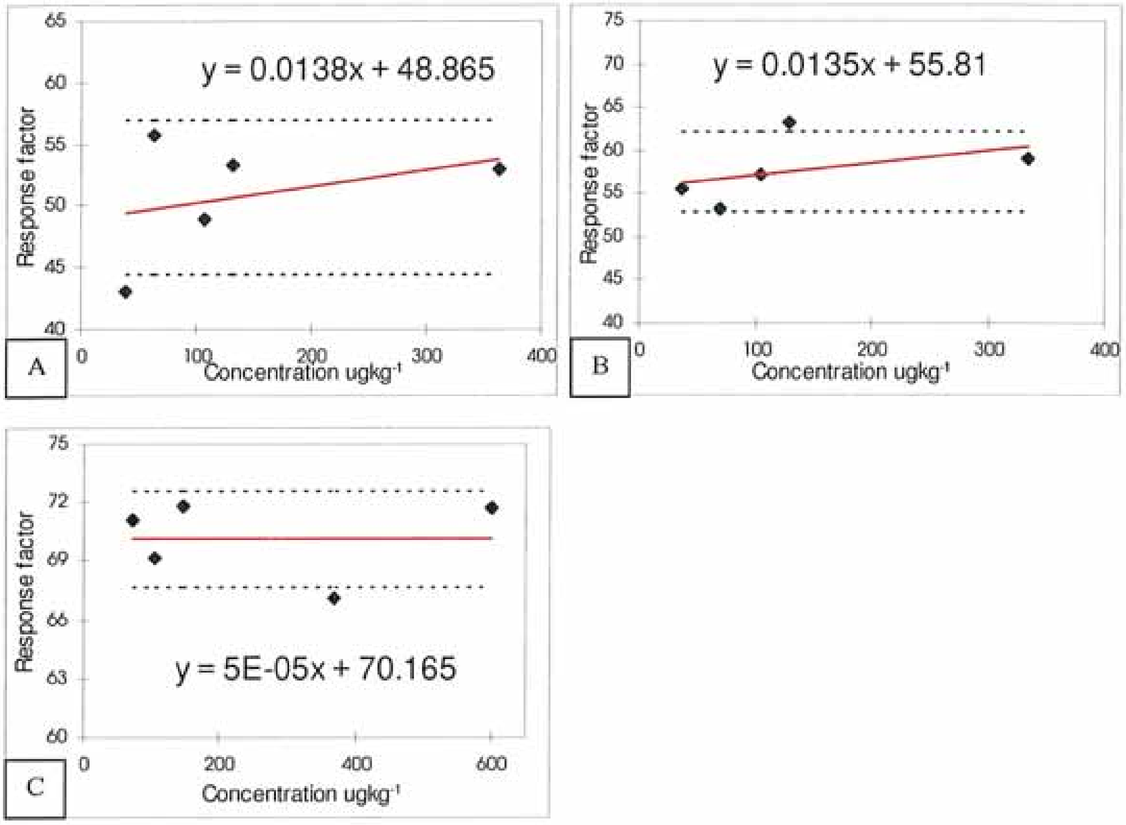


Figure 4.26: Response factor plots for spiked tissue samples for Analysis 1(A), Analysis 2(B) and Analysis 3(C), corresponding to the data in Figures 4.23 –4.25

These plots show that the data points form an almost straight line with a near zero slope. 95% confidence limits were applied to the data and the slopes were found to be ($1.38\times10^{-2} \pm 6.58\times10^{-2}$), ($1.35\times10^{-2} \pm 5.4\times10^{-2}$) and ($5.15\times10^{-5} \pm 1.65\times10^{-2}$) for analysis 1, 2 and 3 respectively. In an ideal situation a zero slope indicates a linear plot. Aditionally an equivalent response factor value would be obtained at each concentration value. Although all the response factor values obtained are not equivalent, it is evident from the plot that all the points are randomly distributed about the mean. The scatter of the response factor points about the mean values are 50.8 ± 7.7, 57.6 ± 5.6, and 70.2 ± 3.1 for analysis 1, 2 and 3 respectively.

A third method was used to examine the linearity of the method. This involved calculating the y-residuals and estimating whether or not the sum of these residuals was zero [22]. A y-residual represents the difference between an experimental value of y and the predicted y-value for the same value of x. The predicted y value, $\hat{y}$, was calculated from the regression line of the standard plot. If a linear calibration plot is appropriate, and if the random errors in the y-values are normally distributed, the residuals $(y-\hat{y})$ themselves should be normally distributed about the value of zero. Theoretically if the sum of the y-residuals is zero then the data points form a linear calibration plot. The residuals for the three separate analyses were calculated and the results can be seen in Table 4.13 and Figure 4.27.

Table 4.13: y-residual data for the three separate analyses

Analysis 1				Analysis 2	Analysis 3
x	y	$\hat{y}$	$y-\hat{y}$	$y-\hat{y}$	$y-\hat{y}$
0	722	323	399	252	424
38.1	1966	2331	-365	-129	38
63.0	3837	3643	194	-414	-140
107.1	5557	5966	-407	-191	183
131.8	7353	7266	87	526	-1244
362.4	19507	19413	94	-43	739
$y = 52.67 + 322.94$		$\sum y-\hat{y}$	0	0	0

From the plots in Figure 4.27 it is evident that the data points are randomly distributed about the x-axis. Since the slopes of ($2.6\text{x}10^{-9}$ ± $7.5\text{x}10^{-2}$), ($3.5\text{x}10^{-8}$ ± $7.6\text{x}10^{-2}$) and ($2.2\text{x}10^{-8}$ ± $6.8\text{x}10^{-2}$) for analysis 1, 2 and 3 respectively, are not significantly different from zero at a 95% level of confidence, the indication is that the best-fit lines are parallel to the x-axis. The intercepts of ($-2.3\text{x}10^{-7}$ ± $7.7\text{x}10^{2}$), ($-2.23\text{x}10^{-4}$ ±$6.9\text{x}10^{2}$) and ($-3.1\text{x}10^{-4}$ ±$1.4\text{x}10^{3}$) are not significantly different from zero at a 95% level of confidence, therefore indicating that the points are randomly distributed about the x-axis. Additionally the sum of the y-residuals is zero for each analysis (Table 4.13) indicating that the data points form a linear calibration plot over the concentration range examined.

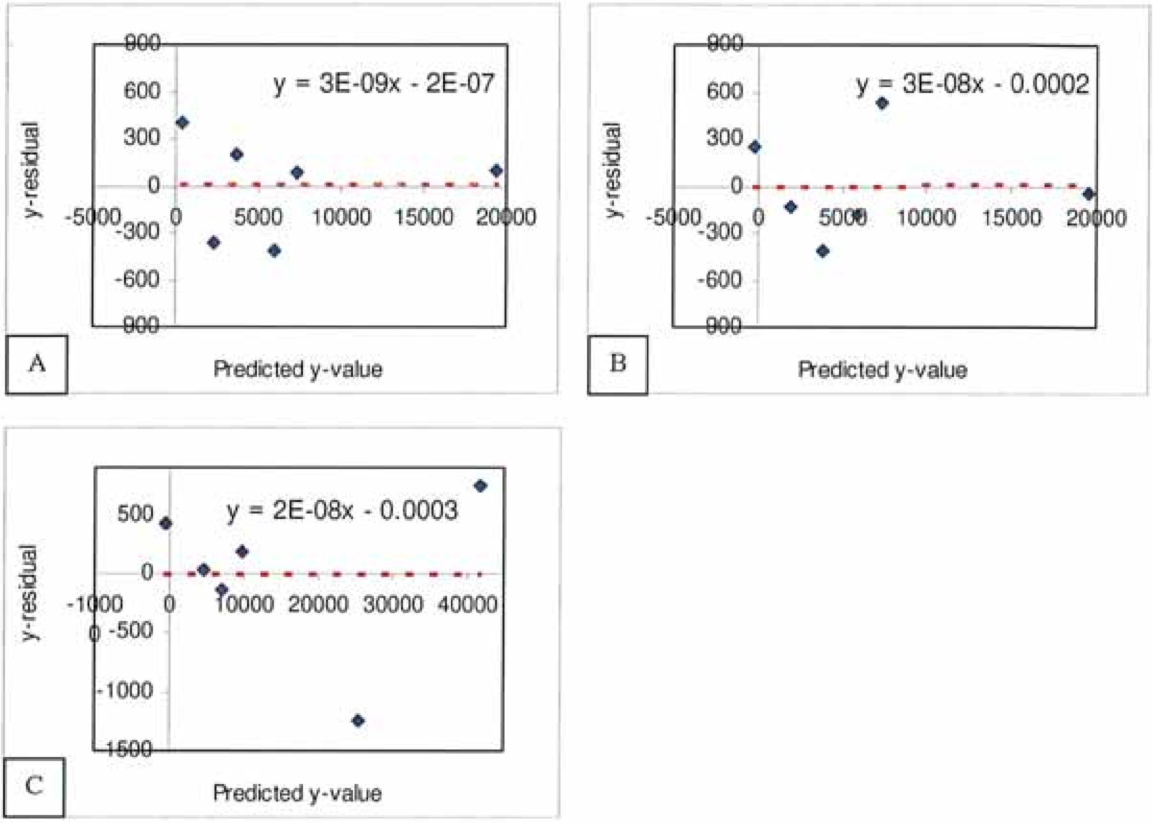


Figure 4.27: Oxytetracycline y-residual plots of spiked tissue samples for Analysis 1 (A), Analysis 2 (B) and Analysis 3 (C).

The studies carried out here show that linearity was obtained for spiked tissue samples over the concentration range of 36-360μgkg^{-1} for three separate analyses carried out independently of each other. However, a higher response was obtained for the samples analysed in analysis 3 when compared to those of analysis 1 and 2. This is indicated by the mean response factor of 70.2 ± 3.1 for analysis 3, compared to 50.8 ± 7.7 and 57.6 ± 5.6 for analysis 1 and 2 respectively (Table 4.13). The higher slope value obtained for the calibration and response factor plots verifies that the response obtained for analysis 3 was higher than that obtained for analysis 1 and 2. This is most probably due to the fact that the three analyses were carried out under separate conditions independently of each other. Slight changes in the mobile phase composition can cause a peak to be either sharper or broader depending on the organic content. Since peak height was used for quantification in this method these changes would have an effect on the response recorded. Standards analysed with the spiked tissue samples in analysis 3 also gave a higher response when compared to those analysed with analysis 1 and 2. The retention time of OTC for analysis 3 was noted to be approximately 20 seconds earlier than those recorded for OTC analysed under the conditions of analysis 1 and 2 which also indicates that the mobile phase was the most likely cause. This higher response however, did not have a significant effect on the linearity of the method as was shown above.

From these studies it can be taken that the linearity of the method from 36μgkg^{-1} to 360μgkg^{-1} is acceptable based on a 100μgkg^{-1} MRL for OTC in salmon tissue.

The limit of detection (LOD) (Section 1.5.7) was estimated by measuring the response obtained for blank tissue samples (n=3) and calculating the LOD based on a S/N = 3. This value was subsituted into the equation of the calibration line to estimate the corresponding concentration value. The equation of the calibration line was $y = 52.68x + 322.94$ and from the information below the LOD was estimated to be 20.1μgkg^{-1}.

Sample number	**Noise Response**	**3 x noise**	**5 x noise**
1	455	1365	2275
2	470	1410	2350
3	458	1374	2290
Mean	461	1383	2305
Concentration /μgkg^{-1}		**20.1**	**37.6**

The limit of quantification (LOQ) (Section 1.5.8) of the method was taken as the S/N = 5. From the data shown this value was estimated to be 37.6μgkg^{-1}. This is the lowest analyte content, which can be quantified as opposed to identified using the above method.

4.9.8 Stability

For routine testing in which many samples are analysed each day, it is often necessary to allow for delays such as instrument breakdowns or overnight analyses. Therefore, the limit of stability of prepared tissue samples and the mobile phase were tested, and storage conditions identified so that the stability can be improved.

4.9.8.1 Prepared tissue sample stability

A calibration plot of spiked tissue samples was prepared containing (38-360)μgkg^{-1} of OTC. These were analysed for three consecutive days using a freshly prepared mobile phase on each of the days. The calibration plot, peak retention time, response and symmetry were all observed and recorded in Table 4.14. The calibration and spiked against measured concentration plots over the three days can be seen in Figures 4.28-4.30.

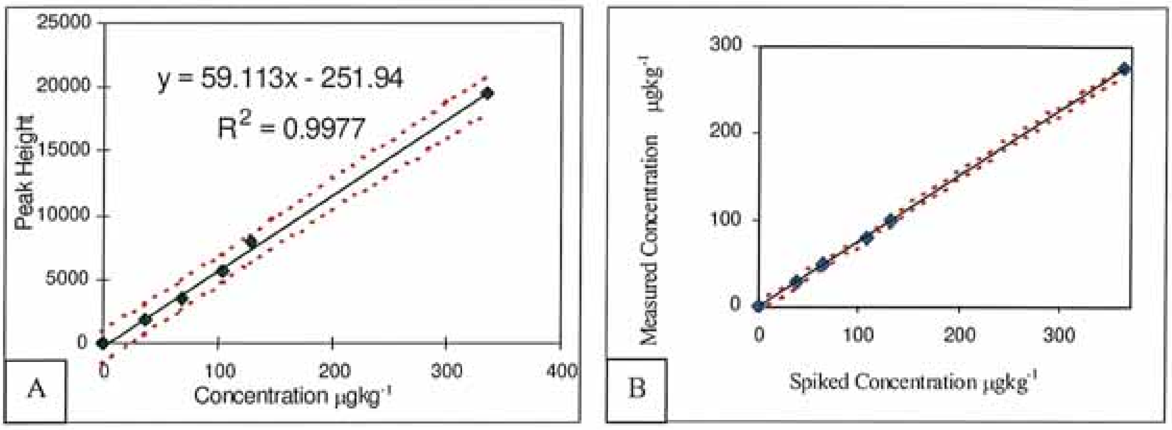


Figure 4.28: Oxytetracycline calibration plot (A) and spiked against measured concentration plot (B) of spiked tissue samples on Day 1 of analysis. The dashed lines represent the upper and lower 95% confidence limits.

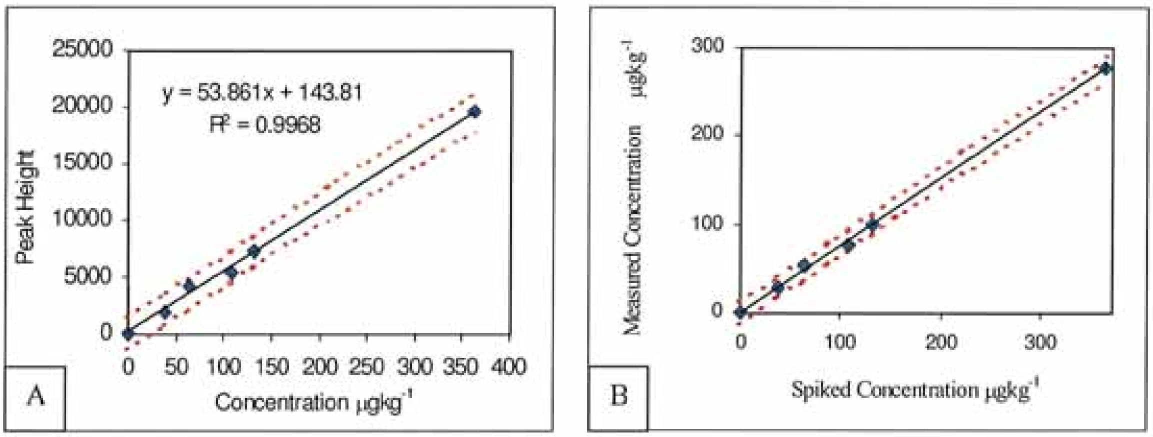


Figure 4.29: Oxytetracycline calibration plot (A) and spiked against measured concentration plot (B) of spiked tissue samples on Day 2 of analysis. The dashed lines represent the upper and lower 95% confidence limits.
A freshly prepared mobile phase was used for this analysis.

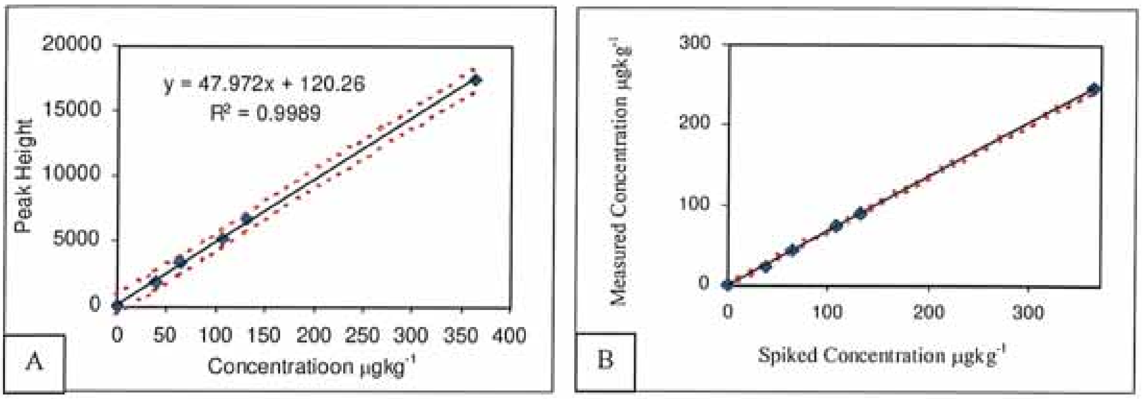


Figure 4.30: Oxytetracycline calibration plot (A) and spiked against measured concentration plot (B) of spiked tissue samples on Day 3 of analysis. The dashed lines represent the upper and lower 95% confidence limits.
A freshly prepared mobile phase was used for this analysis.

Table 4.14: Peak time, symmetry and response data for the prepared spiked tissue, 100µgkg^{-1} stability test. The variation in terms of % RSD is shown for the peak characteristics.

Day	Retention time (minutes)	Variation from Day 1 (seconds)	Peak width (minutes)	Variation from Day 1 (seconds)	A_s
1	4.55		0.46		1.13
2	4.25	18	0.44	2	1.19
3	4.46	5	0.48	1	1.18
Mean	**4.42**		**0.46**		
Standard deviation	**0.152**		**0.023**		
% RSD	**3.4%**		**4.9%**		

It can be seen that the retention time and peak width varied slightly over the three days of analysis but this was most likely due to very slight changes in the mobile phase composition as a new mobile phase was made up on each day of the stability test. The asymmetry factor remained under the required 1.2 value over the three days of the analysis thus verifying that the peak symmetry is satisfactory for spiked tissue samples over three days of analysis.

Table 4.15: Analysis of the calibration plot for OTC spiked tissue samples analysed over three consecutive days.

	R^2	MRL 95% CL	Slope comparison
Day 1	0.9978	97± 7µgkg^{-1}	
Day 2	0.9968	98± 11µgkg^{-1}	$t_{calc} = 0.60$
Day 3	0.9989	88 ± 3µgkg^{-1}	$t_{calc} = 3.17$

From the R^2 values obtained on each day of analysis (Table 4.15) it is evident that the values are all above 0.99 but no particular trend was noted over the three days. Thus this value cannot be used to determine if the spiked tissue samples produced a linear calibration plot over three consecutive days of analysis. Using the spiked against measured concentration plots, the 95% confidence interval was calculated on the MRL for each day of analysis and these are shown in Table 4.15. There does not appear to be a significant difference between the intervals obtained on Day 1 and 2 but the interval for Day 3, 88±3μgkg^{-1} does not contain the true value of 100μgkg^{-1}.

The data in Table 4.16 compares the measured concentration over the three days to the spiked concentration. Since the measured concentration has not been corrected for percentage recovery then these values are lower than the spiked concentration values. Additionally, it is evident from this table that the response obtained on the third day of analysis was lower than that obtained on either day 1 or day 2 at each concentration level, indicating that the response of OTC has decreased over time. To examine this further the sensitivity of the method was compared over time. The slopes of the calibration plots were compared using Equation 1.2 (Table 4.15). Standards analysed on each of the three days of this test to check the performance of the instrument met the sensitivity criteria indicating that the instrument response was acceptable on each day. The critical value of t was found to be 2.31 at $((n_1-2) + (n_2-2)$ degrees of freedom [22]. Between Day 1 and Day 2 $t_{calc} < t_{table}$ and thus the slopes are not significantly different at a 5% level on the second day of analysis. Between Day 1 and Day 3, $t_{calc} > t_{table}$ and therefore the slopes are significantly different between the first and third days of analysis. This indicates that the response obtained on Day 3 was different from that obtained Day 1 at a 95% confidence level. The slope obtained on Day 1 was 59.1, 53.8 on Day 2 while a slope of 47.9 was obtained on Day 3. The response obtained for the spiked tissue samples had decreased by approximately 9% between Day 1 and Day 2 and 20% between Day 1 and Day 3.

Table 4.16: Measured concentrations of spiked tissue samples over three days of analysis

Spiked Concentration (μgkg^{-1})	Measured Concentration (μgkg^{-1}) not corrected for recovery		
	Day 1	Day 2	Day 3
38.1	26.9	27.2	23.9
63.0	48.6	53.7	44.3
107.1	78.6	77.5	72.9
131.8	98.2	98.4	89.6
362.4	275.1	277.1	245.6

On examination of the above data it can be concluded that the method is linear from 38-350μgkg^{-1} for the first two days of the spiked tissue sample stability test at 95% confidence limits. The response obtained decreased significantly at a 95% confidence level, on the third day of analysis. Based on this, the prepared tissue samples should be analysed within two days after the sample preparation is complete. If they are stored in the fridge at 4°C, the prepared tissue samples while still producing a linear plot over two days between 38-350μgkg^{-1}, will degrade and thus the lower concentration values may not be detected. It is possible that over time OTC is degrading to its metabolite 4-*epi*-OTC and this needs to be examined in the future.

4.9.8.2 Mobile phase stability

The mobile phase was tested for stability over time by analysing spiked tissue samples in the form of a calibration plot with the same mobile phase and examining the curve linearity.

Figure 4.28(A) shows the calibration plot for the spiked tissue samples analysed within 18 hours of final sample preparation. The linearity over 38-350μgkg^{-1} was deemed satisfactory as demonstrated by the confidence interval of 97±7μgkg^{-1} obtained for the MRL (Figure 4.28(B)).

While using the same mobile phase the samples were analysed within 42 hours of the completion of the sample preparation step and the relevant plots are shown in Figure 4.31.

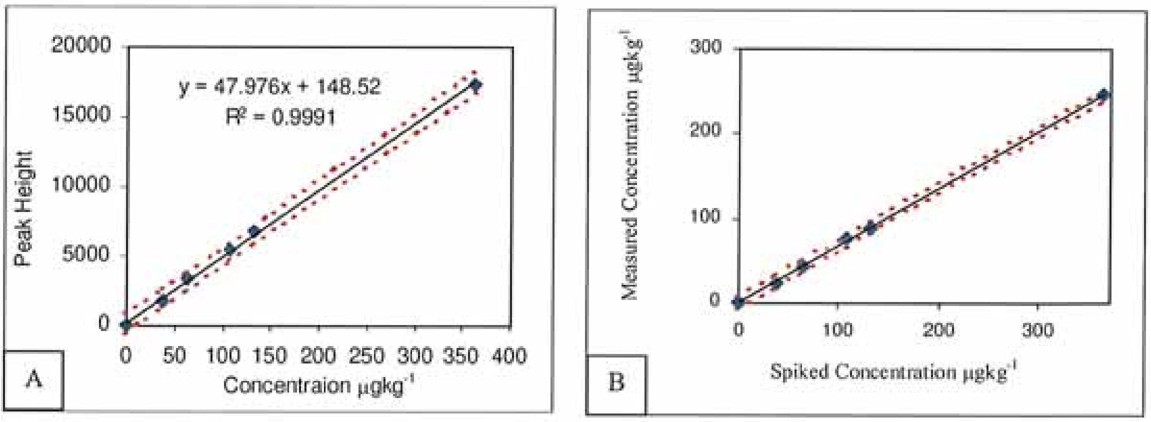


Figure 4.31: Oxytetracycline calibration (A) and spiked against measured concentration (B) plots of spiked tissue samples on Day 2 of the mobile phase stability test. The dashed lines represent the upper and lower 95% confidence limits.
The same mobile phase was used on Day 1 and Day 2 of the analysis.

The spiked against measured concentration plot (Figure 4.31(B)) was used to determine the 95% confidence interval about the MRL of 100μkg^{-1}. This interval was found to be 88±7μkg^{-1} which does not contain the true value of 100μgkg^{-1}. Since the prepared tissue samples have already been deemed satisfactory for analysis in this time period with a freshly prepared mobile phase (Section 4.9.8.1) the fact that the response had decreased was due to the mobile phase. Therefore the method was linear for 42 hours within the range of 38-360μgkg^{-1} only if a freshly prepared mobile phase was used for the analysis.

It should be noted that the asymmetry value on the second day of analysis did not alter significantly from 1.13 on the first day. There was no significant changes in peak retention time, peak width, or peak response for a tissue sample spiked to 100μgkg^{-1} when this was analysed with the same mobile phase for two consecutive days.

4.10 Sample Analysis

Thirty-five farmed salmon samples were obtained randomly from seven major fish farms around the Irish coast. These salmon were of marketable size and were intended for human consumption. The tissue samples were homogenised and stored in acid followed by methanol washed glass sample jars at -30°C prior to analysis. The samples were analysed for OTC in the edible tissue by HPLC.

The analysis of the thirty-five salmon tissue samples for OTC showed levels of OTC, which were below the limit of detection of the analytical method. This limit of 20μgkg^{-1} is below the maximum residue level of 100μgkg^{-1} for OTC in salmon muscle, as set by the European Commission [1] indicating that the samples analysed are safe for human consumption. A sample chromatogram is shown in Figure 4.32.

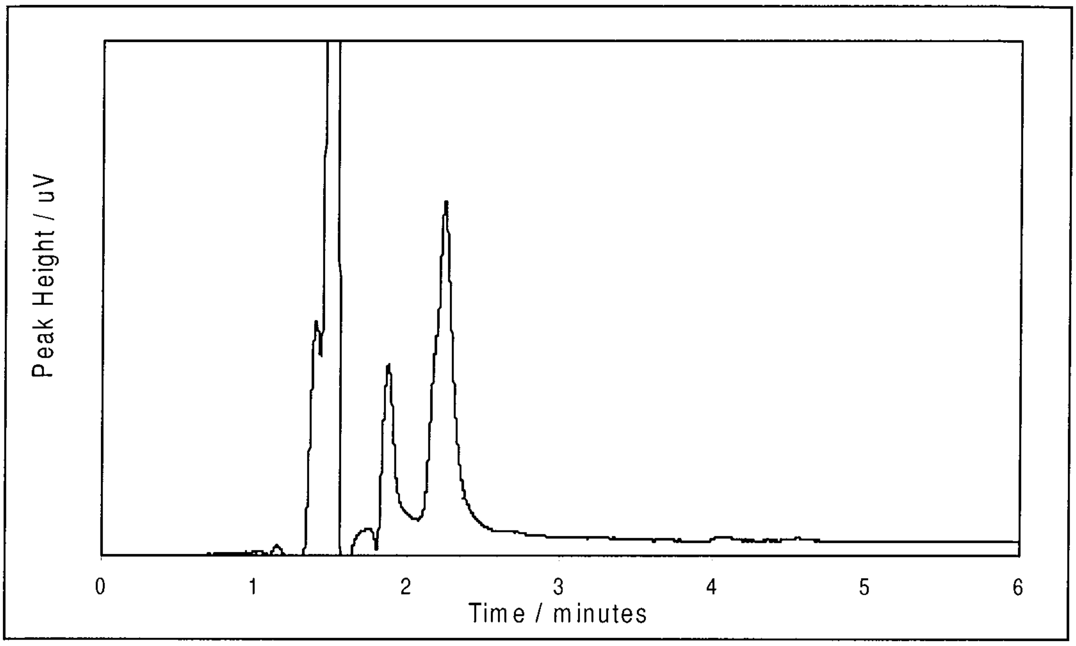


Figure 4.32: Chromatogram of a salmon tissue sample analysed using the method described in Section 4.5 and 4.8.

4.11 Conclusion

Under Council Directive 96/23/EC routine methods must be validated according to the criterion set out in Commission Decision 93/256/EC. The published methods for the determination of OTC in salmon tissue, [6, 12, 14] lack the complete published documentation to demonstrate their ability to meet all these required performance criteria.

The above described method shows clear resolution of OTC from the salmon tissue matrix components within a six minute analytical run. From the chromatogram shown in Figure 4.12, it is evident that the cleanup procedure was effective and thus there should be no difficulties with a short column life due to matrix effects.

The specificity study showed that there was no interference in the chromatogram at the retention time of OTC from the other components listed in Directive 96/23/EEC for routine monitoring. Therefore the analyte retention time can be used as the initial identification of OTC and in the future for confirmation purposes, the fixed wavelength UV detector will be substituted with a DAD detector and the identification confirmed by obtaining the analyte spectra as specified in Commission Decision 93/256/EEC.

The accuracy of the method obtained at the MRL of 100μgkg^{-1} was 77.4±1.5% (n=8). While the recoveries reported in the literature were generally higher than 77.4%, it should be noted that these values were quoted at concentration levels above the MRL for OTC in salmon tissue, while using similar methods i.e. 82% at 200μgkg^{-1} [6] and 87.6% at 1000μgkg^{-1} [4]. However, 77.4% meets the validation criteria set out in the codex guidelines which sets an acceptable accuracy range of 70-110% for spiked tissue samples [18]. The EU guidelines only quote recovery criteria for CRMs and since these do not exist for OTC the Codex guidelines are adhered to [16].

The precision of 1.5% RSD obtained for 8 spiked tissue samples at 100μgkg^{-1} was an improvement on precision values reported in the literature for salmon tissue preparation, i.e. 5%RSD at 200μgkg^{-1} (n=6) [6] and 3%RSD at 1000μgkg^{-1} (n=8) [4].

The LOQ obtained for this method, 37.6μgkg^{-1}, (S/N=5) was also the lowest value measured which gave RSD<2.5% for replicate analysis (n=3) of a spiked tissue sample. This value which is below the MRL is an improvement on the values reported in the literature for salmon tissue, i.e. 100μgkg^{-1} [12], 80μgkg^{-1} [6] and 50μgkg^{-1} [14]. While lower LOQ has been reported for other tissue matrixes (Table 1.4) the value obtained in this method is the lowest obtained for salmon tissue.

While Directive 96/23/EC applies to all farmed finfish, and in Ireland this refers to salmon and trout, the work carried out on in this chapter involved salmon tissue only and trout will be examined in the future. In 1998 Meinertz *et al.* [10] reported a method very similar to that reported in Section 4.5 and 4.8, and demonstrated the ability of this method to be used for six species of fish including salmon, trout, walleye, bass, sturgeon and catfish. The accuracy for salmon was 87.1±3.1% (n=9), and for trout was 85.9±2.5% (n=8) indicating that similar results were obtained for both matrixes. The elution profiles were similar for all species and co-eluting compounds were not detected in the chromatograms of extracts from any species. The chromatographic baselines with the greatest noise were noted for salmon tissue and if this were also to apply to the method described in Section 4.5 and 4.8 then the indication would be that the method could be easily adapted to other fish tissue matrixes including trout.

Commission Decision 93/256/EEC also provides validation criteria on inter-laboratory comparisons of the analytical method. While this work was beyond the scope of this project it will be part of work to be carried out in the future. Inter-laboratory comparisons have however, been made for OTC analysis in pig [23] and bovine [19] tissues but not for salmon or other fish tissues. In 1996 thirteen laboratories analysed bovine tissue for OTC using a method very similar to the proposed method for OTC analysis [19]. The result of this inter-laboratory comparison showed that the method could be applied successfully to the analysis of tetracyclines residues, including OTC, in edible animal tissue at levels of 100-600μgkg^{-1}.

In 1998 fourteen laboratories took part in an inter-laboratory comparison of OTC analysis in pig muscle [23]. The laboratories could use a method of their own choice but all were based on reversed-phase liquid chromatography with different detection modes including UV, DAD and fluorimetric detection after derivatisation. The extraction and clean-up procedures were of three general schemes, one of which was very similar to that proposed for this work. The results showed that all the laboratories obtained satisfactory z-scores in terms of accuracy and only two laboratories obtained a questionable result in terms of repeatability. While the paper did not indicate which clean up procedure gave the best results it did show that the three different methods used in the inter-laboratory study compared well.

The findings in both of these papers indicate that there is a good possibility that the method, which was described, for the sample preparation and analysis of OTC in salmon tissue in this work is capable of performing well in an inter-laboratory comparison. Also the fact that the method reported in 1996, which is very similar to the method described in this work, was recommended and adopted by the AOAC International as an official method is a good indication of its performance [19].

4.12 References

[1] Commission Regulation (EC) No. 508/1999 of 4 March 1999 amending Annexes I to IV of Council Regulation (EEC) No 2377/90 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin.

[2] Pouliquen H, Keita D, and Pinault L. (1992). Determination of oxytetracycline in marine shellfish (*Crassostrea gigas, Ruditapes philippinarum* and *Scrobicultaria plana*) by high-performance liquid chromatography using solid-phase extraction. Journal of chromatography, **627**, 287-293.

[3] Knox J, Jurand J. (1979). Mechanism of reversed-phase seperation of tetracyclines by high-performance liquid chromatography. Journal of chromatography, **186**, 763-782.

[4] Bjorklund H. (1988). Determination of oxytetracycline in fish by high-performance liquid chromatography. Journal of Chromatography, **432**, 381-387.

[5] Cooper AD, Tarbin JA, Farrington WHH, Shearer G. (1998). Effects of extraction and spiking procedures on the determination of incurred residues of oxytetracycline in cattle kidney. Food Additives and Contaminants **15**, No.6, 645-650.

[6] Reimer G, Young L. (1990). Validation of a method for determination of tetracycline antibiotics in salmon muscle tissue. Journal of the Association of Official Analytical Chemists, **73**, No. 5, 813-817.

[7] Brandšteterová E, Kubalec P, Bovanová L, Simko P, Bednáriková A, Macháckova L.(1997). SPE and MSPD as pre-separation techniques for HPLC of tetracyclines in meat, milk and cheese. Z Lebenism Unters Forsch A, **205**, 311-315.

[8] Pouliquen H, Le Bris H, Pinault L. (1994). HPLC Determination of oxolinic acid and oxytetracycline in three types of marine sediments: analytical validation. Quimica Analytica, **13**, Supplement 1, S109-S113.

[9] Oka H, Matsumoto H, Uno K. (1985). Improvement of chemical analysis of antibiotics. VIII. Application of prepacked C18 Cartridge for the analysis of tetracycline residues in animal liver. Journal of Chromatography, **325**, 265-274.

[10] Meinertz JA, Stehly GR, Gingerich WH. (1998). Liquid Chromatographic determination of oxytetracycline in edible fish fillets from six species of fish. Journal of AOAC International, **81**, No. 4, 702-708.

[11] Rogstad A, Hormazabal V, and Yndestad M. (1988). Optimization of solid phase extraction of oxytetracycline from fish tissue and its determination by HPLC. Journal of liquid chromatography, **11**(11), 2337-2347.

[12] Iwaki K, Okumura N, Yamazaki M. (1992). Determination of tetracycline antibiotics by reversed phased liquid chromotagraphy with fluoresence detection. Journal of chromatography, **623**, 153-158.

[13] Nordlander I, Johnsson H. Osterdahl, Bengtgoran. (1987). Oxytetracycline residues in rainbow trout analysed by a rapid HPLC method. Food additives and contaminants, **4**, No. 3, 291-296.

[14] Carignan G, Carrier K, Sved S. (1993). Assay of oxytetracycline residues in salmon muscle by liquid chromatography with ultraviolet detection. Journal of AOAC international, **76**, No. 2, 325-328.

[15] Moretti V, Luzana U, Serrini G, Maria V, Luca G,Valfre F, Albertini A, Bellagamba F. (1994). High performance liquid chromatographic determination of oxytetracycline in channel catfish (*Ictalurus punctatus)* muscle tissue. Analyst, **119**, 2749-2751.

[16] Annex of Commission Decision 93/256/EEC laying down the methods to be used for detecting residues of substances having a hormonal or thyrostatic action.

[17] Dolan JW, Synder LR. (1989). Troubleshooting LC systems. The Humana Press Inc. ISBN 0-89603-151-9.

[18] Codex Alimentarius Volume 3, (1994). Section 2: Recommended international code of practice for control of the use of veterinary drugs.

[19] MacNeil JD, Martz VK, Korsrud GO, Salisbury CDC, Oka H, Epstein RL, Barnes CJ. (1996). Chlortetracycline, oxytetracycline, and tetracycline in edible animal tissues, liquid chromatographic method: Collaborative study. Journal of AOAC International. **79**: (2) 405-417.

[20] Bidlingmeyer BA. (1992). Practical HPLC methodology and applications. Wiley Interscience Publication. ISBN-471-57246-2.

[21] Green JM. (1996). A practical guide to analytical method validation. Analytical Chemistry News and Features May 1, 1996.

[22] Statistics for analytical Chemistry (1993). J.C. Miller and J.N. Miller. 3rd edition. Ellis Horwood PTR Prentice Hall Limited.

[23] Juhel-Gaugain M, Sanders P, Laurenite M, Anger B, Roudaut B, Maris P. (1998). Results of a European interlaboratory study for the determination of oxytetracycline in pig muscle by HPLC. Analyst. **123**: (12) 2767-2771.

CHAPTER 5: ANALYSIS OF SULPHADIAZINE (SD), SULPHAMETHOXAZOLE (SMX) AND TRIMETHOPRIM (TMP) IN SALMON TISSUE

5.1 Introduction

SD, SMX and TMP (Section 1.2.3) which are listed as Group B substances in Directive 96/23/EC are a group of antimicrobial agents that act on gram-negative bacteria by blocking folic acid pathways of susceptible micro-organisms. The MRL for combined sulphonamides in fish tissue samples is $100\mu gkg^{-1}$ while TMP has a MRL of $50\mu gkg^{-1}$ [1]. Thus farmed salmon tissue must be analysed on a routine basis to see if the MRLs are adhered to, otherwise residues of the drug may remain in the tissue intended for human consumption. Therefore routine methods are required for the detection and quantification of SD, SMX and TMP in salmon tissue to monitor residues in slaughtered fish in order to prevent any exposure to the consumer.

SD and SMX are administered to fish in combination with a potentiator, which is TMP (Section 1.2.3). Thus if either SD or SMX was administered to the fish then TMP would have also been administered at the same time. Therefore it is desirable if possible, to have one method which will simultaneously monitor for SD, TMP and SMX.

Following the examination of published methods, (Section 1.5.13), and a further discussion in Section 2.5 a RPHPLC method with DAD, was proposed for the analysis of SD, SMX and TMP in salmon tissue on a routine basis. A C18 column with an ACN based mobile phase was proposed for initial examination in the mobile phase. This chapter describes the optimisation of the LC conditions to find acceptable separation conditions for each analyte.

While general sample preparation techniques were discussed in Section 2.6, the cited literature reported just one method by Gentleman *et al.* [2] which simultaneously extracted and analysed SD and TMP from salmon tissue. This method involved ACN extraction and evaporation of the solvent to yield an aqueous solution, which consisted of lipids and the analytes of interest. This was transferred to a Waters C18 sep-pak and the analytes eluted with MeOH. Recoveries obtained were below the required 70% for spiked tissue samples (Section 1.5.13). The low recoveries were reported to be due to the lipid constituents in the salmon muscle resulting in chromatographic interferences [2].

With the strict measures put in place for the control of veterinary drug residues in farmed animals including fish, the use of multi-residue methods is becoming more desirable. Of the literature cited few such methods [3, 4, 5, 6], which analyse for some of the analytes of interest to Directive 96/23/EC for farmed fish were reported. The sample cleanup technique was different for each of these methods, i.e. liquid-liquid extraction [3], matrix solid phase dispersion (MSPD) [4, 6] and SPE [5].

Reimer *et al.* [6] reported a MSPD method for the analysis of sulphonamides in salmon tissue samples. The slurry was washed with toluene in hexane and the analytes eluted with dichloromethane (DCM). Due to the toxicity and environmental effects of DCM it should be avoided where possible. Low recoveries (66% for SD) were obtained and these were reported to be due to the high fat/oil content of the fish [6]. It may be the case that the lipids were causing chromatographic interferences, and if this was the case then a further hexane extract cleanup to remove the lipids could improve the recoveries.

In addition to the sulphonamides analysed by Reimer *et al.* [6], a similar method reported by Le Boulaire *et al.* [4], included other analytes from Directive 96/23/EC in farmed fish [7], including SMX, chloramphenicol and furazolidone. SMX was identified and quantified to the required validation criteria [8]. The same extraction and cleanup procedure was used for chloramphenicol but GC-MS was recommended in order to reach the required sensitivity. Recoveries of furazolidone at the MRL of 5μgkg^{-1} [8] were reported to be 44% indicating that this can only be used as a screening method for furazolidone [8].

Gehring *et al.* [9] homogenised tissue with 10ml of the HPLC mobile phase, ACN: MeOH: 2% aqueous acetic acid (5:10:85), prior to the addition of 30ml ACN. Consecutive liquid-liquid extractions involving DCM and hexane were carried out on the extract prior to HPLC injection. A similar method was reported by Cannavan *et al.* [10] for the extraction and analysis of TMP from tissue samples. TMP was extracted with a chloroform-acetone mixture followed by an aqueous-hexane extraction to partition the fats into the hexane and use the aqueous layer for analysis. While both methods were validated to the required validation criteria (Section 1.5) the use of chloroform and DCM should be avoided if possible due to their toxic and adverse environmental effects.

Cooper *et al.* [5], reported a SPE method for the identification of SD for screening purposes. This method did not meet the required accuracy (58% recovery from spiked pig kidney at 25μgkg^{-1}) and precision criteria (30% RSD) [8, 11] and thus could not be used for quantification purposes. Chloramphenicol standards were tested in this method and found to give recoveries >80% and thus could be included in this method. Recoveries of 65% (17% RSD) and 50% (15% RSD) were obtained for dimetridazole and furazolidone respectively for spiked pig kidney. These results indicate that while the method could not be used as a quantification procedure for these two analytes it is possible to use the method to screen samples for these substances.

The method reported by Malisch *et al.* [3] involved the homogenisation of the tissue sample in an aqueous buffer followed by the addition of ACN for analyte extraction. This extraction method resulted in a homogeneous mixture and therefore did not consist of coagulates as found by direct extraction with ACN [2]. DCM is usually used to remove the co-extracted water as reported by Gehring *et al.* [9]. However this DCM was replaced by butyl methyl ether-hexane in the method reported by Malisch *et al.* [3]. Defatting of the tissue extract was carried out by a hexane extraction and matrix polar substances were removed with a water/ethyl acetate extraction. This method was validated to the required EU criteria, as discussed in Section 1.5, for the identification and quantification of SD, and SMX using the same extraction, cleanup and HPLC procedure. It was possible to determine OTC using the same extraction and cleanup procedure but with a different HPLC system for screening purposes only, due to the low recoveries obtained. Chloramphenical could also be analysed using the same extraction and cleanup procedure but required GC-ECD in order to achieve the required sensitivity of 1μgkg^{-1}.

Therefore from the above cited literature, the most suitable cleanup procedure for the required purposes was reported for pork tissue by Malisch *et al.* [3]. Therefore it was proposed that this method be adopted for salmon tissue in this work. In addition it was also proposed to include TMP to see if all three analytes could be simultaneously analysed using the same sample preparation and LC conditions.

Under Commission Decision 96/23/EC routine methods for the analysis of veterinary drug residues must be validated according to the criteria set out in Commission Decision 93/256/EEC [8]. These criteria include specificity, injection repeatability, linearity, accuracy, precision, detection limit, quantification limit, range and stability.

Similar criteria are also laid down by the Codex Committee on Residues of Veterinary Drugs in Foods, Volume 3, 1994 [11] and both are discussed in Section 1.5.

This chapter presents a description of the method used and the validation study carried out to demonstrate that the method is fit for its intended purpose by adhering to the criteria set out under Commission Decision 93/256/EEC to allow it to be used for routine residue testing.

5.2 Apparatus and materials

5.2.1 Sample preparation

Scalpel and blades
Hi - speed homogeniser: Breda Scientific
Sample jars

5.2.2 Extraction Process

Balance (5 decimal places)
Polypropylene centrifuge tubes – 50ml
Glass pyrex test tube –25ml
Waters C18 SPE cartridges – 400mg
Vortex mixer - Fisons
Centrifuge - Sorvall T6000
Ori block -08-3 Techne + Si3 sample concentrator - nitrogen purge and water bath at 25^{0}C
Disposable glass insert vials
Glass injection vials-4 ml

5.2.3 Chemicals and Reagents

Acetonitrile: Lab Scan – HPLC
Methanol: Lab Scan - HPLC
Trifluoroacetic acid: Aldrich – Spectrophotometric grade
Triethylamine: Sigma/Aldrich Ltd
SD (S6387), SMX (S7507) and TMP (T7883) 99% purity: Sigma/Aldrich Ltd

5.2.4 HPLC System

Pump: Shimadzu LC - 6A

Column oven: Shimadzu CTO - 6A

Columns: Spherisorb S5ODS2 5μm (250 x 4.6) mm
    Econosphere C18 5μm (250 x 4.6) mm
    Lichrospher C18 5μm (250 x 4.6) mm
    ODS Hypersil 5μm (250 x 4.6) mm

Auto injector and system controller: Shimadzu SCL - 6B

Detector: UV -Vis spectrophotometric detector Shimadzu SPD - 6AV

Data handling: Maxima 820 software (Millipore)

5.2.5 Sample storage

All tissue samples were stored in acid and solvent rinsed sample jars and kept in a freezer at -20°C prior to analysis. Using a hi-speed homogeniser the tissue was homogenised and 15g of this homogenate was used for each analysis.

5.3 Optimisation of chromatographic conditions

The initial chromatographic conditions examined for the analysis of SD, SMX and TMP were a variation of those set out by Kirkland *et al* [12].

5.3.1 Chromatographic conditions for examination

Column: Econosphere C18 5μm (250 x 4.6) mm column, maintained at 25°C, was used instead of the Zorbax column

Oven temperature: 25°C

Mobile phase: 0.02M trifluoroacetic acid: acetonitrile (84:16). The pH of the mobile phase was adjusted to 3.0 with triethylamine (TEA).

Flow rate: 1.0 ml/minute

Detector: UV at 272nm

Injection volume: 50μl

5.3.2 Preparation of standard solutions

The stock standard solution was prepared by dissolving the standards in methanol (1mgml^{-1}). The working standard solutions were prepared by dilution of the stock standard solutions in the mobile phase prior to use. All solutions were stored in the fridge at 4°C and brought to room temperature prior to injection on the HPLC system.

5.3.3 Choice of mobile phase

When the working standard solutions were injected onto the HPLC system, under the conditions described in Section 5.3.1 a retention times of 4.1 minutes, 5.5 minutes, and 7.4 minutes were obtained for SD, TMP and SMX respectively (Figure 5.1). Although the separation between the three analytes was acceptable adequate resolution was not obtained between the solvent peak and the SD analyte peak. This was confirmed by a capacity factor, (Section 2.1.2.1) of 0.4 for the SD analyte peak. Ideally the capacity factor should range from 2-10, [13], in order that the analyte peaks would have acceptable resolution for quantification purposes. The capacity factor is influenced by:

- the strength of the mobile phase
- the strength of the stationary phase
- the temperature.

Thus the temperature of the stationary phase was examined initially to see if the capacity factor could be improved.

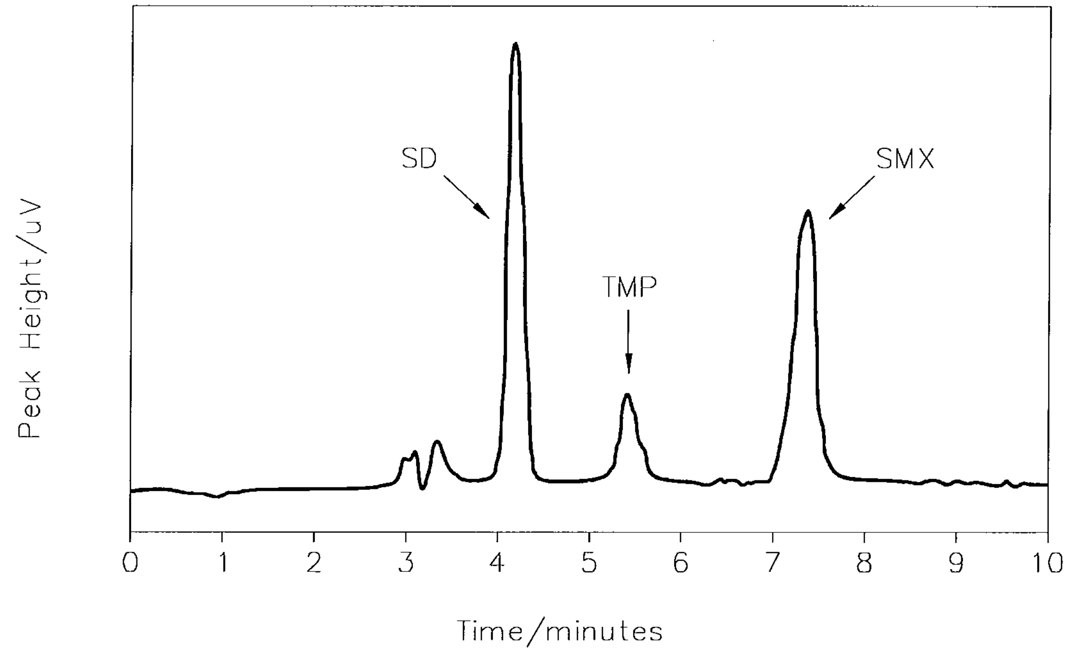


Figure 5.1: Standard solution of SD, TMP and SMX. Analytical conditions: Section 5.3.1.

5.3.4 Effect of column temperature on the capacity factor

To examine the effect of the column oven temperature a standard solution, 1μgml^{-1}, of a mixed standard containing the analytes of interest was injected under the chromatographic conditions (Section 5.3.1) while changing the oven temperature and examining the effect of this on the capacity factor.

Table 5.1: Capacity factors for SD, TMP, and SMX analysed while changing the column oven temperature. Analytical conditions: Section 5.3.1.

Temperature /°C	Sulphadiazine	Trimethoprim	Sulphamethoxazole
35	0.38	0.77	1.32
30	0.40	0.82	1.47
25	0.41	0.84	1.5
20	0.43	0.97	1.78

As can be seen from Table 5.1 the capacity factor decreased for each analyte when the column oven temperature was increased from 30 to 35°C. The capacity factor value increased for each of the three components when the temperature was decreased from 35 to 20°C. However the capacity factor should fall within the range of 2-10, (Section 2.1.2.1), and it is clear that the values in the Table 5.1 are outside this range. Thus the resolution could not be improved by varying the column oven temperature when the analytes were analysed under the above analytical conditions.

5.3.5 Effect of the mobile phase strength on the capacity factor

In order to obtain a better separation (Section 2.1.2) between SD and the solvent peak, the mobile phase polarity had to be increased so that the analytes would be less soluble in the mobile phase and more soluble in the stationary phase. To do this ACN in the mobile phase was reduced from 86% to 12% in incremental amounts of 10% while correspondingly increasing the amount of the aqueous phase. For the mobile phase composition of ACN: TFA in the ratio of 12:88 the capacity factor for SD was 0.59. This shows that the capacity factor could not be significantly improved by reducing the amount of ACN in the mobile phase.

The next step involved substituting the ACN (polarity index 6.2) in the mobile phase with a "weaker" solvent, in terms of polarity, such as methanol (MeOH) (polarity index 6.6). For a mobile phase consisting of 20% MeOH and 80% TFA, the SD peak was not resolved from the solvent peak at 1.5 minutes. The ratio of methanol to TFA in the mobile phase was increased in incremental amounts of 10%, to 80% but it became apparent that the response for each analyte decreased to levels, which would not allow for quantification at the levels of interest. Thus substituting MeOH for ACN in the mobile phase could not increase the capacity factor.

5.3.6 Effect of stationary phase on the capacity factor

Four different stationary phases were examined for the analysis of the three analytes while using the analytical conditions described in Section 5.3.1. These were Econosphere C18, Lichrospher C18, Spherisorb ODS and ODS Hypersil columns. The properties of these columns are summarised in Table 5.2. The mobile phase used in each case consisted of TFA: ACN in the ratio of 86:14 with the pH adjusted to 3.0 with triethylamine. The stationary phase was maintained at 20°C for each of the four columns. The capacity factors, which were obtained for each column, can be seen in Table 5.3.

Table 5.2: Parameters of different types of adsorbents for HPLC

	Econosphere C18	**Lichrospher C18**	**Spherisorb C18**	**Hypersil C18**
Length (mm)	250	250	250	250
Diameter (mm)	4.6	4.6	4.6	4.6
Pore size (Å)	80	100	80	120
Pore Volume (ml/g)	-	1.25	0.5	0.7
Surface area (m^2/g)	200	350	220	170
Carbon loading (%)	15.0	21.4	12.0	10.0
Bonding density ($\mu mole/m^2$)	2.41	3.9	2.72	2.84

Table 5.3: Capacity factors when the standard solutions were analysed using four different stationary phases under the analytical conditions described in Section 5.3.1.

Column	**Sulphadiazine**	**Trimethoprim**	**Sulphamethoxazole**
Econosphere C18	0.43	0.97	1.78
Lichrospher C18	2.1	5.0	11.7
ODS Hypersil	1.5	4.0	8.4
Spherisorb ODS	1.91	4.8	11.5

The capacity factors obtained depend on the stationary phase used as shown in Table 5.3. The optimum values were obtained for each of the three analytes while using either the Lichrospher or Spherisorb columns. This is probably due to the higher surface area of these two columns when compared to the Econosphere and Hypersil columns (Table 5.2). A chromatogram showing the standard solutions analysed on the Lichrospher column can be seen in Figure 5.2(A). However these capacity factor values were obtained for standard solutions, and while the separation for the standard solutions was acceptable it may not be for spiked tissue samples. Therefore the separation for spiked tissue samples needed to be examined and for this purpose the following sample preparation procedure was followed.

5.4 Sample cleanup procedure

A method set out by Gentleman *et al.* [2] was examined for the sample preparation of sulphonamides and TMP in salmon tissue samples. The sample preparation and extraction procedure as set out in this method were as follows:

Salmon tissue (5g) was minced to approximately 1mm cubes and transferred quantitatively to (150 x 18) mm test tubes and ACN (10ml) was added. The samples were homogenised for 1 minute and centrifuged for 10 minutes at 1500r.p.m. The supernatants were decanted to 25ml test tubes. The extraction was repeated with ACN (10ml) and the supernatants were combined, and evaporated under nitrogen to 0.5 - 1.0ml. The volumes were adjusted to 2.0ml with water and the extracts vortex mixed for 1 minute. The samples were centrifuged and the aqueous portion was passed through activated C18 Sep-Pak solid phase extraction cartridges (Waters). The cartridge assemblies consisted of two 400mg cartridges connected in series. These were conditioned with methanol (3ml), and water (5ml). The extract was loaded on to the cartridges and the analytes were eluted with methanol (5ml). This was evaporated to dryness under nitrogen at 37°C and the dry extract was dissolved in the mobile phase (0.5ml), prior to analysis by HPLC.

A chromatogram of the resulting separation can be seen in Figure 5.2(B) where it is evident that the resolution between the matrix components and the SD analyte peak is not acceptable for identification or quantification purposes.

Apart from the difficulties encountered with the resolution of the analyte peaks from the matrix, it was also noted that the response obtained for a 1μgml^{-1} standard solution was approximately equivalent to 250μgkg^{-1} (based on a 5g tissue sample) for SD TMP and SMX respectively. These response values are not adequate for quantification purposes at the levels of interest, i.e. 50μgkg^{-1} for SD, TMP or SMX. Therefore in an attempt to improve both resolution and sensitivity a Liquid Chromatography – Mass Spectrometry (LCMS) method of analysis was examined.

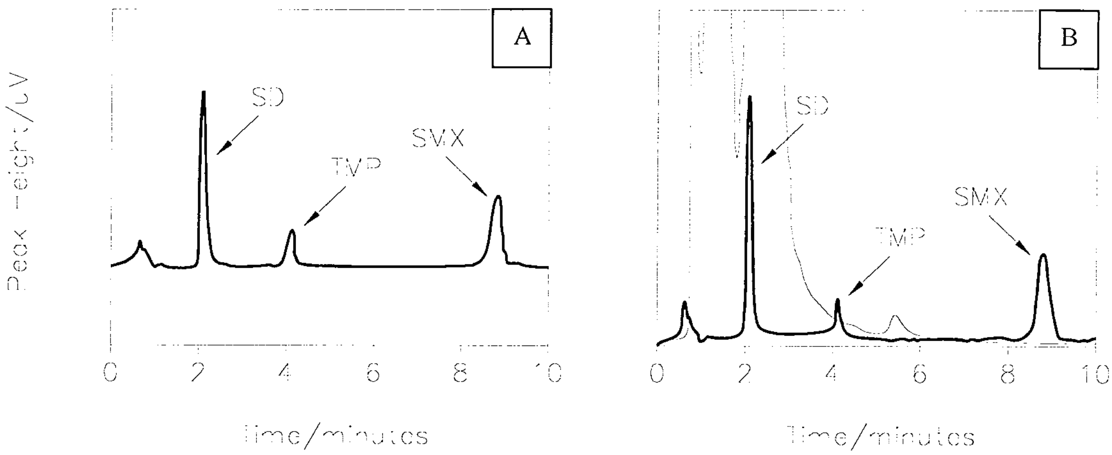


Figure 5.2: Standard solution of SD, TMP and SMX (A), overlayed on a spiked tissue sample (B). Analytical conditions: Section 5.3.6. Cleanup procedure for tissue: Section 5.4.

5.5 Analysis of SD, SMX and TMP by LCMS

5.5.1 Liquid Chromatography Mass Spectrometry (LCMS)

A VG Trio-2000 quadrupole mass spectrometer was used with a liquid chromatography inlet system and an electrospray ionisation source (ESP+). The vacuum was pumped by two water cooled Edwards oil diffusion pumps backed by three Edwards rotary pumps.

5.5.1.1 Instrumentation

The components of the mass spectrometer include a sample inlet system, an ionisation source and acceleration chamber where sample molecules are ionised, fragmented and accelerated into an analyser and an ion detection recording system.

5.5.1.2 Sample inlet system

The mass spectrometer was coupled with a LC system for the purpose of detecting and quantifying the SD, SMX and TMP. The analytes were introduced directly by means of an electrically heated probe inserted through a vacuum lock.

5.5.1.3 Electrospray source

The sample ionisation source used in the method consisted of an ESP+. This type of mass spectrometry allows very large and labile molecules to be analysed routinely. The sample, in solution, emerges from a high voltage capillary tube into a strong electrostatic field at atmospheric pressure producing an aerosol of highly charged droplets (Figure 5.3). Nebulization is assisted with nitrogen gas resulting in sample ions, carrying a positive electronic charge, (MH^+). Vacuum pumps with nitrogen as the drying gas are used to remove the solvent and to achieve the low sample pressures required by the analyser. This process is known as ion evaporation and results in single charged species for introduction into the analyser.

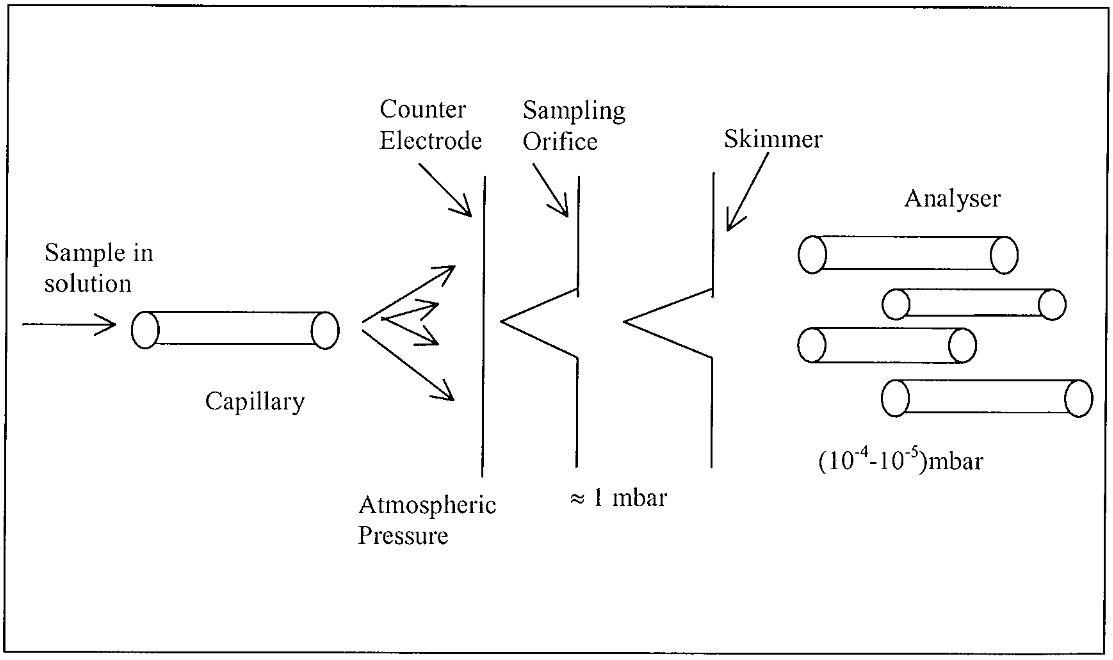


Figure 5.3: Operation of the electrospray source for MS analysis [14].

5.5.1.4 Analyser

After ionisation and fragmentation of the sample, positive ions or fragment ions were accelerated into the analyser chamber by means of a potential gradient and slit system, (Figure 5.3). The positive ions are then separated in space by allowing them to move through an electrostatic field to the detector. A quadrupole analyser is a feature of the VG Trio 2000 system and this consists of a set of four metal rods placed symmetrically around and parallel to the direction of travel of the positive ions. Application of a DC potential and an oscillating RF field across the rods causes all ions except those of a particular mass to charge ratio (m/e), to follow an unstable path leading to collisions with the rods. Ions with the appropriate set m/e value, i.e. 251, 254, 291 and 156, follow a stable path to the detector. By progressive alteration of DC potential and RF field, ions with different m/e values can be made to follow a stable path to the detector, which enables a mass spectrum to be scanned.

5.5.1.5 Ion detection and recording system

Ions from the analyser pass through a slit and impinge on an electron multiplier called the Dynolite^{TM} detector system. Amplification of the signal (x 10) was carried out by changing the load amplifier.

Control of the instrument and acquisition of the data was carried out by the MassLynx Version 2.1 data system. The spectra were also viewed on a cathode ray oscilloscope screen.

5.5.2 Tuning the MS

The instrument was calibrated using the ESP+ with heptacosa {Perfluorotributylamine (PFTBA)} in the range 50-620amu. The mobile phase used initially to separate SD, SMX and TMP consisted of 0.2% trifluoroacetic acid and acetonitrile in the ratio of 84:16. The pH of this mobile phase was adjusted to 3.0 with TEA, which was used to adsorb onto the active sites of the column and thus reduce the TMP peak tailing. The ESP+ was tuned to this mobile phase by looking at the response of 42amu for ACN, 58amu for the trifluoro and 88amu for TEA. The tuning was carried out firstly by adjusting the cone position so that a maximum sensitivity was obtained for 42, 58 and 88amu. The mobile phase flow rate was then adjusted between 0.05 and 0.35ml/minute, and the maximum sensitivity was found to occur at a flow rate of

0.1ml/minute to the detector. The gas flow rates also needed to be adjusted and it was found that a flow rate of 300 l/hr for the drying gas and 12 l/hr for the nebulising gas gave the best results. Finally, in order to tune the mobile phase, the various parameters on the ESP+ controller were adjusted to obtain a maximum sensitivity for the mobile phase ions.

5.5.3 Flow injection analysis of the analytes of interest

Once the mobile phase was tuned the next step involved obtaining a full scan in the range 50-340amu, by flow injection analysis, for the three compounds which were to be analysed (SD, MR: 250, SMX, MR: 253, and TMP, MR: 290). It was found that the best sensitivity was obtained for the SMX peak at 254amu and therefore this was chosen as the compound to which the MS would be tuned. Repeat injections were made of a 10μgml^{-1} solution while changing one parameter for each injection so as to obtain the maximum signal to noise ratio.

5.5.4 Troubleshooting

Once all tuning was completed, the spectrum for a full background scan was examined. It became apparent that the peak of highest sensitivity obtained (87.8amu) was due to the TEA, which was used to adjust the mobile phase pH (Figure 5.4). The TEA was therefore removed from the mobile phase. A full scan was carried out on all three analytes and it was found that the masses to be examined were m/e of 291 for TMP, 251 for SD and 254 for SMX. It also became apparent that fragmentation occurred for both SD and SMX, and this resulted in a peak of mass 156amu.

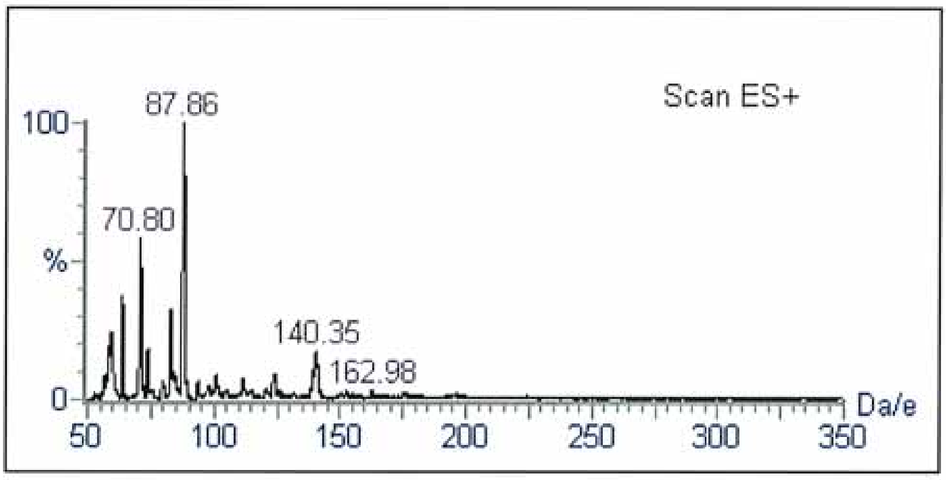


Figure 5.4: Full background scan ESP MS of the mobile phase.

Even though the TEA was removed from the mobile phase the peak at 87.8amu corresponding to TEA was prominent in the mobile phase spectrum. To eliminate this problem the source was removed and cleaned and the system was brought under vacuum. The mobile phase spectrum was again obtained, and no ion was present at 87.8amu indicating that the source cleaning had eliminated the TEA interference in the chromatogram.

5.5.5 Separation of the analytes of interest with no pH adjustment

The separation of the three analytes from the Spherisorb S5 ODS (2) (250 x 4.6) mm, column using the mobile phase consisting of 0.2% TFA: ACN in the ratio of 84:16, pH 1.5, was examined. Since the standards were now being injected on to the HPLC column the flow rate had to be increased, to 1.0 ml/minute in order to obtain a good separation with acceptable peak shape for the compounds of interest. It should be noted that the maximum sensitivity was obtained when the flow rate to the detector was 0.1ml/minute (Section 5.5.2). Therefore a flow splitter was inserted between the column outlet and the detector inlet. The flow rate to the detector was then controlled by the length of the tubing going to waste, and the back- pressure caused by this. This tubing was shortened until a flow rate of 0.1 ml/minute was obtained from the probe going to the detector. The separation obtained under these conditions showed acceptable peaks with regards to resolution and peak shape for both SD and SMX, but the peak obtained for TMP was unacceptable as can be seen in Figure 5.5. The chromatogram at mass 251 shows the spectrum obtained for SD, 254 for SMX and 291 for TMP. The TMP peak, (m/e 291), was broad and tailing and this was most likely due to the absence of an amine which was used to adsorb onto the active sites of the column and thus keep peak tailing to a minimum [12].

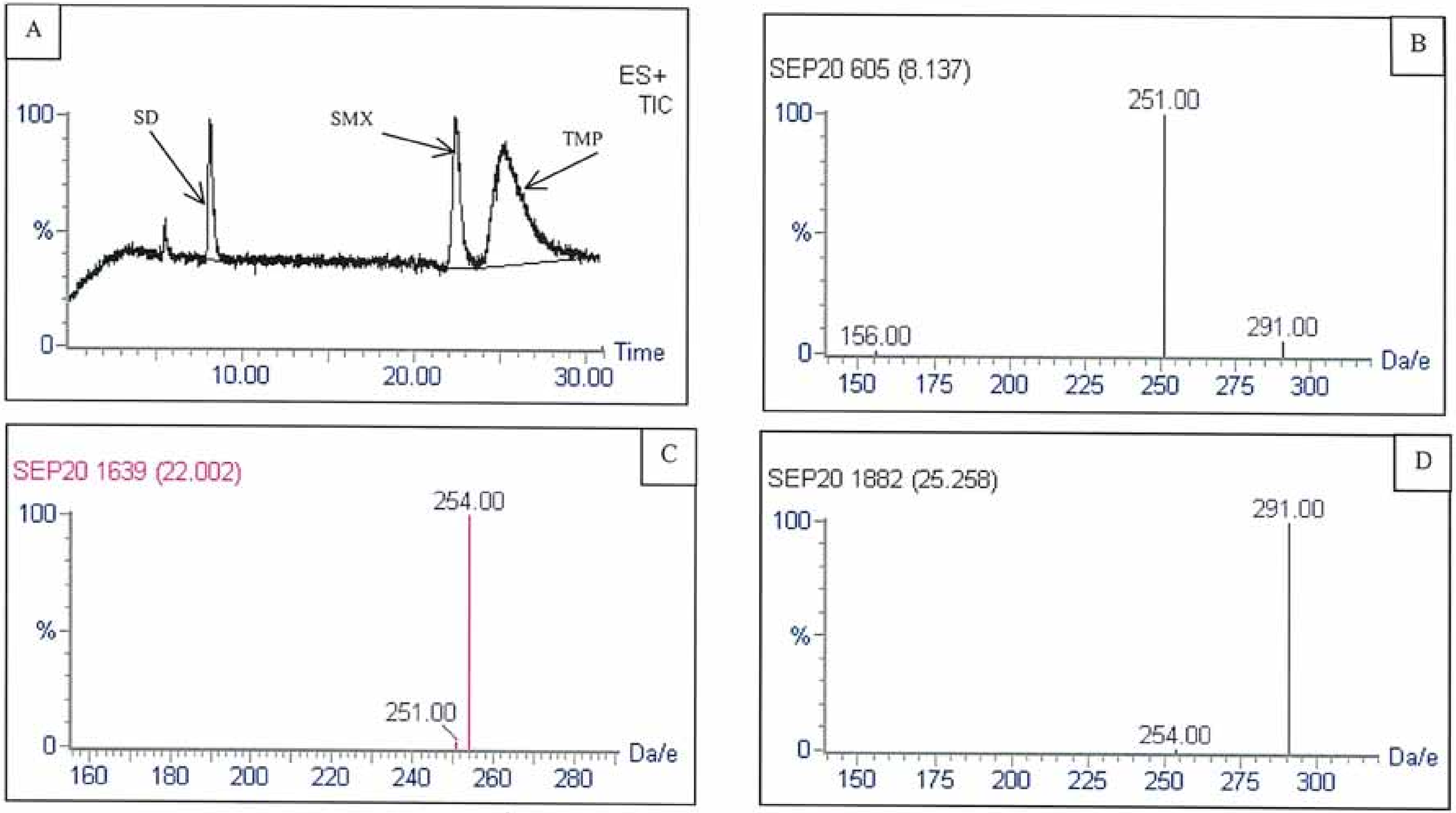


Figure 5.5: TIC for SD, SMX and TMP, 10µgml^{-1}, (A) and the corresponding spectra for SD (B), SMX (C) and TMP (D).

Column: Spherisorb S5 ODS (2) (250 x 4.6) mm. Mobile phase: 0.2% TFA: ACN (84:16), pH 1.5. Flow rate to the detector: 0.1ml/minute.

5.5.6 Choice of mobile phase buffer

For use on the MS and ESP+ source, however, the amine was required to have a high volatility. Three different amines were examined and the results recorded.

Triethylamine, $C_6H_{15}N$ has a molecular weight of 101.19 and a boiling point of 88.8ºC. The background signal for this amine however, interfered in the analysis as discussed above.

Perfluorotriethylamine, (PFTEA) $C_6F_{15}N$, would be a better choice of amine for use on the MS system, due to the presence of the fluoro groups instead of hydrogen. This meant that the amine had greater volatility, (boiling point of 69ºC). However, this amine is not soluble in water or ACN and therefore could not be used to adjust the pH of this mobile phase of interest.

As Dimethylethylamine, (DMEA) C_2H_5N $(CH_3)_2$ has a molecular weight of 73.14, is soluble in water and has a boiling point of 36ºC it is suitable for use on the electrospray source of the MS system.

5.5.7 Separation using the amine to adjust the pH

The mobile phase now used was 0.2% TFA: ACN (84:16) and the pH was adjusted to 3.0 with DMEA. The separation, which was achieved with this mobile phase, can be seen in Figure 5.6, where the TMP peak is acceptable with regards to peak shape. SD and SMX, however, were not detected within 60 minutes with this mobile phase.

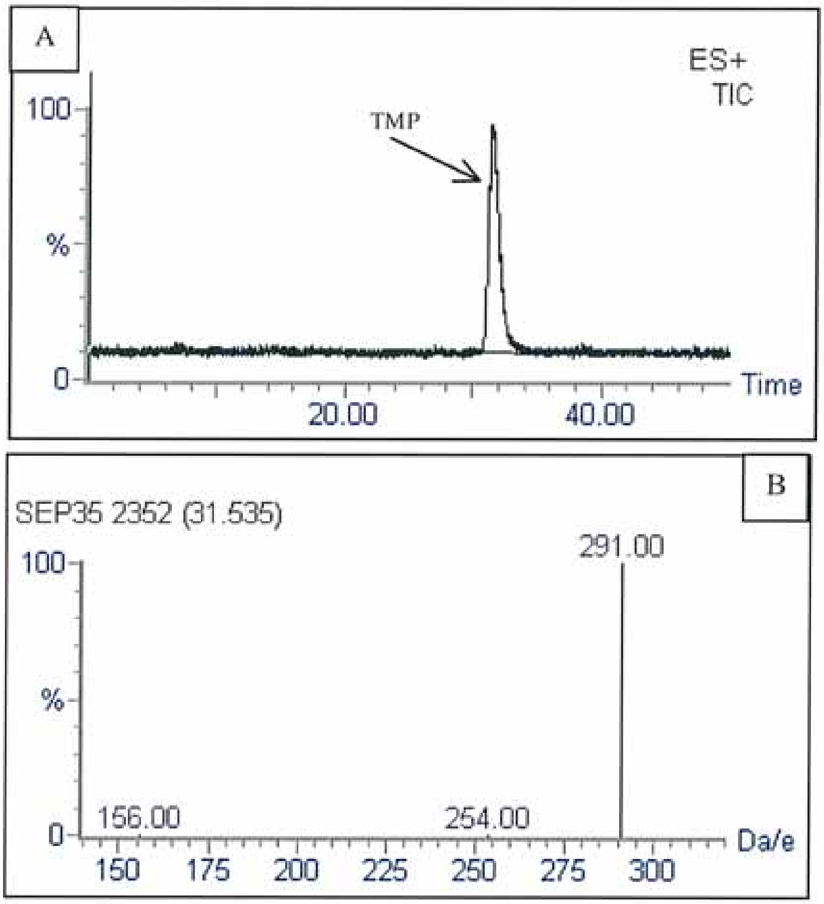


Figure 5.6: TIC chromatogram for a 10µgml^{-1} solution of SD, SMX and TMP (A) and the corresponding spectra showing only TMP present (B). Column: Spherisorb S5 ODS (2) (250 x 4.6) mm. Mobile phase: 0.2% TFA: ACN (84:16), pH adjusted to 3.0 with DMEA. Flow rate to the detector: 0.1ml/minute.

5.5.8 Stationary phase examination

Millibore column:

Using a Spherisorb ODS (2) millibore column, (120 x 2.0) mm the spilt connector was removed and the flow rate was reduced to 0.1 ml/minute in order to attempt to increase the sensitivity of the method further. However when no separation was obtained for the mobile phase 84:16, 0.1% TFA: ACN it was apparent that the ACN was not polar enough for this millibore column. When MeOH, which has a higher polarity than ACN was examined in the mobile phase, with varying ratios from 80% to 20%, no separation was achieved between the analytes.

Hamilton PRP column:

Since a low pH (1.5), was observed for the mobile phase when the DMEA was not used, a Hamilton PRP column was examined as this column can cope with pH values lower than 2.0. However this resulted in the sensitivity of the method dropping considerably and was therefore unsatisfactory for quantification purposes.

5.5.9 Mobile phase examination

Cited Literature (Table 1.5) showed that the mobile phases used to analyse sulphonamides consisted of either an acid with ACN or a buffer used with ACN. The acid: ACN mobile phase has been discussed and therefore the buffer: ACN combination needed to be examined.

The most appropriate mobile phase in the literature was reported by Porter [15] and consisted of 0.01M ammonium acetate: ACN: MeOH in the ratio of 85:8:7, pH 8.4. As before the flow-rate to the detector was maintained at 0.1 ml/minute. Following the tuning process the separation shown in Figure 5.7 for SD, SMX and TMP was achieved.

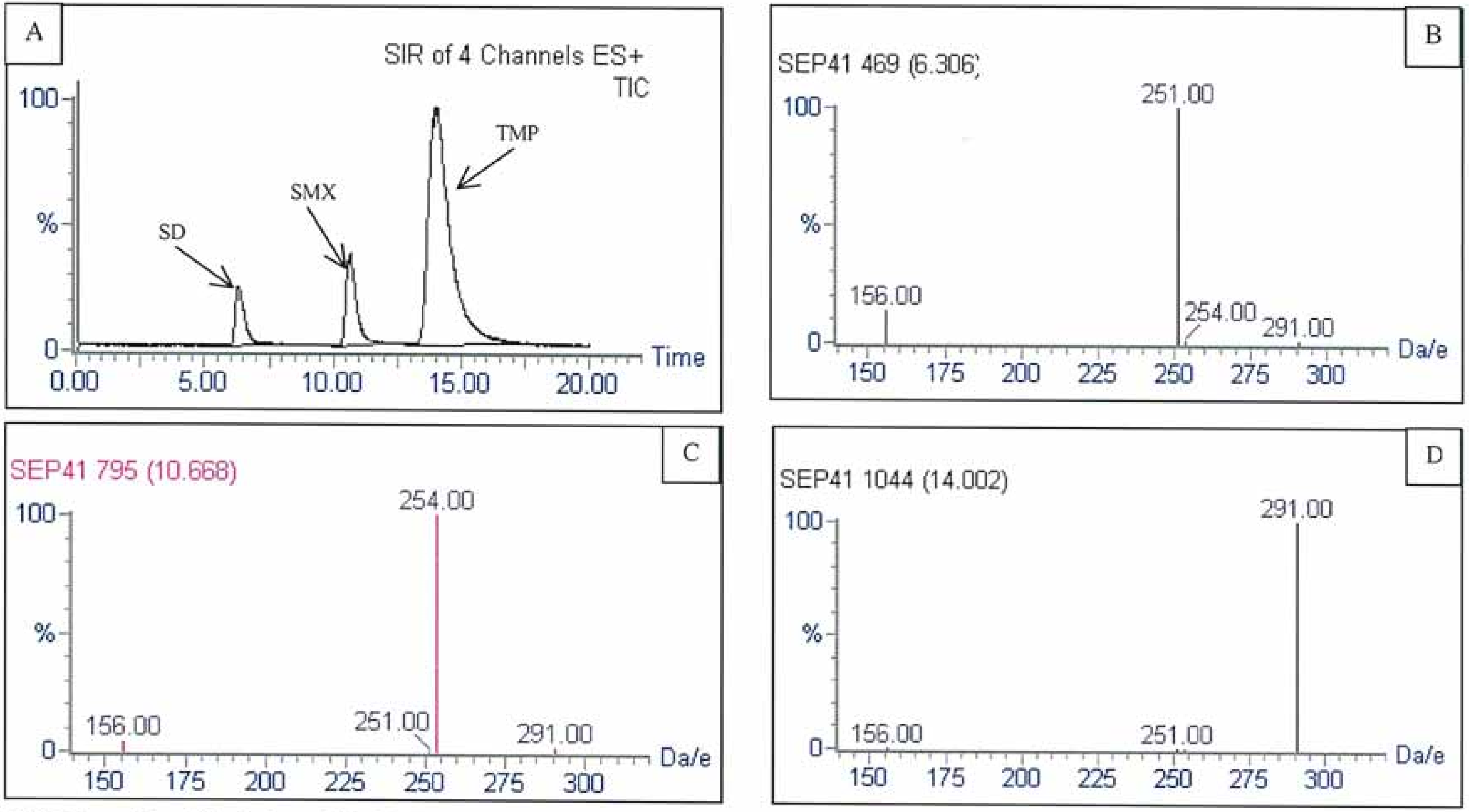


Figure: 5.7: TIC for a 10µgml^{-1} solution of SD, SMX and TMP (A), and the corresponding spectra for SD (B), SMX (C) and TMP (D).
Column: Spherisorb S5 ODS (2) (250 x 4.6) mm. Mobile phase: 0.01M ammonium acetate: ACN: MeOH (85:8:7), pH 8.4.Flow rate to the detector: 0.1ml/minute

5.5.10 Sensitivity

The LOD was calculated based on S/N=3, for each of the three analytes. Based on a 10g tissue sample, the LOD for SD, SMX and TMP was approximately 330μgkg^{-1}, 250μgkg^{-1} and 100μgkg^{-1} respectively when analysed under the conditions described in Figure 5.7. However, since the combined MRL for SD and SMX in tissue samples is 100μgkg^{-1} [1], it is apparent that the LOD obtained under the conditions described are not adequate for the detection and quantification of SD or SMX at the concentration levels of interest.

For TMP the MRL in tissue samples is 50μgkg^{-1} [1], and thus it is also apparent that the LOD of 100μgkg^{-1} is not adequate for the required purposes.

5.5.11 Confirmation and Conclusion

GC-MS requires extensive sample pre-treatment and analyte derivatisation, which makes it a rather time-consuming confirmation method [16]. This indicates the importance of LC-MS as a confirmation method for SD, SMX and TMP analysis in salmon tissue. Several LC-MS methods have been reported for the confirmation analysis of various sulphonamides primarily in kidney and milk [16]. However published work on the LC-MS analysis of SD, SMX and TMP in tissue matrixes is limited. SD analysis by LC-MS in kidney tissue [15] and salmon tissue [17] has been reported in 1994 and 1996 respectively. TMP analysis by LC-MS in tissue samples has also been reported in 1997 [10].

Porter [15] reported sensitivity problems due to matrix interferences for the analysis of SD while using ESP+ LC-MS. The mobile phase used in this work was the same as that examined in Figure 5.7, while the column was a Novapak C18 (150 x 3.9) mm. The sensitivity problems were overcome by using a MS-MS detection system. However, a MS-MS system was not available for this work and therefore this possibility could not be examined.

Gehring *et al.* [17] reported the application of liquid chromatography with atmospheric pressure chemical ionisation mass spectrometry (LC-APCI-MS) to the analysis of SD in salmon tissue. This method showed acceptable criteria for a regulatory confirmation method and the LOQ was found to be 1μgkg^{-1}. Since the MRL for combined sulphonamides is 100μgkg^{-1} [1], then the LOQ quoted in this method is acceptable for the required purposes. LC-APCI-MS was not available and could not be examined for SD analysis in this study.

Cannavan *et al.* [10] reported the successful application of LC-MS with thermospray chemical ionisation to TMP analysis in bovine tissue samples. The LC conditions consisted of a Partisil 5 ODS 3μm, (4.6 x 250) mm column and the mobile phase was ACN: H_2O: glacial acetic acid (25:73.7:1.3) containing ammonium acetate (0.05M). No peak tailing problems were reported for TMP under these conditions. The accuracy of this method was 73.1 ± 3.6% (n=4), while the LOQ was quoted to be 25μgkg^{-1}. Since the MRL for TMP is 50μgkg^{-1} in tissue samples, then the LOQ obtained in this method was acceptable for the required purposes. However, thermospray chemical ionisation was not available for this work.

It can be seen that the LCMS electrospray examined here did not give adequate sensitivity for the required purposes for the analysis of the three analytes. For all three analytes the best sensitivity was obtained with the ESP+ LC-MS used with a Spherisorb S5 ODS (250 x 4.6) mm column and the mobile phase which consisted of 0.01M ammonium acetate: ACN: MeOH (85:8:7), pH 8.4 at a flow rate of 0.1ml/minute to the detector. However the LOD obtained (330μgkg^{-1}, 250μgkg^{-1} and 100μgkg^{-1} for SD, SMX and TMP respectively) were not adequate for the detection of the analytes at the required MRL.

Therefore it was concluded that the electrospray MS method examined could not be used for quantification of the analytes at the levels of interest. However it could be used as a confirmatory method to determine the presence of and identify the analytes if they were present in the sample at concentration levels greater than 330μgkg^{-1}, 250μgkg^{-1} and 100μgkg^{-1} for SD, SMX and TMP respectively.

5.6 Examination of a method by Malisch *et al.* [3]

5.6.1 Apparatus and materials

5.6.1.1 Sample preparation

Scalpel and blades
Hi - speed homogeniser: Breda Scientific
Sample jars

5.6.1.2 Extraction Process

Balance (5 decimal places)
Glass beakers –250mls
Glass funnels
Graduated cylinder –100ml
Conical flask –250ml
Whatmann GF/C Filter paper-15cm diameter
Separating funnel –250ml
Silanised glass wool
Round bottom flask –250ml
Glass pyrex test tube –50ml
Vortex mixer - Fisons
Centrifuge - Sorvall T6000
Ori block -08-3 Techne + Si3 sample concentrator - nitrogen purge and water bath at 25^0C
Disposable glass insert vials
Glass injection vials-4 ml

5.6.1.3 Chemicals and Reagents

Buffer pH 6.0 (0.1M citric acid, 0.1M Na_2HPO_4: dissolve 21g of citric acid-monohydrate and 18g $Na_2HPO_4.2H_2O$ in about 900ml water, adjust the pH with 5M NaOH, fill up with water to 1 Litre. This was stored in the fridge at 4°C while not in use.
Acetonitrile: Lab Scan – HPLC
Sodium chloride: Riedel de Haen- reagent grade
Isohexane: Lab Scan – Super purity
Methyl tert butyl ether: Lab Scan – Super purity

Anhydrous sodium sulphate: Merck – Super purity grade. This was placed in the muffle furnace at 550ºC overnight before use.
Ethylene glycol: BDH – Analar Grade 99.5%
Ethyl acetate: Lab Scan – Super purity
SD (S6307), SMX (S7507) and TMP (T7883) 99% purity: Sigma/Aldrich Ltd
EDM (45474) Pestanal: Riedel-deHaen

5.6.1.4 HPLC System

Pump: Shimadzu LC - 6A
Column oven: Shimadzu CTO - 6A
Column: Spherisorb S5ODS2 5μm (250 x 4.6) mm
Auto injector and system controller: Shimadzu SCL - 6B
Detector: UV -Vis spectrophotometric detector Shimadzu SPD - 6AV
HP DAD Series 1100
Data handling: HP chemstations Version A.06.01

5.6.2 Sample Preparation

The procedure set out by Malisch *et al.* [3] was followed in this analysis.

Step 1: Sample preparation

The frozen tissue sample was allowed to defrost in the fridge overnight. The meat was cut into pieces and it was minced in an acid washed glass sample jar with the Breda-Scientific blender. The samples were kept cool before the ACN extraction, especially after homogenisation. The time between homogenisation and extraction was kept to a minimum.

Step 2: Extraction

The homogenised tissue sample (15g) was accurately weighed into a 250ml beaker. While the tissue sample was being weighed the extraction buffer was taken from the fridge and placed on the bench at room temperature. The tissue was spiked to the required concentration level with the standards diluted in ACN (Section 5.6.3). Using a 50ml graduated cylinder, cold extraction buffer (30ml) was added to the tissue. The tissue sample was homogenised using the Ultra turrax homogeniser for approximately 15 seconds. Using a 100ml graduated cylinder ACN (90ml) was measured out. 40ml of this ACN was added to the tissue extract and this was homogenised for 20 seconds. The remaining 50ml ACN was added to the extract and the homogenisation was continued for a further approximately 20 seconds. The probe was washed with water for 20 seconds. The water was discarded and the probe was again washed with a further 80ml of water. This water solution was again discarded and the next tissue sample was extracted.

Step 3: Separation of water

The tissue extract was decanted through a folded filter paper, fitted into a glass funnel, into a conical flask. Using the 100ml graduated cylinder, 90ml of the tissue extract, (corresponding to 10g of tissue) was measured into a 250ml separating funnel. Sodium chloride (4g) was added into the separating funnel and it was shaken vigorously for 30 seconds. Using the 100ml graduated cylinder tert butyl methyl ether: isohexane (80:20) (30ml) was measured into the separating funnel. The separating funnel was shaken and at this stage there should not be any NaCl visible in the funnel. Silanised glass wool was placed into the neck of the glass funnel which were used earlier for filtration, and approximately 10g of anhydrous Na_2SO_4 were placed on top of this. The lower water phase was run off from the separating funnel to waste into the conical flasks, which were used earlier. After some seconds the separating funnel was swirled slowly and any remaining water was allowed to collect and run off to waste. The weight of a 250ml round bottom flask was recorded. The top organic layer was filtered through the Na_2SO_4 and the glass wool into a 250ml round bottom flask. The separating funnel was rinsed with ACN (10ml) and this was also filtered through the Na_2SO_4 and the glass wool into a 250ml round bottom flask. Eethylene glycol: ACN (1:2) (6ml) were added to the round bottom flask using a 5ml pipette. The weight of the round bottom flask containing the sample solution was recorded. The round bottom flask was placed on the rotary evaporator at 40°C. The pressure was maintained at approximately 300 bar for five minutes and was then reduced to approximately 250 bar. This pressure was maintained for 15 minutes and was then reduced to approximately 180 bar for 10 minutes, 170 bar for a further 15 minutes. At this stage the final residue was 2-3g.

Step 4: Cleansing by distribution

The weight of a 25ml pyrex test tube was recorded. The residue from the 250ml round bottom flask was vortex mixed to dissolve the visible residues. The residue was decanted into a 50ml polypropylene centrifuge tube. The 250ml round bottom flask was washed with the following solutions in succession:

1. 10ml hexane
2. 15ml hexane
3. 3ml ACN
4. 0.5ml buffer + 2ml H_2O

The hexane was measured with a 25ml graduated cylinder while the ACN, buffer and H_2O were measured with a 5ml Gilson pipette. Each wash solution was transferred into the 50ml centrifuge tube and this was shaken for 30 seconds. The upper hexane layer was removed by glass pipette to waste. Water (3ml) was added using a 5ml Gilson pipette. NaCl (500mg) was added directly to the centrifuge tube and this was shaken and the pressure released. Ethyl acetate (15ml) was added to the 250ml round bottom flask and this was rinsed into the centrifuge tube. This was shaken and the upper ethyl acetate layer was removed with a glass pipette to the 25ml test tube. Another 15ml ethyl acetate was added to the 50ml centrifuge tube and this was shaken briefly. The ethyl acetate layers were combined in the test tube and any water, which was below the ethyl acetate layer in the test tube, was removed to waste. Ethylene glycol: ACN (1:2) (600μl) was added to the test tube and the weight was recorded. This was then evaporated under a gentle stream of nitrogen at 40°C, until the final volume weighed approximately 500mg. The extract was adjusted with water to a final weight of 1.05g (equivalent to 1.0ml). This was vortex mixed for 30 seconds, and filtered through a 0.45μm ministart filter prior to HPLC injection.

5.6.3 Spiking the tissue sample

Tissue sample (15g) was accurately weighed into a 250ml beaker. The standards used to spike the tissue were prepared in ACN according to the procedure described in Section 5.81. To spike the tissue to 100μgkg^{-1} with SD and SMX 150μl of the 10μgml^{-1} standard solution were accurately measured into the beaker using a Microman M250 pipette. To spike the tissue to 50μgkg^{-1} with TMP, 75μl of the 10μgml^{-1} standard solution were accurately measured into the beaker containing the tissue with a Microman M100 pipette. This was allowed to stand for 30 minutes and the preparation procedure described in Section 5.6.2 was followed.

Using the spiked tissue samples the cleanup procedure was examined and the recoveries obtained can be seen in Table 5.4.

Table 5.4: Percentage recoveries and % RSD for tissue samples spiked to 100μgkg^{-1} with SD and SMX and 50μgkg^{-1} with TMP, which were analysed according to the procedure described in Section 5.6.2.

	Sulphadiazine	Sulphamethoxazole	Trimethoprim
% Recovery	92.9	89.1	41.1
Standard Deviation	9.05	7.5	3.5
% RSD	9.75	8.36	8.43

5.6.4 Examination of the low TMP percentage recoveries

The low recoveries obtained for TMP need to be examined and the source of the losses identified. To do this each step of the cleanup procedure was examined individually with standard solutions and the results are shown in Table 5.5.

Table 5.5: Percentage recoveries and % RSD for standards, 0.1μg of SD and SMX and 0.05μg of TMP, which were analysed according to the procedure described in Section 5.6.2.

	Sulphadiazine	Sulphamethoxazole	Trimethoprim
Complete procedure	95.3 (6.4)	103.5 (5.6)	66.5 (7.1)
Step 4	98.1 (2.1)	98.7 (1.0)	47.5 (9.3)
Step 4 without NaCl	95.7 (5.2)	99.9 (2.2)	88.3 (8.1)

Step 4 involved taking 0.1μg of SD and SMX and 0.05μg of TMP, dissolving it in ACN (3ml), buffer (0.5ml) and water (5ml), adding NaCl (500mg) and extracting this twice with ethyl acetate (15ml). The ethyl acetate layers were combined into a 50ml pyrex test tube and ethylene glycol: ACN (1:2) (600μl) were added. This was evaporated to ethylene glycol dryness under a gentle stream of nitrogen at 40°C and the final volume adjusted to 1.0ml with water prior to analysis by HPLC. When this procedure was followed 47.5% of the TMP added was recovered. However when this procedure was repeated without adding the NaCl salt to separate out the aqueous and organic layers a recovery of 88.3% was obtained for TMP. Therefore it appears that the TMP is lost to the aqueous layer containing the NaCl salt. This needed to be improved either by washing the NaCl with ACN prior to discarding it to waste, or using an alternative to NaCl to separate out the two layers. By not adding any salt to the extract the aqueous and organic layers did separate out after 3 hours but this is not practicable for routine analysis.

The recoveries obtained for SD and SMX, prepared according to the procedure in Section 5.6.2, meet the criteria set out in the Annex of Directive 93/256/EEC of 70-110% for tissue samples spiked to $100gkg^{-1}$. Precision and as accuracy are further discussed in Section 5.10.5 and 5.10.6 respectively.

5.6.5 Effect of ethylene glycol on the baseline noise

It was found that the ethylene glycol caused interfering peaks in the chromatogram baseline over time and this is discussed further in Section 5.8.6. The purpose of the ethylene glycol was as a keeper in the evaporation steps. It was replaced with water and this was found to be effective. For the final solution injected onto the HPLC system 0.02M CH_3COONa was examined instead of ethylene glycol: water and the resulting chromatograms were found to be satisfactory.

The final sample preparation procedure used is summarised in Section 5.7.

5.7 Sample Preparation Procedure

Muscle (15g) Homogenised

Spike with standard (150µl) in ACN
Add 30ml buffer
Homogenise for 30 seconds
Add 40ml ACN and homogenise
Add 50ml of ACN and homogenise
Filter and transfer 90ml to a separation funnel

Add 4g NaCl
Add 30ml butyl methyl ether: hexane (8:2)

Shake
Discard the lower water layer to waste

Filter the organic layer through Na_2SO_4 and glass wool into a 250ml RBF
Add 3ml water

Evaporate to 2-3g residue by rotary evaporation

Decant the residue to a 50ml centrifuge tube and wash the RBF with:
Hexane –10ml and 15ml,
ACN – 3ml,
Buffer and water – 0.5ml and 2ml

Combine the phases and shake
Remove the upper hexane layer to waste

Add 3ml water,
Add 500mg of NaCl,
Rinse the 250ml RBF with ethyl acetate (15ml) and add to the aqueous extract

Shake and remove the ethyl acetate layer to a 25ml pyrex tube

Re-extract with ethyl acetate (15ml)
Add 500µl water to the combined ethyl acetate layers

Evaporate the residue accurately down to less than 0.5g under a gentle stream of nitrogen

Fill up with 0.02M CH_3COONa to 1g

Filter using a 0.45µm ministart filter

HPLC Analysis

5.8 Optimisation of chromatographic conditions for SD, SMX and TMP analysis

The initial chromatographic conditions examined for the analysis of SD, SMX and TMP analysis were set out by Malisch *et al.* [3]. The column used was a Spherisorb ODS (2) 5µm (250 x 4.6) mm in conjunction with a gradient profile mobile phase. Ethidimuron (EDM) was chosen as an internal standard from the Malisch *et al.* [3] muti-residue method as it elutes close to the SD peak in this method. It is not used for treating farmed fish and was therefore included in the method while optimising the chromatographic conditions.

5.8.1 Preparation of standard solutions

A stock standard solution was prepared in ACN at a concentration of $1mgml^{-1}$ by dissolving 25mg of each of the three analytes and the internal standard in 25ml of ACN. This solution was sonicated for 5 minutes and was stored in the fridge at 4°C. Working standards were prepared by serial dilution of the stock standard in 0.02M CH_3COONa adjusted to pH = 3.5 with glacial acetic acid before use. All solutions were stored in the fridge at 4°C prior to use.

5.8.2 Chromatographic conditions for the analysis of SD, SMX, TMP and EDM

Detector: HP Photo-diode array (DAD) at 265nm, 262nm, 267nm, and 276nm for SD, TMP, SMX and EDM respectively.

Column: Spherisorb ODS (2) (250 x 4.6) mm

Temperature: 40°C

Injection volume: 100µl

Flow rate: 2ml/minute

Mobile phase: Gradient – A: ACN: H2O (70:30)
B: 0.02M CH_3COONa pH adjusted to 4.8

5.8.3 Optimisation of the gradient profile

The gradient profile as set out by Malisch *et al.* [3] can be seen in Figure 5.8(A). It should be noted however that this profile was used to separate out 14 analytes and for the purposes of this method we only need to look at two of these 14 analytes, SD and SMX. TMP and EDM (used as internal standard) were also included in this method. From an isocratic elution at 80% B and 20% A, all four analytes eluted from the column but the resolution obtained needed to be improved. In addition to obtaining a sharp peak for TMP it was necessary to add TEA to the mobile phase to adsorb onto the active sites on the column and prevent the TMP tailing. This value was set at 0.1% initially.

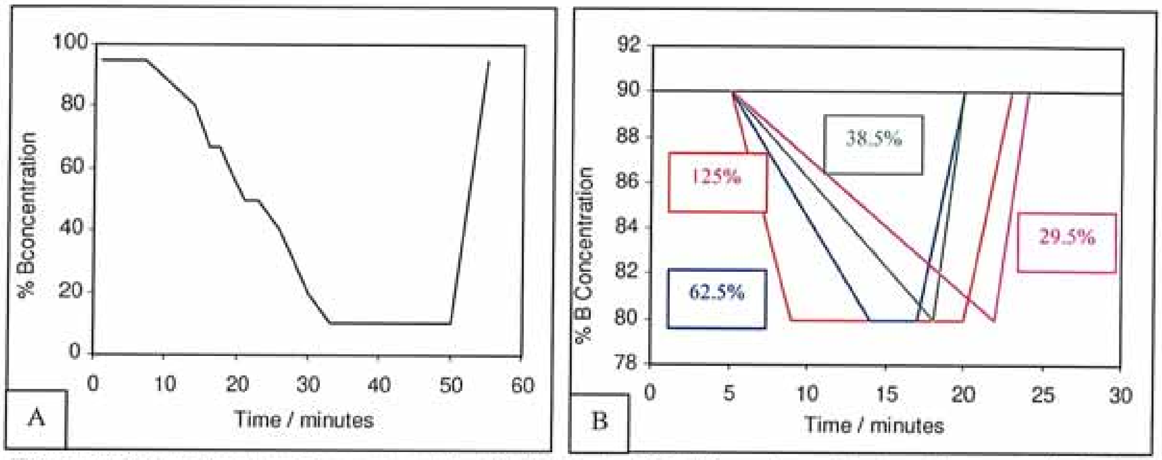


Figure 5.8: Gradient profile as set out by Malisch *et al.* [3] (A), and gradient profiles examined for the 4 analytes of interest (B).

Four different gradient profiles were examined as shown in Figure 5.8(B) where the % rate of change of the mobile phase composition was changed from 125% to 29.5%.

% ROC = change in composition (%) / volume over which the change took place

The chromatograms obtained (Figure 5.9) show that the resolution between the SMX and EDM improved by decreasing the %ROC but the width of the TMP peak was unacceptable using these gradients. Table 5.6 shows the capacity factors, selectivity, peak width and resolution for all the peaks analysed under the four separate gradient profiles.

Table 5.6: Capacity factor, selectivity, peak width and resolution of the analyte peaks obtained for different gradient runs.

	Sulphadiazine	Sulphamethoxazole	Ethidimuron	Trimethoprim
Gradient 1: % ROC = 125%, N = 8475				
Capacity factor	3.06	9.88	10.09	11.88
Selectivity		3.22	1.02	1.17
Peak Width (minutes)	0.62	0.52	0.31	1.04
Resolution		12.05	0.71	2.06
Gradient 2: % ROC = 62.5%, N = 5668				
Capacity factor	2.66	9.75	10.26	12.42
Selectivity		3.66	1.05	1.21
Peak Width (minutes)	0.6	0.73	0.73	1.25
Resolution		14.38	0.86	2.55
Gradient 3: % ROC = 38.5%, N = 4284				
Capacity factor	2.66	10.14	10.81	13.51
Selectivity		2.81	1.07	1.25
Peak Width (minutes)	0.6	0.83	1.14	1.35
Resolution		15.21	0.81	3.04
Gradient 4: % ROC = 29.5%, N = 4100				
Capacity factor	2.66	10.34	11.21	14.45
Selectivity		3.88	1.08	1.29
Peak Width (minutes)	0.6	1.15	0.93	1.56
Resolution		15.64	1.17	3.26

From the data in Table 5.6 and the chromatograms in Figure 5.9 it is evident that the resolution between SD and SMX is acceptable while SMX and EDM need better resolution. However, the resolution between these two compounds did improve as the %ROC in the gradient run was decreased, but the number of theoretical plates decreased for the same gradient runs. In order to improve this resolution and increase the number of theoretical plates, the gradient was changed by increasing the % ROC in the run. TMP was resolved from all the peaks but it is evident that the peak width (1.56 minutes) is not acceptable. Therefore the amount of TEA in the mobile phase was increased from 0.1-0.3%, in an attempt to reduce peak tailing of the TMP peak. Since the TEA in the organic phase of the mobile phase was increased the pH of the aqueous phase was reduced from 4.8 to 3.5.

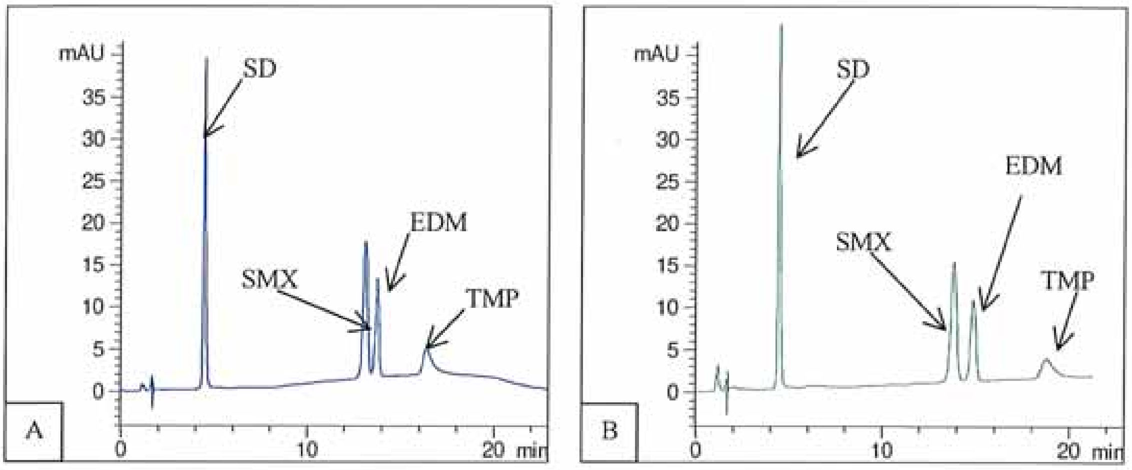


Figure 5.9: Separation of SD, SMX, EDM and TMP with a 125% ROC (A) and a 29.5% ROC (B), Table 5.6. Chromatographic Conditions: Section 5.8.2.

5.8.4 Effects of different amounts of TEA in the mobile phase on the resulting resolution

The gradient run can be seen in Figure 5.10(A) while Figure 5.10(B) shows the chromatogram obtained under those conditions. The changes in this gradient run resulted in a significant increase in the number of theoretical plates and also in the resolution obtained between the SMX and EDM peaks, (Table 5.7). The resolution is now acceptable for the criteria specified [13]. The increase in the TEA in the mobile phase caused the TMP peak to elute earlier and this reduced the resolution between the EDM and TMP from 3.26 to 0.66. However the peak width did improve from 1.56 to 0.75 minutes and in order to improve this further the amount of TEA in the mobile phase was increased incrementally and the results recorded (Table 5.8).

Table 5.7: Capacity factor, selectivity, peak width and resolution of the analyte peaks. Mobile phase A: ACN: H_2O: TEA (69.85:29.85:0.3), B: 0.02M CH_3COONa pH 3.5 with glacial acetic acid.

	Sulphadiazine	**Sulphamethoxazole**	**Ethidimuron**	**Trimethoprim**
Gradient: % ROC = 58.5%, N = 15133				
Capacity factor	2.88	10.76	11.69	12.08
Selectivity		3.74	1.09	1.03
Peak Width (minutes)	0.7	0.9	0.55	0.75
Resolution		15.0	2.55	0.66

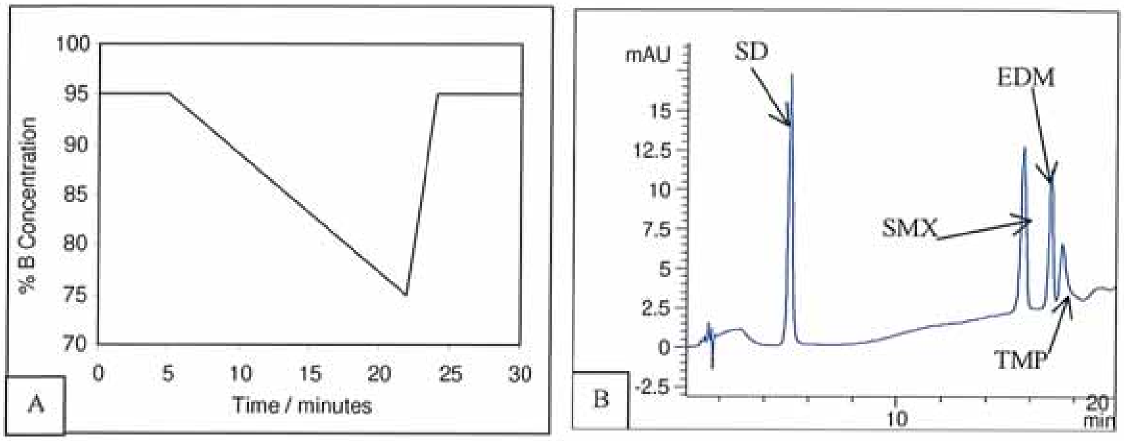


Figure 5.10: Gradient profile for 58.8% ROC (A) and a chromatogram of the resulting separation (B) (Table 5.8). Chromatographic Conditions: Section 5.8.2.

Table 5.8: Capacity factor, selectivity, peak width and resolution of the analyte peaks obtained for different mobile phase compositions analysed under the gradient run shown in Figure 5.11. Mobile phase B: 0.02M CH_3COONa pH 3.5 with glacial acetic acid, A: ACN: H_2O: TEA – the ratio of TEA was increased by correspondingly reducing the ratio of the other two components.

	Sulphadiazine	**Trimethoprim**	**Sulphamethoxazole**	**Ethidimuron**
Triethylamine: 1.5% N = 12858				
Capacity factor	2.81	10.58	10.58	16.01
Selectivity		3.77	-	1.51
Peak Width (minutes)	0.72	1.11		0.6
Resolution		14.7	0	13.6
Triethylamine: 3.9% N = 10942				
Capacity factor	2.26	8.18	9.0	9.92
Selectivity		3.62	1.1	1.1
Peak Width (minutes)	0.8	0.7	0.8	0.65
Resolution		11.65	1.77	1.0
Triethylamine: 5.6% N = 9604				
Capacity factor	3.55	9.6	10.23	11.37
Selectivity		2.7	1.06	1.11
Peak Width (minutes)	0.8	0.8	0.8	0.6
Resolution		9.5	1.14	2.23
Triethylamine: 6.0% N = 8633				
Capacity factor	3.47	9.52	10.24	11.31
Selectivity		2.74	1.08	1.11
Peak Width (minutes)	1.0	0.8	0.8	0.75
Resolution		9.11	1.5	2.13

The increase of TEA in the mobile phase had the effect of reducing the number of theoretical plates but not significantly. More importantly however, the TEA shifted the retention time of the TMP as is evident from the capacity factors and the chromatograms (Figure 5.9 and 5.10). This was caused by the TEA adsorbing onto the active sites on the column, resulting in the TMP having fewer sites to interact with and therefore was eluted earlier in the chromatographic run. The TMP eluted between the SD and SMX (Figure 5.11), as opposed to eluting last of the four peaks as previously obtained (Figure 5.10). The peak width of TMP was considerably decreased, from 1.56 minutes (Figure 5.9), to 0.8 minutes (Figure 5.11), with the increase of TEA in the mobile phase. It was also noted that the increase in TEA in the mobile phase did not have a linear effect on the resolution obtained between the TMP and SMX analyte peaks as can be seen in Table 5.8. Therefore increasing the amount of TEA further in the mobile phase will not have any significant effect on the resolution obtained between the TMP and SMX.

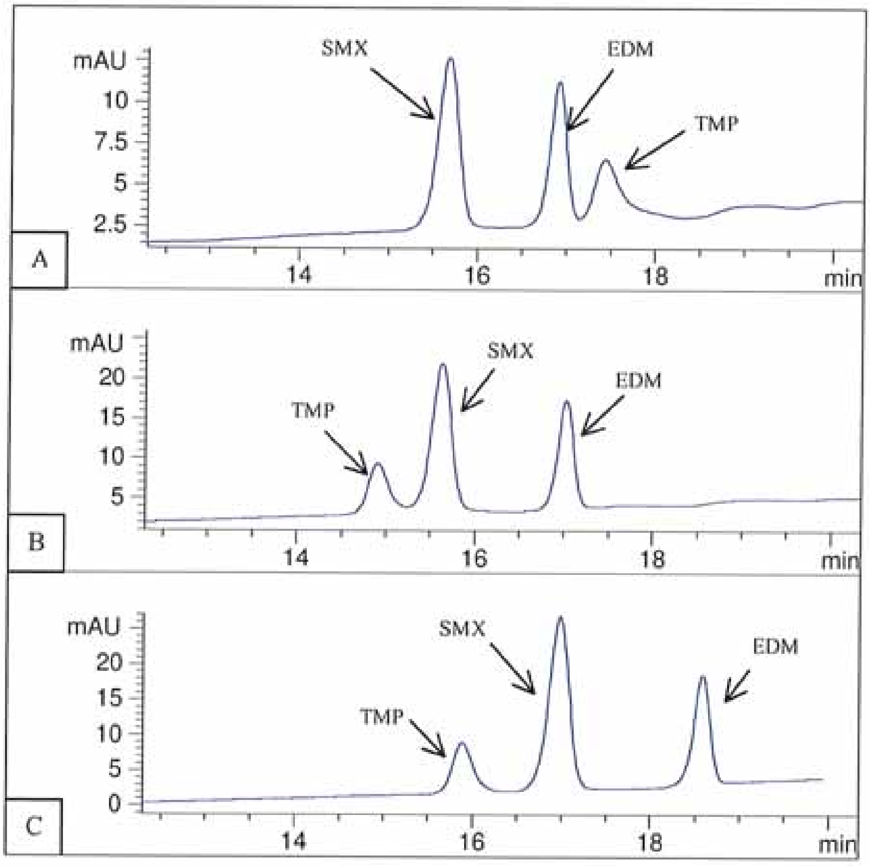


Figure 5.11: Overlay of standard analytes which were analysed with different amounts of triethylamine in the mobile phase- (A): 0.3%, (B): 3.9%, (C): 6%, Table 5.7 and Table 5.8. Chromatographic Conditions: Section 5.8.2.

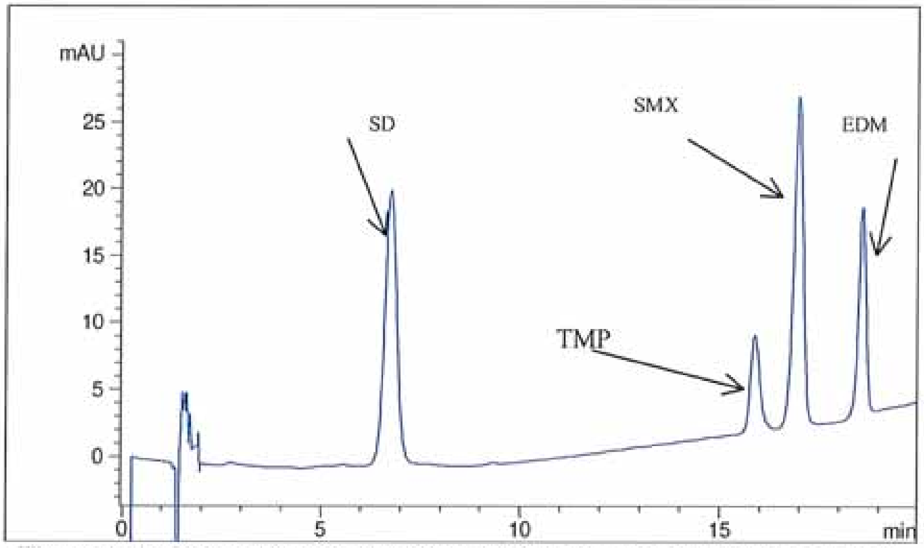


Figure 5.12: Chromatogram of the four analytes shows acceptable resolution for quantification purposes. Data shown in Table 5.8, (TEA = 6%). Chromatographic Conditions: Section 5.8.2.

5.8.5 Final optimisation of the gradient conditions

In an attempt to utilise the gradient to its maximum and reduce the time between the elution of the SD and TMP peaks and thus reduce the total analysis time the percentage change in the gradient was increased over the same time.

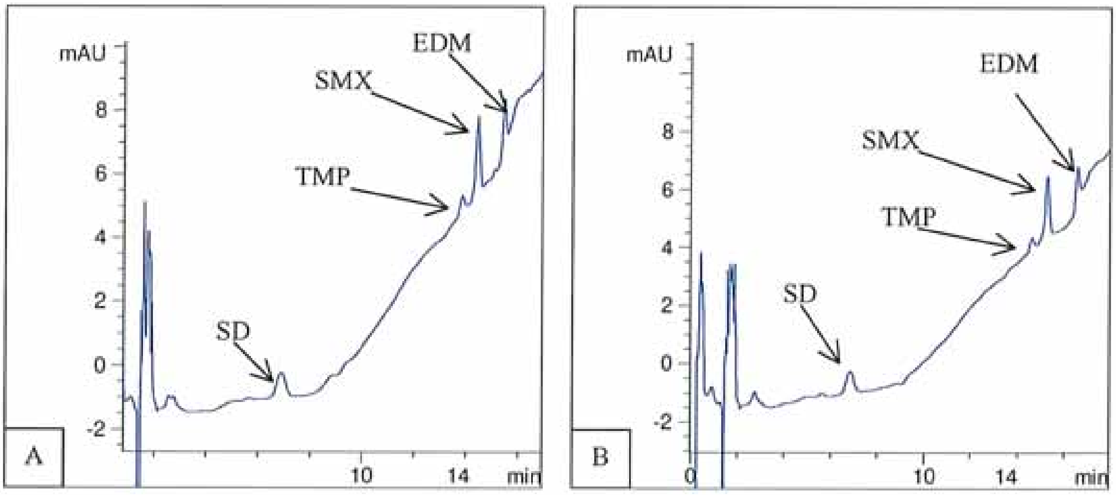


Figure 5.13: Standard analytes obtained with a 95-50% gradient over 17 minutes (A) and with a 95-65% gradient over 17 minutes (B). Chromatographic Conditions: Section 5.8.2.

Instead of having a gradient from 95-75% over 17 minutes a gradient from 95-50% was examined over the same 17 minutes. The result of this can be seen in Figure 5.13(A) where it is evident that the analytes eluted closer to the SD when compared to that in

Figure 5.12. However, the increase in the slope of the baseline due to the increased rate of gradient, is significant and would make quantification under those conditions very difficult. The same trend was noted for a gradient of 95-65% over 17 minutes as can be seen in Figure 5.13(B).

In a further attempt to reduce the analysis time, a gradient of 95-85% over 1 minute was examined and the resulting chromatogram can be seen in Figure 5.14(A). From this it is evident that the analysis time has reduced from 20 minutes (Figure 5.12), to 16 minutes (Figure 5.14) while still maintaining adequate resolution.

A spiked tissue sample was prepared according to the procedure described in Section 5.7 and was analysed using the chromatographic conditions described in Section 5.9. An overlay of the spiked tissue sample on a blank tissue sample can be seen in Figure 5.15.

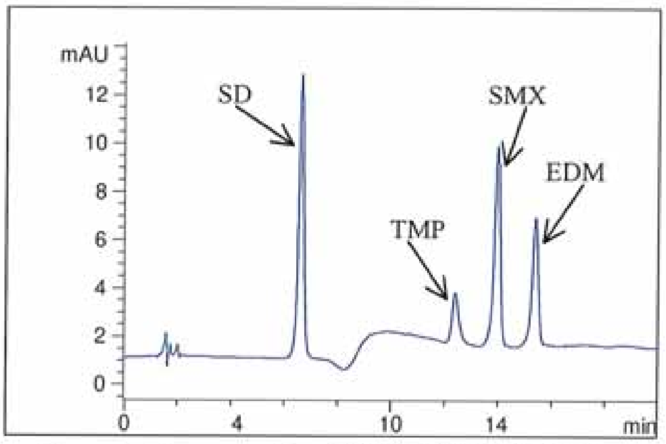


Figure: 5.14: separation of SD, TMP, SMX, EDM standards under the conditions described in Section 5.9.

5.8.6 Examination of the sample solution

Under the conditions described by Malisch *et al.* [3], the standards and the prepared tissue samples were injected in a mixture of ethylene glycol: water (1:1). The ethylene glycol was used in the sample preparation procedure to prevent the evaporation of the sample to dryness and thus the loss of the analytes [3]. However, when ethylene glycol was examined in this work, extra peaks in the baseline of the chromatogram became evident over time. These interfering peaks caused difficulties in the quantification of the analytes and as a result the precision of the recoveries obtained was high. An examination of each step of the method pointed towards the ethylene glycol as being the source of this interference. A blank solution of ethylene glycol: water (1:1) was injected immediately after opening the bottle of Analar grade ethylene glycol. The LOD of the method was determined based on a S/N=3, and thus using the response obtained at the analyte retention times, a LOD and %RSD for each analyte was calculated and are shown in Table 5.9. This data confirms the increases in the response for a blank solution over 15 days and also the decrease in the precision obtained. This was most likely due to the short shelf life of the ethylene glycol. The manufacturers however, recommend a three-month shelf life for ethylene glycol once the bottle was open, but under these conditions ethylene glycol was found to be inadequate for the required purposes in this method.

As an alternative to ethylene glycol for sample and standard injection, the mobile phase component B, 0.02M CH_3COONa, pH=3.5, was examined. A blank solution of this was injected and the LOD for each analyte was calculated and recorded in Table 5.9. From the %RSD on these values it is apparent that the precision obtained for the acetate buffer is higher than that obtained for the ethylene glycol and thus this was chosen as the solution for sample and standard injection.

As ethylene glycol was used as a keeper in the sample preparation procedure at the evaporation steps, an alternative was required. Water was chosen, and at every step ethylene glycol was used in the procedure it was replaced with water and this was found to be effective to prevent the evaporation of the sample to dryness.

Table 5.9: LOD and (%RSD) for each analyte determined using ethylene glycol: water at two different stages and sodium acetate buffer, (n=3)

	SD /μgkg^{-1}	TMP/μgkg^{-1}	SMX/μgkg^{-1}	EDM/μgkg^{-1}
Ethylene glycol : water (Day 1)	0.9 (5.0%)	1.0 (3.5%)	2.0 (4.2%)	5.2 (3.8%)
Ethylene glycol : water (Day 15)	3.2 (10.2%)	2.8 (11.3%)	5.4 (15.2%)	7.0 (9.6%)
0.02M CH_3COONa	1.0 (1.1%)	1.0 (1.8%)	1.6 (1.5%)	5.4 (2.0%)

5.9 Final Chromatographic conditions

The final chromatographic conditions used to obtain the chromatogram in Figure 5.14 and 5.15 consist of the following:

Column: Spherisorb S5 ODS (2) (250 x 4.6) mm

Temperature: 40°C

Injection volume: 100µl in 0.02M CH_3COONa pH = 3.5 with glacial acetic acid

Detector: DAD at 265nm, 262nm, 267nm and 276nm for SD, TMP, SMX and EDM respectively.

Mobile phase: Gradient of A = ACN: H_2O: TEA (67:27:6) pH = 12.3

B = 0.02M CH_3COONa, the pH was adjusted to 3.5 with glacial acetic acid

The gradient started with 95% B, constant for 5 minutes, then the gradient to 85% B over 1 minute, this was held constant for 12 minutes and then back to 95% over two minutes, constant for 5 minutes.

Flow rate: 2ml/minute

5.10 Method validation

Under Directive 96/23/EC routine methods must be validated for the detection and quantification of veterinary drug residues in farmed salmon [18]. The European Union, 93/256/EEC, has laid down criteria for the detection of analytes in test samples for routine methods [8]. These criteria include specificity, injection repeatability, linearity, accuracy, precision, detection limit, quantification limit, range and stability. Similar criteria are also laid down by the Codex Committee on Residues of Veterinary Drugs in Foods, Volume 3, 1994 [11]. These criteria were used as guidelines, for the validation of the method for SD, SMX and TMP residues in salmon tissue and have been discussed in Section 1.5.1. It became apparent during this study that the precision, while still adhering to the EU criteria of <15% RSD for repeated analysis, could be improved. Thus an internal standard was identified from the Malisch *et al.* [3] multi-residue method. This analyte was EDM and it was treated in the same manner as the analytes of interest during the validation study in order to determine its suitability as an internal standard for the method under examination.

5.10.1 Specificity

For chromatographic methods, developing a separation involves demonstrating specificity, which is the ability of the method to accurately measure the analyte response in the presence of all sample components. The response of the analyte in the test mixture, i.e. fish tissue which contains the analyte and all other sample components was compared to the response of a standard solution containing only the analyte. Fish tissue, was spiked with 1.5μg of SD, SMX and EDM and 0.75μg of TMP to yield a concentration of $100\mu gkg^{-1}$ of the sulphonamides and EDM, and $50\mu gkg^{-1}$ TMP in the fish tissue. In order for fish tissue to be fit for human consumption a MRL for the combined sulphonamides was set at $100\mu gkg^{-1}$ and $50\mu gkg^{-1}$ for TMP [1]. The spiked tissue was brought through the cleanup procedure and analysed under the same conditions as described for the samples (Section 5.7). The resulting chromatogram, which was obtained using the analytical conditions in Section 5.9, of a spiked tissue sample overlayed on a blank tissue sample can be seen in Figure 5.15.

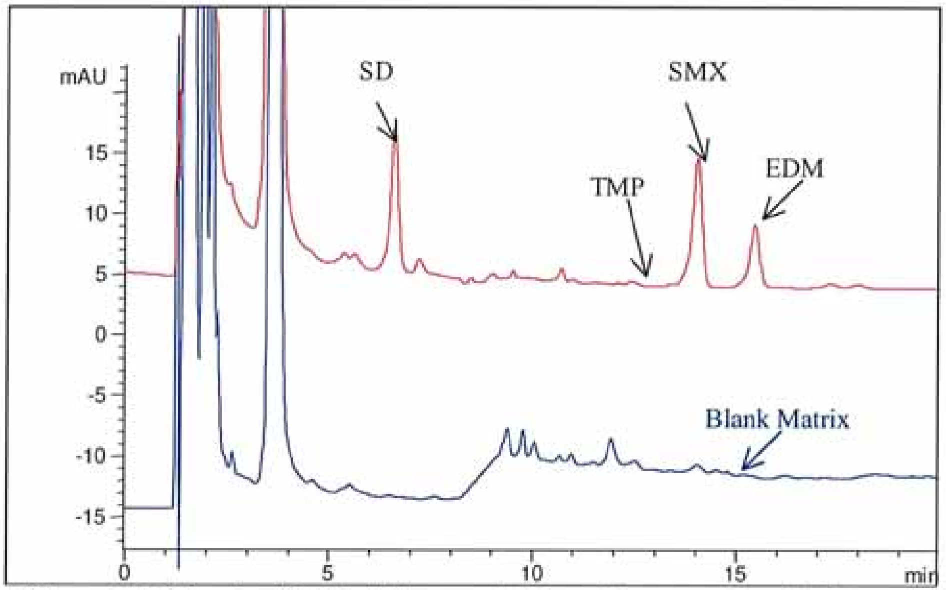


Figure 5.15: Overlay of a blank tissue matrix on a tissue sample spiked to 100μgkg^{-1} with SD, SMX, EDM and 50μgkg^{-1} TMP. Analytical conditions: Section 5.9.

From Figure 5.15 it can be seen that there are no matrix interferences from the blank tissue sample at the retention times of any of the analyte peaks. The analyte peaks have baseline chromatographic resolution of greater than the required 1.5 value [13], from each other.

To confirm the identification of the analyte peaks, the spectra of each analyte was obtained using the DAD detector and compared to those of the standard analytes which were stored in the library. The resulting overlay of the spectra can be seen in Figure 5.16. In an ideal situation the match of the analyte spectra in the matrix to the standard analyte spectra stored in the library would be 1000. Values of 999.6, 999.3, 999.2 and 999.9 were obtained for SD, TMP, SMX and EDM respectively. This indicates that the spectra obtained for SD, TMP, SMX and EDM in spiked tissue samples were pure spectra.

Commission Decision 93/256/EC has lay down criteria for using DAD as a confirmation method. These criteria state that the absorption maxima in the spectrum of the analyte should be at the same wavelengths as those of the standard analyte within ±2nm. Also the spectrum above 220nm should not be visually different from that of the standard analyte and the difference between the absorbance of the two spectra should at no point be greater than 10%. From Figure 5.16, it can be seen that these criteria are met for each of the four analytes examined.

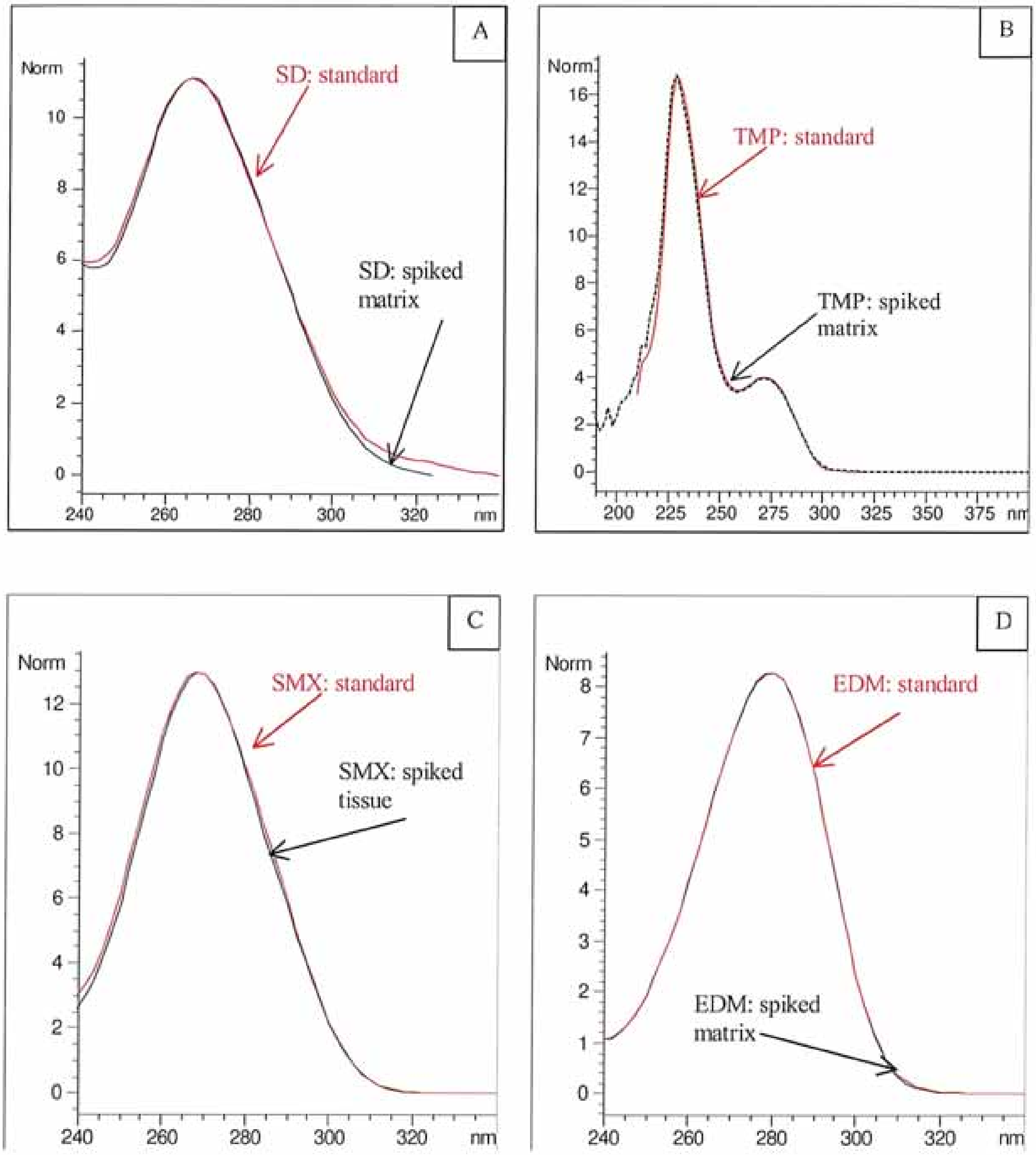


Figure: 5.16: Overlay of a spectra from a spiked tissue sample on a standard analyte spectra for SD (A), TMP (B), SMX (C) and EDM (D).

Other analytes, which are used to treat farmed fish in Ireland including oxytetracycline, oxolinic acid, ivermectin, cypermethrin and dichlorvos were analysed under the HPLC conditions optimised in this method (Section 5.9). It was found that they did not interfere with the retention times of any of the analytes analysed in this method. From this specificity evaluation the chromatographic parameters were set and these were used in all further validation studies. These analytical conditions are described in Section 5.9.

5.10.2 Injection repeatability

In this study five standard injections from the same sample vial were made to test the performance of the chromatographic instrument. The standard calibration plots were used to calculate the concentration of the standard solution for each analyte (Figure 5.19). The upper and lower 95% confidence limits were calculated for the calculated concentration values using Equation 1.1 (Table 5.10). Due to the resolution between some of the matrix components and the analyte peaks it became apparent that it would be necessary to use peak height as the method of quantification as opposed to peak area in this method (Figure 5.15).

Table 5.10: Concentration based on peak height for the instrument repeatability study showing the mean, standard deviation, %RSD and the upper and lower 95% confidence limits for SD, TMP, SMX and EDM.

	SD (μgml^{-1})	TMP (μgml^{-1})	SMX (μgml^{-1})	EDM (μgml^{-1})
Mean	0.472	0.419	0.553	0.518
SD	0.081	0.015	0.045	0.029
% RSD	1.81	2.38	1.55	1.95
95% UCL	0.482	0.431	0.565	0.543
95% LCL	0.462	0.407	0.541	0.500

The %RSD for each analyte was below the required value of 2.5% [19], thus indicating acceptable precision for the injection repeatability (Table 5.10). The results show that the five replicates which were injected are evenly distributed on both sides of the mean value for each of the four analytes i.e. 0.473±2.0% for SD, 0.419±3.1% for TMP, 0.553±1.4% for SMX and 0.519±2.5% for EDM (Figure 5.17). Therefore peak height was suitable as the response for each of the four analytes for subsequent validation studies.

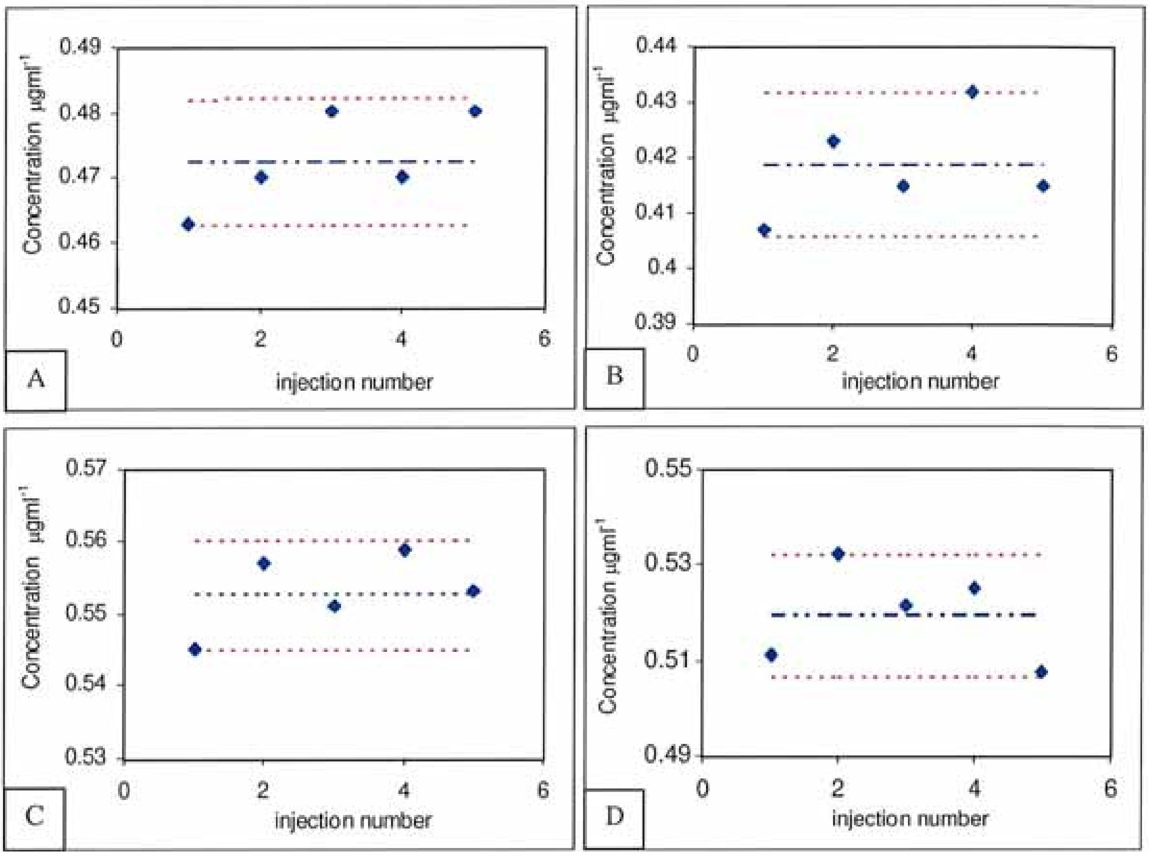


Figure 5.17: Injection repeatability study showing the mean concentration obtained (-----) and the upper and lower 95% confidence limits (-------) for SD (A), TMP (B), SMX (C) and EDM (D).

5.10.3 Linearity

A linearity study checks if the sample solutions are in a concentration range where the analyte response is linearly proportional to concentration. Standard solutions of SD, TMP, SMX and EDM were prepared over a wide concentration range and analysed by RPHPLC (Section 5.9). In order to find a concentration range for which linearity, accuracy, and precision studies will be examined the linear range of the method must first be determined. A plot of the concentration of the standard solutions against the peak height measurements, can be seen in Figure 5.18^1 while the corresponding response factor plots are shown in Figure 5.18^2.

Using the data from Figure 5.18^1, the response factors were calculated and from these plots (Figure 5.18^2) the linear range of the method for each analyte was determined (Section 1.5.5). In an ideal situation a uniform response factor value would be obtained for each concentration level for the plots in Figure 5.18^2. However, in practice this is not always the situation and therefore criteria were set to determine the linear range for this method.

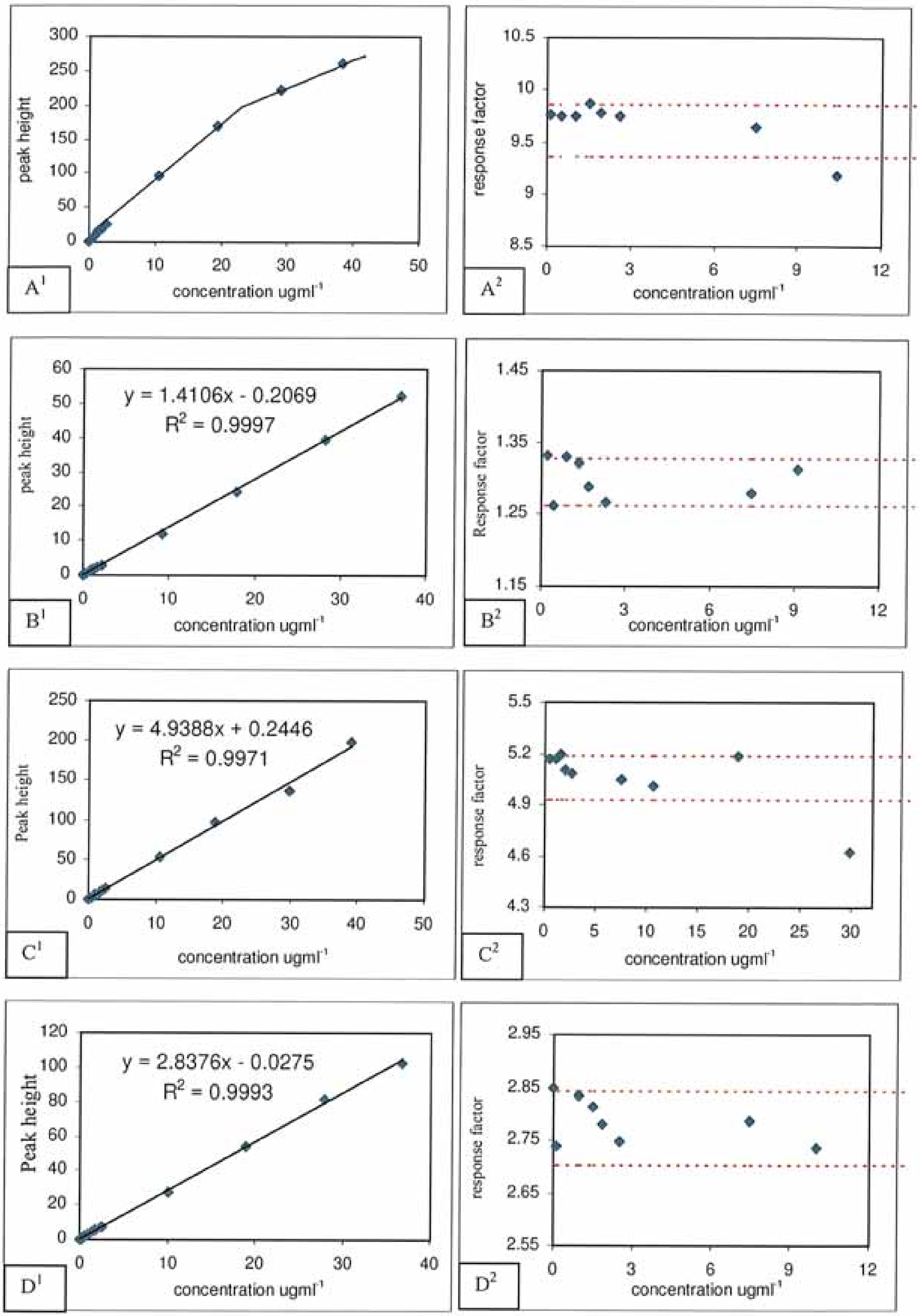


Figure 5.18: Linearity study showing calibration (¹) and corresponding response factor (²) plots for SD (A), TMP (B), SMX (C), and EDM (D) respectively. The ± 2.5% limits on the response factor plots are indicated by (------).

For a concentration level to be included in the linear range of the method, its corresponding response factor value must lie within the mean response factor value ± 2.5%. However due to the uncertainty of response factor values at higher and lower concentrations, the mean value is set based on the maximum response factor value obtained (S_{max}) and 95% of this value. The corresponding response factor value for any concentraion must lie within these two limits to be included in the linear range of the method. From Figure 5.18, the linear range was found to be 0.1-10.4μgml^{-1}, 0.20-11.1μgml^{-1}, 0.15-26.2μgml^{-1}, 0.098-10.2μgml^{-1}, corresponding to 10-1040μgkg^{-1}, 20.5-910μgkg^{-1}, 15.2-750μgkg^{-1}, 9.8-1020μgkg^{-1} for SD, TMP, SMX and EDM respectively.

The least squares line and y-residual plots for standard solutions for each analyte were examined from 50-200% of the MRL for each analyte. These concentration levels are included in the linear range of the method. The correlation coefficient of the regression data and the variance of this data provide a mathematical measure of linearity. A correlation coefficient of > 0.999 is generally considered as evidence of acceptable fit of the data to the regression line. The calibration plots showing peak height against concentration were examined (Figure 5.19).

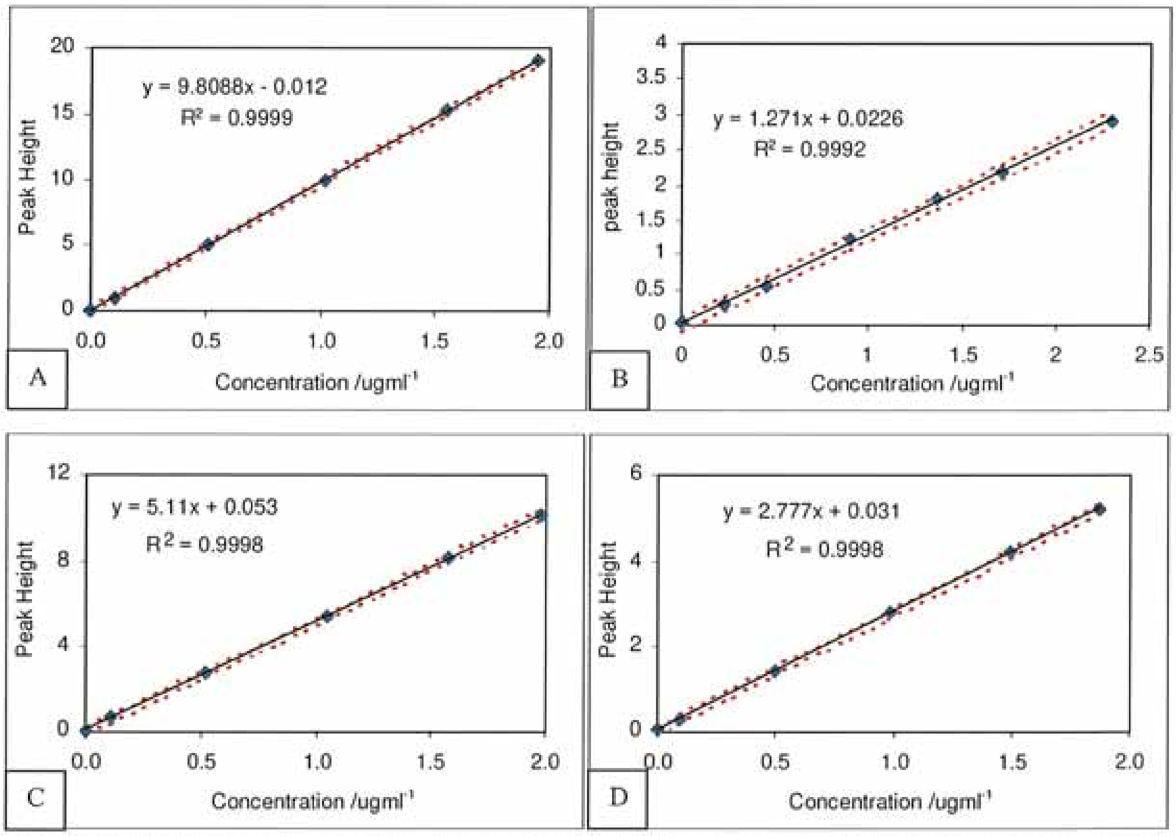


Figure 5.19: Calibration plots for standard solutions of concentration values within the linear range of the method for SD (A), TMP (B), SMX (C) and EDM (D). The upper and lower 95% confidence limits are indicated by (-----).

From these plots it is evident that there is a good fit of the data points to the least squares line and this is confirmed by the coefficient of variation values of 0.9999, 0.9992, 0.9998 and 0.9998 for SD, TMP, SMX and EDM respectively.

To determine if the method is capable of detecting levels of the analyte at the MRL, the y-intercept of the calibration plots in Figure 5.19 were compared to the expected response of a sample at the MRL. Table 5.11 summarises the results for each of the four analytes of interest. The expected response at the MRL of 100μgkg^{-1} for SD is approximately 10 and the y-intercept is 0.12% of this value. If the y-intercept response is ≤2% of the expected value then the method is capable of detecting residues at this concentration level [19]. Thus from Table 5.11, it is evident that the current method will detect residues at the MRL for the analytes of interest.

Table 5.11: Comparison of the y-intercept to the analyte response at the MRL

Analyte	y-intercept	Expected response at the MRL	Intercept as a % of the MRL response
SD	-0.012	10 (100μgkg^{-1})	0.12%
TMP	0.0226	1.2 (50μgkg^{-1})	1.88%
SMX	0.053	5.4 (100μgkg^{-1})	0.98%
EDM	0.031	2.9 (100μgkg^{-1})	1.07%

5.10.4 Calculations

1.46μg of SD was added to 15g of tissue. This was extracted with 120ml solution. 90mls of this was taken through the complete clean-up procedure and dissolved in 1ml of buffer prior to injection, i.e. 66.66% of the sample which is equivalent to 0.975*μg* in 10g of tissue. This yielded a 97.5μgkg^{-1} sample. The peak height obtained for this sample was *11.95*.

From a calibration plot of SD standards against peak height a straight line was obtained. The equation of this line was: $y = 11.73x + 0.19$

When the height for the sample was substituted into this equation a concentration value corresponding to 1.00μgml^{-1} was obtained.

$$\text{i.e. } 11.95 = 11.73x + 0.19$$

$$x = 1.00\mu g ml^{-1}$$

The final volume was 1ml, therefore *1.00µg was recovered.*

The percentage recovery is:

$$\% \text{ recovery} = \frac{ug \text{ recovered}}{ug \text{ added}} \times 100$$

- (1.00/0.975) *100 = 102.5%

5.10.5 Precision

The precision of a method is the amount of scatter in the results obtained from multiple analyses of a homogeneous sample. To be meaningful the precision studies were performed using the exact sample and standard preparation procedures that were optimised and reported in Section 5.7 and 5.9. The first type of precision study is the instrument precision or injection repeatability, which was dealt with in Section 5.10.2. The second type of precision study is the repeatability or intra-assay precision. Intra-assay precision data was obtained by repeatedly analysing, in one laboratory in one day, aliquots of a homogeneous sample each of, which was independently prepared according to the method procedure.

In order to carry out the intra-assay precision study, replicate samples were prepared containing the MRL of each of the components. Fish tissues samples (15g), were spiked with 100µgkg^{-1} of SD, SMX, EDM and 50µgkg^{-1} TMP and each sample was independently prepared according to the sample procedure. These samples were analysed by HPLC and percentage recoveries recorded.

Table 5.12: Percentage recoveries and % RSD for tissue samples spiked to 100µgkg^{-1} (n=6)

	SD	TMP	SMX	EDM
Mean % Recovery	92.8	41.1	89.1	88.3
Standard Deviation	9.0	3.5	7.5	1.0
% RSD	9.7	8.4	8.4	11.4

For six replicates of a tissue sample spiked to 100µgkg^{-1} the mean percentage recovery obtained was 92.9% for SD, 41.1% for TMP, 89.1% for SMX and 88.3% for EDM. The respective range of recoveries were 81.5-104.9%, 35.8-46.0%, 78.3-100.8%, and 83.1-106.7% with %RSD of 9.7%, 8.4%, 8.4%, and 11.4% for SD, TMP, SMX and EDM.

The range of recoveries reported for pork samples spiked to 100μgkg^{-1} were 64-152.4%, 77.2-114.9% and 63.8-133% for SD, SMX and EDM respectively [3]. When these are compared to the range of recoveries reported for salmon tissue it is evident that those obtained for salmon are closer than those reported for meat samples [3]. It should be noted however that 8 different analysts reported data for the recoveries obtained for the meat samples [3], while one analyst carried out all the analyses for the data reported above for the salmon tissue samples. The %RSD obtained for each analyte is below the required 15% value as quoted in the Codex guidelines for spiked tissue samples [11].

5.10.6 Accuracy

Accuracy is defined as the proximity of a measured value to the true value and it is affected by systematic errors. Systematic error or bias remains constant or varies in a predictable way over a series of measurements and it can be corrected for if detected.

Accuracy may often be expressed as % recovery by the assay method, of known, spiked amounts of analyte. Tissue samples were spiked with each of the three analytes and the internal standard to five different concentration levels about the MRL and these were analysed according to the method in Section 5.7 and 5.9. The spiked samples were run in random order, to subject the method to whatever uncontrolled variables, which may have been operating. The same analyst carried out all the analyses to eliminate between analyst variations. When all the analyses were carried out, the data was then evaluated (Table 5.13).

Table 5.13: Percentage recoveries for spiked tissue samples analysed over the concentration range of 25-200*μgkg^{-1}*.

Sulphadiazine		Trimethoprim		Sulphamethoxazole		Ethidimuron	
μgkg^{-1}	% Recovery	μgkg^{-1}	% Recovery	μgkg^{-1}	% Recovery	μgkg^{-1}	% Recovery
23.92	97.7	24.31	-	24.52	80.6	24.33	106.7
48.94	106.6	49.72	42.6	50.15	88.5	49.77	83.1
97.51	102.8	99.08	46.0	99.92	98.1	99.17	98.0
146.04	99.8	148.38	41.3	149.65	85.6	148.52	85.3
195.87	101.9	199.01	40.3	200.72	93.6	199.21	86.7
Mean	101.8	**Mean**	42.5	**Mean**	89.3	**Mean**	91.9
SD	3.34	**SD**	2.48	**SD**	6.18	**SD**	10.05
%RSD	3.3	**%RSD**	5.8	**%RSD**	7.6	**%RSD**	10.9

For SD the percentage recoveries obtained were 101.8 ± 4.8% over the concentration range of 25-200% of the MRL. The %RSD on five spiked concentration levels was 3.3%. This indicates that the results for SD are both accurate, values close to 100% and also precise $\leq$ 15%RSD [11]. The suggested accuracy requirements for spiked tissue samples at $\leq 100\mu gkg^{-1}$, as set out by the Codex Alimentarius [11] is 70-110%, while the EU guidelines only quote criteria for CRM and these do not exist for sulphonamides in salmon tissue.

The accuracy obtained for TMP is clearly not acceptable and was discussed in Section 5.6.4.

As for SD, the accuracy obtained for SMX and EDM meets the Codex criteria for accuracy with recoveries of 89.3±8.7% for SMX and 91.9±14.8% for EDM. However the precision of the results obtained for both SMX and EDM indicated by the %RSD values of 7.6 and 10.9% respectively while meeting the required guidelines of $\leq$15% are not as good as that obtained for SD, i.e. 3.3% RSD for SD. This is most likely due to the small matrix interferences in the chromatogram, which elute close to the SMX and EDM peaks, while clear resolution was obtained for SD. The %RSD reported for SD, SMX and EDM in pork tissue spiked to $100\mu gkg^{-1}$ were 13.0%, 12.2% and 7.7% respectively indicating that the precision results obtained for the spiked salmon tissue samples were an improvement on those reported for spiked pork tissue [3].

5.10.7 Range and Limits of Detection and Quantification

The range of the analytical method was determined on the basis that the concentration value of most interest in the method is the MRL of $100\mu gkg^{-1}$ for SD, SMX and $50\mu gkg^{-1}$ for TMP. Therefore, to determine if the amount of analytes found in salmon samples are below the MRL, it was necessary to prepare a calibration plot of spiked tissue samples over the range of 25-200% of $100\mu gkg^{-1}$. The range of an analytical method is defined as the concentration interval over which acceptable accuracy, linearity and precision are obtained. The range was determined using data from the linearity accuracy and precision studies, which were carried out earlier. The linear range was found to be $0.1\text{-}10.4\mu gml^{-1}$, $0.20\text{-}11.1\mu gml^{-1}$, $0.15\text{-}26.2\mu gml^{-1}$, $0.098\text{-}10.2\mu gml^{-1}$, corresponding to $10\text{-}1040\mu gkg^{-1}$, $20.5\text{-}910\mu gkg^{-1}$, $15.2\text{-}750\mu gml^{-1}$, $9.8\text{-}1020\mu gkg^{-1}$ for SD, TMP, SMX and EDM respectively. Since these ranges contain the MRL values for each analyte they were deemed acceptable for the required analysis.

The accuracy of the method was deemed satisfactory for each analyte except TMP, but this analyte was still examined in this study (Section 5.10.6). The precision was deemed acceptable for the repeated analysis of a salmon tissue sample spiked to 100μgkg^{-1} for SD, TMP, SMX and EDM (Section 5.10.5). Thus in order to find the range of the method, the only remaining factor to be examined was the analysis and linearity of a calibration plot of spiked tissue samples over the concentration range of 25-200% of 100μgkg^{-1} for each analyte.

The spiked tissue plots were prepared by spiking (5 x 15g) of tissue with different known levels of each analyte. The spiked tissue samples plus a blank were extracted and analysed according to the method procedure (Section 5.7). The calibration plot was examined to determine if a straight line with correlation coefficient of ≥ 0.999 was obtained. A response factor and y-residual plot was examined and once acceptable linearity was obtained the range and the limit of quantification of the method were determined.

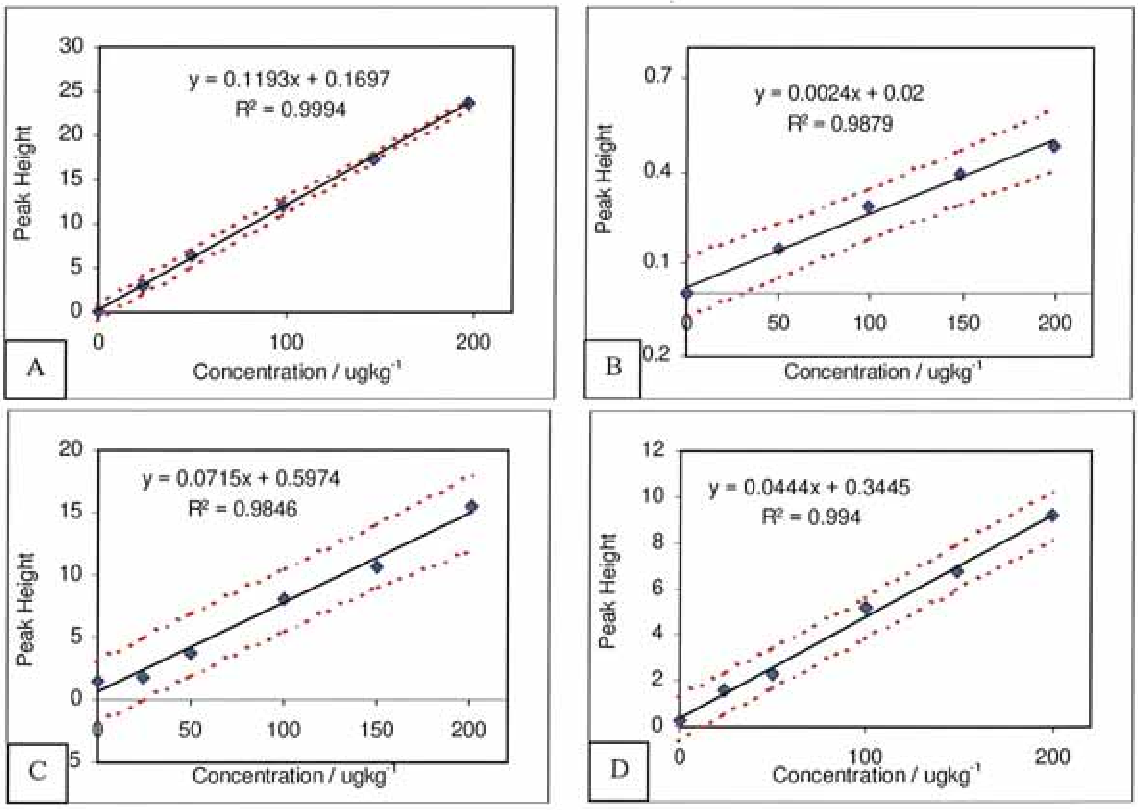


Figure 5.20: Calibration plots for spiked tissue samples for SD (A), TMP (B), SMX (C), and EDM (D). The 95% confidence limits are indicated by (----).

The calibration plots (Figure 5.20) show a linear response, all points are within the 95% confidence limits, over the concentration range of 24-200μgkg^{-1} for SD, SMX and EDM and 50-200μgkg^{-1} for TMP. The R^2 values obtained were 0.9994 for SD, 0.9879 for TMP, 0.9846 for SMX and 0.994 for EDM. Since for an ideal calibration plot the R^2 value would be 1, this indicates that the value obtained for SD is acceptable, indicating a linear calibration plot. For TMP, SMX and EDM however, the values obtained indicate that there is more of a scatter in the points about the line although the response still appears to be essentially linear.

Using the plots of spiked concentration against measured concentration the confidence interval at the MRL was estimated for each analyte (Figure 5.21). These values were corrected for percentage recovery and the intervals were found to be 99±5.5μgkg^{-1}, 52±9.5μgkg^{-1}, 92.7±18μgkg^{-1} and 91±17.5μgkg^{-1} for SD, TMP, SMX and EDM respectively. Further work is required in an attempt to improve the confidence interval about the MRL for TMP, SMX and EDM.

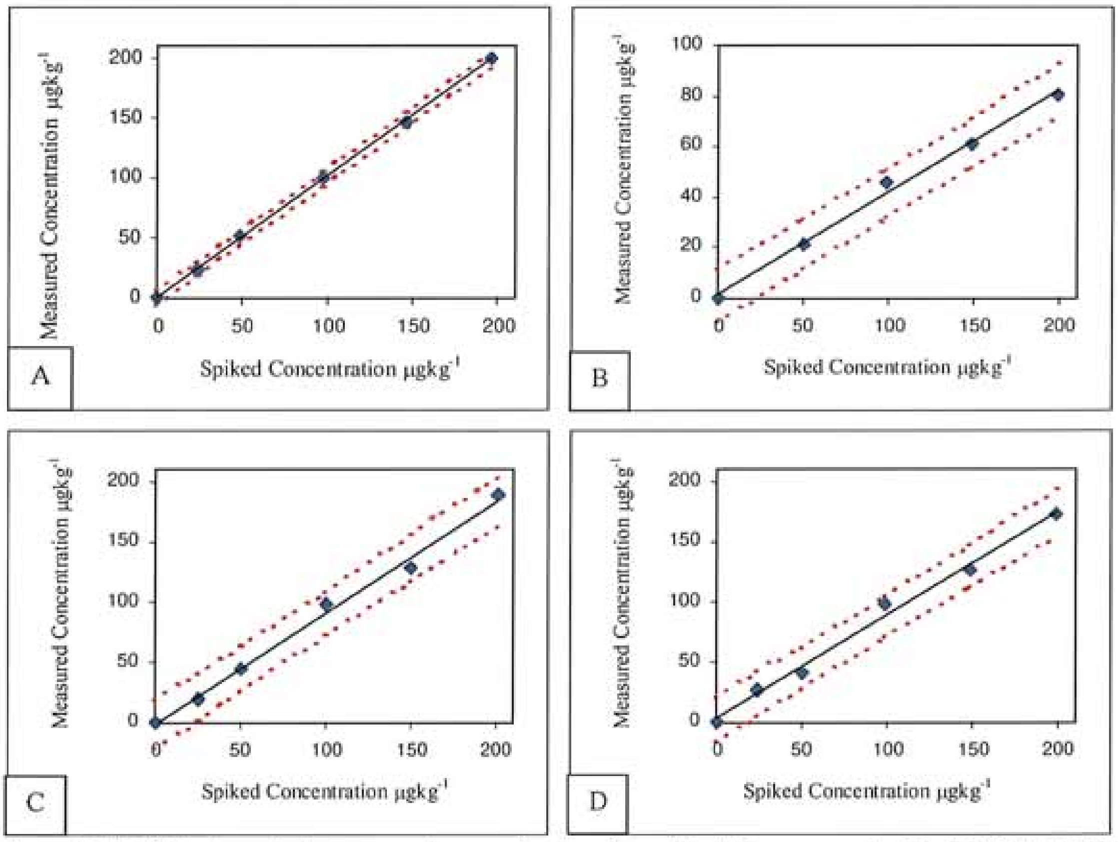


Figure 5.21: Spiked against measured concentration for spiked tissue samples for SD (A), TMP (B), SMX (C), and EDM (D). The 95% confidence limits are indicated by (----).

The response factor plots for each of the four analytes can be seen in Figure 5.22 where it is evident that the data points are randomly distributed about the mean response factor value for each analyte. In an ideal situation an equivalent response would be obtained for all the concentration values and the data points would form a straight line with a zero slope. The scatter on the response factors obtained are the 0.123±0.008, 0.0028±0.0006, 0.0745±0.0099 and 0.0507±0.017 for SD, TMP, SMX and EDM respectively at 99% confidence. The slopes obtained at a 95% level of confidence were ($-7.02\times10^{-6} \pm 1.03\times10^{-4}$), ($-1.69\times10^{-6} \pm 3.37\times10^{-6}$), ($1.29\times10^{-4} \pm 2.05\times10^{-4}$) and ($-1.27\times10^{-5} \pm 1.21\times10^{-4}$) fro SD, TMP, SMX and EDM respectively. These indicate a linear response was obtained over the concentration range examined for each analyte.

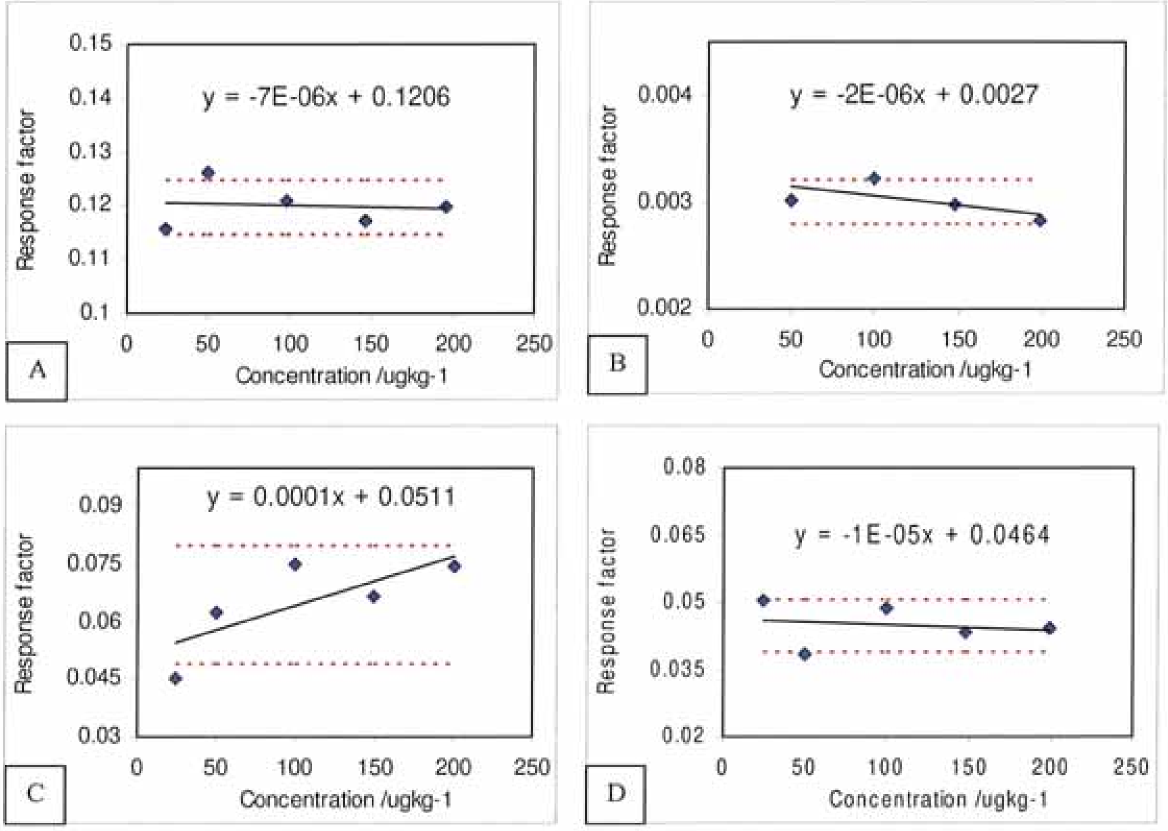


Figure 5.22: Response factor plots for spiked tissue samples on Day 1 of analysis for SD (A), TMP (B), SMX (C), EDM (D). The 95% confidence limits are indicated by (----) while the 99% confidence intervals are indicated by (----).

Linearity was further examined by plotting the y-residual against the predicted y-value for each analyte (Section 1.5.4.2). Visual examination of the y-residual plots indicates that the data points are randomly distributed about zero (Figure 5.23). The $\sum$y-residual was found to be equal to zero for each analyte indicating a linear response was obtained. The slopes obtained at a 95% level of confidence, ($-6.7\text{x}10^{-5} \pm 3.9\text{x}10^{-2}$), ($1.1\text{x}10^{-4} \pm 2.0\text{x}10^{-1}$), ($6.7\text{x}10^{-3} \pm 2.0\text{x}10^{-1}$) and ($3.0\text{x}10^{-5} \pm 1.2\text{x}10^{-1}$) for SD, TMP, SMX and EDM respectively), indicate the data points form a best-fit line which is parallel to the x-axis. The intercepts on these plots, which at 95% confidence are also close to zero, ($6.9\text{x}10^{-4} \pm 5.2\text{x}10^{-1}$), ($-7.7\text{x}10^{-6} \pm 6.4\text{x}10^{-2}$), ($-4.6\text{x}10^{-2} \pm 1.69$) and ($-1.1\text{x}10^{-4} \pm 6.4\text{x}10\text{-}1$) for SD, TMP, SMX and EDM respectively, indicate that the points are randomly distributed about the x-axis.

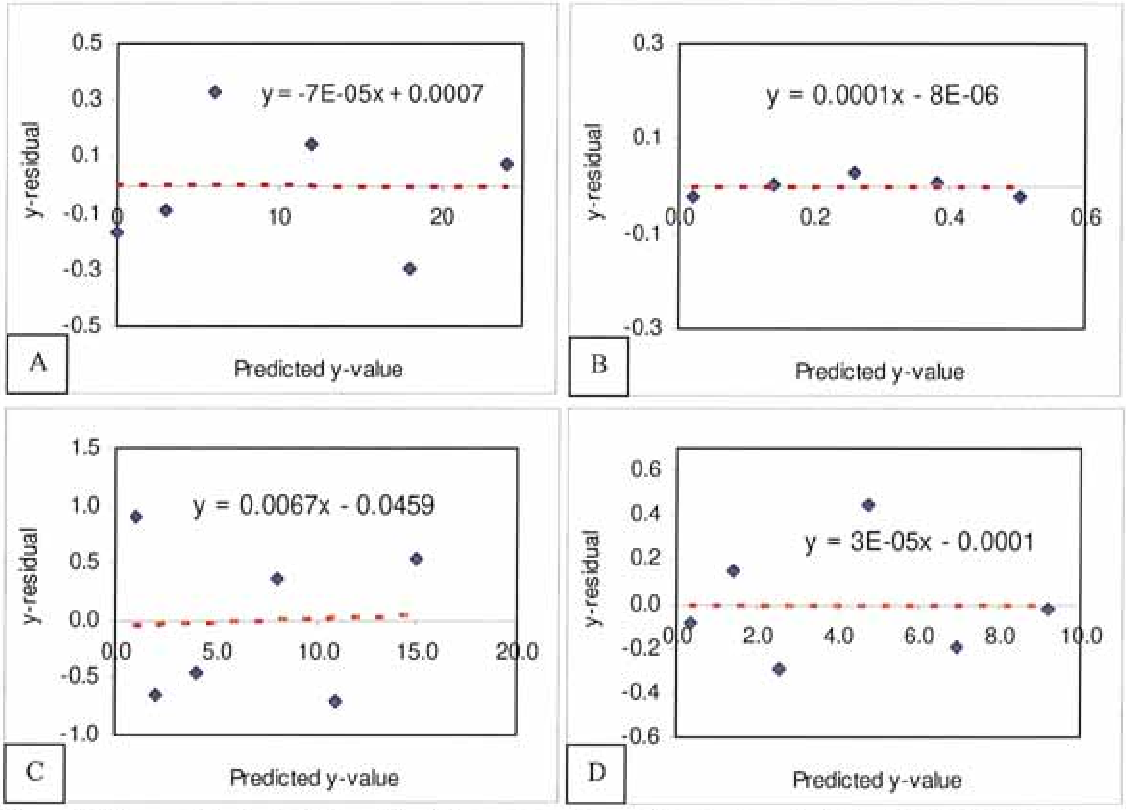


Figure 5.23: y-residual plots for spiked tissue samples on Day 1 of analysis for SD (A), TMP (B), SMX (C), EDM (D)

The above studies indicate that linearity was obtained for each analyte over the concentration range examined (24-196μgkg^{-1}, 50-200μgkg^{-1}, 24-200μgkg^{-1} and 24-200μgkg^{-1} for SD, TMP, SMX and EDM respectively). This range is acceptable for SD, SMX and EDM as the concentration of most interest for these analytes is 100μgkg^{-1}. For

TMP however, it would be required that the lower level on the range be approximately 25μgkg^{-1} to allow for quantification at half the MRL value of 50μgkg^{-1}.

This method is capable of quantifying levels of a standard solution of TMP equivalent to this required 25μgkg^{-1} level. However, since the recoveries from the cleanup procedure are low for the spiked tissue samples then the lower level obtained was above the required value for TMP.

The limit of detection (LOD) (Section 1.5.7) was estimated by measuring the response obtained for blank tissue samples (n=3) and calculating the LOD based on a S/N = 3. This value was subsituted into the equation of the calibration line to estimate the corresponding concentration value. The estimated LOD values for each analyte is shown below.

Analyte	**Mean Noise Response**	**LOD: S/N = 3 /μgkg^{-1}**	**LOQ: S/N = 5 /μgkg^{-1}**
SD	0.0963	1.0	2.6
SMX	0.2373	1.6	8.2
TMP	0.0075	1.0	7.3
EDM	0.1948	5.4	14.2

The limit of quantification (LOQ) (Section 1.5.8) for each analyte was taken as the S/N = 5. The estimated values for each analyte based on this method are shown above. Since the MRL for the combined sulphonamides is 100μgkg^{-1}, then the value of 2.6μgkg^{-1} and 8.2μgkg^{-1} obtained for SD and SMX respectively are satisfactory for the required purposes. The MRL of TMP is 50μgkg^{-1} and therfore the estimated LOQ of 7.3μgkg^{-1} based on the above method is satisfactory. Additional work is required to improve the accuracy and precision of EDM before using it as an internal standard. However the information gained in this study, (LOD of 5.4μgkg^{-1} and LOQ of 14.2μgkg^{-1}), is useful to have and will be required for additional work on this method.

5.10.8 Stability

For routine testing in which many samples are analysed each day, it is often necessary to allow for delays such as instrument breakdowns or overnight analyses. Therefore, the limit of stability of prepared tissue samples was tested while they were being stored in the fridge at 4°C.

A calibration plot of spiked tissue samples was prepared containing 24-200µgkg^{-1} SD, TMP, SMX and EDM according to the procedure (Section 5.7). These were analysed for three consecutive days using a freshly prepared mobile phase on each of the days. The calibration and spiked against measured concentration plots were examined on each of the three days of analysis for each analyte.

5.10.8.1 SD sample stability

For SD, the calibration plots and spiked against measured concentration plots are shown in Figure 5.20(A) and 5.21(A) for Day 1, Figure 5.24 for Day 2 and Figure 5.25 and Day 3.

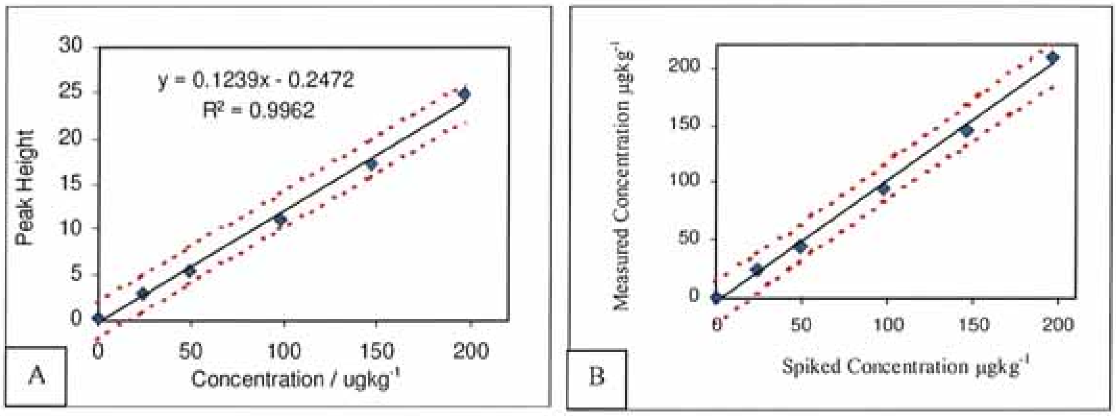


Figure 5.24: Calibration (A) and spiked against measured concentration (B) plots for SD on Day 2 of analysis. The upper and lower 95% confidence limits are indicated by (----).

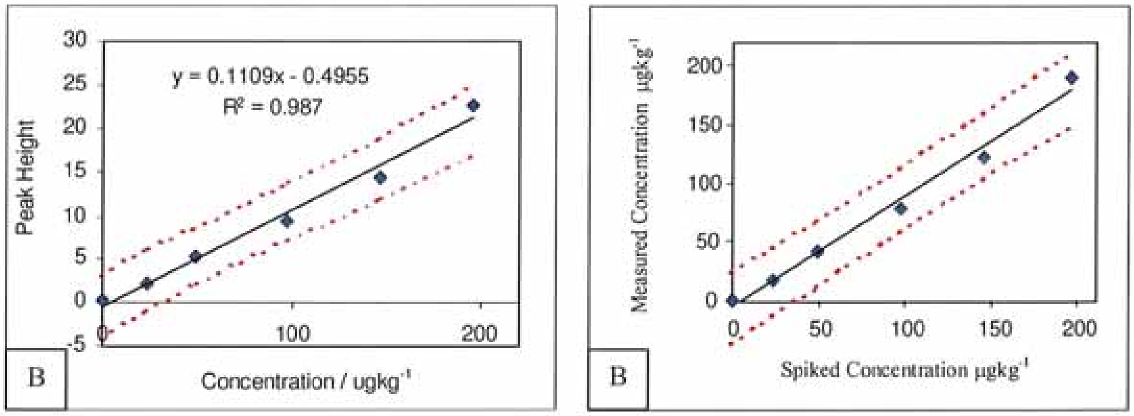


Figure 5.25: Calibration (A) and spiked against measured concentration (B) plots for SD on Day 3 of analysis. The upper and lower 95% confidence limits are indicated by (----).

The R^2 values on the calibration plots decrease from 0.9994 on Day 1 to 0.9962 and 0.987 on Day 2 and day 3 respectively. Since ideally this value would be equal to 1 then the values obtained indicate that over time there is an in increase in the scatter of the points about the line although the response still appears to be linear. To get a better idea if this deviation was significant, the 95% confidence intervals about the MRL of 100μgkg^{-1} were examined using the spiked against measured concentration plots (Figure 5.21(A), 5.24(B) and 5.25(B)). A correction factor for percentage recoveries was applied to these values and the confidence intervals were found to be 99±5.5μgkg^{-1}, 105±16μgkg^{-1} and 91.5±27μgkg^{-1} for Day 1, 2 and 3 respectively. While the confidence intervals increased over the three days of analysis, the response obtained does not appear to have altered significantly.

Standards analysed on each of the three days of this test to check the performance of the instrument met the sensitivity criteria indicating that the instrument response was acceptable on each day. Between Day 1 and Day 2 t_{calc} was found to 1.12 while t_{calc} was found to be 1.28 between Day 1 and Day 3. Using $((n_1-2) + (n_2-2))$ degrees of freedom the critical value of t was found to be 2.31 [20]. Since $t_{calc} < t_{table}$ for both comparisons then it can be concluded that the slopes do not differ significantly at a 5% level of confidence. Thus the response obtained did not alter significantly over the three days of analysis.

Therefore based on these studies for SD, it was recommended that the prepared tissue samples should be analysed immediately after the sample preparation procedure is completed. If they are stored in the fridge at 4°C and analysed within three days then a wider confidence interval will be obtained on the calibration plot.

5.10.8.2 TMP sample stability

For TMP, acceptable accuracy was not obtained in the clean-up procedure but even so, it was examined in this study to see if any trends were noted over the three days of analysis of the prepared spiked tissue samples. The calibration and spiked against measured concentration plots ±95% confidence intervals are shown in Figure 5.20(B) and 5.21(B) for Day 1, Figure 5.26 for Day 2, and Figure 5.27 for Day 3.

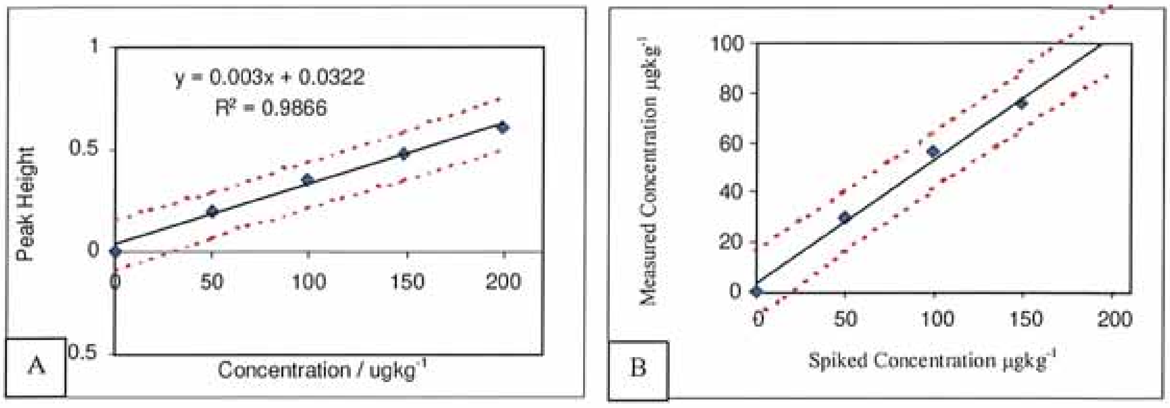


Figure 5.26: Calibration (A) and spiked against measured concentration (B) plots for TMP on Day 2 of analysis. The upper and lower 95% confidence limits are indicated by (----).

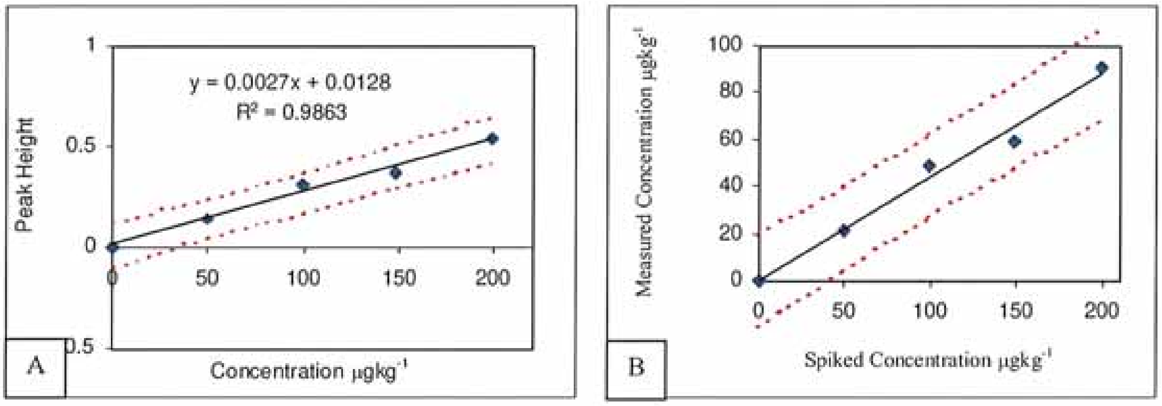


Figure 5.27: Calibration (A) and spiked against measured concentration (B) plots for TMP on Day 3 of analysis. The upper and lower 95% confidence limits are indicated by (----).

The R^2 values on the calibration plots were 0.9879, 0.9866 and 0.9863 on Day 1, 2 and 3 respectively. These values obtained indicate that while there is a scatter of the points about the line, it remains constant over the three days. The 95% confidence intervals about the MRL of 50μgkg^{-1} were examined using the spiked against measured concentration plots (Figure 5.21(B), 5.26(B) and 5.27(B)). A correction factor for percentage recoveries was applied to these values and the confidence intervals were found to be 51.6±9.5μgkg^{-1}, 65±12μgkg^{-1} and 51.6±18.5μgkg^{-1} for Day 1, 2 and 3 respectively. The confidence intervals increased over the three days of analysis, but it was also noted that the interval obtained on the second day (53-77μgkg^{-1}) did not contain the true value of 50μgkg^{-1}.

To examine if the sensitivity of the method varied at a 95% confidence level over time, the slopes of the linear regression line on the calibration plots were compared (Equation 1.2). Standards analysed on each of the three days of this test to check the performance of the instrument met the sensitivity criteria indicating that the instrument response was acceptable on each day. Between Day 1 and Day 2 t_{calc} was found to 1.746 while t_{calc} was found to be 0.904 between Day 1 and Day 3 for the spiked tissue samples. Using $((n_1-2) + (n_2-2))$ degrees of freedom the critical value of t was found to be 2.45 [20]. Since $t_{calc} < t_{table}$ for both comparisons then it was concluded that the slopes did not differ significantly at a 5% level of confidence.

Therefore based on these studies it was difficult to recommend a time period within which to analyse the prepared tissue samples for TMP after the clean up procedure is complete. However more efforts should be made to improve the accuracy of the TMP analysis in salmon tissue and also to use the EDM as the internal standard in an attempt to improve the precision. Thus this stability test should be repeated for TMP when acceptable accuracy values are obtained.

5.10.8.3 SMX sample stability

For SMX, the calibration and spiked against measured concentration plots ±95% confidence intervals were examined (Figure 5.20(C) and 5.21(C) for Day 1, Figure 5.28 for Day 2, and Figure 5.29 for Day 3).

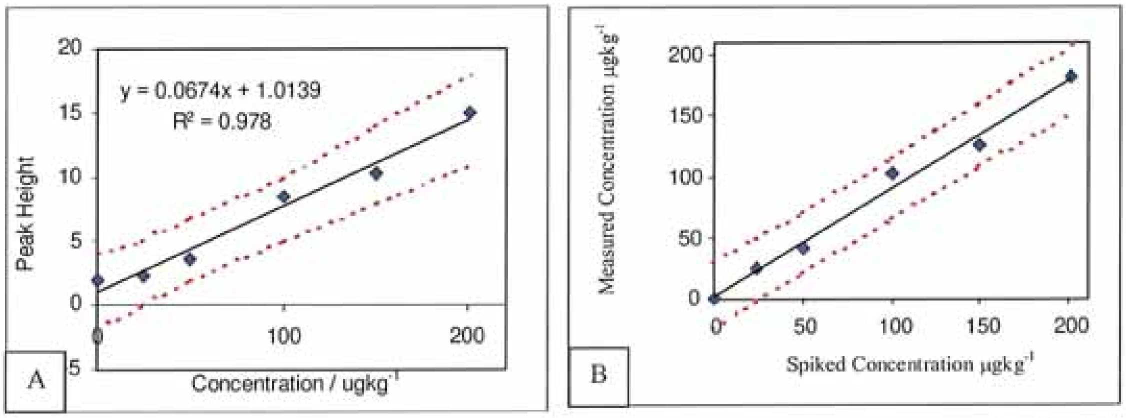


Figure 5.28: Calibration (A) and spiked against measured concentration (B) plots for SMX on Day 2 of analysis. The upper and lower 95% confidence limits are indicated by (----).

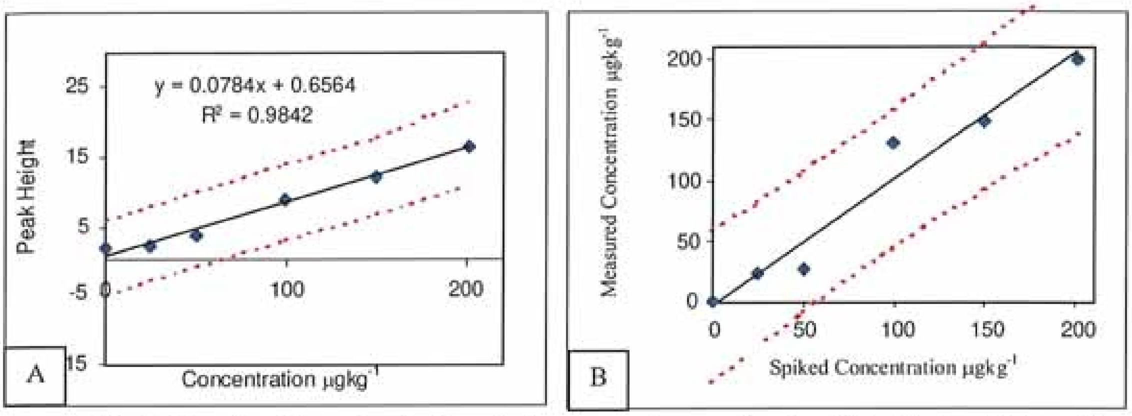


Figure 5.29: Calibration (A) and spiked against measured concentration (B) plots for SMX on Day 3 of analysis. The upper and lower 95% confidence limits are indicated by (----).

The R^2 values on the calibration plots were below 0.99 for SMX (0.9846, 0.978 and 0.9842 on Day 1, 2 and 3 respectively). It is hoped that when the EDM will be used as the internal standard in the future that this value will improve and there will be a better fit of the data points to the linear regression line.

The 95% confidence intervals about the MRL of $100\mu gkg^{-1}$ were examined using the spiked against measured concentration plots (Figure 5.21(C), 5.28(B) and 5.29(B)). A correction factor for percentage recoveries was applied to these values and the confidence intervals were found to be $92.7\pm18\mu gkg^{-1}$, $93\pm25\mu gkg^{-1}$ and $103\pm58\mu gkg^{-1}$ for Day 1, 2 and 3 respectively. While the confidence intervals increased over the three days of analysis, the response obtained does not appear to have altered significantly.

To examine if the sensitivity of the method varied at a 95% confidence level over time, the slopes of the linear regression line on the calibration plots were compared (Equation 1.2). Standards analysed on each of the three days of this test to check the performance of the instrument met the sensitivity criteria indicating that the instrument response was acceptable on each day. Between Day 1 and Day 2, t_{calc} was found to 0.607 while t_{calc} was found to be 0.618 between Day 1 and Day 3. Using $((n_1-2) + (n_2-2))$ degrees of freedom the critical value of t was found to be 2.31 [20]. Since $t_{calc} < t_{table}$ for both comparisons then it can be concluded that the slopes do not differ significantly at a 5% level of confidence. Thus the response obtained has not altered significantly over the three days of analysis.

Based on these studies it was recommended that the prepared tissue samples containing SMX should be analysed immediately after the sample preparation procedure is complete. If they are stored in the fridge at 4°C and analysed within 3 days, a wider confidence interval will be obtained on the result. The confidence intervals about 100µgkg^{-1} were found to be ±19.4%, ±26.8%, and ±56.3% on Day 1, 2 and 3 respectively. Therefore additional work is required on the accuracy and precision of the method in an attempt to reduce these intervals.

5.10.8.4 EDM sample stability

For EDM, the calibration and spiked against measured concentration plots ±95% confidence intervals (Figure 5.20(D) and 5.21(D) for Day 1, Figure 5.30 for Day 2, and Figure 5.31 for Day 3) were examined.

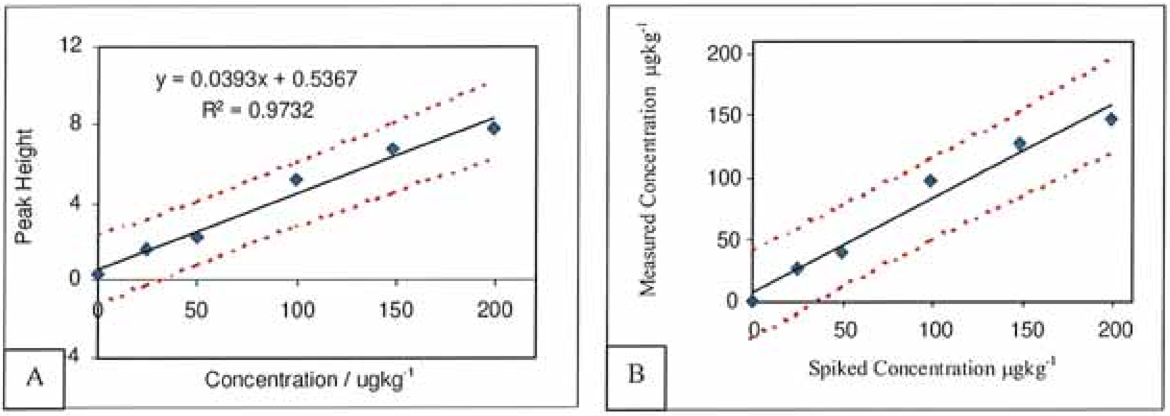


Figure 5.30: Calibration (A) and spiked against measured concentration (B) plots for EDM on Day 2 of analysis. The upper and lower 95% confidence limits are indicated by (----).

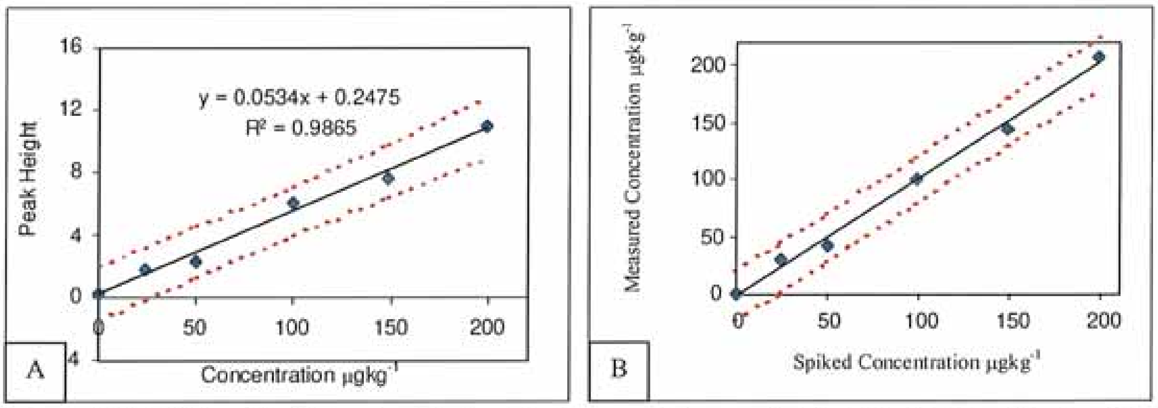


Figure 5.31: Calibration (A) and spiked against measured concentration (B) plots for EDM on Day 3 of analysis. The upper and lower 95% confidence limits are indicated by (----).

The R^2 values obtained for the calibration plots are 0.994, 0.9732 and 0.9865 on Day 1, 2 and 3 respectively. While the plots are essentially linear the values obtained indicate that there is a random scatter of the points about the line over time. To get a better idea if this scatter was significant, the 95% confidence intervals about 100µgkg^{-1} were examined using the spiked against measured concentration plots (Figure 5.21(D), 5.30(B) and

5.31(B)). A correction factor for percentage recoveries was applied to these values and the confidence intervals were found to be 85±17.5μgkg^{-1}, 83±33μgkg^{-1} and 103±20μgkg^{-1} for Day 1, 2 and 3 respectively. The confidence intervals obtained, like the R^2 values showed no trend over the time period studied. However, the response obtained on the third day does appear to be significantly higher than that obtained on either Day1 or Day 2.

To verify this, the sensitivity of the method was examined by comparing the slopes of the linear regression line on the calibration plots (Equation 1.2). Standards analysed on each of the three days of this test to check the performance of the instrument met the sensitivity criteria indicating that the instrument response was acceptable on each day. Between Day 1 and Day 2, t_{calc} was found to 1.38 while t_{calc} was found to be 2.52 between Day 1 and Day 3. Using $((n_1-2) + (n_2-2))$ degrees of freedom the critical value of t [20], was found to be 2.31. Since $t_{calc} < t_{table}$ between Day 1 and Day 2 the slopes do not differ significantly at a 5% level of confidence. However, $t_{calc} > t_{table}$ between Day 1 and Day 3 and thus there is significant difference between the slopes at a 5% confidence level, indicating that the response obtained has changed over the three days of analysis. Since this was not due to variations in the instrument response the spiked tissue samples were not stable on the third day of analysis.

Therefore based on these studies for EDM, it was recommended that the prepared tissue samples should be analysed immediately after the sample preparation procedure is completed. If they are stored in the fridge at 4°C and analysed within three days then a wider confidence interval will be obtained on the calibration plot.

5.11 Suitability of EDM as an internal standard

EDM was found to elute in the same chromatographic run as SD, TMP and SMX while having adequate resolution from all of the analytes (Figure 5.15). Similar results were reported for SD, SMX and EDM for injection repeatability (Section 5.10.2) linear range and correlation coefficient for standard solution (Section 5.10.3). The accuracy for EDM was similar to that obtained for SD and SMX over 25-200μgkg^{-1} while the %RSD on this data was 3.3%, 7.6% and 10.9% for SD, SMX and EDM respectively (Section 5.10.6). The precision of the repeated analysis of tissue samples spiked to 100μgkg^{-1} was similar for all three analytes (Section 5.10.5) while the linearity of EDM over 25-200% of the 100μgkg^{-1} MRL was adequate for the required purposes (Section 5.10.6). Finally, from the stability of the prepared tissue samples test, it was concluded that all the analytes must be analysed immediately after the sample preparation procedure is complete. This is due to the fact that all the analytes will be in the same solution prior to analysis, and SD and EDM (Section 5.10.8.1, 5.10.8.4), must be analysed immediately after the sample preparation procedure is complete. Therefore from the above study it is apparent that EDM behaves similarly to both SD and SMX and therefore can be used as the method internal standard in the future.

5.12 Conclusion

The aim of this work was to develop a routine method for the detection and quantification of SD, SMX, and TMP in farmed salmon tissue under Directive 96/23/EC. As part of this Directive it is necessary that the method be validated according to the criteria set out in Commission Decision 93/256/EEC. In the cited literature (Section 1.5.13) it was found that only one method examined the simultaneous determination of SD and TMP in salmon tissue, [2], while the simultaneous determination of all three analytes was not cited in any of the literature methods for salmon tissue samples.

LC-MS was examined as a possible method for the simultaneous determination of the three analytes of interest. The results of this study showed that while it was possible to analyse for all three analytes within the same chromatographic run under the analytical conditions examined (Section 5.5) the required sensitivity was not obtained for any of the three analytes. The LOD was found to be 330μgkg^{-1}, 250μgkg^{-1} and 100μgkg^{-1} for SD, SMX and TMP respectively. Since the MRL of SD and SMX is 100μgkg^{-1} combined, and TMP has a MRL of 50μgkg^{-1}, then it is apparent that the LODs obtained are not satisfactory for the required purposes. The examination of MS-MS as opposed to MS has been reported to yield better sensitivity for SD and SMX in kidney matrix [15]. Since MS-MS was not available during this work then it was not possible to examine this technique for salmon tissue. In 1997, a LC-APCI-MS method was reported for the analysis of TMP in tissue samples [10]. This method quoted a LOD of 25μgkg^{-1} and recoveries greater than the required 70%. APCI-MS system was not however available for this work.

The method as described by Malisch *et al.* [3], for the analysis of sulphonamides in pork tissue was applied to salmon tissue in this work. Ideally a single method was required for the analysis of SD, TMP and SMX in salmon tissue and therefore TMP was included in the method. The analytical conditions were modified and adequate resolution was obtained within the same chromatographic run for all three analytes as shown in Figure

5.15. The specificity study showed that there was no interference in the chromatogram at the retention time of SD, TMP, SMX or EDM from the other components listed in Directive 96/23/EEC for routine monitoring. Therefore the analyte retention times can be used as the initial identification of each analyte and the DAD detector was used to confirm the identification by obtaining the analyte spectra as required in Commission Decision 93/256/EEC.

The accuracy of the method obtained at the MRL of 100μgkg^{-1} (n=6), was 92.8±9.7%, 41.1±8.4% and 89.1±8.4% for SD, TMP and SMX respectively. The recoveries obtained for SD and SMX correlate with those reported for pork tissue by Malisch *et al.* [3] i.e. 100% (n=64) for SD and 100.7% (n=9) for SMX. The precision obtained on those results for salmon tissue was an improvement on the same values reported for the pork samples, i.e. 13% RSD for SD and 12.2% RSD for SMX. It should be noted however, that the results reported for the pork tissue by Malisch *et al.* [3] were part of an inter-laboratory study and thus any data quoted was obtained from 8 different laboratories while the data reported for this work was obtained in the same laboratory from one analyst. The %RSD obtained for SD, SMX and TMP do not exceed 15% thus meeting the requirements of the EU [8].

The Codex guidelines, [11], specify that recoveries between 70-110% must be obtained for tissue samples spiked with 100μgkg^{-1}. Recoveries obtained for SD and SMX in this study meet these required criteria. For TMP however, the recoveries obtained do not meet the required criteria for accuracy studies and this was discussed in Section 5.6.4. The EU guidelines [8] only quote recovery criteria for CRMs and since these do not exist for SD, SMX or TMP in salmon tissue the Codex guidelines are adhered to.

The LOQ (S/N=5) obtained for this method was 2.6μgkg^{-1}, 8.2μgkg^{-1} and 7.3μgkg^{-1} for SD, SMX and TMP respectively. These values for SD and SMX were lower than any reported in the cited literature for salmon or trout tissue, i.e. 100μgkg^{-1} for salmon [2, 6], 125μgkg^{-1} for salmon and 50μgkg^{-1} for trout [21]. However a LOQ of 5μgkg^{-1} was also reported for salmon tissue [9] while using post-column derivatisation and fluorescence detection. Therefore if lower LOQs are required in the future then the fluorescence and DAD could possibly be put in series for the quantification and confirmation of the analytes respectively. Commission Decision 93/256/EEC also provides validation criteria on inter-laboratory comparisons of the analytical method. While this work was beyond the scope of this project it will be part of work to be carried out in the future.

The results reported in this chapter were obtained without the use of an internal standard. The method reported by Malisch *et al.* [3] for pork samples was a multi-residue method including the use of EDM as an internal standard. Since this analyte is not used in the treatment of farmed fish it was examined as a possible internal standard. During the validation study EDM was treated the same as the three analytes of interest and was found to give similar results as those obtained for SD and SMX, as shown in Section 5.10. Therefore the next step to be carried out in this method is to use the EDM as the internal standard and to see if this will improve the precision of the results obtained.

From the results reported in this chapter on the work carried out to date, the method reported by Malisch *et al.* [3] for pork tissue was successfully applied to salmon tissue for both SD and SMX. Recoveries for TMP however, were below the required 70%, but even so it may be possible to use this method to screen for TMP while at the same time quantifying SD and SMX in the tissue sample.

If the accuracy of the method described for TMP analysis cannot be improved then consideration will be given to a method reported by Brandsteterova *et al.* [22] in 1997. This is the first successful SPE-HPLC method for the analysis of TMP in tissue samples reported in the literature to date. It involves extraction into an aqueous buffer followed by SPE for sample cleanup and pre-concentration. The recoveries reported were 86±5% at $50\mu gkg^{-1}$ while the LOD was $15\mu gkg^{-1}$ [22].

5.13 References

[1] Commission Regulation (EC) No. 508/1999 of 4 March 1999 amending Annexes I to IV of Council Regulation (EEC) No 2377/90 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin.

[2] Gentleman M, Burt H. (1993). High-performance liquid chromatographic determination of sulphadiazine and trimethoprim in Chinook salmon muscle tissue. Journal of Chromatography, **633**, 105-110.

[3] Malisch R, Bourgeois B, and Lippold R. (1992). Multiresidue analysis of selected chemotherapeutics and antiparasitics. Part 1: Determination of sulphonamides and their N4-Acetyl-metabolites, chloramphenicol and nicarbazin in meat. Deutsche Lebensmittel-Rundschau, **88**, Jahrg, Heft 7, 205-216.

[4] Le Boulaire S, Bauduret J-C, Andre F.(1997). Veterinary drug residues survey in meat: An HPLC method with a matrix solid phase dispersion extraction. Journal of Agricultural and Food Chemistry, **45**, 2134-2142.

[5] Cooper A.D., Creaser C.S., Farrington W.H.H., Tarbin J.A., Shearer G. (1995). Development of multi-residue methodology for the HPLC determination of veterinary drugs in animal tissues. Food Additives and Contaminants, **12,** 167-176.

[6] Reimer G, Suarez A. (1992). Liquid chromatographic confirmatory method for five sulphonamides in salmon muscle tissue by matrix solid phase dispersion. Journal of AOAC International **75**, No. 6, 979-981.

[7] Residue testing plan for Ireland for 1998 drawn up in accordance with Council Directive 96/23/EC on measures to monitor certain substances and residues thereof in animals and animal products. Depatrment of Agriculture and Food, Dublin 2, March 1998.

[8] Annex of Commission Decision 93/256/EEC laying down the methods to be used for detecting residues of substances having a hormonal or thyrostatic action. Council Directive 93/256/EEC.

[9] Gehring TA, Rushing LG, Thompson HC jr. (1997). Determination of sulfonamides in edible salmon tissue by liquid chromatography with postcolumn derivatization and fluorescence detection. Journal of AOAC International. **80**, No.4, 751-755.

[10] Cannavan A, Hewitt SA, Floyd SD, Kennedy DG. (1997). Determination of trimethoprim in tissues using liquid chromatography-thermospray mass spectrometry. Analyst **122** (11), 1379-1381.

[11] Codex Alimentarius Volume 3, (1994). Section 2: Recommended international code of practice for control of the use of veterinary drugs.

[12] Kirkland KM., McCombs DA, Kirkland JJ. (1994). Rapid, high-resolution high-performance liquid chromatographic analysis of antibiotics. Journal of Chromatography A, **660**, 327-337.

[13] Bidlingmeyer BA. (1992). Practical HPLC methodology and applications. Wiley Interscience Publication. ISBN –471-57246-2.

[14] VG Trio-2000 Users guide. Fisons instruments VG Biotech.

[15] Porter S. (1994). Confirmation of sulphonamide residues in kidney tissue by Liquid Chromatography-Mass Spectrometry. Analyst, **119,** 2753-2756.

[16] Niessen W.M.A. (1998). Analysis of antibiotics by liquid chromatography-mass spectrometry, A Review. Journal of Chromatography, A, **812**, 53-75.

[17] Gehring T.A., Rushing L.G., Churchwell M. I., Doerge D.R., McErlane K.M., Thomson H.C. (1996). HPLC determination of Sulphadiazine in Coho Salmon (*Oncorhynchus kisutch*) with confirmation by liquid chromatography with atmospheric pressure chemical ionisation mass spectrometry. Journal of Agriculture and Food Chemistry, **44**, 3164-3170.

[18] Council Directive 96/23/EC on measures to monitor certain substances and residues thereof in live animals and animal products.

[19] Green JM. (1996). A practical guide to analytical method validation. Analytical Chemistry News and Features, 305-309.

[20] Miller JC, Miller JN. (1993). Statistics for analytical Chemistry. 3rd edition. Ellis Horwood PTR Prentice Hall Limited, ISBN 0-13-030990-7.

[21] Ikai Y, Oka H, Kawamura N, Hayakawa J, Yamada M. (1991). Application of an amino cartridge to the determination of residual sulphonamide antibacterials in meat, fish and egg. Journal of Chromatography, **541**, 393-400.

[22] Brandsteterova E, Kubalec P, Machackova L.(1997). HPLC determination of trimethoprim in meat and milk with an SPE preseparation procedure. Zeitschrift fuer Lebensmittel Untersuchung und Forschung, A. Food Research and Technology, **204**(5), 341-344.

CHAPTER 6: INITIAL INVESTIGATIONS INTO THE USE OF SIZE EXCLUSION CHROMATOGRAPHY FOR THE ANALYSIS OF VETERINARY DRUG RESIDUES IN SALMON TISSUE.

6.1 Introduction

Size exclusion chromatography (SEC) is a LC mode where molecules are separated according to their molecular size. Small molecules can penetrate the pores of the gel packing while the large molecules do not or only partially penetrate the pores and are therefore separated from the smaller molecules and elute from the column first (Section 2.7).

The methods discussed so far in this work have been validated using the EU guidelines of Commission Decision 93/256/EEC (Chapters 3, 4, and 5). These methods, which can identify and quantify the specified analytes to the required validation criteria involve many cleanup steps and are thus time consuming [1]. For OA (Chapter 3) and sulphonamides (Chapter 5) six samples can be analysed in two days while for OTC (Chapter 4) twelve samples can be analysed over a two-day period. While microbiological methods are used for screening tissue samples for veterinary drug residues, these methods can often only detect a group of compounds rather than individual analytes. Thus there is a definite need for a quick screening method which can identify veterinary drug residues at the concentration levels of interest.

Thin layer chromatography has been reported as a screening method for sulphonamides and tetracyclines in tissue samples. The cleanup procedure used for this method however, was the same procedure that was used in the final confirmation method, which consisted of HPLC with DAD detection [2]. Thus the time required to prepare the tissue samples was the same for both the screening and the confirmation methods and therefore there was no time advantage to using this screening method.

In an attempt to obtain a quick screening method, which could analyse a large number of tissue samples in a relatively short time period, SEC coupled with a UV detector was examined as a separation technique for various veterinary drug residues in salmon tissue including ivermectin, OTC, cypermethrin, TMP, OA, SMX and SD.

In the past GPC has been used as a clean up technique for OCPs, and PCBs in fat [3], and lipophilic organic pollutants, musk xylene and musk ketone metabolites, pesticides and triazine herbicides in the marine environment. [4, 5, 6, 7, 8]. However, published work using GPC as a clean-up technique for veterinary drug residues in fish tissue is limited [9, 10].

Biancotto *et al.* [11] reported a SEC method using Bio-beads SX-3 resin with a tetrahydrofuran mobile phase and DAD detection, for the analysis of sulphonamides, nitrofurans and growth promoters in animal feed. The linear range of the method however, was reported to be 1000-100,000μgkg^{-1} while the LOD was 1000μgkg^{-1}. These values are clearly above the MRLs for any of the compounds of interest for this work (Table 1.2).

Holtmannspotter *et al.* [9] reported a SEC method for the determination of sulphonamides and chloramphenicol from tissue samples, but did not show any SEC chromatograms. Therefore it was difficult to determine if the resolution between the analytes and the matrix components was sufficient for the required purposes. However, the same SEC conditions were used in 1987 by Petz *et al.* [10] for the analysis of OTC, OA, SD and SMX in tissue samples. Petz *et al.* [10] compared Sephadez LH-20, bio beads s-x, and Fractogel PGM 3000 GPC columns for the sample preparation of veterinary drug residues in meat. The Sephadex LH-20 system which was used with MeOH: 0.01M Acetic acid as the mobile phase was reported as the most efficient system. When the GPC chromatograms were examined, however, it was found that the compounds which were of interest to this work (SD, SMX, and OTC) all eluted at the same time as the tissue matrix. Since the aim of this work was to examine SEC as a relatively quick screening method for veterinary drug residues while using UV detection, the analytical conditions described by Petz *et al.* [10] were not suitable.

This chapter describes the examination of SEC with UV detection as a screening method for the analysis of SD, SMX, TMP, OA, OTC ivermectin and cypermethrin in salmon tissue. Tissue samples were extracted and injected onto the SEC column, which was coupled with a UV detector. This monitored the separation which took place, and gave some indication as to the resolution obtained and how suitable this was for the identification of the analytes in the tissue matrix.

Using this separation technique different SEC gel-packings were examined and the mobile phase conditions optimised to separate veterinary drug residues from each other and from salmon tissue matrix for identification purposes. This technique was also examined as a sample cleanup method as the UV detector was not always sensitive enough to detect the analytes at the required concentration levels. Therefore the fraction of eluent containing the analyte eluting from the column was identified and collected for analysis by HPLC using the confirmation methods, which have already been developed and validated.

6.2 Apparatus and materials

Column: PLgel 5μm, 50Å, (300 x 7.5) mm.
Detector: L-400 UV Merck Hitachi.
Pump: Merck Hitachi L-6200 Intelligent Pump.
Software: Anleitung zum HP-GPC-Program.
Injection volume: 200μl in the mobile phase.
Flow rate: 1.5ml/minute.
Pressure: 51 bar for ethyl acetate: cyclohexane (50: 50), 40 bar for dichloromethane, and 47 bar for toluene.

6.2.1 SEC using a PLgel column

PLgel is a highly cross-linked spherical polystyrene/divinylbenzene matrix. The PLgel column used was (300 x 7.5) mm, MR range 1000-10000amu, and consisted of 5μm, 50Å particles. Various mobile phases were examined for the separation of the seven analytes on the PLgel GPC column. For this purpose SD, SMX, TMP, OA were dissolved in acetonitrile (ACN), OTC and ivermectin were dissolved in methanol (MeOH) while cypermethrin was dissolved in ethyl acetate. Stock standard solutions were then diluted in the GPC mobile phase prior to injection. The UV detector set at 254nm and the elution time of the standard and solvent peaks were recorded. The relative retention times of the analyte peaks to the solvent peaks were calculated for three different mobile phases and recorded in Table 6.1.

Table 6.1: Relative retention times for the analytes compared to the solvent peaks when testing different mobile phases on a PLgel (300 x 7.6) mm 5μm, 50 Å GPC column.

Analyte/Mobile phase	**Ethyl acetate: cyclohexane (50 : 50)**	**Dichloromethane**	**Toluene**
Sulphadiazine	1.06	0.87	0.8 – 1.2
Sulphamethoxazole	1.02	0.96	0.8 – 1.2
Trimethoprim	1.02	0.97	0.8 – 1.2
Oxolinic Acid	1.35	0.84	0.8 – 1.2
Oxytetracycline	-	1.10	0.8 – 1.2
Ivermectin	-	0.89	0.8 – 1.2
Cypermethrin	0.8 – 1.2	0.8 – 1.2	0.8 – 1.2

As toluene has a high UV absorbency at 254nm there was a high background absorbance at this wavelength and thus the analytes could not be detected by UV. Therefore various fractions of the eluent coming from the GPC column were collected blindly and analysed by RPHPLC. From this it was determined which fraction contained the analyte and the relative retention times were calculated and recorded. The data in Table 6.1 shows that the relative retention times of the analyte to solvent peaks are very low for each of the mobile phases tested and thus no resolution occurred between solvent and analyte peaks. When using ethyl acetate: cyclohexane (50:50), it can be seen that the analyte peaks eluted after the solvent peak. Also it was noted that while using this mobile phase no analyte peaks were observed for OTC or ivermectin. When using dichloromethane as the mobile phase the analyte peaks eluted before the solvent peak, but again it is apparent the resolution is not satisfactory for the purpose required. As a peak for cypermethrin was not observed while using a UV detector at 254nm under the conditions described above it was necessary to use the GC-MS system to obtain a chromatogram for cypermethrin.

6.3 GCMS of Cypermethrin

6.3.1 Apparatus

Varian GC, model 3400 with a 1075 split/splitless capillary injector.
Magnum Finnigan Matt Mass Spectrometer.
Column: J&W DB1 fused silica capillary column (250 x 0.25) mm.
Auto sampler: CTC Analytics Liquid Sampler A200SE.
Carrier gas: Helium.
Temperature program:
80ºC for 2 minutes, 260ºC: 15ºC/minute, 280ºC: 3ºC/minute.
Hold at 280°C for 15 minutes.

6.3.2 Results

Cypermethrin fractions were collected from the GPC and analysed on the GCMS system. EI-MS was performed with a source temperature of 180ºC and electron energy of 70eV. A full scan from m/z of 50 to m/z of 450 was carried out on the analyte and it was found that the ions associated with cypermethrin had m/z of 127, 163, and 181, Figure 6.1(B). The chromatogram shows that poor resolution was obtained from the four isomers in the cypermethrin standard (Figure 6.1(A)). Poorly resolved peaks were obtained for only three of the isomers and the fourth isomer was unresolved. The results of this test showed that the change in mobile phase from ethyl acetate: cyclohexane (50:50) to dichloromethane and toluene did not improve the resolution between the solvent and analyte peaks. This was confirmed when the relative retention times of the analyte to solvent peaks for all three mobile phases were found to range from 0.8 to 1.2.

Subsequent investigations on GCECD showed that resolution was obtained between the four isomers of cypermethrin using a J+W 0.5μm DB 5Ms (30m x 0.25mm) column but this work will be reported elsewhere.

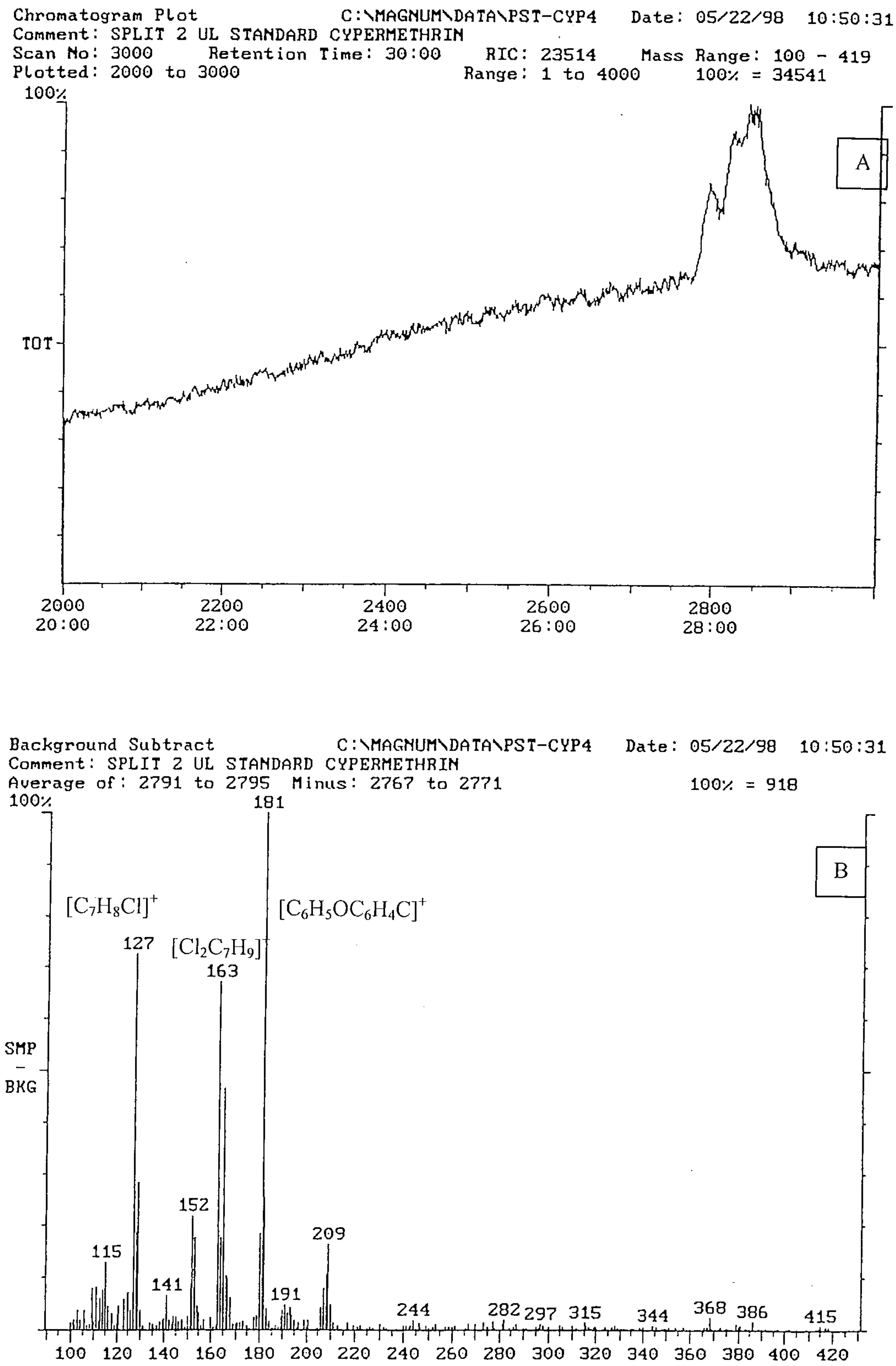


Figure 6.1: GCMS chromatogram, (A) and corresponding ion spectrum, (B) of a standard cypermethrin solution. Analytical conditions: Section 6.3.1.

6.4 SEC Column Choice

The GPC separation of veterinary drug residues in their standard form has been examined and reported while using a Sphadex LH-20 GPC column [13]. This paper reported the GPC separation of various veterinary drug residues including SD, SMX and OTC to be unsatisfactory for identification and sample clean-up purposes. The column used, Sphadex LH-20 (39.5 x 2.7) cm, had a molecular weight cut off of 5000, while the mobile phase was 0.01M methanolic acetic acid at 5.0ml/minute.

Since the Sphadex LH-20 material did not give acceptable results, a Fractogel TSK HW-40(S) supplied by Merck was used to pack a column for SEC purposes in this work. The material which is a copolymer of oligoethylene glycol, has a molecular weight range of 100-1000amu and particle size of 40μm. This was washed with MeOH followed by sonication in MeOH for 15 minutes prior to the column packing procedure. A column (150 x 4.6) mm was packed with the Fractogel material and was then conditioned with MeOH prior to use to determine its stability. After injecting MeOH until consistency in the chromatograms and a stable pressure of 60 bar was obtained the column was deemed satisfactory for use.

6.4.1 Apparatus

Pump: Gynkotek Model 480.
Degaser: Degasys DG-1310 uniflows.
Injector: Gynkotek Gina 50.
Oven: Gynketok at 24°C.
Detector: Gynkotek UVD3405.
Software: Gynkotek HPLC.
Injection volume: 20μl.

Based on the method described by Petz *et al.* [10], the effect of acetic acid in the mobile phase on resolution was investigated. Various mobile phases consisting of MeOH with various concentrations of acetic acid ranging from 0% to 2% were prepared and used to elute the standard analytes from the Fractogel TSK SEC column. The relative retention and the resolution (Equation 6.1) for the standard analyte peaks were calculated for each of these mobile phases and were recorded in Table 6.2.

Table 6.2: Retention time, relative retention time, peak width and resolution of analytes to solvent peaks for standard solutions under different mobile phase conditions.

Mobile Phase	Sulphadiazine	Sulphamethoxazole	Trimethoprim	Oxolinic Acid	Oxytetracycline	Ivermectin	Cypermethrin
MeOH: Relative Retention time.	1.67	1.55	-	2.51	-	0.91	1.99
Retention Time / minutes	21.37	19.70	-	32.10	-	10.03	13.63
Peak Width /mls	2.90	2.00	-	5.00	-	1.60	4.60
Resolution	3.95	3.33	-	1.62	-	-	-
0.06% Acetic Acid / MeOH: Relative Retention time.	1.67	1.55	1.21	1.83	-	0.89	1.12
Retention Time / minutes	16.10	14.97	11.70	17.63	-	7.43	9.97
Peak Width /mls	1.80	1.80	1.20	4.40	-	1.20	2.20
Resolution	3.55	3.12	-	3.81	-	-	-
0.1% Acetic Acid / MeOH: Relative Retention time.	1.65	1.54	-	1.86	-	0.8	1.15
Retention Time / minutes	14.1	13.1	-	15.5	-	6.7	8.9
Peak Width /mls	2.13	2.91	-	4.9	-	1.14	1.89
Resolution	3.80	2.93	-	3.45	-	2.90	0.86

Table 6.2 cont'd: Retention time, relative retention time, peak width and resolution of analytes to solvent peaks for standard solutions under different mobile phase conditions.

Mobile Phase	**Sulphadiazine**	**Sulphamethoxazole**	**Trimethoprim**	**Oxolinic Acid**	**Oxytetracycline**	**Ivermectin**	**Cypermethrin**
0.5% Acetic Acid / MeOH: Relative Retention time.	1.67	1.55	1.15	1.80	1.45	0.91	1.13
Retention Time / minutes	13.9	13.0	10.1	14.8	13.5	6.7	8.9
Peak Width /mls	2.34	3.36	2.49	5.67	1.65	0.81	2.34
Resolution	3.93	3.06	0.65	2.86	-	2.96	0.92
2% Acetic Acid / MeOH: Relative Retention time.	1.65	1.55	1.16	1.73	0.88	0.79	0.86
Retention Time / minutes	13.9	13.0	8.4	14.56	7.4	6.7	7.8
Peak Width /mls	2.43	3.12	3.18	6.0	7.2	0.78	0.96
Resolution	3.66	3.28	-	3.05	-	2.66	0.86

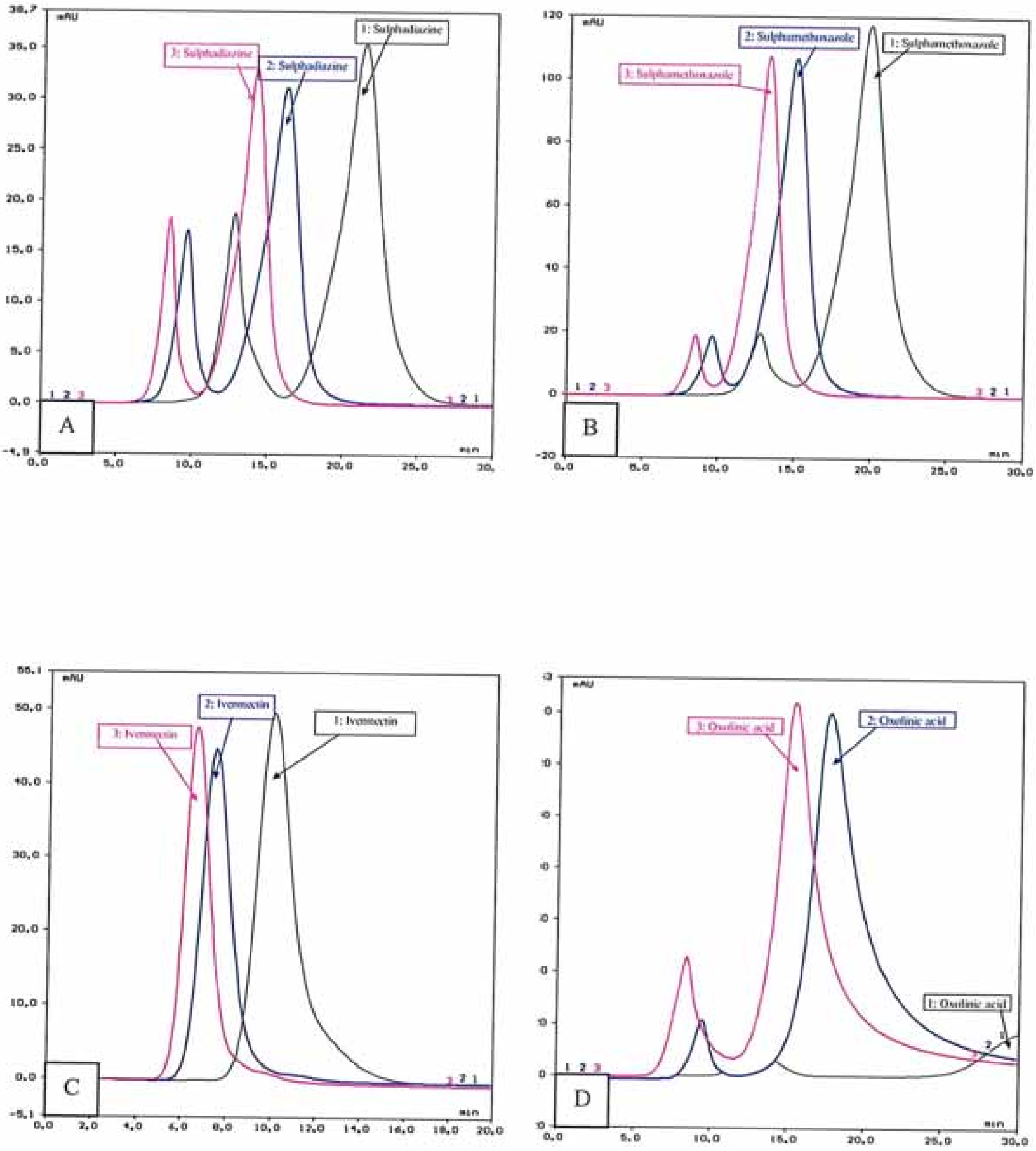


Figure 6.2: SEC chromatogram of Sulphadiazine (A), Sulphamethoxazole (B), Ivermectin (C) and Oxolinic acid (D) standards. Column: Fractogel TSK HW-40(S). Mobile phase: 1: 0.06% acetic acid in MeOH, 2: 0.5% acetic acid in MeOH, 3: 2% acetic acid in MeOH. Detection: UV @ 254nm.

$$R_S = \frac{\Delta R_T}{\frac{1}{2}(W_A + W_B)} \qquad \text{Equation 6.1}$$

R_S: Resolution

ΔR_T: Difference in retention time between peak A and peak B

W_A: Width of peak A

W_B: Width of peak B.

For the mobile phase consisting of 100% MeOH it was apparent that the resolution of the standard from the solvent peak was satisfactory for SD, SMX and OA. However this was not the case for TMP, OTC, ivermectin and cypermethrin. When the data from the mobile phases containing the acetic acid was examined it was noted that again the resolution of the analyte peak from the solvent peak for TMP, OTC and cypermethrin was not satisfactory (Table 6.2). Also it must be pointed out that the addition of different amounts of acetic acid to the mobile phase, ranging from 0.06% to 2%, did not have a significant effect on the resolution of the standard SD and SMX peaks from the solvent peaks (Figure 6.2(A) and 6.2(B)). For ivermectin, however the resolution was best when the mobile phase contained 2% acetic acid (Figure 6.2(C)) and for OA this occurred with a mobile phase containing 0.06% acetic acid (Figure 6.2(D)).

6.4.2 Initial examination of tissue samples

So far only the resolution of the standard analyte peaks from the solvent peak has been discussed. However, what is more important is to examine the resolution of the analyte peaks from the matrix peaks of the tissue samples. Therefore salmon tissue samples were extracted with different extraction solvents depending on the analyte of interest.

For OTC, SD, SMX, and TMP the tissue was extracted with an aqueous acetate buffer (pH of 6.0) and ACN. Sodium chloride was added to the extract to separate out the ACN from the aqueous layer and this volume was reduced by rotary evaporation to dryness and re-suspended in MeOH (3mls). This was diluted by approximately a factor of 10 before injection on to the SEC column. For OA the tissue was extracted with ACN containing 1.4% ammonia solution. This was reduced to dryness prior to re-suspension in MeOH. For ivermectin the tissue was extracted with MeOH and the volume was reduced to 3mls prior to injection. Finally tissue was extracted with a mixture of acetone: hexane for cypermethrin analysis. This volume was reduced to dryness and re-suspended in MeOH prior to injection. The blank matrices were spiked to 100μgkg^{-1} with the relevant analyte and were injected onto the SEC column. The resolution between the analyte peak and the matrix peaks is shown in Table 6.3 where it is evident that the trends observed are similar to those recorded in Table 6.2 for the standard analytes and the solvent peaks.

Table 6.3: Retention time, relative retention time, peak width and resolution of analytes to matrix peaks for spiked tissue samples under different mobile phase conditions

Mobile Phase	**Sulphadiazine**	**Sulphamethoxazole**	**Trimethoprim**	**Oxolinic Acid**	**Oxytetracycline**	**Ivermectin**	**Cypermethrin**
0.1% Acetic Acid / MeOH: Relative Retention time.	1.2	1.13	0.95	1.3	-	0.81	0.77
Retention Time / minutes	14.1	13.23	11.23	15.3	-	6.7	9.00
Peak Width /mls	2.94	3.42	3.96	4.95	-	2.01	1.90
Resolution	1.12	0.75	-	1.9	-	3.54	1.30
0.5% Acetic Acid / MeOH: Relative Retention time.	1.2	1.13	-	1.29	-	0.83	0.77
Retention Time / minutes	13.9	13.23	-	15.1	-	6.7	8.90
Peak Width /mls	2.34	3.42	-	4.98	-	0.6	2.34
Resolution	1.26	0.72		2.0	-	3.5	1.26
2% Acetic Acid / MeOH: Relative Retention time.	1.19	1.13	0.75	1.25		0.57	0.79
Retention Time / minutes	13.9	13.16	7.56	14.6		6.77	9.1
Peak Width /mls	0.6	4.23	1.29	4.8		0.72	2.1
Resolution	1.29	0.77	1.4	1.64		3.57	1.2

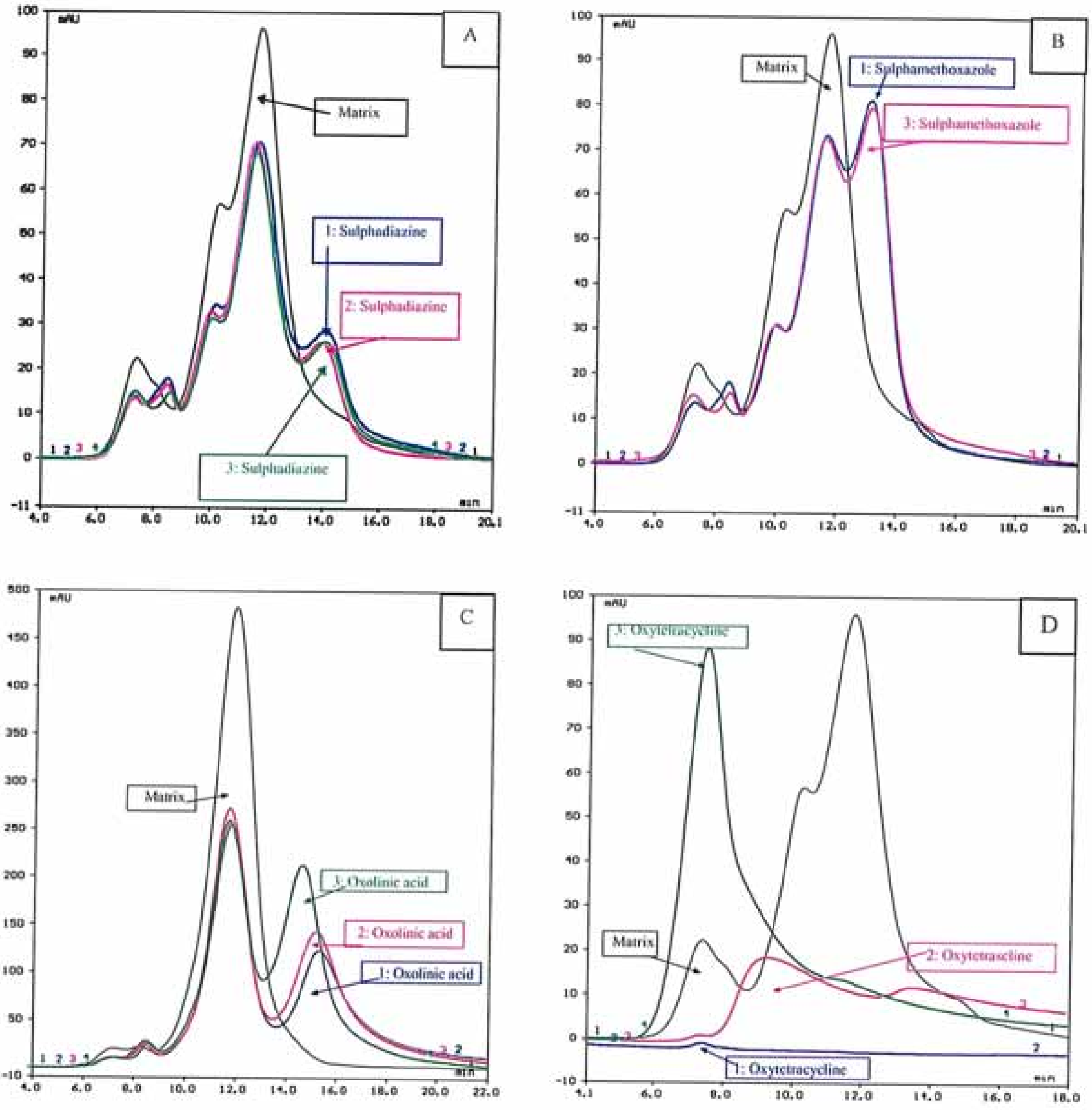


Figure: 6.3: SEC chromatogram of a tissue sample spiked with sulphadiazine (A), sulphamethoxazole (B), Oxolinic acid (C), Oxytetracycline (D). Column: Fractogel TSK HW-40(S). Mobile phase: 1: 0.1% acetic acid in MeOH, 2: 0.5% acetic acid in MeOH, 3: 2% acetic acid in MeOH. Detection: UV @ 254nm.

For SD and SMX the resolution is unaffected by the presence of the acetic acid in the mobile phase (Figure 6.3(A) and 6.3 (B)). Again for OA the resolution improved by the addition of acetic acid to the mobile phase (Figure 6.3C) while for ivermectin, TMP, OTC and cypermethrin optimum separation occurs with 2% acetic acid in the mobile phase (Figure 6.3(D) –(G) and Table 6.3).

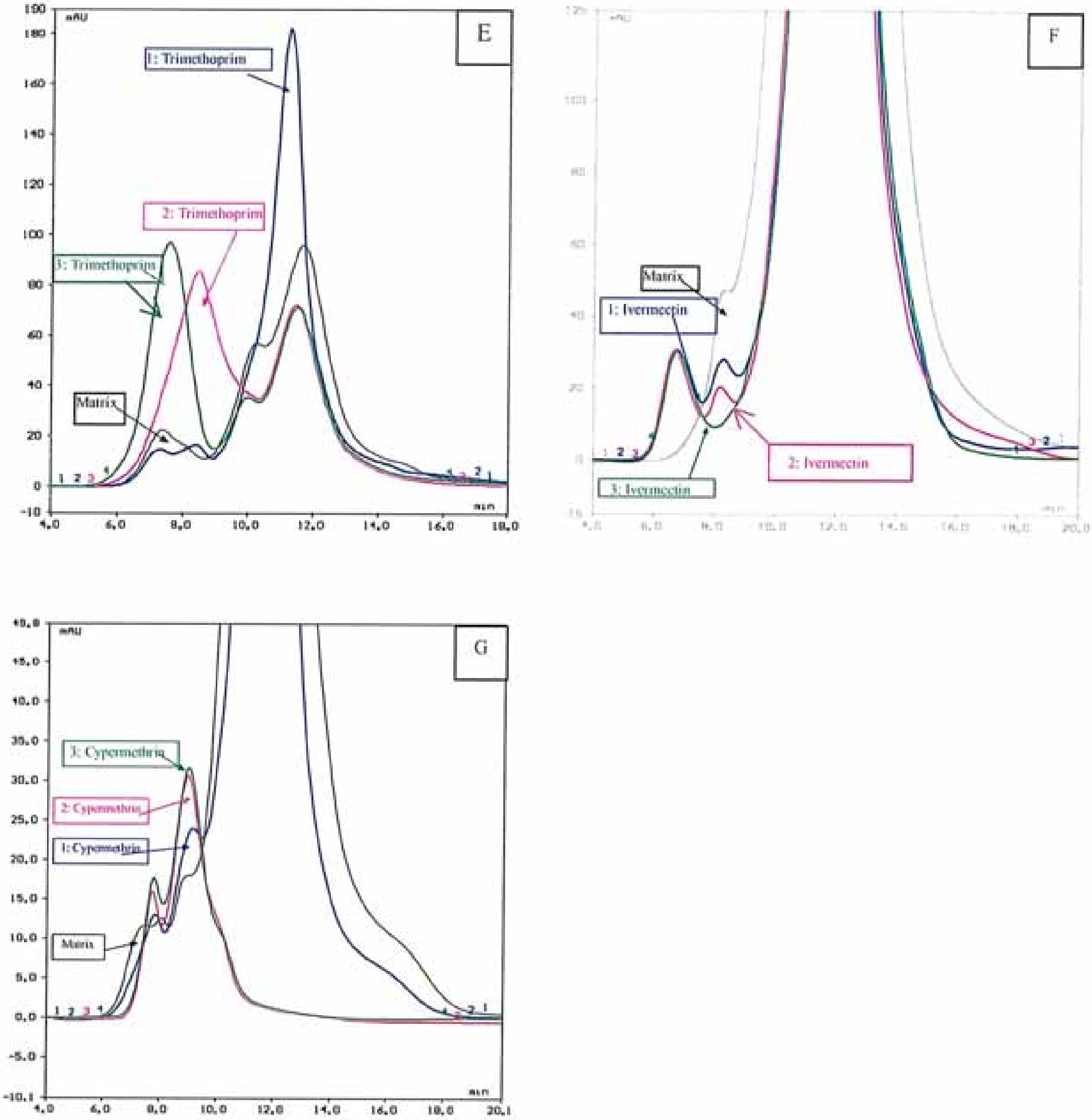


Figure: 6.3 cont'd: SEC chromatogram of a tissue sample spiked with trimethoprim (E), ivermectin (F), cypermethrin (G). Column: Fractogel TSK HW-40(S). Mobile phase: 1: 0.1% acetic acid in MeOH, 2: 0.5% acetic acid in MeOH, 3: 2% acetic acid in MeOH. Detection: UV @ 254nm.

6.4.3 Effect of water in the mobile phase

A tertiary gradient pump was used to mix three components of the mobile phase in different ratios and examine the effect of these ratios on the resolution. The acid levels in the mobile phase were kept constant at 0.1%, 0.5% and 2.0% while the percentage MeOH and water were varied. The retention time, relative retention data, peak width and resolution from the matrix peaks can be seen in Table 6.4.

Table 6.4: Retention time, relative retention time, peak width and resolution of analytes to matrix peaks for spiked tissue samples under different mobile phase conditions

MeOH : 2% methanolic Acetic Acid : H_2O	Sulphadiazine	Sulphamethoxazole	Trimethoprim	Oxolinic Acid	Oxytetracycline	Ivermectin	Cypermethrin
68: 30: 2. Relative Retention time.	1.24	1.19	0.87	1.26	0.80	0.58	0.81
Retention Time / minutes	20.40	19.60	14.40	21.30	13.20	9.90	13.90
Peak Width /mls	1.38	1.86	2.60	2.94	3.40	0.68	4.20
Resolution	1.53	1.24	0.72	2.00	1.26	3.79	1.57
65: 30: 5. Relative Retention time.	1.24	1.21	0.84	1.25	0.90	0.60	0.88
Retention Time / minutes	19.70	19.20	13.40	20.10	14.40	9.60	14.10
Peak Width /mls	1.14	1.88	2.20	2.92	3.26	0.70	1.94
Resolution	1.69	1.43	1.11	1.90	0.63	3.70	0,95
60: 30: 10. Relative Retention time.	1.26	1.27	0.89	1.30	1.05	0.64	1.02
Retention Time / minutes	25.20	25.50	17.80	25.90	20.90	12.70	20.40
Peak Width /mls	1.49	1.84	3.00	4.41	4.69	0.80	4.62
Resolution	1.76	1.86	0.82	2.18	0.25	3.13	0.17
8: 90: 2. Relative Retention time.	1.22	1.17	0.73	1.24	0.70	0.59	0.83
Retention Time / minutes	20.10	19.30	12.00	20.40	11.40	9.80	13.70
Peak Width /mls	0.84	1.56	3.20	2.88	4.40	0.70	3.80
Resolution	1.80	1.36	2.25	1.88	2.76	3.83	1.41
5: 90: 5. Relative Retention time.	1.23	1.20	0.76	1.25	0.78	0.61	0.90
Retention Time / minutes	19.30	18.90	12.00	19.60	12.30	9.50	14.20
Peak Width /mls	3.75	2.40	3.74	2.88	3.88	0.74	3.92
Resolution	1.67	1.45	2.18	1.90	1.66	3.50	0.69

Table 6.4 cont'd: Retention time, relative retention time, peak width and resolution of analytes to matrix peaks for spiked tissue samples under different mobile phase conditions

MeOH: 2% methanolic Acetic Acid: H_2O	**Sulphadiazine**	**Sulphamethoxazole**	**Trimethoprim**	**Oxolinic Acid**	**Oxytetracycline**	**Ivermectin**	**Cypermethrin**
0: 90: 10. Relative Retention time.	1.24	1.26	0.80	1.27	0.89	0.63	1.00
Retention Time / minutes	25.00	25.30	16.20	25.40	17.90	12.60	19.90
Peak Width /mls	1.50	1.74	1.95	4.35	4.95	0.97	4.23
Resolution	1.63	1.76	1.59	2.04	0.71	3.17	0.00
93: 5: 2. Relative Retention time.	1.23	1.28	0.84	1.27	0.95	0.57	0.81
Retention Time / minutes	20.40	19.60	13.90	21.50	15.80	9.80	13.90
Peak Width /mls	1.66	2.36	1.76	2.46	2.92	0.68	3.86
Resolution	1.76	1.39	1.63	2.09	0.46	4.02	1.58
90: 5: 5. Relative Retention time.	1.24	1.22	0.85	1.28	1.19	0.60	0.90
Retention Time / minutes	19.60	19.20	13.40	20.30	18.80	9.60	14.30
Peak Width /mls	1.14	1.70	2.22	2.58	3.00	0.66	3.56
Resolution	1.62	1.42	1.29	2.26	0.97	3.63	0.81
85: 5: 10. Relative Retention time.	1.27	1.27	1.33	1.33	1.50	0.63	1.04
Retention Time / minutes	25.23	25.4	26.60	26.5	29.9	12.7	20.7
Peak Width /mls	1.35	1.45	2.19	4.9	3.6	0.68	13.9
Resolution	1.94	1.96	2.27	2.51	1.45	3.33	0.29

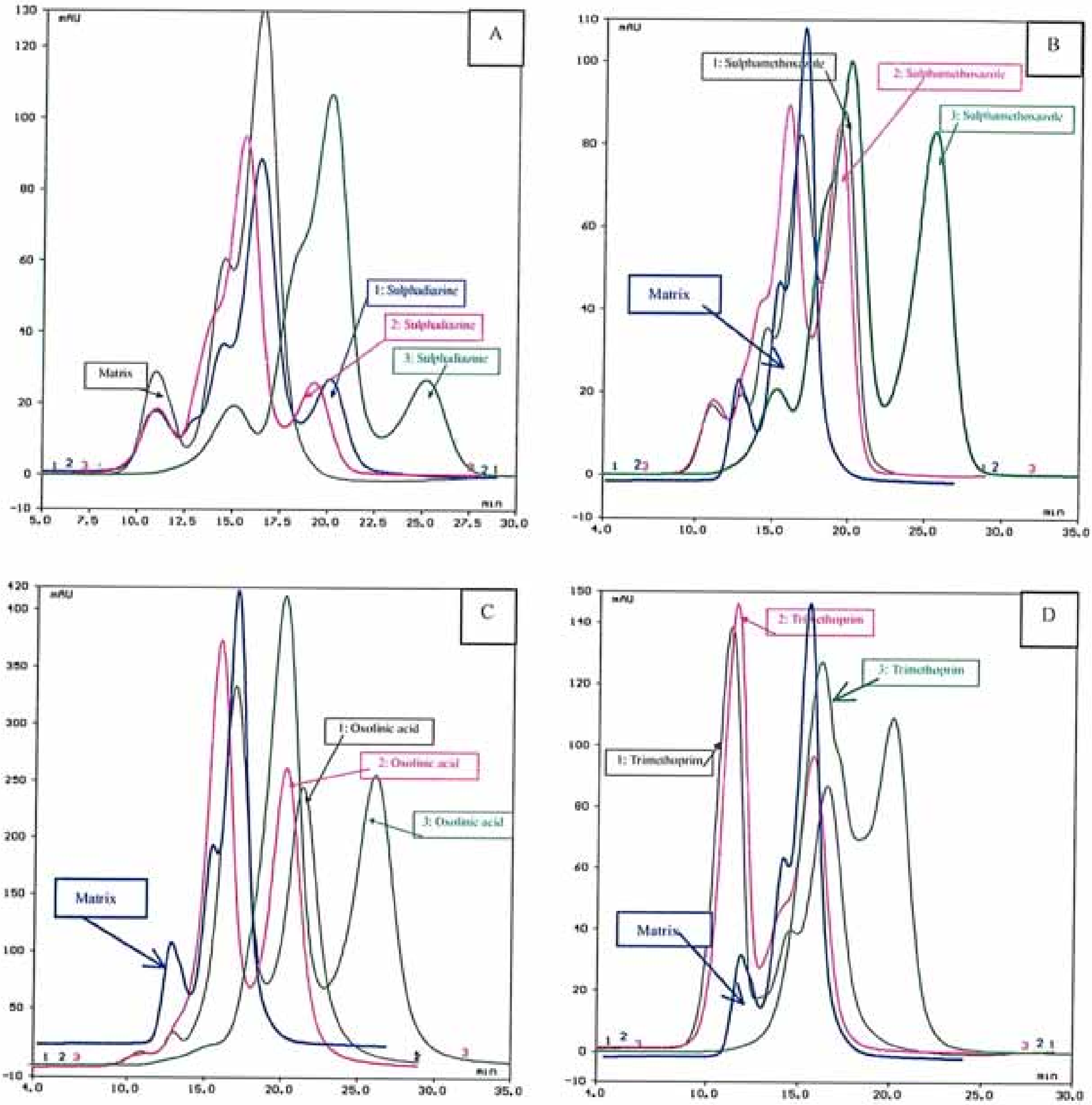


Figure: 6.4: SEC chromatogram of a tissue sample spiked with sulphadiazine (A), sulphamethoxazole (B), oxolinic acid (C), trimethoprim (D). Column: Fractogel TSK HW-40(S). Mobile phase: MeOH: 0.01M methanolic acetic acid: H_2O. 1: 93:5:2, 2: 90:5:5, 3: 85:5:10. Detection: UV @ 254nm.

The addition of water to the mobile phase had a significant improvement on the resolution of the analyte peaks from the matrix peaks in the SEC chromatograms. For SD, SMX and OA (Figure 6.4(A)-(C), Table 6.4) the best separation was obtained when the mobile phase contained 10% water, 85% MeOH and 5% (0.01M methanolic acetic acid). The separation here could possibly be improved if more water was added to the mobile phase. The resolution between TMP and OTC peaks from the matrix peaks improved when water was added to the mobile phase due to the increase in the sharpness of the analyte peaks. The optimum resolution for TMP and OTC was obtained when the mobile phase consisted of 8% MeOH, 90% (0.01M methanolic acetic acid) and 2% water (Figure 6.4(D)-(E)).

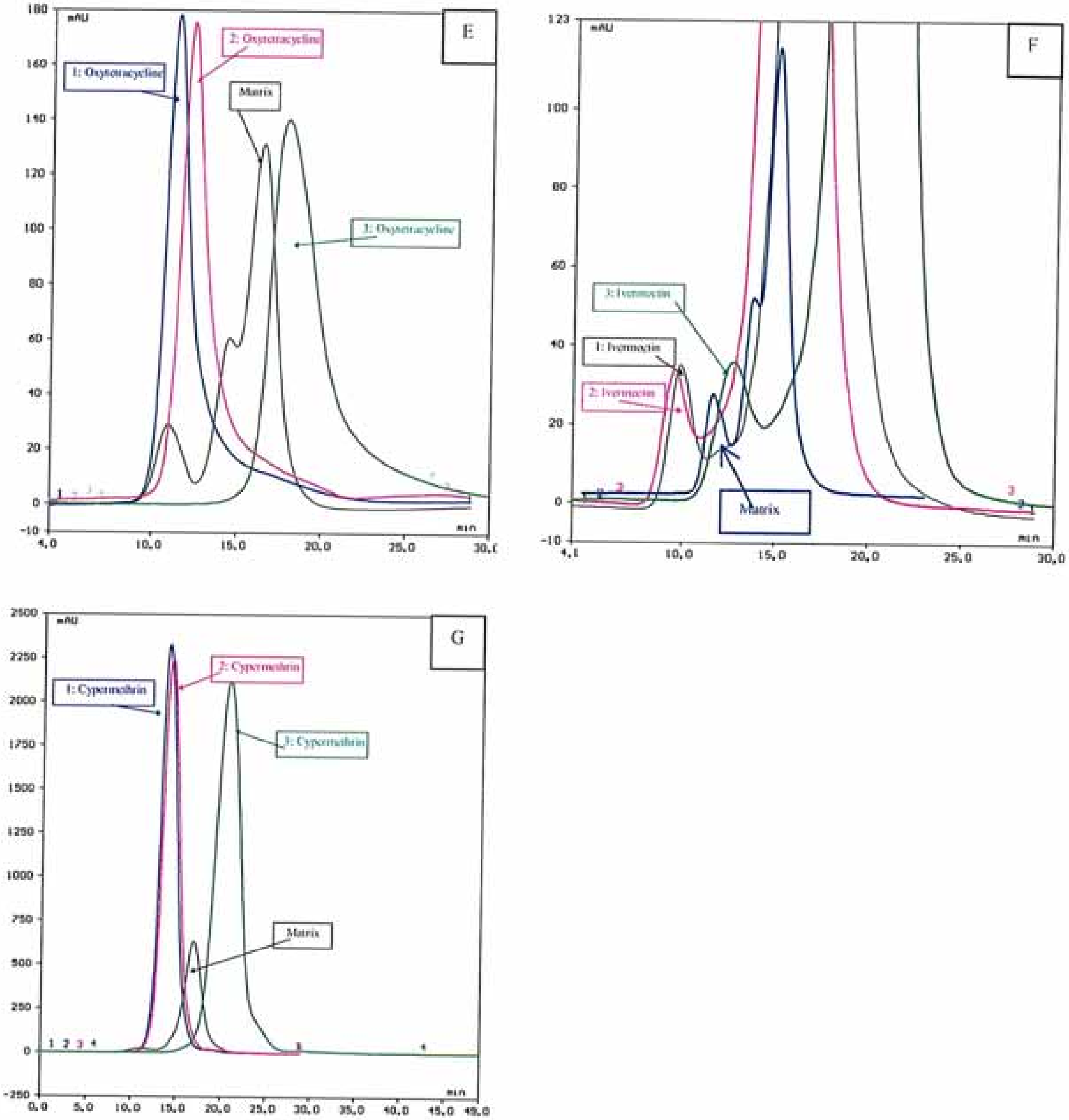


Figure: 6.4 cont'd: SEC chromatogram of a tissue sample spiked with oxytetracycline (E), ivermectin (F), and cypermethrin (G). Column: Fractogel TSK HW-40(S). Mobile phase: MeOH: 0.01M methanolic acetic acid: H_2O. 1: 93:5:2, 2: 90:5:5, 3: 85:5:10. Detection: UV @ 254nm

The asymmetry of the ivermectin and cypermethrin peaks also improved when water was added to the mobile phase as can be seen when Figures 6.4(F)-(G) are compared to Figures 6.3(F)-(G). Optimum resolution was obtained for both ivermectin and cypermethrin when the SEC mobile phase consisted of 93% MeOH, 5% 0.01M methanolic acetic acid and 2% water.

6.5 Scaling up to a larger sample size

The SEC work described in this chapter so far was carried out using a Fractogel TSK HW-40(S) column (150 x 4.6) mm. However, it was apparent that if the required detection limits were to be obtained larger sample sizes must be injected and thus a larger column was required. As the Fractogel TSK HW-40 (S) gel was no longer being manufactured an alternative was required. The best alternative available to Fractogel was Toyopearl HW-40(S) with a 30μm particle size, a 50Å pore size and a MR range of 100-10000amu. This resin consists of semi-rigid, spherical beads synthesised by a copolymerization of ethylene glycol and methacrylate type polymers. The Toyopearl resin has hydrophilic surfaces due to the presence of hydroxyl groups, which provide attachments points for other functional groups [14]. Therefore this material was used to pack two columns with dimensions of (250 x 4.6) mm and (300 x 10) mm and the resolution obtained on both columns using this new Toyopearl TSK HW 40(S) gel was compared.

6.5.1 Effect of a larger column

Column efficiency was estimated by calculating the number of theoretical plates (Section 2.1.2.3) when using 100% MeOH as the mobile phase. For the (250 x 4.6) mm column, N was found to be 521 while for the (300 x 10) mm column, N was found to be 3277.

A comparison between the resolution obtained on the smaller and larger columns containing the Toyopearl HW-40(S) gel was made and can be seen in Table 6.5. This table also compares the resolution obtained using the Fractogel TSK HW-40(S) and Toyopearl HW-40(S) gels.

Table 6.5: Resolution between analytes and matrix components on different size columns containing the Fractogel TSK and Toyopearl HW-40(S) gels. The mobile phase conditions were water: MeOH: 0.01M methanolic acetic acid in the ratio of 10: 85: 5 for SD, SMX and OA, 2: 8: 90 for TMP and OTC and 2: 93: 5 for ivermectin and cypermethrin. Detection: UV at 254 nm

Column	SD	SMX	OA	Ivermectin	Cypermethrin	TMP	OTC
Fractogel TSK (150 x 4.6) mm	1.9	2.0	2.5	4.0	1.6	2.3	2.8
Toyopearl HW-40 (250 x 4.6) mm	1.9	1.7	2.0	2.0	1.7	1.2	0.4
Toyopearl HW-40 (300 x 10) mm	5.1	4.6	5.1	3.9	3.3	2.0	1.6

The data in Table 6.5 show that similar resolution was obtained while using the Fractogel TSK and Toyopearl HW-40(S) columns for SD, SMX OA and cypermethrin. For ivermectin, OTC and TMP however better resolution was obtained on the Fractogel column when compared to the Toyopearl (250 x 4.6) mm column. The Toyopearl HW-40(S) gel was scaled up from a (250 x 4.6) mm to a (300 x 10) mm column. It can be clearly seen from the data in Table 6.5 that the resolution was improved considerably with the use of a larger column for SD, SMX, OA and cypermethrin. Similar resolution was recorded for both the Fractogel and the Toyopearl (300 x 10) mm columns, for ivermectin and TMP. For OTC however, resolution using the Fractogel column was better than that obtained on the Toyopearl (300 x 10) mm column.

6.5.2 Effect of volume injected on column efficiency

In order to determine what sample volume could be injected on the Toyopearl HW-40(S) (300 x 10) mm column different volumes of SD standards, ranging from (500-5000)μl were injected and the column efficiency was examined. The results can be seen in Table 6.6, where it is evident that the asymmetry of the peak changed from having a peak front to a more symmetrical shape at an injection volume of 5000μl. Also the column efficiency increased and reached a maximum at 1500μl but then decreased as the injection volume was further increased. This trend can also be seen in Figure 6.5, where the peak for 5000μl shows a shape consistent with sample overload. Based on the information obtained from Table 6.6 and Figure 6.5, it was evident that a good compromise between column efficiency and analyte detection limits would be obtained if a 2000μl sample was injected.

Table 6.6: Asymmetry values and column efficiency for different injection volumes of sulphadiazine standards.

Injection Volume (μl)	Asymmetry factor	Column efficiency (N)
500	0.55	256
1000	0.66	285
1500	0.69	386
2000	0.75	378
2500	0.78	336
5000	0.93	88

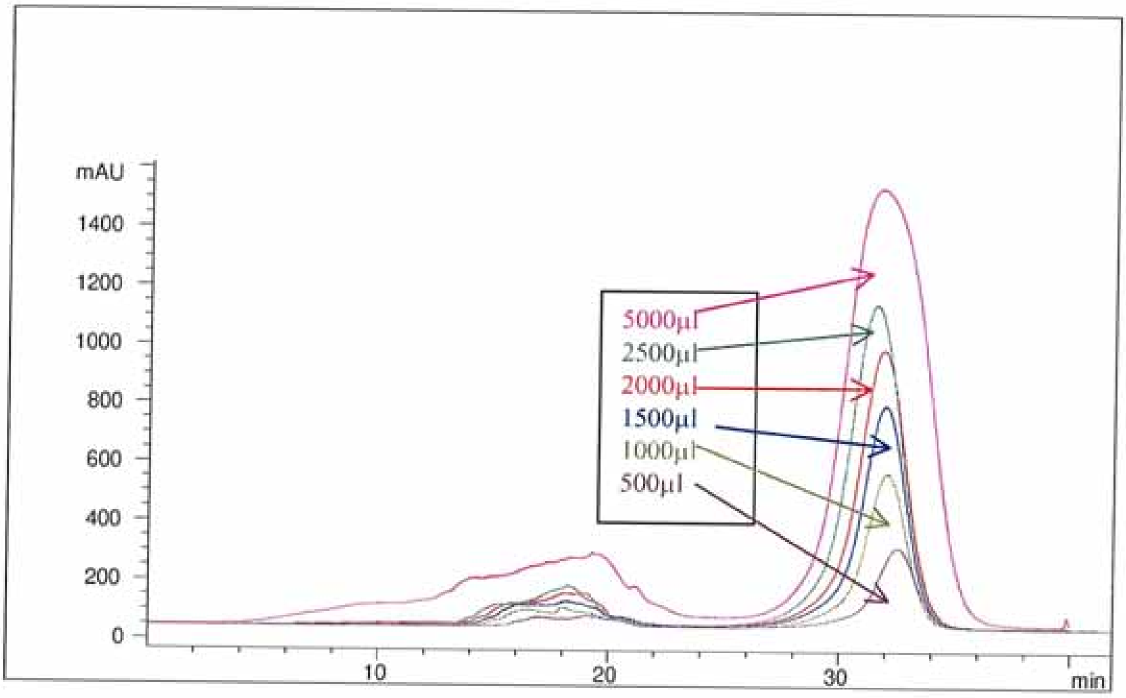


Figure 6.5: Injection volumes (500-5000)μl of sulphadiazine standards injected under the conditions described in Section 6.8.1.4.

From the discussion in Section 6.4.3, it is clear that the addition of water to the mobile phase had a significant improvement on the resolution obtained between the analyte and matrix peaks for the SEC. It also became apparent in this section that certain analytes could be analysed together with the same mobile phase, while other analytes required a change in the ratio of the mobile phase components in order for resolution to be obtained. It was found that the optimum resolution between the analytes and the matrix components occurred with a mobile phase consisting of MeOH: 0.01M Methanolic acetic acid: water in the ratio of 8: 90: 2 for OTC and TMP, 93: 5: 2 for ivermectin and cypermethrin and 85: 5: 10 for SD, SMX and OA. Thus the analytes were further examined for the SEC method in groups, based on the ratio of mobile phase components which were used to obtain resolution between these particular analytes and the matrix components.

6.6 SEC for Oxytetracycline and Trimethoprim in Salmon Tissue

6.6.1 Apparatus and materials

6.6.1.1 Sample preparation

Scalpel and blades
Hi - speed homogeniser: Breda Scientific
Glass sample jars

6.6.1.2 Extraction Process

Balance (5 decimal places)
Vortex mixer - Fisons
Centrifuge - Sorvall T6000
200ml polypropylene centrifuge tube
Ori block -08-3 Techne + Si3 sample concentrator - nitrogen purge and water bath at 30^0C
Pyrex test tubes: 25mls

6.6.1.3 Chemicals and Reagents

Buffer pH 6.0(0.1M citric acid, 0.1M Na_2HPO_4: dissolve 21g citric acid mono hydrate and 18g $Na_2HPO_4.2H_2O$ in about 900ml water, adjust the pH with conc. NaOH, and fill up to 1L with water.)
Acetonitrile: Lab Scan - Pestiscan
Sodium chloride: Merck
Oxytetracycline hydrogen chloride, trimethoprim: Sigma

6.6.1.4 SEC System

Pump: Shimadzu LC - 6A
Column oven: Shimadzu CTO - 6A
Column: Toyopearl TSK HW 40(S) (300 x 10) mm

Auto injector: Rainin-Dynamax Al-200

Detector: UV -Vis spectrophotometric detector -Shimadzu SPD - 6AV

Data handling: HP Chemstations version A.02.05.

6.6.1.5 Sample storage

All tissue samples were stored in acid followed by MeOH rinsed glass sample jars and kept in a freezer at -20°C prior to analysis. Using a hi-speed homogeniser the tissue was homogenised and the required weight of this homogenate was used for each analysis.

6.6.2 Sample preparation procedure

Homogenised tissue sample (15g) was accurately weighed into a 200ml polypropylene tube. Buffer pH 6.0 (30ml) was added and this was homogenised for 2 minutes. ACN (90ml) was added in 2 portions and this was homogenised after each addition. The sample was then filtered through a Whatman No. 1 filter paper and the volume corresponding to 10g of tissue sample (90ml) was measured into a separating funnel. Sodium chloride (4g) was added and the separating funnel was shaken intensively for 30 seconds. The lower aqueous layer was removed to waste. The organic layer was transferred to a 500ml round bottom flask and the solution evaporated to dryness using the rotary evaporator at 40°C. The residue was dissolved in the SEC mobile phase (10mls). Prior to injection on the SEC system, 100μl of a 10μgml^{-1} standard OTC and TMP solution (dissolved in MeOH) were added to yield a 100μgkg^{-1} sample.

6.6.3 Analytical conditions for OTC and TMP

Column: Toyopearl TSK HW 40(S), (300 x 10) mm

Flow rate: 1 ml/minute

Temperature: 25°C

Injection volume: 2mls.

Detector: Shimadzu UV@272 nm.

6.6.4 Mobile phase optimisation for OTC and TMP

Table 6.5 compares the resolution obtained on the Fractogel and Toyopearl columns when using a mobile phase consisting of MeOH: 0.01M methanolic acetic acid: water in the ratio of 8: 90: 2. From this data it was evident that better resolution was obtained for OTC when using the Fractogel column while similar resolution was obtained on both columns for TMP when using the mobile phase described. Since the Fractogel packing was no longer available it was necessary to optimise the mobile phase conditions to obtain maximum resolution between the matrix components and the analytes while using the Toyopearl TSK HW-40(S) column. Table 6.7 contains the resolution data for OTC and TMP under different mobile phase conditions.

Table 6.7: Resolution of analytes from the tissue matrix, obtained for oxytetracycline and trimethoprim for different ratios of the mobile phase components using the Toyopearl TSK HW40(S), (300 x 10) mm column

% Acetic Acid	% Water	% Methanol	R_S: OTC	R_S: TMP
0.5	0	99.5	-	-
0.5	2	97.5	0.92	1.08
0.5	4	95.5	-	0.53
1.0	2	97.0	1.27	1.40
1.5	2	96.5	1.43	1.66
2.0	2	96.0	1.36	1.95
2.5	2	95.5	1.22	1.86
3.0	2	95.0	1.62	1.86
3.5	2	94.5	1.42	1.64

The mobile phase conditions were optimised by keeping the percentage of one component constant while altering the other two. With zero percent water in the mobile phase no resolution was obtained from the matrix for either analytes. When the water content was adjusted to 2% by keeping the acetic acid content constant, at 0.5% a small degree of resolution was obtained for both analytes. However, when the water was increased further to 4%, while still maintaining the acid content at 0.5%, the degree of resolution decreased for both analytes. Therefore the optimum amount of water in the mobile phase was 2% for both analytes. The acetic acid content was then increased from (0.5- 3.5)% by maintaining the water content at 2% and adjusting the MeOH content. For OTC the optimum mobile phase conditions were found to consist of 3% acetic acid, 2% water and 95% MeOH, while for TMP the optimum mobile phase consisted of 2% acetic acid, 2% water and 96% MeOH. A chromatogram of these conditions can be seen in Figure 6.6(A) for OTC and Figure 6.6(B) for TMP.

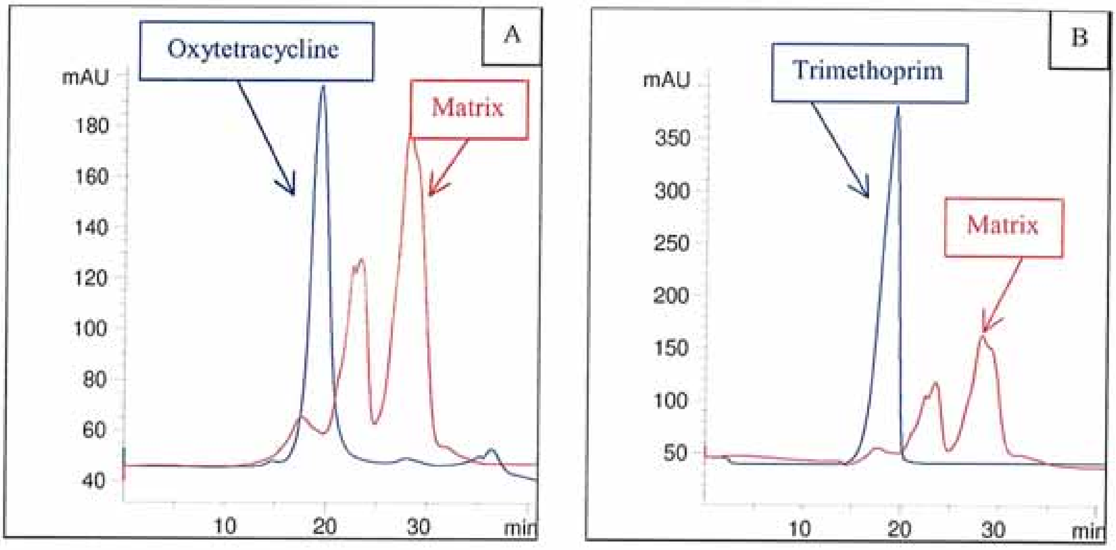


Figure 6.6: SEC of oxytetracycline (100μgkg^{-1}) (A) and trimethoprim (100μgkg^{-1}) (B) in salmon tissue. Mobile phase: Acetic acid: MeOH: H_2O 3:95:2 (A) and 2:96:2 (B). Flow rate: 1ml/minute. Column: Toyopearl TSK HW 40(S), (300x10) mm. Temperature: 25°C. Detector: UV @ 272nm.

6.6.5 Conclusion of SEC analysis of OTC and TMP

The chromatograms show that the resolution between the analyte and the matrix peak is not adequate for identification purposes for either analyte. The eluent fractions corresponding to the analyte peaks need to be collected and analysed by the confirmatory HPLC methods (Chapter 4 and 5). Thus SEC could be examined as a sample preparation and cleanup technique. However, the fraction collector was not available at the time this method was examined. The concentration of the analytes in the above chromatograms was 100μgkg^{-1} for each analyte which is the MRL for OTC and twice the MRL for TMP.

6.7 SEC for sulphadiazine, sulphamethoxazole and oxolinic acid in Salmon Tissue

6.7.1 Apparatus and materials

6.7.1.1 Sample preparation

Scalpel and blades

Hi - speed homogeniser: Breda Scientific

Glass sample jars

6.7.1.2 Extraction Process

Balance (5 decimal places)

Vortex mixer - Fisons

Centrifuge - Sorvall T6000

200ml polypropylene centrifuge tube

Rotary evaporator with vacuum controller – Resona Technics

Ori block -08-3 Techne + Si3 sample concentrator - nitrogen purge and water bath at 30°C

Pyrex test tubes: 25mls

6.7.1.3 Chemicals and Reagents

Buffer pH 6.0(0.1M citric acid, 0.1M Na_2HPO_4: dissolve 21g citric acid mono hydrate and 18g $Na_2HPO_4.2H_2O$ in about 900ml water, adjust the pH with conc. NaOH, and fill up to 1L with water.)

Acetonitrile: Lab Scan - Pestiscan

Butyl methyl ether/hexane (80/20): Labscan - HPLC grade

Ammonia solution: Reidel de Haen

Sodium chloride: Merck

Sulphadiazine, sulphamethoxazole, oxolinic acid: Sigma

6.7.1.4 SEC System

Pump: Shimadzu LC - 6A

Column oven: Shimadzu CTO - 6A

Column: Toyopearl TSK HW 40(S) (300 x 10)mm

Auto injector: Rainin-Dynamax Al-200

Detector: UV -Vis spectrophotometric detector -Shimadzu SPD - 6AV

Data handling: HP Chemstations version A.02.05.

6.7.1.5 Sample storage

All tissue samples were stored in acid followed by MeOH rinsed sample jars and kept in a freezer at -20°C prior to analysis. Using a hi-speed homogeniser the tissue was homogenised and the required weight of this homogenate was used for each analysis.

6.7.2 Sample preparation procedure

While SD, SMX and OA can be analysed by SEC under the same analytical conditions, two sample preparation procedures were required- one for SD and SMX and one for OA.

6.7.2.1 Extraction of SD and SMX

Homogenised tissue sample (15g) was accurately weighed into a 200ml polypropylene tube. 150μl of a $10\mu gml^{-1}$ standard SD and SMX solution (dissolved in ACN) was added to the tissue to yield a $100\mu gkg^{-1}$ sample. This was allowed to sit for 30 minutes prior to extraction. Buffer pH 6.0 (30ml) was added and this was homogenised for 2 minutes. ACN (90ml) was added in 2 portions and this was homogenised after each addition. The sample was then filtered through a Whatman No. 1 filter paper and the volume corresponding to 10g of tissue sample (90ml) was measured into a separating funnel. Sodium chloride (4g) was added and the separating funnel was shaken intensively for 30 seconds. Butyl methyl ether/ hexane mixture (30ml) was added and the sample shaken for 30 seconds. The lower aqueous layer was removed to waste. The organic layer was transferred to a 500ml round bottom flask and the solution evaporated to dryness using the rotary evaporator at 40°C. The residue was dissolved in the SEC mobile phase (10mls) prior to injection on the SEC system.

6.7.2.2 Extraction procedure for OA

Homogenised tissue sample (10g) was accurately weighed into a 50ml polypropylene centrifuge tube. 50μl of a $10\mu gml^{-1}$ standard OA solution (dissolved in ACN) was added to the tissue to yield a $50\mu gkg^{-1}$ sample. This was allowed to sit for 30 minutes prior to extraction. ACN (20mls) and ammonia solution (3mls) were added to the tissue sample. This was homogenised for 1 minute followed by centrifugation at 2700r.p.m. for 10 minutes. The supernatant was decanted into a 25ml pyrex test tube and evaporated to dryness at 30°C under a gentle stream of nitrogen. The residue was dissolved in 10mls of the SEC mobile phase prior to injection onto the SEC column.

6.7.3 Analytical conditions for SD, SMX and OA

Column: Toyopearl TSK HW 40(S), (300 x 10) mm
Mobile phase: MeOH: 1% aqueous acetic acid (90:10)
Flow rate: 1 ml/minute
Temperature: 25°C
Injection volume: 2mls
Detector: 272 nm

6.7.4 Results for SD and SMX

A chromatogram of SD and SMX, which were prepared under the above described conditions, can be seen in Figure 6.7(A)-(B) respectively. These show two separate tissue samples spiked to100μgkg^{-1} with each of the two respective analytes. For SD and SMX spiked tissue samples, the analyte peaks are resolved from the matrix and the sensitivity is adequate for a 100μgkg^{-1} spike level.

Since the MRL for combined sulphonamides in fish tissue is 100μgkg^{-1} [15], it was necessary to examine a tissue sample spiked to 50μgkg^{-1} with SD and SMX for sensitivity and resolution. The resulting chromatogram can be seen in Figure 6.7(C)-(D) for the two analytes respectively. It is clear from these chromatograms that the analytes can be identified by retention time at 50μgkg^{-1} for both SD and SMX.

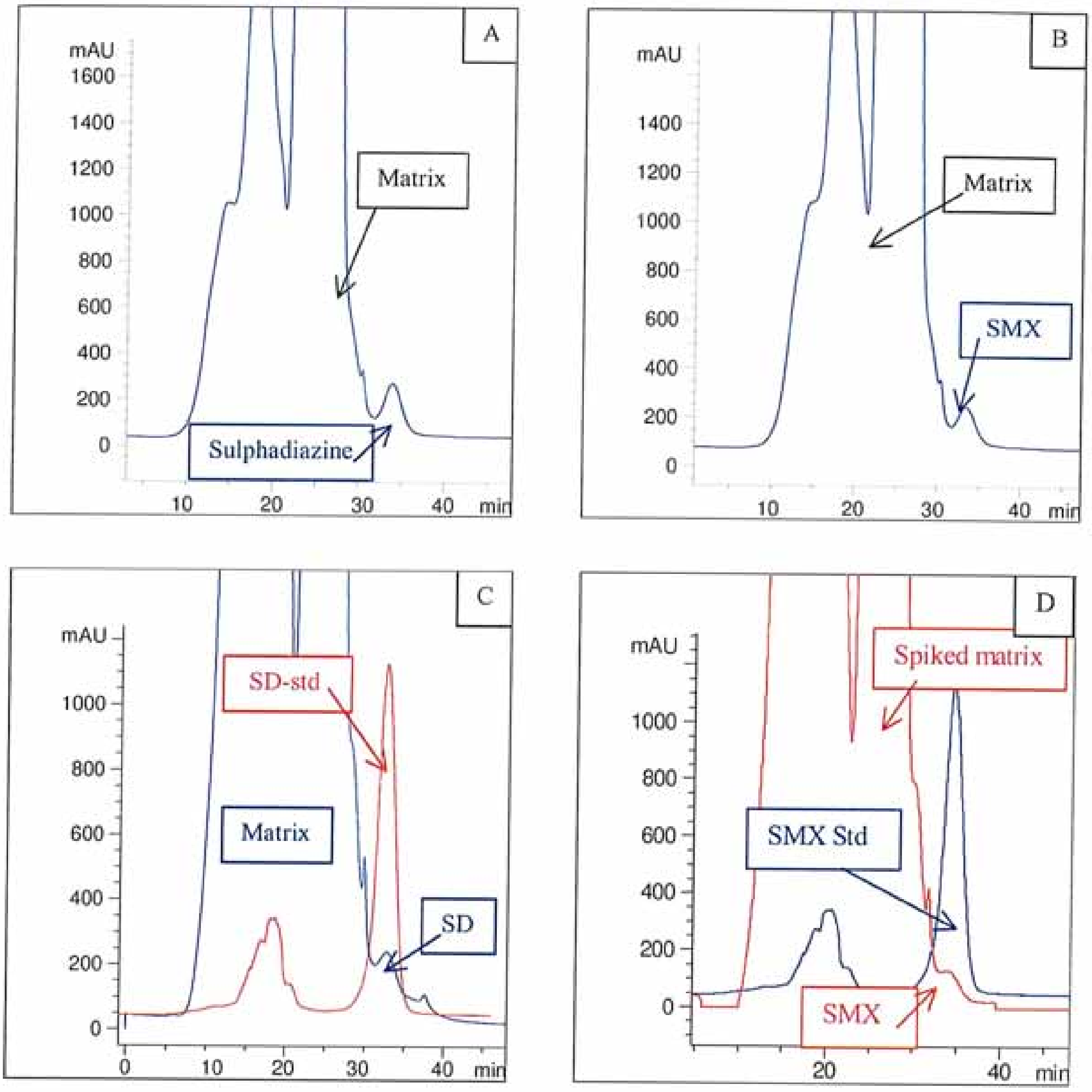


Figure 6.7: SEC of a salmon tissue spiked to 100μgkg^{-1} with sulphadiazine (A) and sulphamethoxazole (B), and spiked to 50μgkg^{-1} with sulphadiazine overlayed on a standard solution (C) and sulphamethoxazole (D).
Mobile phase: MeOH: 1% aqueous acetic acid (90:10). Flow rate: 1ml/minute. Column: Toyopearl TSK HW 40(S), (300 x 10) mm, Temperature: 25°C. Detector: UV@272nm.

6.7.5 Results for OA

A chromatogram of OA spiked to 50μgkg^{-1}, and prepared according to the above described extraction procedure (Section 6.7.2.2) was analysed under the SEC conditions described (Section 6.7.3) and the resulting chromatogram can be seen in Figure 6.8(A). From this chromatogram it is evident that the resolution is relatively poor and needs to be examined further.

6.7.6 Effect of liquid- liquid extractions prior to SEC

In an attempt to improve the resolution between the OA and matrix peaks in the chromatogram different liquid-liquid partitions were examined as possible clean-up techniques.

A blank tissue sample was prepared according to the procedure described (Section 6.7.2.2). When the sample was evaporated to dryness, the residue was dissolved in MeOH (10mls). A liquid-liquid extraction with hexane (10mls) was carried out in an attempt to remove the lipids from the sample. The hexane layer was discarded and the sample in MeOH was injected. The resulting chromatograms can be seen in Figure 6.8(B), where it is apparent that there is a reduction in the absorption at the start of the chromatogram for the tissue sample treated with hexane.

However the hexane wash does not improve the resolution between the analyte and matrix peaks as the analyte peak elutes at the latter end of the chromatogram. Therefore it was necessary to examine a method for removing the matrix components (possibly proteins) at the latter end of the chromatogram. This was examined by taking the evaporated tissue sample from Section 6.7.2.2 and dissolving it in water (3mls). Ethyl acetate (8mls) was added and this was vortex mixed for 30 seconds. The ethyl acetate was removed and the procedure repeated twice more. The ethyl acetate layers were evaporated to dryness and diluted in the mobile phase prior to injection. The resulting chromatogram is shown in Figure 6.8(C).

The chromatograms show that the matrix components from the latter end of the chromatogram are reduced considerably and thus the resolution between OA and matrix components was improved. In the water /ethyl acetate partition the matrix components which remained in the water were most likely polar. The OA was extracted from the water into the ethyl acetate for analysis. Additionally the water /ethyl acetate partition was more efficient than the hexane wash for removing the large absorbing matrix components from the beginning of the chromatogram. Therefore from the point of view of both having a cleaner tissue extract and also improving resolution between the matrix and analyte components the water /ethyl acetate partition was more efficient than the hexane wash.

A tissue sample spiked to 50μgkg^{-1} with OA was brought through this procedure and a clearly resolved OA peak can be seen at 35 minutes (Figure 6.8(C)).

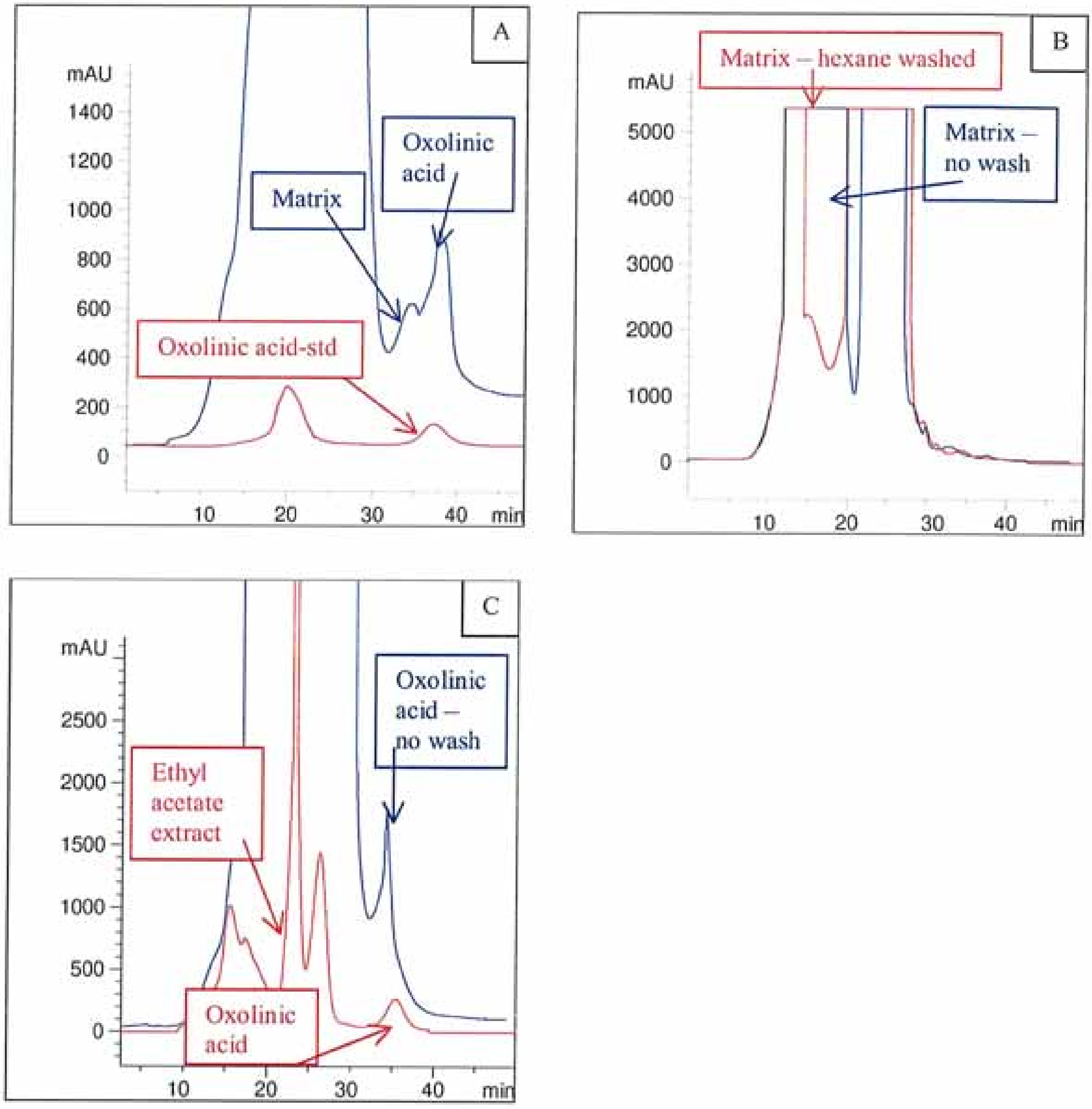


Figure 6.8: SEC of salmon tissue spiked to 50µgkg^{-1} with oxolinic acid, overlayed on a standard solution (A), washed with hexane prior to injection overlayed on a tissue which was not washed (B), and extracted with ethyl acetate prior to injection overlayed on a tissue sample which was not washed (C).
Mobile Phase: MeOH: 1% aqueous acetic acid (90:10). Flow rate: 1ml/minute.
Column: Toyopearl TSK HW 40(S), (300 x 10) mm, Temperature: 25°C. Detector: UV@272nm.

A tissue sample spiked to 50µgkg^{-1} with SD was also examined under both of the wash conditions described above. The resulting chromatogram, which shows a decrease in matrix effects but no improvement in resolution, can be seen in Figure 6.9(A) for the hexane wash. Some improvement in resolution was evident however for the tissue sample subjected to the water /ethyl acetate partition as can be seen in Figure 6.9(B).

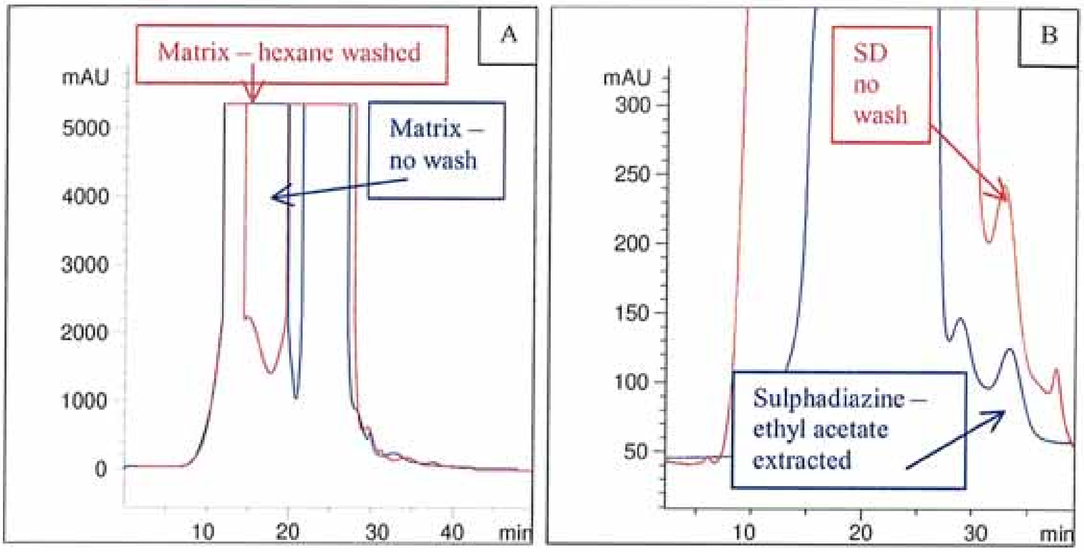


Figure 6.9: Overlay of an unwashed spiked sulphadiazine tissue sample, 50μgkg^{-1}, on a spiked sulphadiazine tissue sample, 50μgkg^{-1}, washed with hexane prior to injection (A) and extracted with ethyl acetate prior to injection (B).
Mobile phase: MeOH: 1% aqueous acetic acid (90:10). Flow rate: 1ml/minute. Column: Toyopearl TSK HW 40(S), (300 x 10) mm, Temperature: 25°C. Detector: UV@272nm.
R_s for the unwashed sample: 2.45, Hexane washed R_s = 2.5 (A), Ethyl acetate washed R_s = 2.68 (B).

6.7.7 Conclusion for SEC analysis of SD, SMX and OA

The aim of this work was to find a quick and effective method for screening for various veterinary drug residues in salmon tissue. It was evident from the work discussed that the resolution obtained without any wash steps for SD and SMX was sufficient for the identification of both analytes at 50μgkg^{-1}. The inclusion of the hexane wash in this procedure did not improve the resolution obtained for OA, SD or SMX in salmon tissue. The water /ethyl acetate partition did however improve the resolution and therefore should be included in the sample preparation procedure.

In the future, however a photodiode array detector (DAD) will be required to obtain the spectra for these analyte peaks to aid in identification. Since a DAD was not available when this work was carried out then further examination of this method was postponed.

6.8 SEC for Cypermethrin and Ivermectin in Salmon Tissue

6.8.1 Apparatus and materials

6.8.1.1 Sample preparation

Scalpel and blades

Hi - speed homogeniser: Breda Scientific

Glass sample jars

6.8.1.2 Extraction Process

Balance (5 decimal places)

Vortex mixer - Fisons

Centrifuge - Sorvall T6000

50ml polypropylene centrifuge tube

Ori block -08-3 Techne + Si3 sample concentrator - nitrogen purge and water bath at 30^0C

Pyrex test tubes: 25mls

Bond Elut: C8 SPE cartridge-1g (6mls)

6.8.1.3 Chemicals and Reagents

Methanol: Lab Scan-Pestiscan

Acetonitrile: Lab Scan-Pestiscan

Hexane: Lab Scan-Pestiscan

Acetone: Lab Scan –Pestiscan

Ivermectin, cypermethrin: Sigma

6.8.1.4 SEC System

Pump: Shimadzu LC - 6A

Column oven: Shimadzu CTO - 6A

Column: Toyopearl TSK HW 40(S) (300 x 1.0) mm

Auto injector: Rainin-Dynamax Al-200

Detector: UV -Vis spectrophotometric detector -Shimadzu SPD - 6AV

Data handling: HP Chemstations version A.02.05.

6.8.1.5 Sample storage

All tissue samples were stored in acid followed by MeOH rinsed sample jars and kept in a freezer at -20°C prior to analysis. Using a hi-speed homogeniser the tissue was homogenised and the required weight of this homogenate was used for each analysis.

6.8.2 Sample preparation procedure

The extraction procedures for both ivermectin and cypermethrin differ and thus samples must be prepared individually for each analyte.

6.8.2.1 Extraction of ivermectin

Homogenised tissue (10g) was spiked with ivermectin standard (50μl of a 10μgml^{-1} solution dissolved in MeOH) to yield a 50μgkg^{-1} sample. This was allowed to sit for 30 minutes prior to extraction with MeOH (40mls). The MeOH was evaporated to dryness using the rotary evaporator and the residue was dissolved in the SEC mobile phase (10mls) prior to injection on the SEC column

6.8.2.2 Extraction of cypermethrin

Homogenised tissue (10g) was extracted with hexane: acetone (2:1), 20mls. The hexane: acetone was evaporated to dryness using the rotary evaporator and the residue was dissolved in the SEC mobile phase (10mls). This was spiked with 50μl of a 10μgml^{-1} cypermethrin standard, diluted in ethyl acetate, prior to injection on the SEC column.

6.8.3 Analytical conditions for ivermectin and cypermethrin

Column: Toyopearl TSK HW 40(S), (300 x 10) mm

Mobile phase: MeOH: Acetic acid: H_2O (97.9:0.1:2)

Flow rate: 1ml/minute

Temperature: 25°C

Injection volume: 2mls

Detection: 254nm and 0.001 aufs.

6.8.4 Results for cypermethrin

A chromatogram of a tissue sample spiked with cypermethrin, which was prepared under the conditions described in Section 6.8.2.2, can be seen in Figure 6.10(A). The overlay of this with a cypermethrin standard shows that no resolution between the tissue and the analyte peak was obtained and thus cypermethrin could not be identified in this way (Figure 6.10(A)).

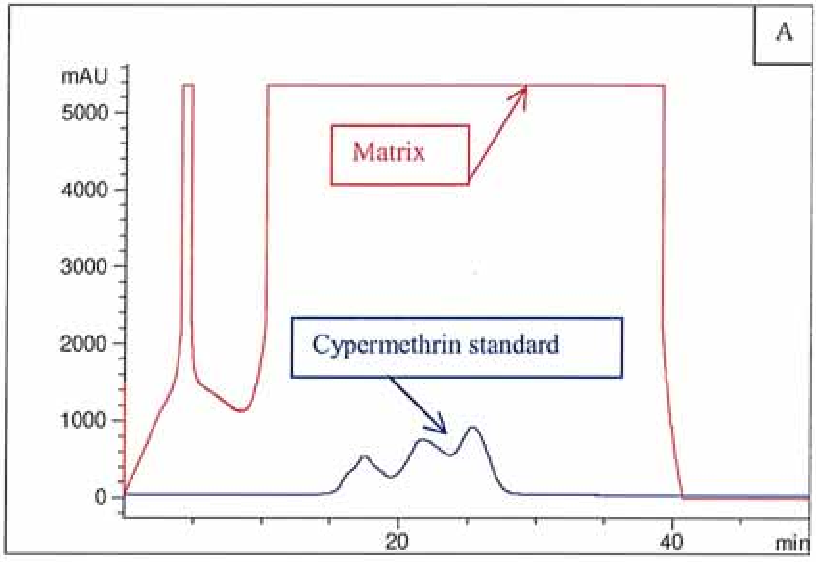


Figure 6.10(A): SEC of salmon tissue spiked to 50μgkg^{-1} with cypermethrin, overlayed on a standard cypermethrin solution. Analytical conditions: Section 6.8.3.

6.8.4.1 Examination of liquid-liquid extractions prior to SEC

In an attempt to improve the resolution the spiked tissue sample from Section 6.8.2.2 was evaporated to dryness and dissolved in water (5mls). Since cypermethrin is soluble in ethyl acetate and a water /ethyl acetate partition reduced the matrix effects in the chromatogram considerably (Figure 6.8(C)), this step was examined here. The aqueous layer was extracted twice with ethyl acetate (8mls) which was evaporated to dryness under a gentle stream of nitrogen, and dissolved in the SEC mobile phase prior to injection. An overlay of the resulting chromatogram on a standard solution of cypermethrin can be seen in Figure 6.10(B). This shows a cleaner tissue matrix but adequate resolution was not obtained.

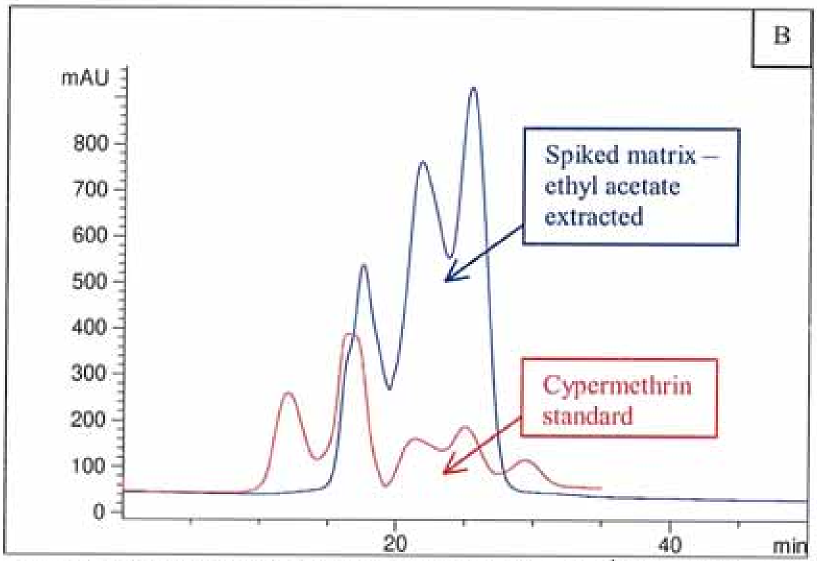


Figure 6.10(B): SEC of spiked salmon tissue (50µgkg^{-1} with cypermethrin) washed with ethyl acetate overlayed on a standard cypermethrin solution. Analytical Conditions: Section 6.8.3.

6.8.5 Conclusion for SEC of Cypermethrin

From the studies carried out on the SEC analysis of cypermethrin it was evident that the UV detector did not have adequate sensitivity for the identification of cypermethrin in tissue samples. Since GC ECD is the conventional method for cypermethrin identification and quantification, the collection of the eluent from the size exclusion column for analysis by this method needs to be examined in the future. Additionally the SEC conditions examined did not appear to have adequate resolution for the identification of cypermethrin but in order to confirm this analysis GC ECD is required. If this method proves not to have adequate resolution for identification purposes then other possibilities could be examined. This could for example involve taking the tissue sample from Section 6.8.2.2 through the SEC system and collect the fraction where the cypermethrin is expected to eluate. This could then be brought through a SPE cartridge to remove some of the matrix components and therefore make the cleanup procedure effective for identification and quantification by GC ECD. A method such as this would be quicker than the traditional liquid-liquid extraction and SPE procedures, which are used in the confirmation method for cypermethrin analysis in the laboratory.

6.8.6 Results for ivermectin

A chromatogram of a tissue sample spiked with ivermectin, which was prepared under the conditions, described in Section 6.8.2.1 can be seen in Figure 6.11. The overlay of this with a standard ivermectin solution shows that the resolution between the tissue and the analyte peak is not satisfactory for identification purposes. Similar to cypermethrin, it should be noted that the UV detector was not sensitive enough to detect ivermectin at the required concentration levels. Therefore the work carried out here involved examining the SEC process as a possible cleanup technique for identification and quantification by LC with fluorescence detection. The separation in Figure 6.11 is not sufficient for an adequate cleanup technique for ivermectin analysis in salmon tissue.

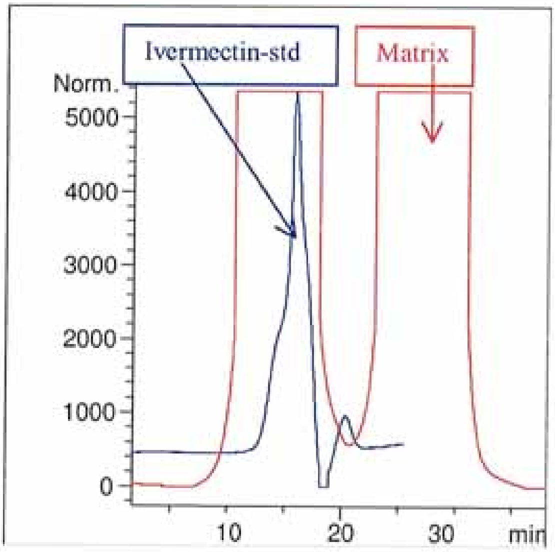


Figure 6.11: SEC of spiked salmon tissue ($50\mu g kg^{-1}$ with ivermectin) overlayed on a standard solution. Analytical conditions: Section 6.9.3.

6.8.6.1 Collection of eluent fractions

If the eluent fraction containing the ivermectin peak was collected for analysis with fluorescence detection then this eluent must be taken to dryness so that the analyte can be derivatised for HPLC injection. The mobile phase from the SEC consists of MeOH: H_2O: Acetic acid in the ratio of 97.9:2:0.1. Due to the aqueous content of this mobile phase the eluent could not be evaporated to dryness. Therefore it was necessary to find an alternative such as a SPE cartridge to take the sample through and then eluate the analyte with an organic solvent which can be evaporated to dryness.

From the HPLC confirmation method for ivermectin in the laboratory the sample in MeOH: H_2O (75:25) can be brought through a Varian C8 SPE cartridge and ivermectin eluted with ACN.

6.8.7 Examination of the mobile phase

The mobile phase of the SEC system contains 0.1% acid and in order to load the eluent onto the C8 SPE cartridge the analyte must be dissolved in MeOH: H_2O (75:25). Therefore the SEC mobile phase was examined to see if the ivermectin could be separated from the matrix components when the mobile phase contained only two components (MeOH and H_2O). Different ratios of the two mobile phase components were examined and an overlay of these can be seen in Figure 6.12(A)-(D). Ivermectin eluted at the start of the matrix components for the mobile phase consisting of MeOH: H_2O (80:20). As the MeOH was reduced and the water content increased correspondingly, the elution time of the ivermectin moved to the end of the matrix. Eventually a situation was reached where the ivermectin peak was not seen at all as shown in Figure 6.12(D) for the mobile phase consisting if MeOH: H_2O (30:70). Thus from the chromatograms it was apparent that the best resolution, while not ideal, was obtained with a mobile phase containing MeOH: H_2O (40:60) (Figure 6.12(C)). The column oven was maintained at 45°C under these conditions. With this mobile phase the eluent fraction containing the ivermectin was collected and adjusted to consist of MeOH: H_2O (75:25) prior to being loaded on the SPE cartridge. Ivermectin was then eluted from the cartridge with ACN, which was evaporated to dryness prior to derivatisation for fluorescence detection.

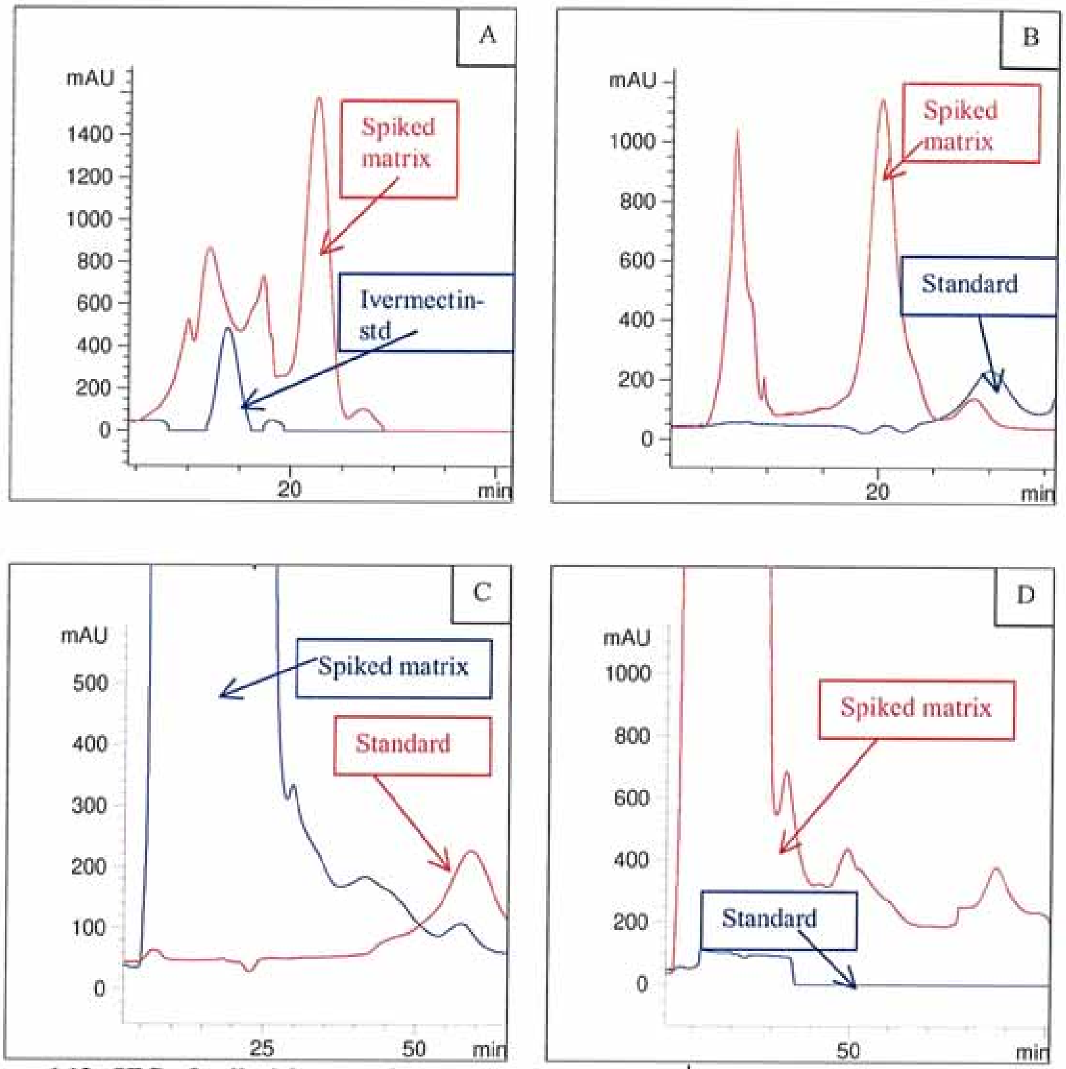


Figure 6.12: SEC of spiked ivermectin salmon tissue (50μgkg^{-1}) overlayed on a standard solution. Mobile phase: MeOH: H_2O 80:20 (A), 50:50 (B), 40:60 (C) and 30:70 (D)

6.8.7.1 Method procedure

Homogenised tissue sample (10g) was extracted three times with MeOH (20, 20, 20mls). The tissue sample was filtered using a Whatman No. 1 filter paper. The filtrate was evaporated to approximately 4mls using the rotary evaporator at 40°C and the volume was adjusted to 10mls with water. The weight of the final solution was recorded and 2mls was injected onto the SEC column. The weight of the remaining solution was also recorded for calculation purposes. The eluent fraction, containing the ivermectin (Section 6.8.7.2), from the SEC column was collected and the volume adjusted so that the solution consisted of MeOH: H_2O (25:75). The C8 SPE cartridge was conditioned with MeOH, ACN and H_2O (5mls of each) and the sample was loaded onto the cartridge. Once the sample was brought through the cartridge it was dried with air for 5 minutes. Ivermectin was then eluted from the cartridge with ACN (10mls). This was evaporated to dryness under a gentle stream of nitrogen at 35°C. Derivatizing agent was added to the residue and the sample analysed by HPLC with fluorescence detection [16].

6.8.7.2 Fraction collection

The fraction collector was programmed to collect all the eluent from the SEC column in 4 minute fractions and all were analysed to find where the ivermectin eluted. From this test ivermectin was found to eluate in a (1 x 8)ml fraction between 16-24 minutes.

6.8.8 Results

The spiked tissue sample was brought through the procedure and the collected fraction was analysed with fluorescence detection. A resulting chromatogram of the fluorescence analysis can be seen in Figure 6.13. This chromatogram is overlayed on a fluorescence detection chromatogram obtained from the confirmation method. The sample was homogenised and extracted with methanol, purified using liquid-liquid and solid phase extraction techniques. The resultant residue was derivatized and analysed by HPLC with fluorescence detection [16].

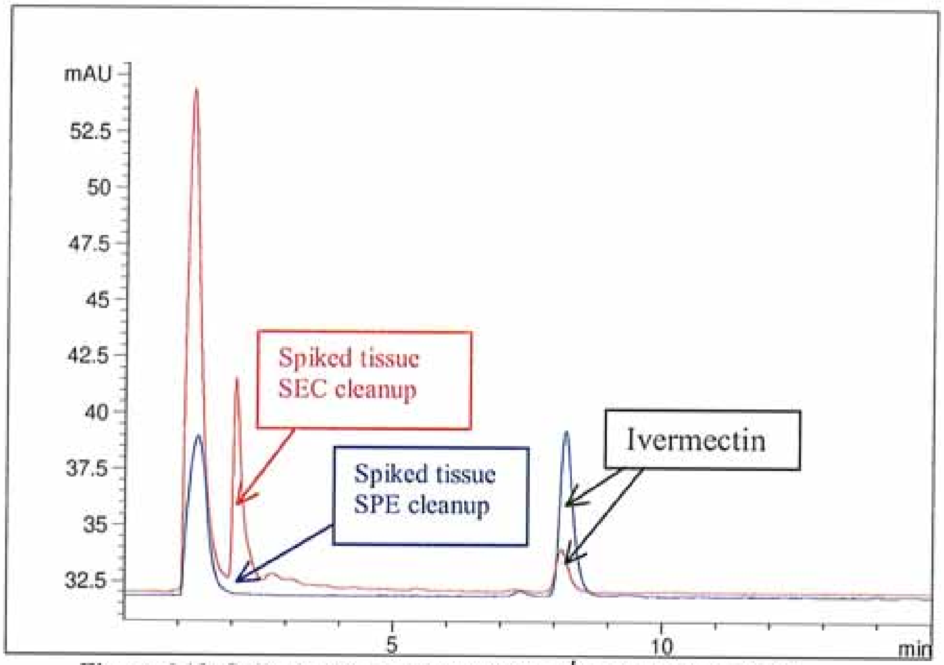


Figure 6.13: Spiked salmon tissue (40μgkg^{-1}) prepared by SEC overlayed on a spiked tissue sample (40μgkg^{-1}) prepared by SPE [16].

When a spiked tissue sample was brought through the SEC column it became apparent that the recoveries obtained were 70%, <3%RSD (n=5). To determine where the losses were occurring in the method each step was examined individually and the results are recorded in Table 6.8.

Table 6.8: Percentage recoveries obtained from each step of the procedure for ivermectin spiked tissue samples analysed by SEC.

Spiked tissue / Absolute concentration (ng)	Evaporation of ACN	C8 SPE cartridge	SEC	Complete procedure
25.6	99	97	70.1	72.1
106.1	102	102	72.4	73.4
210.2	101	99	70.3	71.3
255.3	98	98	73.1	70.6

The above results were calculated based on ivermectin standards used to prepare a calibration plot, $y=1.25x + 1.46$; $R^2 = 0.9996$, Figure 6.14.

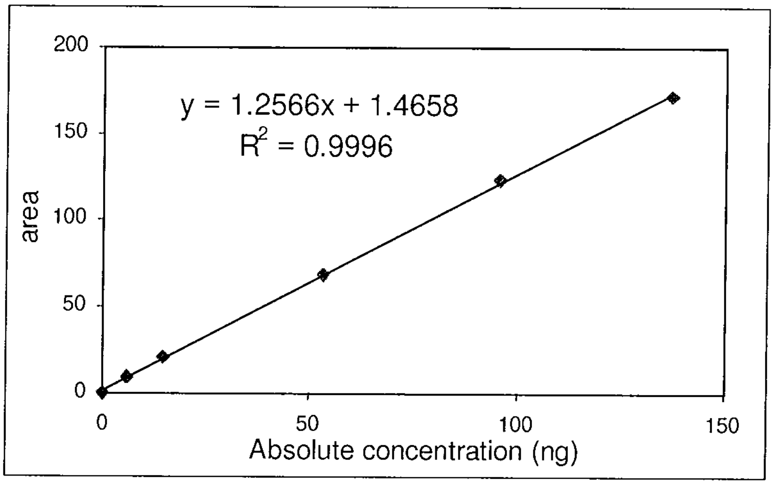


Figure 6.14: Standard calibration plot of ivermectin showing peak area plotted against concentration.

From Table 6.8 it is clearly evident that the losses in percentage recoveries obtained occur in the SEC step of the procedure. To test if the matrix is having any effect on this once the sample is injected onto the column, standard ivermectin solutions were injected onto the column and the percentage recoveries obtained were recorded in Table 6.9.

Table 6.9: Percentage recoveries obtained from each step of the procedure for ivermectin standards analysed by SEC.

Standard Absolute Concentration (ng)	Evaporation of ACN	C8 SPE cartridge	SEC	Complete procedure
1.5	98	101	-	-
2.3	103	103	-	-
5.6	102	102	-	-
8.2	98	98	15.6	16.3
10.4	99	99	71.2	72.4
18	101	101	76.5	70.6
60.2	102	102	70.2	71.5
225.6	97	97	71.4	70.8

From the results in Table 6.9 it appears that the recoveries obtained are approximately 70% for standard absolute concentrations equivalent to 10ng and over. However, for the lower concentration values examined, it is evident that no recovery of ivermectin was obtained from the SEC column. While percentage recoveries of 70% are deemed acceptable for spiked tissue samples in the Codex guidelines no recovery of ivermectin was obtained at low concentration levels of 10ng (5μgkg^{-1}) and less. Since these are the concentration levels of interest this is a cause for concern. It could be possible that the column requires a background level of ivermectin to absorb onto the packing to use up active sites. If this were the case however, the column would be saturated at this stage since most of the samples injected to date consisted of 100μgkg^{-1} and over.

6.8.9 SEC of Ivermectin - Conclusion

The SEC method examined for the analysis of ivermectin in salmon tissue resulted in an effective cleanup technique which was evident when the chromatogram was compared to that of an ivermectin tissue sample prepared and analysed using the conventional SPE method (Figure 6.13). However, recoveries of approximately 70% were recorded for tissue samples spiked to 10ng absolute concentration (5μgkg^{-1}). Below this concentration level, which are the values of interest to this method, no ivermectin was recovered from the SEC column. Therefore this problem will have to be addressed in the future. To get some indication of possible explanations however, SEC methods from the literature were examined further.

It has been reported that the pore size of the packing material has an effect on the recovery of the analyte obtained from the column [17]. For the GPC separation of humic acid substances under the same mobile phase conditions, the recovery was 88%

on a 100Å pore size column, 64% on a 1000Å pore size column, and 23% on a 4000Å pore size column. In order to examine this theory for the purposes of ivermectin analysis a packing material with a pore size less than 50Å would need to be examined. However, the smallest pore size available in the Toyopearl HW resins is 50Å, thus to examine this theory a different resin type needs to identified.

For humic acid substances it has also been reported that the recovery increased from 87.8% for the 1st injection to 91.4% in subsequent runs [17]. The recoveries approached a constant rate after 4 consecutive injections, indicating that the reactive sites on the column are now covered in humic molecules. A washing procedure of the column between consecutive injections, combined with a gradient elution profile of the mobile phase consisting of ACN: H_2O resulted in no irreversibly adsorbed molecules being retained on the column and thus 100% recoveries were obtained. However recoveries obtained for ivermectin were approximately 70% and these did not increase with consecutive injections. A washing procedure like that used for the humic acid substances could be examined to see if this had any effect on the recoveries. It was also noted that when the mobile phase ACN/H_2O for humic substances, was replaced with H_2O/MeOH the percentage recoveries obtained declined. This was reported to be due to low elution strength of MeOH compared to the interactions of the HS substances with the stationary phase [17]. This could also be a possibility for the ivermectin method and therefore ACN could in the future be examined in the mobile phase rather than MeOH.

6.9 Conclusion

The aim of this work was to find a quick and effective screening method for veterinary drug residues in salmon tissue samples. Varying degrees of success were found for different analytes of interest.

From Section 6.6 the separation obtained by SEC with UV detection for TMP and OTC was not acceptable for identification by retention times at the MRL. However, the resolution obtained would probably be sufficient if the eluent fraction containing the analytes was collected from the size exclusion column and the final residue analysis made by HPLC with DAD detection. This however, needs to be examined in the future and if it proved to be successful then the SEC would be used as a cleanup technique for screening purposes and therefore reducing the time required to prepare the samples, when compared to the conventional methods. In order to confirm this however, further work is required on this method.

Under the conditions described in Section 6.7 the use of SEC with UV detection, as a screening method for SD, SMX and OA was successful at 50μgkg^{-1}. This method is capable of screening 16 samples in one overnight run. When compared to a two day requirement for 6 samples for both OA and sulphonamides while using the conventional methods (Chapters 3 and 5 respectively) the SEC method provides definite advantages. However, since all three analytes examined under these conditions elute between 31 and 38 minutes then a specificity study is required to ensure that adequate resolution is obtained between the three analytes for identification by retention time. DAD would be very useful for this study and this should be examined in the future.

From Section 6.8 it was evident that the SEC separation obtained for cypermethrin was probably not adequate under the conditions described for GC ECD detection as this detection mode requires a very clean sample prior to injection. However this can only be confirmed if the eluate fraction from the size exclusion column containing the analyte is collected and analysed by GC ECD. Access to the fraction collector was not possible when this work was being examined but it should be carried out in the future.

Ivermectin cannot be analysed by UV detection due to sensitivity problems. For fluorescence detection the sample must be taken to dryness prior to derivatisation. Therefore SEC was examined as a cleanup technique and the fraction of eluent from the size exclusion column was collected for analysis by fluorescence detection (Section 6.8.7.1). This SEC method resulted in adequate separation of the analyte from the matrix components for a screening method. This was evident from the overlay of the chromatogram prepared by SEC on that prepared by the conventional method (Figure 6.13). It was found however, that recoveries obtained were approximately 70% (Table 6.9). Additionally the fact that no recoveries were obtained for concentrations <5μgkg^{-1} is very important. Since this SEC method is capable of screening for 18 samples in 1½ days compared to 12 samples in two days by the conventional method then this problem should be examined in the future.

The work discussed here involved the initial examination of SEC as both a screening and cleanup technique for various veterinary drug residues. From the above discussion it is evident that this method could be successful as both a screening and cleanup technique for the analytes examined but additional work is required and should be carried out in the future to determine the full extent of the capabilities of this method.

6.10 References

[1] Annex of Commission Decision 93/256/EEC laying down the methods to be used for detecting residues of substances having a hormonal or thyrostatic action.

[2] Malisch R, Bourgeois B, and Lippold R. (1992). Multiresidue analysis of selected chemotherapeutics and antiparasitics. Part 1: Determination of sulphonamides and their N4-Acetyl-metabolites, chloramphenicol and nicarbazin in meat. Deutsche Lebensmittel-Rundschau, 88, Jahrg, Heft 7, 206-215.

[3] Rimkus G.G, Rummler M, Nausch I. (1996). Gel permeation chromatography-high-performance liquid chromatography combination as an automated clean-up technique for the multiresidue analysis of fats. Journal of Chromatography, A. **737**, 9-14.

[4] Bester K, Hunherfuss H, Lange W, Theobald N. (1997). Results of non-target screening of lipophilic organic pollutants in the German Bight I: benzothiazoles. The Science of the Total Environment **207**, 111-118.

[5] Gatermann R, Hühnerfuss H, Rimkus G, Attar A, Kettrup A. (1998). Occurrence of musk xylene and musk ketone metabolites in the aquatic environment. Chemosphere, **38**, No.11, 2535-2547.

[6] Bester K, Hühnerfuss H. (1997). Improvements of a combined size exclusion chromatography and solid phase extraction approach for the clean-up of marine sediment samples for trace analysis of pesticides. Fresenius Journal of Analytical Chemistry **358**, 630-634.

[7] Bester K, Hühnerfuss H. (1996). Triazine herbicide concentrations in the german Wadden Sea. Chemosphere, **32**, No.10, 1919-1928.

[8] Bicking M.K.L., Wilson R.L, (1991). High performance size exclusion chromatography in environmental method development. 1. The effect of mobile phase and temperature on the retention of dioxins, furans, and polychlorinated biphenyls. Chemosphere, **22**, Nos.5-6, 421-435.

[9] Holtmannspötter H, Their H. (1982). Analysemethode für Rückstände von sechs Sulphonamiden und Chloramphenicol durch Gaschromatographie an Glaskapillaren. Deutsche Lebensmittel-Rundschau, **78**, 347-350.

[10] Petz M, Meetschen U. (1987). Untersuchungen zur Gelchromatographie (GPC) als Reinigungsverfahren in der Rückständsanalytik von Tierarzneimitteln. Z. Lebensm. Unters. Forsch, **184**, 85-90.

[11] Biancotto G, Angeletti R, Piro R. (1996). Detection of veterinary drugs in foodstuffs using gel permeation. Analyst, **121**, 229-232.

[12] Class T.J. (1992). Environmental analysis of cypermethrin and its degradation products after forestry applications. International Journal of Environmental and Analytical Chemistry, **49**, 189-205.

[13] Analytik von Rückständen Pharmakologisch Wirkamer Stoffe. (1989). Band 13 der Schriftenreihe Lebensmittelchemie, Lebensmittelqualität. Herausgeber: Fachgruppe, "Lebensmittelchemie und gerichtliche Chemie" in der GDCh. Behr's Verlag, 34-61.

[14] Phenomenex product notes. Size Exclusion Chromatography, Bulk Polymeric Material.

[15] Commission Regulation (EC) No. 508/1999 of 4 March 1999 amending Annexes I to IV of Council Regulation (EEC) No 2377/90 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin.

[16] De Montigny P, Shim J, Pivichny J. (1990). Liquid chromatographic determination of ivermectin in animal plasma with trifluoroacetic anhydride and N-methylimidazole as the derivatisation reagent. Journal of Pharmaceutical and Biomedical Analysis **8**, No. 6, 507-511.

[17] Woelki G, Friedrich S, Hanschmann G, Salzer R. (1997). HPLC fractionation and structural dynamics of humic acids. Fresenius Journal of Analytical Chemistry **357**, 548-552.

CHAPTER 7: CONCLUSION

Under Council Directive 96/23/EC member states are required to develop and implement a plan to monitor, on a routine basis, residues in animals and animal products including fish and fish products [1]. The aim of this work was to develop analytical methods, which could be used for the analysis of veterinary drug residues in farmed salmon tissue under this Directive. These analytical methods will be used to ensure that residues in fish tissue are below the recommended Maximum Residue Level (MRL) where these are set. In the case where no MRL is set for a particular chemical in fish tissue, the method must be capable of detecting any residue present and therefore low limits of detection (LOD) and quantification (LOQ) are required. Additionally under Council Directive 96/23/EC the methods must be validated according to the criteria set out in Annex 1 of Commission Decision 93/256/EEC [2] which help to evaluate the performance of the analytical method.

Oxolinic acid (OA), Oxytetracycline (OTC), Sulphadiazine (SD), Sulphamethoxazole (SMX) and Trimethoprim (TMP) are listed as a Group B substances under Directive 96/23/EC. The EU MRL for OTC in fish tissue is 100μgkg^{-1} [3], while for combined sulphonamides the MRL is 100μgkg^{-1} and 50μgkg^{-1} for TMP [3]. Subsequent to this work being carried out a MRL of 300μgkg^{-1} was set by the EMEA for OA in fish tissue [4]. In this work three High Performance Liquid Chromatography (HPLC) methods were optimised and validated according to the criteria of Commission Decision 93/256/EEC. A separate method was used for the analysis of OA and OTC while a method for the simultaneous analysis of SD, SMX and TMP was developed.

Extraction of the analytes from the tissue samples was achieved using either sonication or ultra turrax homogenisation. OA was extracted into acetonitrile (ACN), at basic pH while OTC, SD, SMX and TMP were extracted into aqueous buffers. The sample clean-up procedures to remove the interfering components involved liquid – liquid extraction using various solutions for OA, SD, SMX and TMP while a Bond Elut C18 solid phase extraction cartridge was used for OTC. For all three methods the samples were pre-concentrated prior to analysis by HPLC with UV or diode array detection (DAD).

The method optimised and validated for OA meets the specified validation criteria, and therefore can be used for routine analysis, under Directive 96/23/EC. Published methods however, [5, 6, 7, 8, 9, 10, 11] lack the complete documentation to meet these criteria. The precision obtained in this method was improved from 5.7% RSD (n=8), [8] to 2.1% RSD (n=5). The LOQ was 11.3μgkg^{-1} (S/N=5) while in the literature, similar values are quoted [8, 9, 10].

It has been reported that interference from the salmon tissue matrix occurs in the chromatogram at the same retention time as that of OA while using UV detection [10] and thus fluorescence detection was recommended instead of UV. However confirmation of the analyte as required by Commission Decision 93/256/EEC can be achieved using DAD. Therefore the chromatography conditions of this method were optimised to obtain resolution using DAD as confirmation.

Subsequent to this work Maxwell *et al.* [12], published a paper reporting the successful use of aqueous dialysis for the analysis of OA in chicken tissue. This technique was compared to an AOAC peer verified method, [10], and it was found that the volumes of $CHCl_3$ used were reduced from 125ml [10] to 25ml [12]. The method optimised in this work however, involves the use of 16mls of $CHCL_3$. While it would be desirable to have eliminated the use $CHCl_3$ altogether, the volume used in this work was considerably reduced compared to the literature methods [10, 12]. Additionally a SEC method capable of detecting residues of OA in salmon tissue at 50μgkg^{-1} was developed as a quick screening technique. While this method did not involve the use of $CHCl_3$ further work is required to establish if the SEC can be used as a sample preparation procedure for quantification of OA in tissue samples.

While complete documentation to meet the validation criteria is lacking in published methods cited for OTC analysis in salmon tissue, [14, 16, 17], the method optimised in this work was found to meet the criteria examined. The recoveries of the method obtained at the MRL of 100μgkg^{-1} was 77.4±1.5% (n=8). While the recoveries reported in the literature were generally higher than 77.4%, it should be noted that these values were quoted at concentration levels above the MRL for OTC in salmon tissue, while using similar methods i.e. 82% at 200μgkg^{-1} [14] and 87.6% at 1000μgkg^{-1} [13]. However, 77.4% recovery obtained meets the validation criteria of 70-110% for spiked tissue samples as set out in the codex [18]. The precision of 1.5% RSD at 100μgkg^{-1} (n=8) was an improvement on values reported in the literature for salmon tissue i.e.

5%RSD at 200μgkg^{-1} (n=6) [14] and 3%RSD at 1000μgkg^{-1} (n=8). The LOQ obtained for this method, 37.6μgkg^{-1}, (S/N=5) which is below the MRL, is also an improvement on values reported in the literature for salmon tissue, i.e. 100μgkg^{-1} [16], 80μgkg^{-1} [14] and 50μgkg^{-1} [17].

While Directive 96/23/EC applies to all farmed finfish, and in Ireland this refers to salmon and trout, the work carried out here involved salmon tissue only. In 1998 Meinertz *et al.* [15] reported a method very similar to that reported in Section 4.5 and 4.8, and demonstrated the ability of this method to be used for six species of fish including salmon, trout, walleye, bass, sturgeon and catfish. The recoveries for salmon was 87.1±3.1% (n=9), and for trout was 85.9±2.5% (n=8) indicating that similar results were obtained for both matrixes. The elution profiles were similar for all species and co-eluting compounds were not detected in the chromatograms of extracts from any species. The chromatographic baselines with the greatest noise were noted for salmon tissue and if this were also to apply to the method described in Section 4.5 and 4.8 then the indication would be that the method could be easily adapted to other fish tissue matrixes including trout. Additionally a method reported in 1996, which is very similar to that described in this work, was recommended and adopted by the AOAC International as an official method and this is also a good indication of its performance [19].

LC-MS was examined for the simultaneous determination of the SD, SMX, and TMP. This study showed that while it was possible to analyse for all three analytes within the same chromatographic run the required sensitivity was not obtained. The examination of MS-MS as opposed to MS has been reported to yield better sensitivity for SD and SMX in kidney matrix [20]. This technique however, was not available during this work. In 1997, a LC-APCI-MS method was reported for the analysis of TMP in tissue samples [21]. This method quoted a LOD of 25μgkg^{-1} and recoveries greater than the required 70%. APCI-MS system was not however available for examination during this work.

In the cited literature (Section 1.5.13) it was found that only HPLC one method examined the simultaneous determination of SD and TMP in salmon tissue, [22], while the simultaneous determination of all three analytes in salmon tissue was not cited in any of the literature methods. Optimisation of the analytical conditions as described by Malisch *et al.* [23] was carried out in this work and a HPLC separation of SD, SMX and TMP was achieved in one analytical run. This method [23] for the analysis of sulphonamides in pork tissue was then adapted successfully to salmon tissue for SD, SMX and TMP. The recoveries of the method obtained at the MRL of 100μgkg^{-1} (n=6), was 92.8±9.7%, 41.1±8.4% and 89.1±8.4% for SD, TMP and SMX respectively. These correlate for SD and SMX, with those reported for pork tissue by Malisch *et al.* [23] i.e. 100% (n=64) for SD and 100.7% (n=9) for SMX. The precision obtained on those results for salmon tissue was an improvement on the same values reported for the pork samples, i.e. 13% RSD for SD and 12.2% RSD for SMX. The LOQ (S/N=5) obtained was 2.6μgkg^{-1}, 8.2μgkg^{-1} and 7.3μgkg^{-1} for SD, SMX and TMP respectively. The values for SD and SMX were lower than any reported in the cited literature for salmon or trout tissue using UV detection, i.e. 100μgkg^{-1} for salmon [22, 24], 125μgkg^{-1} for salmon and 50μgkg^{-1} for trout [25]. Therefore the method reported by Malisch *et al.* [23] for pork tissue was successfully applied to salmon tissue for both SD and SMX. Recoveries for TMP however, were below the required 70%, but even so it may be possible to use this method to screen for TMP while at the same time quantifying SD and SMX in the tissue sample.

The methods discussed so far, which can identify and quantify the specified analytes to the required validation criteria involve many cleanup steps, are labour intensive and are thus time consuming. For OA and sulphonamides six samples can be analysed in two days while for OTC twelve samples can be analysed over a two-day period. Therefore a chemical screening method which can identify veterinary drug residues at the concentration levels of interest was required.

A SEC method with UV detection was successfully developed as a screening method for SD, SMX and OA at 50μgkg^{-1}. This method is capable of screening 16 samples below the required MRL values in one overnight run. When compared to two days for 6 samples for both OA and sulphonamide analysis, while using the conventional methods, the SEC method developed provides definite advantages.

SEC was also examined as a cleanup technique for ivermectin samples followed by analysis with fluorescence detection. This SEC method resulted in adequate separation of the analyte from the matrix components. It was found however, that recoveries obtained were approximately 70% and no recoveries were obtained for concentrations <5μgkg^{-1}. Since this SEC method can screen 18 samples in 1½ days compared to 12 samples in two days by the conventional method, and is also much less labour intensive, then this problem should be addressed in the future.

The three HPLC methods optimised and validated in this work can be successfully used for routine monitoring under Council Directive 96/23/EC. These methods will contribute towards the implementation of a National Monitoring programme in Ireland for farmed fish. Therefore the quality of the fish will be monitored on an ongoing basis and this will increase consumer confidence with regards to food safety. However, further work on these methods involving inter-laboratory comparisons and proficiency testing schemes, where these are available, will be required in the future.

While the HPLC methods examined were validated to the required criteria and can be used routinely, they are for the most part, labour intensive and time consuming. The SEC methods developed in this work however, overcome these difficulties. Samples can be prepared and injected on the SEC system overnight and screening results recorded. It is also possible to use the fraction collector and pre-concentrate the samples for quantification by the conventional analytical techniques. This technique which has been used for pesticide, herbicide and organic pollutant analysis in environmental samples has not been widely applied for analysing veterinary drug residues. The GPC analysis of veterinary drug residues in tissue samples has been addressed in 1982 [26] and 1987 [27] but no publications since this date were obtained from the cited literature.

From the work carried out here however, it is evident that this technique could be successful for both screening and sample cleanup for the analytes examined but additional work is required and should be carried out in the future to determine the full extent of its capabilities.

7.1 References

[1] Council Directive 96/23/EC on measures to monitor certain substances and residues thereof in live animals and animal products.

[2] Annex of Commission Decision 93/256/EEC laying down the methods to be used for detecting residues of substances having a hormonal or thyrostatic action.

[3] Commission Regulation (EC) No. 508/1999 of 4 March 1999 amending Annexes I to IV of Council Regulation (EEC) No 2377/90 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin Annex of Commission Decision 93/256/EEC laying down the methods to be used for detecting residues of substances having a hormonal or thyrostatic action.

[4] EMEA (1998). The European Agency for the Evaluation of Medicinal Products, Veterinary Medicines Evaluation Unit. Committee for veterinary medicinal products, Oxolinic Acid (extension to salmonidae). Summary report. EMEA/MRL/436/98-Final.

[5] Pouliquen H, Pinault L, Le Bris H. (1994). Determination of oxolinic acid in seawater, marine sediment, and Japanese oyster (Crassostrea Gigas) by high-performance liquid chromatography. Journal of liquid chromatography, **17**(4), 929-945.

[6] Ikai Y, Oka H, Kawamura N, Yamada M, Nakazawa H. (1989). Improvement of chemical analysis of antibiotics. XV1. Simple and rapid determination of residual pyridone-carboxylic acid antibacterials in fish using a prepacked amino cartridge. Journal of Chromatography, **477**, 397-406.

[7] Rose MD, Bygrave J, Stubbings GWF. (1998). Extension of multi-residue methodology to include the determination of quinolones in food. Analyst, **123**(12), 2789-2796.

[8] Steffenak I, Hormazabel V, and Yndestad M. (1991). Rapid assay for the simultaneous determination of residues of oxolinic acid and flumequine in fish tissues by high-performance liquid chromatography. Journal of liquid chromatography, **14**(1), 61-70.

[9] Carignan G, Larocque L, Sved S. (1991). Assay of Oxolinic acid residues in salmon muscle by liquid chromatography with fluorescence detection: Interlaboratory study. Journal of the Association of Official Analytical Chemists, **74**, No. 6, 906-909.

[10] Munns R, Turnipseed S, Peenning A, Roybal E, Holland D, Long A, Plakas S. (1998). Liquid chromatographic determination of flumequine, nalidixic acid, oxolinic acid and piromidic acid residues in catfish (*Ictalurus punctatus*). Journal of AOAC International, **81**, No. 4, 825-839.

[11] Bjorklund H. (1990). Analysis of oxolinic acid in fish by high-performance liquid chromatography. Journal of Chromatography, Biomedical Applications, **530**, 75-82.

[12] Eng G.Y, Maxwell R.J, Cohen E, Piotrowski E.G, Fiddler W. (1998). Determination of flumequine and oxolinic acid in fortified chicken tissue using on-line dialysis and high-performance liquid chromatography with fluorescence detection. Journal of Chromatography A, **799**, 249-354.

[13] Bjorklund H. (1988). Determination of oxytetracycline in fish by high-performance liquid chromatography. Journal of Chromatography, **432**, 381-387.

[14] Reimer G, Young L. (1990). Validation of a method for determination of tetracycline antibiotics in salmon muscle tissue. Journal of the Association of Official Analytical Chemists, **73**, No. 5, 813-817.

[15] Meinertz JA, Stehly GR, Gingerich WH. (1998). Liquid Chromatographic determination of oxytetracycline in edible fish fillets from six species of fish. Journal of AOAC International, **81**, No. 4, 702-708.

[16] Iwaki K, Okumura N, Yamazaki M. (1992). Determination of tetracycline antibiotics by reversed phased liquid chromotagraphy with fluoresence detection. Journal of chromatography, **623**, 153-158.

[17] Carignan G, Carrier K, Sved S. (1993). Assay of oxytetracycline residues in salmon muscle by liquid chromatography with ultraviolet detection. Journal of AOAC international, **76**, No. 2, 325-328.

[18] Codex Alimentarius Volume 3, (1994). Section 2: Recommended international code of practice for control of the use of veterinary drugs.

[19] MacNeil JD, Martz VK, Korsrud GO, Salisbury CDC, Oka H, Epstein RL, Barnes CJ. (1996). Chlortetracycline, oxytetracycline, and tetracycline in edible animal tissues, liquid chromatographic method: Collaborative study. Journal of AOAC International. **79**: (2) 405-417.

[20] Porter S. (1994). Confirmation of sulphonamide residues in kidney tissue by Liquid Chromatography-Mass Spectrometry. Analyst, **119**, 2753-2756.

[21] Cannavan A, Hewitt SA, Floyd SD, Kennedy DG. (1997). Determination of trimethoprim in tissues using liquid chromatography-thermospray mass spectrometry. Analyst **122** (11), 1379-1381.

[22] Gentleman M, Burt H. (1993). High-performance liquid chromatographic determination of sulphadiazine and trimethoprim in Chinook salmon muscle tissue. Journal of Chromatography, **633**, 105-110.

[23] Malisch R, Bourgeois B, and Lippold R. (1992). Multiresidue analysis of selected chemotherapeutics and antiparasitics. Part 1: Determination of sulphonamides and their N4-Acetyl-metabolites, chloramphenicol and nicarbazin in meat. Deutsche Lebensmittel-Rundschau, 88, Jahrg, Heft **7**, 205-216.

[24] Reimer G, Suarez A. (1992). Liquid chromatographic confirmatory method for five sulphonamides in salmon muscle tissue by matrix solid phase dispersion. Journal of AOAC International **75**, No. 6, 979-981.

[25] Ikai Y, Oka H, Kawamura N, Hayakawa J, Yamada M. (1991). Application of an amino cartridge to the determination of residual sulphonamide antibacterials in meat, fish and egg. Journal of Chromatography, **541**, 393-400.

[26] Holtmannspötter H, Their H. (1982). Analysemethode für Rückstände von sechs Sulphonamiden und Chloramphenicol durch Gaschromatographie an Glaskapillaren. Deutsche Lebensmittel-Rundschau, **78**, 347-350.

[27] Petz M, Meetschen U. (1987). Untersuchungen zur Gelchromatographie (GPC) als Reinigungsverfahren in der Rückständsanalytik von Tierarzneimitteln. Z. Lebensm. Unters. Forsch, **184**, 85-90.

APPENDIX 1: FINAL OPTIMISED ANALYTICAL METHODS

ANALYSIS OF OXYTETRACYCLINE IN FISH TISSUE BY HPLC

1.1 MATERIALS

1.1.1 Chemicals

Citric acid monohydrate	GPR grade -BDH
Oxalic acid dihydrate	BDH
Disodium EDTA dihydrate	May and Baker
Disodium hydrogen phosphate dodecahydrate	Merck
Acetonitrile	Pestiscan grade- Lab Scan
N, N-Dimethylformamide	Riedel-de Haen
Methanol	HPLC grade -Lab Scan
Oxytetracycline	(O5875), 99% purity-Sigma/Aldrich
Water	All water used is deionised and purified.

1.1.2 Reagents

1.1.2.1 0.1M citric acid	Weigh precisely 2.1g citric acid monohydrate into a 250cm^3 beaker. Add approx. 50cm^3 water to dissolve. Transfer quantitatively to a 100cm^3 volumetric flask. Wash the beaker with water and transfer the washings to the flask making up to a volume of 100cm^3. Can be stored for up to 12 months at room temperature.

1.1.2.2 0.2M disodium hydrogen phosphate	Weigh precisely 3.58g disodium hydrogen phosphate into a 250cm^3 beaker. Add approx. 25cm^3 water to dissolve. Transfer quantitatively to a 50cm^3 volumetric flask. Wash the beaker with water and transfer the washings to the flask making up to a volume of 50cm^3. Can be stored for up to 12 months at room temperature.
1.1.2.3 McIlvaine buffer (pH 3.0)	Volumetrically transfer 79.5cm^3 of 0.1M citric acid and 20.5cm^3 of 0.2M disodium hydrogen phosphate to a stoppered conical flask. Can be stored for up to 12 months at room temperature.
1.1.2.4 0.1M Na_2EDTA McIlvaine buffer (pH 4.0)	Precisely weigh 37.224g of $Na_2EDTA.2H_2O$ into a 500cm^3 beaker. Add approx. 250cm^3 water to dissolve. Adjust the pH to (4.0±0.1) with McIlvaine buffer (pH 3.0). Transfer quantitatively to a 1000cm^3 volumetric flask Wash the beaker with water and transfer the washings to the flask making up to a volume of 1000cm^3. Can be stored for up to 12 months at room temperature.
1.1.2.5 0.01M Oxalic acid	Precisely weigh 1.26g Oxalic acid into a 500cm^3 beaker. Add approx. 250cm^3 water to dissolve. Transfer quantitatively to a 1000cm^3 volumetric flask. Wash the beaker with water and transfer the washings to the flask making up to a volume of 1000cm^3. Can be stored for up to 12 months at room temperature.

1.1.2.6 Mobile phase	Volumetrically, using a 500cm^3 graduated cylinder, prepare a solution in the ratio of ACN: DMF: Oxalic acid (0.01M), 23:10:67. Filter through a millipore 0.45μm filter and degas by sonication or by helium purge.

1.1.3 Standards

1.1.3.1 Stock standard, 1000ppm. Standards are made up on a weight/ weight basis.	Weigh precisely OTC (25)mg into a 25cm^3 volumetric flask. Dissolve in MeOH and sonicate for 5 minutes. Make up to volume with MeOH. Can be stored between (2.5-7)°C for 1 month.
1.1.3.2 Fortification standard, 10ppm. Standards are made up on a weight/ weight basis.	Pipette 1cm^3 of the stock standard to a 100cm^3 volumetric flask. Dilute to the mark with MeOH and sonicate. Can be stored between (2.5-7)°C for 1 month.
1.1.3.3 Working standards for calibration plot, 50, 75, 100, 125, 150μgkg^{-1} equivalent. Standards are made up on a weight/ weight basis.	Pipette 150μl, 225μl, 300μl, 375μl and 450μl of the fortification standard (1.3.2) into five separate 10cm^3 volumetric flasks. Dilute up to the mark with 0.01M oxalic acid and sonicate. Store in the fridge between (2.5-7)°C for 1 month.

1.1.4 EQUIPMENT

1.1.4.1 Preparation of reagents

Balance to 5 decimal places	Sartorius
Beakers	Glass, 250cm^3, 500cm^3
Volumetric flasks	Glass, 10±0.03cm^3, 25±0.04cm^3, 50±0.4cm^3, 100±0.8cm^3, 1000±0.4cm^3
Graduated cylinders	Glass, 100cm^3, 500cm^3
Conical flask	Glass and stoppered, 250cm^3

pH meter	WTW
0.45μm filter	Millipore, disposable
Pipettes	Gilson and Micromann

Sample Preparation

Scalpel and blades	Sterile and disposable
Hi - speed homogeniser	Breda Scientific
Sample jars	Glass, solvent washed

Extraction Process

Balance to 3 decimal places	Sartorious
Centrifuge tubes	Polypropylene, 15cm^3 and 50cm^3
Homogeniser	Ultra turrax
Vortex mixer	Fisons
Centrifuge	Sorvall T6000
Bond Elut SPE cartridges	Varian C18, 500mg- 3cm^3
Vacuum-elut box	Varian
Bond Elut accessories	Adapters, polypropylene syringe barrels, 5cm^3
Filter papers	Whatman No. 1 -30mm diameter
Test tubes	Glass, brown 10cm^3
Evaporation manifold with nitrogen and water bath	Ori block -08-3 Techne + Si3 sample concentrator water bath at 25±5^{0}C
0.45μm filters	Ministart, disposable
HPLC insert vials	Disposable glass, 300μl
HPLC vials	Glass, 4cm^3
Pasteur pipettes	Glass

1.1.4.2 HPLC system

Pump	Shimadzu LC - 6A or equivalent
Column oven	Shimadzu CTO - 6A or equivalent
Auto injector and system controller	Shimadzu SCL - 6B or equivalent
UV Detector	Shimadzu SPD - 6AV or equivalent

Data handling	Maxima 820 software – Millipore or equivalent
Columns	Waters Spherisorb S5ODS2 5μm (250 x 4.6)mm column and Phenomenex Sphereclone C18 (30 x 4.6)mm, 5μm all guard columns

1.1.4.3 HPLC conditions

Mobile phase	See section 1.1.2.6
Flow rate	2.0 ml/minute
Run time	8 minutes
Injection volume	50 μl in 0.01M oxalic acid
Oven Temperature	25 ± 5°C
Detector	UV @ 365nm and 0.002 AUFS

1.2. PROCEDURE

1.2.1 Sample throughput

1.2.1.1 A sample analysis batch comprises of:

- One reagent blank: This will be treated exactly the same as the sample except that it will contain no tissue sample.
- Two fortified tissue samples at 100μgkg^{-1} (MRL)
- 9 test samples

1.2.2 Sample storage

1.2.2.1 All tissue samples were stored in MeOH rinsed sample jars and kept in a freezer at –20 ± 5°C prior to analysis

1.2.2.2 Using the homogeniser the tissue was homogenised and 3±0.1g of the homogenate was used for each analysis.

1.2.3 Spiking the tissue sample

1.2.3.1 Accurately weigh 30μl of the fortifying solution (section 1.3.2) using the Micromann pipette into a 50ml polypropylene tube. Record the weight on the sample sheet.

1.2.3.2 Evaporate the MeOH to dryness at room temperature under a gentle stream of nitrogen.

1.2.3.3 Accurately weigh 3±0.1g of the homogenised blank tissue sample into this 50ml centrifuge tube. Record the weight on the sample sheet.

1.2.3.4 Vortex mix for 1 minute.

1.2.3.5 Allow to sit for 15 minutes prior to extraction.

1.2.4 Extraction

1.2.4.1 Weigh out 3.0 ± 0.1g of the test sample accurately into the 50cm^3 centrifuge tube. Record the weights on the sample sheet

1.2.4.2 Using the Gilson pipette transfer cold McIlvaine buffer, pH 4.0 (12cm^3) to the tubes containing the sample and vortex mix for 30 seconds followed by sonication for 5 minutes.

1.2.4.3 Centrifuge for 10 minutes at 2700 rpm and decant the supernatant into another 50cm^3 polypropylene tube.

1.2.4.4 Repeat steps 5.5.2 and 5.5.3 with a further 12 and 6cm^3 of the extraction buffer.

1.2.4.5 Combine the extracts and vortex mix for 30 seconds to ensure an even distribution of the analyte in the aqueous extract

1.2.4.6 Centrifuge the combined supernatants for a further 10 minutes at 2700 rpm and allow to sit in the fridge at (2.5-7)°C overnight.

1.2.4.7 Weigh the combined extracts to 3 decimal places and centrifuge again for 10 minutes at 2700 rpm

1.2.4.8 Assemble the Vac-elut box connected to a vacuum line, C18 Bond-Elut cartridges, adaptors and 5cm^3 reservoirs fitted with Whatmann No. 1 filter paper.

1.2.4.9 Condition the cartridge with 0.01M methanolic oxalic acid (5cm^3) followed by McIlvaine buffer pH 4.0 (5cm^3). Do not allow the columns to run dry prior to loading the sample.

1.2.4.10 Pass as much of the combined sample extract as possible through the cartridge using a glass pipette tip.

1.2.4.11 Weight of the remainder of the extract in the centrifuge tube and record to three decimal places on the sample sheet.

1.2.4.12 Wash the cartridge with water (30mls), and elute the analyte from the cartridge with 0.01M methanolic oxalic acid (5cm^3) using the Gilson pipette into a brown glass test tube.

1.2.4.13 Evaporate to dryness at (25 ±5)°C under a gentle stream of nitrogen

1.2.4.14 Re-dissolve in 0.01M oxalic acid (0.5 ±0.05)cm^3.

1.2.4.15 Filter using a Ministart 0.45μm filter

1.2.4.16 Collect the filtrate into a small insert vial and place this into a 4ml vial prior to analysis by RPHPLC.

1.3 Preparation of working standards

1.3.1 Transfer the five working standards (section 1.1.3) for the calibration plot to the HPLC vials.

1.4 HPLC Analysis

1.4.1 Condition the HPLC column as required prior to analysis

1.4.2 Purge the auto-injector as required prior to analysis

1.4.3 Inject the sample batch in the following order:

- a 100μgkg^{-1} equivalent standard
- reagent blank
- working standards for the standard curve
- fortified control sample to assess recovery
- a 100μgkg^{-1} equivalent standard
- test samples
- a 100μgkg^{-1} equivalent standard
- include a 100μgkg^{-1} equivalent standard every five samples

1.4.4 On completion of the analysis condition the column and HPLC for storage

1.5 RESULTS

1.5.1 Results are expressed in μg OTC per kilogram of tissue (μgkg^{-1}, ppb)

1.5.2 Recoveries

1.5.2.1 Recovery data from the fortified controls is used to determine the acceptance, or rejection, of an analytical batch.

1.5.2.2 If the batch % recovery is outside the validated performance criteria, (Chapter 4, section 4.9.6), the batch recovery obtained is used for screening analysis, while for confirmatory analysis the batch must be re-analysed.

1.5.2.3 % recovery (recovery factor) is calculated as follows:

- The peak height obtained for the fortified sample is substituted into the equation of the best-fit calibration line and a concentration in $\mu g kg^{-1}$ is estimated (Example shown in Chapter 4, section 4.9.4)
- $$\%\text{recovery} = \frac{\text{concentration recovered ug/kg}}{\text{concentration added ug/kg}} \times 100$$

1.6 Peak Identification

1.6.1 Peak retention time is used as the criteria for identifying a positive or negative sample.

1.6.2 The mean retention time of the OTC peak in the standards sandwiching the samples is calculated.

1.6.3 Identification of the OTC in the test and fortified samples is made by comparing the retention times of the peaks found in the samples with the mean retention time from the standards. The peak present in the sample must be within ±5% of the mean retention time of the standards for the sample to be declared suspect positive. Otherwise the sample is reported negative.

1.6.4 If a peak is present in the reagent blank at or above the LOD and within ±5% of the mean retention time for the standards, the batch must be re-analysed.

1.6.5 If any standard or fortified control contains no OTC peak then the batch must be re-analysed.

1.7 Quantification

1.7.1 All samples containing a suspect peak with a response less than $20 \mu g kg^{-1}$ (LOD) are reported as negative. OTC may be present but not detectable.

1.7.2 Samples with a response greater than $20 \mu g kg^{-1}$ (LOD) are selected for confirmation.

1.7.3 The concentration is calculated as follows:

- The peak height obtained for the test sample is substituted into the equation of the best-fit calibration line and a concentration in μgkg^{-1} is estimated (Example is shown in Chapter 4, section 4.9.4)
- All samples are corrected for % recovery using the specified recovery factor (section 1.5.2).

1.7.4 Samples confirmed with a value above the LOQ but below the MRL are reported as negative with their confirmed concentration to 1 decimal place

1.7.5 Samples confirmed with a value above the MRL are reported as positive with their confirmed concentration to 1 decimal place

FLOW DIAGRAM OF THE METHOD USED FOR THE DETERMINATION OF OXYTETRACYCLINE RESIDUES IN FARMED FISH TISSUES

Homogenised fish tissue 3 g

Extract with 0.1 M Na_2EDTA McIlvaine Buffer (pH 4) 12, 12, 6mls

Centrifuge each extract for 10 minutes at 2700 rpm.

Combine the supernatants and vortex mix for 1 minute followed by centrifugation for 10 minutes.

Allow to sit in the fridge overnight.

Condition the Bond Elut SPE cartridge with 0.01M methanolic oxalic acid (5ml) and Buffer pH 4 (5ml).

Pass the sample through the SPE cartridge.

Wash the SPE cartridge with water (30ml)

Elute with 0.01M methanolic oxalic acid (5ml).

Evaporate to dryness at 25°C under nitrogen.

Reconstitute to 0.5ml with the 0.01M oxalic acid.

Analysis by RPHPLC

APPENDIX 2: FINAL OPTIMISED ANALYTICAL METHODS

ANALYSIS OF OXOLINIC ACID IN FISH TISSUE BY HPLC

2.1. MATERIALS

2.1.1 Chemicals

Orthophosphoric acid	Riedel - deHaen 85%
Ammonia	35% BDH
Chloroform	Super purity, Romil
Sodium hydroxide pellets	General purpose, BDH
Acetonitrile	Pestiscan grade- Lab Scan
Sodium chloride:	Extra pure, Merck
Tetrahydrofuran	HPLC grade -Lab Scan
Oxolinic acid (O0877)	99% purity-Sigma/Aldrich
Water	All water used is deionised and purified.

2.1.2 Reagents

2.1.2.1 0.03M sodium hydroxide	Weigh precisely 0.12g of NaOH into a 250cm^3 beaker. Add approx. 50cm^3 water to dissolve. Transfer quantitatively to a 100cm^3 volumetric flask. Wash the beaker with water and transfer the washings to the flask making up to a volume of 100cm^3. Can be stored for up to 12 months at room temperature.
2.1.2.2 5M Sodium Chloride	Weigh precisely 14.62g NaCl into a 250cm^3 beaker. Add approx. 25cm^3 water to dissolve. Transfer quantitatively to a 50cm^3 volumetric flask. Wash the beaker with water and transfer the washings to the flask making up to a volume of 50cm^3. Can be stored for up to 12 months at room temperature.

2.1.2.3 0.02M Phosphoric acid	Weigh precisely 3.92g H_3PO_4 into a $1000cm^3$ volumetric flask containing approx. $100cm^3$ water. Make up to a volume of $1000cm^3$ with water.
2.1.2.4 Mobile phase	Volumetrically, using a $500cm^3$ graduated cylinder, prepare a solution in the ratio of H_3PO_4 (0.02M): THF: IPA, 78:17:5. Filter through a millipore 0.45μm filter and degas by sonication or by helium purge.

2.1.3 Standards

2.1.3.1 Stock standard, 1000ppm. Standards are made up on a weight/ weight basis.	Weigh precisely OA (25)mg into a $25cm^3$ volumetric flask. Dissolve in 0.03M NaOH and sonicate for 5 minutes. Make up to volume with 0.03M NaOH. Can be stored between (2.5-7)°C for 1 month.
2132 Fortification standard, 10ppm. Standards are made up on a weight/ weight basis.	Pipette $1cm^3$ of the stock standard to a $100cm^3$ volumetric flask. Dilute to the mark with ACN and sonicate for 5 minutes. Can be stored between (2.5-7)°C for 1 month.
2.1.3.3 Working standards for calibration plot, 25, 50, 75, 100, 125 μgkg^{-1} equivalent. Standards are made up on a weight/ weight basis.	Pipette 75μl, 150μl, 225μl, 300μl and 375μl of the fortification standard (2.1.3.2) into five separate $10cm^3$ volumetric flasks. Dilute up to the mark with the mobile phase and sonicate. Make up fresh prior to use.

2.1.4 EQUIPMENT

2.1.4.1 Preparation of reagents

Balance to 5 decimal places	Sartorius or equivalent
Beakers	Glass, 250cm^3
Volumetric flasks	Glass, 10±0.03cm^3, 25±0.04cm^3, 50±0.4cm^3, 100±0.8cm^3, 1000±0.4cm^3
Graduated cylinders	Glass, 100cm^3, 500cm^3
0.45μm filter	Millipore, disposable
Pipettes	Gilson and Micromann or equivalent

Sample Preparation

Scalpel and blades	Sterile and disposable
Hi - speed homogeniser	Breda Scientific
Sample jars	Glass, solvent washed

Extraction Process

Balance to 3 decimal places	Sartorious
Centrifuge tubes	Polypropylene, 50cm^3
Vortex mixer	Fisons
Centrifuge	Sorvall T6000
Test tubes	Glass, pyrex 10cm^3, 25cm^3
Evaporation manifold with nitrogen and water bath	Ori block -08-3 Techne + Si3 sample concentrator water bath at (40 ± 5)^{0}C
0.45μm filters	Ministart, disposable
HPLC vials	Glass, disposable 4cm^3 and 300μl insert vials
Pasteur pipettes	Glass

2.1.4.2 HPLC system

Pump	Shimadzu LC - 6A
Column oven	Shimadzu CTO - 6A
Auto injector and system controller	Shimadzu SCL - 6B
UV Detector	Shimadzu SPD - 6AV

Data handling	Maxima 820 software – Millipore
Columns	Lichrospher RP 18 - 5μm Endcapped (125x4.6)mm Lichrospher RP-18 Endcapped (7.5x4.6)mm guard column

2.1.4.3 HPLC conditions

Mobile phase	See section 2.1.2.4
Flow rate	2.0 ml/minute
Run time	6 minutes
Injection volume	50μl
Oven Temperature	35 ± 5°C
Detector	UV @ 262nm and 0.05AUFS

2.2. PROCEDURE

2.2.1 Sample throughput

2.2.1.1 A sample analysis batch comprises of:

- One reagent blank: This will be treated exactly the same as the sample except that it will contain no tissue sample.
- Two fortified tissue samples at $75\mu gkg^{-1}$
- 10 test samples

2.2.2 Sample storage

2.2.2.1 All tissue samples were stored in MeOH rinsed sample jars and kept in a freezer at –20 ± 5°C prior to analysis

2.2.2.2 Using the homogeniser the tissue was homogenised using the ultra turrax homogeniser.

2.2.3 Spiking the tissue sample

2.2.3.1 Accurately weigh 3 ± 0.1g of the homogenised blank tissue sample into this 50ml centrifuge tube. Record the weight.

2.2.3.2 Weigh 225μl of the fortifying solution (section 2.1.3.2) using the Micromann pipette into a 50ml polypropylene tube. Record the weight.

2.2.3.3 Vortex mix for 30 seconds

2.2.3.4 Allow to sit for 15 minutes prior to extraction.

2.2.4 Extraction

2.2.4.1 Weigh out 3.0 ± 0.1g of the homogenised test sample accurately into the 50cm^3 centrifuge tube. Record the weights on the sample sheet

2.2.4.2 Using the Gilson pipette transfer ammonia solution (1cm^3) and ACN (5.7cm^3) to the tubes containing the sample and vortex mix for 1 minute followed by sonication for 5 minutes.

2.2.4.3 Centrifuge for 4 minutes at 2500rpm and weigh the supernatant.

2.2.4.4 Using the Gilson pipette remove the supernatant (5cm^3) to a 25cm^3 pyrex test tube and record the weight of this. The calculations for this section of the procedure were carried out on a weight/weight basis.

2.2.4.5 Add 5M NaCl (3cm^3) using the Gilson pipette and vortex mix for 20 seconds.

2.2.4.6 Centrifuge for 2 minutes at 1500r.p.m.

2.2.4.7 Transfer the lower NaCl layer to a 25cm^3 pyrex tube using a glass pasteur pipette.

2.2.4.8 Repeat the extraction with a further 3cm^3 of 5M NaCl and centrifuge for 2 minutes at 1500r.p.m.

2.2.4.9 Remove the lower aqueous NaCl layer using the glass pasteur pipette, and transfer to the 25ml test tube containing the initial NaCl extract.

2.2.4.10 Vortex mix the aqueous layers for 20 seconds and discard the ACN layer.

2.2.4.11 Add 85% H_3PO_4 (2cm^3) and $CHCl_3$ (8cm^3) to the 25cm^3 tube containing the combined aqueous NaCl layers.

2.2.4.12 Vortex mix for 20 seconds and centrifuge for 3 minutes at 1500r.p.m.

2.2.4.13 Transfer the lower $CHCl_3$ layer to another 25cm^3 pyrex tube, using the glass pasteur pipette. Take care not to remove any of the aqueous solution from the test tube.

2.2.4.14 Re-extract the upper aqueous layer with a further 8cm^3 of $CHCl_3$.

2.2.4.15 After centrifugation remove the lower $CHCl_3$ layer from beneath the aqueous layer using the glass pasteur pipette, this time taking care not to leave any of the $CHCl_3$ behind.

2.2.4.16 Combine both $CHCl_3$ extracts and vortex mix for 30 seconds followed by centrifugation at 1500r.p.m. for 2 minutes.

2.2.4.17 Using a pipette, and taking care not to take any aqueous solution, which may be in the test tube, transfer 4cm^3 of the $CHCl_3$ solution to a disposable glass vial.

2.2.4.18 Evaporate to dryness under a gentle stream of nitrogen at 40 ± 5°C.

2.2.4.19 Add 0.75cm^3 of the mobile phase to this vial and vortex mix for 1 minute.

2.2.4.20 Filter using a Ministart 0.45μm filter

2.2.4.21 Collect the filtrate into a small insert vial and place this into a 4ml vial prior to analysis by RPHPLC.

2.3 Preparation of working standards

2.3.1 Transfer the five working standards of 25, 50, 75, 100, 125 μgkg^{-1} equivalent, (section 2.1.3) for the calibration plot to the HPLC vials.

2.4 HPLC Analysis

2.4.1 Condition the HPLC column as required prior to analysis

2.4.2 Purge the auto-injector as required prior to analysis

2.4.3 Inject the sample batch in the following order:

- a 75 μgkg^{-1} equivalent standard
- reagent blank
- working standards for the standard curve
- fortified control sample to assess recovery
- a 75 μgkg^{-1} equivalent standard
- test samples
- a 75 μgkg^{-1} equivalent standard
- include a 75 μgkg^{-1} equivalent standard every five samples

2.4.4 On completion of the analysis condition the column and HPLC for storage

2.5 RESULTS

2.5.1 Results are expressed in μg OA per kilogram of tissue (μgkg^{-1}, ppb)

2.5.2 Recoveries

2.5.2.1 Recovery data from the fortified controls is used to determine the acceptance, or rejection, of an analytical batch.

2.5.2.2 If the batch % recovery is outside the validated performance criteria, (Chapter 3, section 3.7.6), the batch recovery obtained is used for screening analysis, while for confirmatory analysis the batch must be re-analysed.

2.5.2.3 % recovery (recovery factor) is calculated as follows:

- The peak height obtained for the fortified sample is substituted into the equation of the best-fit calibration line and a concentration in μgkg^{-1} is estimated (Example shown in Chapter 3, section 3.7.4)
- $\%\text{recovery} = \dfrac{\text{concentration recovered ug/kg}}{\text{concentration added ug/kg}} \times 100$

2.6 Peak Identification

2.6.1 Peak retention time is used as the criteria for identifying a positive or negative sample.

2.6.2 The mean retention time of the OA peak in the standards sandwiching the samples is calculated.

2.6.3 Identification of the OA in the test and fortified samples is made by comparing the retention times of the peaks found in the samples with the mean retention time from the standards. The peak present in the sample must be within ±5% of the mean retention time of the standards for the sample to be declared suspect positive. Otherwise the sample is reported negative.

2.6.4 If a peak is present in the reagent blank at or above the LOD and within ±5% of the mean retention time for the standards, the batch must be re-analysed.

2.6.5 If any standard or fortified control contains no OA peak then the batch must be re-analysed.

2.7 Quantification

2.7.1 All samples containing a suspect peak with a response less than $10\mu gkg^{-1}$ (LOD) are reported as negative. OA may be present but not detectable.

2.7.2 Samples with a response greater than $10\mu gkg^{-1}$ (LOD) are selected for confirmation.

2.7.3 The concentration is calculated as follows:

- The peak height obtained for the test sample is substituted into the equation of the best-fit calibration line and a concentration in μgkg^{-1} is estimated (Example is shown in Chapter 3, section 3.7.4)
- All samples are corrected for % recovery using the specified recovery factor (section 2.5.2).

2.7.4 Samples confirmed with a value above the LOD but below the LOQ are reported as negative with their confirmed concentration to 1 decimal place

2.7.5 Samples confirmed with a value above the LOQ are reported as positive with their confirmed concentration to 1 decimal place

FLOW DIAGRAM OF THE METHOD USED FOR THE DETERMINATION OF OXOLINIC ACID RESIDUES IN FARMED FISH TISSUES

Muscle (3g) Homogenised

Spike with standard (275µl) in ACN.
Vortex mix for 30 seconds in a 50ml centrifuge tube.

Add NH_3 (1ml).
Add ACN(5.7ml).
Mix for 1 minute at the whirlimixer.
Centrifuge for 4 minutes at 2500r.p.m.

Supernatant (5 ml) of ACN to a 10 ml pyrex tube.

Discard the residue

Add 5 M NaCl (3ml).
Vortex mix for 20 seconds.
Centrifuge for 2 minutes at 1500r.p.m.
Remove the lower aqueous layer to a 25ml pyrex tube.
Repeat the extraction and combine the aqueous layers.

Discard the upper ACN layer

Add 85% H_3PO_4 (2ml) and $CHCl_3$ (8ml).
Vortex mix for 20 seconds
Centrifuge for 3 minutes at 1500r.p.m.
Transfer the lower $CHCL_3$ layer to another 25ml pyrex test tube.
Repeat the extraction of the aqueous layer.

Discard the H_2O

Combine the two $CHCl_3$ layers.
Vortex mix for 30 seconds.
Transfer 4ml of the $CHCl_3$ to a disposable glass vial.
Evaporate to dryness at 40°C.
Dissolve in mobile phase (0.75ml).

HPLC Analysis

APPENDIX 3: FINAL OPTIMISED ANALYTICAL METHODS

ANALYSIS OF SULPHADIAZINE, SULPHAMETHOXAZOLE, AND TRIMETHOPRIM IN FISH TISSUE BY HPLC

3.1. MATERIALS

3.1.1 Chemicals

Ethyl acetate	Super purity, Lab Scan
Anhydrous sodium sulphate	Super purity, Merck This was placed in the muffle furnace at 550ºC overnight before use.
Methyl tert butyl ether	Super purity, Lab Scan
Acetonitrile	HPLC grade- Lab Scan
Citric acid monohydrate	GPR grade -BDH
Disodium hydrogen phosphate dihydrate	Merck
Sodium hydroxide pellets	General purpose, BDH
Sodium acetate	General purpose, BDH
Glacial acetic acid	99.8% purity, Riedel-de Haen
Sodium Chloride	Reagent grade, Riedel-de Haen
Isohexane	Pestican grade -Lab Scan
Sulphadiazine (S6307)	99% purity-Sigma/Aldrich
Sulphamethoxazole (S7507)	99% purity-Sigma/Aldrich
Trimethoprim (T7883)	99% purity-Sigma/Aldrich
Ethidimuron (45474)	Pestanal- Riedel-deHaen
Water	All water used is deionised and purified.

3.1.2 Reagents

3.1.2.1 5M Sodium Hydroxide	Weigh precisely 20g NaOH into a 250cm^3 beaker. Add approx. 75cm^3 water to dissolve. Transfer quantitatively to a 100cm^3 volumetric flask. Wash the beaker with water and transfer the washings to the flask making up to a volume of 100cm^3. Can be stored for up to 12 months at room temperature.
3.1.2.2 Buffer pH 6.0: 0.1M citric acid and 0.1M Na_2HPO_4	Weigh precisely 21g of citric acid monohydrate and 18g of $Na_2HPO_4.2H_2O$ in about 900ml water. Adjust the pH to 6.0 with 5M NaOH. Transfer quantitatively to a 1000cm^3 volumetric flask. Wash the beaker with water and transfer the washings to the flask making up to a volume of 1000cm^3. Can be stored for up to 12 months between (2.5-7)°C.
3.1.2.3 0.02M CH_3COONa	Precisely weigh 2.72g CH_3COONa into a 1000cm^3 beaker. Add approx. 800cm^3 water to dissolve. Adjust to pH = 3.5 with glacial acetic acid. Transfer quantitatively to a 1000cm^3 volumetric flask. Wash the beaker with water and transfer the washings to the flask making up to a volume of 1000cm^3. Can be stored for up to 12 months at room temperature.
3.1.2.4 Mobile phase A	Volumetrically, using a 500cm^3 graduated cylinder, prepare a solution in the ratio of ACN: H_2O: TEA, 67:27:6. Filter through a millipore 0.45μm filter and degas by sonication or by helium purge.

3.1.2.5	Mobile phase B	0.02M CH_3COONa, the pH was adjusted to 3.5 with glacial acetic acid (section 3.1.2.3)
3.1.2.6	Mobile phase Gradient	The gradient started with 95% B, constant for 5 minutes, then the gradient to 85% B over 1 minute, this was held constant for 12 minutes and then back to 95% over two minutes, constant for 5 minutes.

3.1.3 Standards

3.1.3.1	Stock standard, 1000ppm. Standards are made up on a weight/ weight basis.	Weigh precisely SD, SMX, TMP 25mg into a 25cm^3 volumetric flask. Dissolve in ACN and sonicate for 5 minutes. Make up to volume with ACN. Can be stored between (2.5-7)°C for 1 month.
3.1.3.2	Internal standard, 1000ppm Standards are made up on a weight/ weight basis	Weigh accurately EDM 25mg into a 25cm^3 volumetric flask. Dissolve in ACN and sonicate for 5 minutes. Make up to volume with ACN. Can be stored between (2.5-7)°C for 1 month.
3.1.3.3	Fortification standard, 10ppm. Standards are made up on a weight/ weight basis.	Pipette 1cm^3 of the stock standard to a 100cm^3 volumetric flask. Dilute to the mark with ACN and sonicate. Can be stored between (2.5-7)°C for 1 month.
3.1.3.4	Fortification Internal standard, 100ppm Standards are made up on a weight/ weight basis	Pipette 1cm^3 of the stock internal standard to a 100cm^3 volumetric flask. Dilute to the mark with ACN and sonicate. Can be stored between (2.5-7)°C for 1 month.

3.1.3.5	Working standards for calibration plot, 50, 75, 100, 125, 150µgkg^{-1} equivalent. Standards are made up on a weight/ weight basis.	Pipette 150µl, 225µl, 300µl, 375µl and 450µl of the fortification standard (1.3.2) into five separate 10cm^3 volumetric flasks. Add 300µl of the fortification internal standard to each flask. Dilute up to the mark with 0.02M CH$_3$COONa and sonicate. Store in the fridge between (2.5-7)°C for 1 month.

3.1.4 EQUIPMENT

3.1.4.1 Preparation of reagents

Balance to 5 decimal places	Sartorius or equivalent
Beakers	Glass, 250cm^3, 1000cm^3
Volumetric flasks	Glass, 10±0.03cm^3, 25±0.04cm^3, 50±0.4cm^3, 100±0.8cm^3, 1000±0.4cm^3
Graduated cylinders	Glass, 25±0.37cm^3, 100±0.75cm^3, 500±3.75cm^3
pH meter	WTW or equivalent
0.45µm filter	Millipore, disposable
Pipettes	Gilson and Micromann or equivalent

Sample Preparation

Scalpel and blades	Sterile and disposable
Hi - speed homogeniser	Breda Scientific
Sample jars	Glass, solvent washed

Extraction Process

Balance to 3 decimal places	Sartorious or equivalent
Homogeniser	Ultra turrax or equivalent
Vortex mixer	Fisons or equivalent
Centrifuge	Sorvall T6000 or equivalent
Test tubes	Glass pyrex, 25cm^3
Evaporation manifold with nitrogen and water bath	Ori block -08-3 Techne + Si3 sample concentrator water bath at 25^{0}C or equivalent

Beakers	Glass, 250cm^3
Funnels	Glass
Graduated cylinder	Glass, 100cm^3
Conical flask	Glass, 250cm^3
Filter paper	15cm diameter, Whatmann GF/C
Separating funnel	Glass, 250cm^3
Silanised glass wool	
Round bottom flask	Glass, 250cm^3
0.45μm filters	Ministart, disposable
HPLC insert vials	Disposable glass, 300μl
HPLC vials	Glass, 4cm^3
Pasteur pipettes	Glass

3.1.4.2 HPLC system

Pump	Shimadzu LC - 6A
Column oven	Shimadzu CTO - 6A
Auto injector and system controller	Shimadzu SCL - 6B
Detector	HP DAD Series 1100
Data handling	HP chemstations Version A.06.
Columns	Waters Spherisorb S5ODS2 5μm (250 x 4.6)mm column

3.1.4.3 HPLC conditions

Mobile phase	See Section 3.1.2.6
Flow rate	2.0 ml/minute
Run time	20 minutes
Injection volume	100μl in 0.02M CH_3COONa
Oven Temperature	40 ± 5°C
Detector	DAD at 265nm, 262nm, 267nm and 276nm for SD, TMP, SMX and EDM respectively.

3.2. PROCEDURE

3.2.1 Sample throughput

3.2.1.1 A sample analysis batch comprises of:

- One reagent blank: This will be treated exactly the same as the sample except that it will contain no tissue sample.
- One fortified tissue samples at the MRL
- 4 test samples

3.2.2 Sample storage

3.2.2.1 All tissue samples were stored in MeOH rinsed sample jars and kept in a freezer at –20 ± 5°C prior to analysis

3.2.2.2 Using the homogeniser the tissue was homogenised and 15±0.1g of the homogenate was used for each analysis.

3.2.3 Spiking the tissue sample

3.2.3.1 Accurately weigh 15±0.1g of the homogenised blank tissue sample into a 250ml beaker. Record the weight on the sample sheet.

3.2.3.2 Accurately weigh 150μl of the fortifying solution and the fortifying internal standard solution (section 1.3.2) using the Micromann pipette to the tissue sample. Record the weight on the sample sheet.

3.2.3.3 Allow to sit for 15 minutes prior to extraction.

3.2.4 Sample preparation

3.2.4.1 Defrost the frozen tissue sample in the fridge overnight.

3.2.4.2 The samples should be kept cool before the ACN extraction, especially after homogenisation. The time between homogenisation and extraction was kept to a minimum.

3.2.5 Extraction

3.2.5.1 Accurately weigh the homogenised tissue sample (15±0.1)g into a 250ml beaker.

3.2.5.2 While the tissue sample is being weighed remove the extraction buffer from the fridge and place on the bench at room temperature.

3.2.5.3 Using a 50ml graduated cylinder, add cold extraction buffer (30ml) to the tissue.

3.2.5.4 Homogenise the tissue sample using the Ultra turrax homogeniser for approximately 15 seconds.

3.2.5.5 Using a 100ml graduated cylinder measure out ACN (90ml)

3.2.5.6 Add approx. 40ml of this to the homogenate and homogenise for 20 seconds.

3.2.5.7 Add the remaining 50ml ACN to the extract and continue for a further approximately 20 seconds.

3.2.5.8 Wash the probe with water for 20 seconds.

3.2.5.9 Discard the water and wash the probe with a further 80ml of water.

3.2.5.10 Discard this water solution and extract the next tissue sample.

3.2.6 Separation of water

3.2.6.1 Decant the tissue extract through a folded filter paper, fitted into a glass funnel, into a conical flask.

3.2.6.2 Using the 100ml graduated cylinder, measure 90ml of the tissue extract, (corresponding to 10g of tissue) into a 250ml separating funnel.

3.2.6.3 Add Sodium chloride (approx.4g) into the separating funnel and shake vigorously for 30 seconds.

3.2.6.4 Measure tert butyl methyl ether: isohexane (80:20) (30ml) using the 100ml graduated cylinder into the separating funnel.

3.2.6.5 Shake the separating funnel, at this stage there should not be any NaCl visible in the funnel.

3.2.6.6 Place silanised glass wool into the neck of the glass funnel which were used earlier for filtration, and place approximately 10g of anhydrous Na_2SO_4 on top of this.

3.2.6.7 Allow the lower water phase to run off from the separating funnel to waste into the conical flasks.

3.2.6.8 Swirl the separating funnel slowly after some seconds and allow any remaining water to collect and run off to waste.

3.2.6.9 Record the weight of a 250ml round bottom flask.

3.2.6.10 Filter the top organic layer through the Na_2SO_4 and the glass wool into a 250ml round bottom flask.

3.2.6.11 Rinse the separating funnel with ACN (10ml) and filter this through the Na_2SO_4 and the glass wool into a 250ml round bottom flask.

3.2.6.12 Add water (3ml) to the round bottom flask using a Gilson 5ml pipette.

3.2.6.13 Record the weight of the round bottom flask containing the sample solution.

3.2.6.14 Place the round bottom flask on the rotary evaporator at 40±5°C. Maintain the pressure at approximately 300 bar for five minutes and was then reduce to approximately 250 bar. Maintain this pressure for 15 minutes and then reduce to approximately 180 bar for 10 minutes, 170 bar for a further 15 minutes. At this stage the final residue should be 2-3g.

3.2.7 Cleansing by distribution

3.2.7.1 Record the weight of a 25ml pyrex test tube.

3.2.7.2 Vortex mix the residue from the 250ml round bottom flask to dissolve the visible residues.

3.2.7.3 Decant the residue into a 50ml polypropylene centrifuge tube.

3.2.7.4 Wash the 250ml round bottom flask with the following solutions in succession:

- 10ml hexane
- 15ml hexane
- 3ml ACN
- 0.5ml buffer + 2ml H_2O

3.2.7.5 Measure the hexane with a 25ml graduated cylinder while the ACN, buffer and H_2O were measured with a 5ml Gilson pipette.

3.2.7.6 Transfer each wash solution into the 50ml centrifuge tube and shake this for 30 seconds.

3.2.7.7 Remove the upper hexane layer by glass pipette to waste.

3.2.7.8 Add water (3ml) using a 5ml Gilson pipette.

3.2.7.9 Add NaCl (approx. 500mg) directly to the centrifuge tube, shake and release the pressure.

3.2.7.10 Using the 25ml graduated cylinder add Ethyl acetate (15ml) to the 250ml round bottom flask and rinse this into the centrifuge tube.

3.2.7.11 Shake and remove the upper ethyl acetate layer with a glass pipette to the 25ml test tube.

3.2.7.12 Using the 25ml graduated cylinder add another 15ml ethyl acetate to the 50ml centrifuge tube and shake this briefly.

3.2.7.13 Combine the ethyl acetate layers in the test tube and remove any water, which is below the ethyl acetate layer in the test tube, to waste.

3.2.7.14 Add water (300μl) to the test tube and record the weight using the micromann pipette.

3.2.7.15 Evaporate this under a gentle stream of nitrogen at 40±5°C, until the final volume weighs approximately 500mg.

3.2.7.16 Adjust the extract with water to a final weight of 1.05g (equivalent to 1.0ml).

3.2.7.17 Vortex mix for 30 seconds, and filter through a 0.45μm ministart filter prior to HPLC injection.

3.3. Preparation of working standards

3.3.1 Transfer the five working standards (section 3.1.3) for the calibration plot to the HPLC vials.

3.4 HPLC Analysis

3.4.1 Condition the HPLC column as required prior to analysis

3.4.2 Purge the auto-injector as required prior to analysis

3.4.3 Inject the sample batch in the following order:

- a 100μgkg^{-1} equivalent standard
- reagent blank
- working standards for the standard curve
- fortified control sample to assess recovery
- a 100μgkg^{-1} equivalent standard
- test samples
- a 100μgkg^{-1} equivalent standard
- include a 100μgkg^{-1} equivalent standard every five samples

3.4.4 On completion of the analysis condition the column and HPLC for storage

3.5. RESULTS

3.5.1 Results are expressed in μg SD, SMX, and TMP per kilogram of tissue ($\mu g kg^{-1}$, ppb)

3.6. Recoveries

3.6.1 Recovery data from the fortified controls is used to determine the acceptance, or rejection, of an analytical batch.

3.6.2 If the batch % recovery is outside the validated performance criteria, (Chapter 5, section 5.10.6), the batch recovery obtained is used for screening analysis, while for confirmatory analysis the batch must be re-analysed.

3.6.3 % recovery (recovery factor) is calculated as follows:

- Using the internal standard the peak height obtained for the fortified sample is substituted into the equation of the best-fit calibration line and a concentration in $\mu g kg^{-1}$ is estimated (Example shown in Chapter 5, section 5.10.4)
- $\%\text{recovery} = \dfrac{\text{concentration recovered ug/kg}}{\text{concentration added ug/kg}} \times 100$

3.7. Peak Identification

3.7.1 Peak retention time is used as the criteria for identifying a positive or negative sample.

3.7.2 The mean retention time of each analyte peak in the standards sandwiching the samples is calculated.

3.7.3 Identification of the analyte peaks in the test and fortified samples is made by comparing the retention times of the peaks found in the samples with the mean retention time from the standards. The peaks present in the sample must be within ±5% of the mean retention time of the standards for the sample to be declared suspect positive. Otherwise the sample is reported negative.

3.7.4 If a peak is present in the reagent blank at or above the LOD and within ±5% of the mean retention time for the standards, the batch must be re-analysed.

3.7.5 If any standard or fortified control contains no analyte peaks then the batch must be re-analysed.

3.8. Quantification

3.8.1 All samples containing a suspect peak with a response less than the LOD for each analyte (Chapter 5, section 5.10.7) are reported as negative. Analytes may be present but not detectable.

3.8.2 Samples with a response greater than the LOD are selected for confirmation.

3.8.3 The concentration is calculated as follows:

- Using the internal standard the peak height obtained for the test sample is substituted into the equation of the best-fit calibration line for each analyte and a concentration in μgkg^{-1} is estimated (Example is shown in Chapter 5, section 5.10.4)
- All samples are corrected for % recovery using the specified recovery factor (section 3.6).

3.8.4 Samples confirmed with a value above the LOQ but below the MRL are reported as negative with their confirmed concentration to 1 decimal place

3.8.5 Samples confirmed with a value above the MRL are reported as positive with their confirmed concentration to 1 decimal place

FLOW DIAGRAM OF THE METHOD USED FOR THE DETERMINATION OF SULPHADIAZINE, SULPHAMETHOXAZOLE AND TRIMETHOPRIM RESIDUES IN FARMED FISH TISSUES

Muscle (15g) Homogenised

Spike with standard (150µl) in ACN
Add 30ml buffer
Homogenise for 30 seconds
Add 40ml ACN and homogenise
Add 50ml of ACN and homogenise
Filter and transfer 90ml to a separation funnel

Add 4g NaCl
Add 30ml butyl methyl ether: hexane (8:2)

Shake
Discard the lower water layer to waste

Filter the organic layer through Na_2SO_4 and glass wool into a 250ml RBF
Add 3ml water

Evaporate to 2-3g residue by rotary evaporation

Decant the residue to a 50ml centrifuge tube and wash the RBF with:
Hexane –10ml and 15ml,
ACN – 3ml,
Buffer and water – 0.5ml and 2ml

Combine the phases and shake
Remove the upper hexane layer to waste

Add 3ml water,
Add 500mg of NaCl,
Rinse the 250ml RBF with ethyl acetate (15ml) and add to the aqueous extract

Shake and remove the ethyl acetate layer to a 25ml pyrex tube

Re-extract with ethyl acetate (15ml)
Add 500µl water to the combined ethyl acetate layers

Evaporate the residue accurately down to less than 0.5g under a gentle stream of nitrogen

Fill up with 0.02M CH_3COONa to 1g

Filter using a 0.45µm ministart filter

HPLC Analysis