THE TRANSPORT AND FATE OF FLUOXETINE HYDROCHLORIDE, DIAZEPAM AND THEIR HUMAN METABOLITES IN SEWAGE SLUDGE-AMENDED SOIL

By

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ABSTRACT

The European Union (EU) banned disposal of sewage sludge (SS) at sea in 1998. Since that time the application rate of SS to land has risen significantly and is set to rise further. Fifty-two percent of SS was disposed to land in the UK in 2000. Land application is thus possibly an *important transport route* for SS-associated organic chemicals into the environment.

There are now over 3000 different pharmaceutical ingredients in use in the EU and the last decade has also seen an increase in reports of pharmacologically active compounds in the environment (e.g. in watercourses, open ocean, soil). Regardless of this there is *still a significant lack of knowledge as regards* the transport and fate of pharmaceuticals in the environment, particularly in soils. The present project therefore investigated the biotic fate of the selective serotonin re-uptake inhibitor (SSRI), Prozac® (Fluoxetine HCl), and the 1,4-benzodiazepine, Valium® (Diazepam) and their major human metabolites Norfluoxetine HCl, Temazepam, Oxazepam and Nordiazepam *in a UK SS-amended soil.*

Extraction techniques, such as solid phase extraction, for the analytes from a range of matrices (water, soil and plant material) were developed, which allowed subsequent analysis using developed high performance liquid chromatography – electrospray ionization – multistage mass spectrometry (HPLC-ESI-MS^n) techniques. Ratio calibration *using deuterated internal standards* allowed the generation of quantitative data. The pharmaceuticals were found to be resistant to biodegradation in both liquid culture studies (60 days), and even after prolonged exposure in SS-amended soil (>200 days; Fluoxetine HCl only). Oxazepam was the only 1,4-benzodiazepine studied which underwent biotic transformation (~ 80%) in liquid culture studies. Evidence to *support the theory that the transformation* product seen was a 1,4-benzodiazepine tautomer, is presented.

Results of what is believed to be one of the first examples of research into pharmaceutical uptake by plants are presented. In a preliminary tissue culture study the translocation of Fluoxetine HCl into *Brassica* stems (5% uptake) and leaves (3% uptake) confirmed that *plant uptake of some pharmaceuticals* may be a potential transport route in the environment. The stability of the pharmaceuticals under environmentally relevant conditions has implications for the consequent accumulation in SS-amended soils and possible subsequent uptake into plants grown on the soils.

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
ANOVA	Analysis of variance
APCI	Atmospheric pressure chemical ionisation
ASE	Accelerated solvent extraction
BCF	Bioconcentration factor
BHP	Bacteriohopanepolyol
CBT	Closed bottle test
CID	Collision induced dissociation
DDMS	Data-dependent mass spectrometry
DGGE	Denaturing gradient gel electrophoresis
DO	Dissolved oxygen
DOC	Dissolved organic carbon
DT_{xx}	Dissipation time for xx percent of target analyte
EC_{xx}	Effective concentration for xx percent of population
EIC	Environmental introduction concentration
EPA	Environmental protection agency
ESI	Electrospray ionisation
FDA	Food and drug administration
GABA	Aminobutyric acid
GC	Gas chromatography
HPLC	High performance liquid chromatography
HPLC-ESI-MS^n	High performance liquid chromatography - electrospray ionisation - multistage mass spectrometry
5-HT	5-hydroxytryptophan
IPA	Isopropyl alcohol (propan-2-ol)
IS	Internal standard
IT	Ion trap
k'	capacity factor
$Kbiol_{xx}$	Biodegradation constant for xx percentage of compound
Kd_{xx}	Partition coefficient (matrix / matrices stated)
Koc	Partition coefficient, normalised against organic carbon content
Kow	Octanol-water partition coefficient
LC_{xx}	Lethal concentration for xx percent of population
LLE	Liquid-liquid extraction
LOD	Limit of detection
Log P	Log of polarity constant
LOQ	Limit of quantification

MAE	Microwave assisted extraction
MDL	Method detection limit
MeOH	Methanol
Milli-Q	Milli-Q system purified water
MOST	Modified OECD screening test
MS	Mass spectrometry
MSM	Minimal salts media
NMR	Nuclear magnetic resonance spectroscopy
OECD	Organisation for economic co-operation and development
PCB	Polychlorinated Biphenyl
PEC	Predicted environmental concentration
PLE	Pressurised liquid extraction
PNEC	Predicted no-effect concentration
PPCPs	Pharmaceuticals and personal care products
ppm	Parts per million
QqQ	Triple quadruople
QSAR	Quantitative structure activity relationship
RP	Reverse phase
% RSD	Percentage relative standard deviation
Rt	Retention time
S:N	Signal to noise ratio
SAX	Strong anion exchange
SIM	Selected ion monitoring
SPE	Solid phase extraction
SRM	Selected reaction monitoring
SS	Sewage sludge
SSRI	Selective serotonin reuptake inhibitor
STW	Sewage treatment works
SWW	South West Water plc
$t_{1/2}$	Half life
T/C	Tissue culture
tds	tonnes dry solids
TSA	Tryptone soya agar
USE	Ultrasonic solvent extraction
UV-DAD	Ultra-violet - diode array detector
WWTP	Waste water treatment plant
ZWT	Zahn-Wellens test

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AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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Relevant scientific conferences were regularly attended throughout the period of study at which research was regularly presented. Consultation with other institutions occurred (e.g. Rothamsted Research and University of Newcastle) and several publications are to be prepared.

Presentations and Conferences Attended:

4th European Meeting of Environmental Chemistry, 10th – 13th December, 2003, Plymouth, UK. Attended.

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C.E. West, C.H. Redshaw, S.J. Rowland, C.A. Lewis, S.T. Belt, M.J. Hetheridge, G. Roberts and Q. Lui. *Cheering up the Environment: The Environmental Fate of Prozac and Valium.* **16th Meeting of the British Organic Geochemical Society (BOGS)**, 14th – 15th July 2004, Nottingham, UK. Poster presentation. Awarded best student poster prize.

C.E. West, C.H. Redshaw, S.J. Rowland, C.A. Lewis, S.T. Belt, M.J. Hetheridge, G. Roberts and Q. Lui. *Shedding light on the Environmental Fate of Prozac and Valium.* **5th European Meeting of Environmental Chemistry (EMEC)**, 15th – 18th December 2004, Bari, Italy. Poster presentation. Awarded best student poster prize.

C.E. West, C.H. Redshaw, S.J. Rowland, C.A. Lewis, S.T. Belt, M.J. Hetheridge, G. Roberts and Q. Lui. *Shedding light on the Environmental Fate of Prozac and Valium.* **Society of Chemical Industry: Pharmaceuticals in the Environment**, 1st March 2005, London, UK. Poster presentation.

C.H. Redshaw, S.J. Rowland, C. Whitby, M. Frenzel, M.J. Hetheridge and G. Roberts. *Unearthing the Fate of Prozac, Valium and their Human Metabolites in Sewage Sludge Amended Soils.* **6th European Meeting of Environmental Chemistry (EMEC)**, 6th – 10th December, 2005, Belgrade, Serbia and Montenegro. Oral presentation.

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Signed.....

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CHAPTER ONE

INTRODUCTION

1. Introduction

1.1. Background

During the last decade the occurrence of pharmaceuticals and personal care products (PPCPs) in the environment has been repeatedly documented (Ayscough *et al.*, 2000; Halling-Sorensen *et al.*, 1998; Heberer, 2002; Ternes, 1998; Daughton and Ternes, 1999). A range of studies has shown that pharmaceuticals are neither completely removed by sewage treatment works (STW) processes, nor completely degraded in the environment; hence the environmental occurrence of PPCPs is perhaps of little surprise (Carballa *et al.*, 2004; Golet *et al.*, 2002; Heberer *et al.*, 2001).

As concern grows in the public eye (CBC, 2004; Townsend, 2004) UK agencies such as Department of Environment, Food and Rural Affairs (DEFRA, 2005) and The Environment Agency (EA, 2005a) are expressing their concerns about the risks of pharmaceuticals within sewage sludge entering the environment. In the EU the POSEIDON project, described as an 'assessment of technologies for the removal of pharmaceuticals and personal care products in sewage and drinking water to improve the indirect potable water reuse', aimed to provide solutions to some of these concerns and was completed in 2004 (Ternes, 2004). The latter project assessed a range of different methods for wastewater collection and treatment to remove PPCPs, including flocculation, use of activated carbon, various oxidation processes and membrane filter technologies. It was concluded that ozone-based oxidation treatments, which are inexpensive but high energy processes, are capable of removing many PPCPs from waste water. However, small scale waterworks without advanced technologies would not remove the more polar compounds. Therefore it was

recommended that for drinking water treatment, ozonation, activated carbon filtration and UV or ozone advanced oxidation should be used to prevent PPCPs entering drinking water (Ternes, 2004).

However, even following the POSEIDON study, relatively little is known about the fate and transport of pharmaceuticals in the environment; although several possible exposure routes have been identified. As human metabolism of pharmaceuticals is incomplete, both parent pharmaceuticals and metabolites may be excreted. These compounds enter either the aqueous or the sludge phase of sewage, depending upon their individual physico-chemical characteristics. Both abiotic and biotic processes may then act upon these compounds during STW processes, but incomplete degradation will result in discharge of pharmaceuticals into waterways or in the terrestrial environment as a component of solid sewage sludge when it is used either as an agricultural fertilizer or sent to landfill. Ayscough *et al.,* (2000) highlight the processes occurring in domestic sewage, in STW and within the aquatic and terrestrial compartments. The processes that may occur along this transport route include; hydrolysis, biodegradation (aerobic and anaerobic), adsorption, direct and indirect photodegradation, volatilisation, run-off and leaching. Such transport routes into the environment are summarised in Figure 1.1.

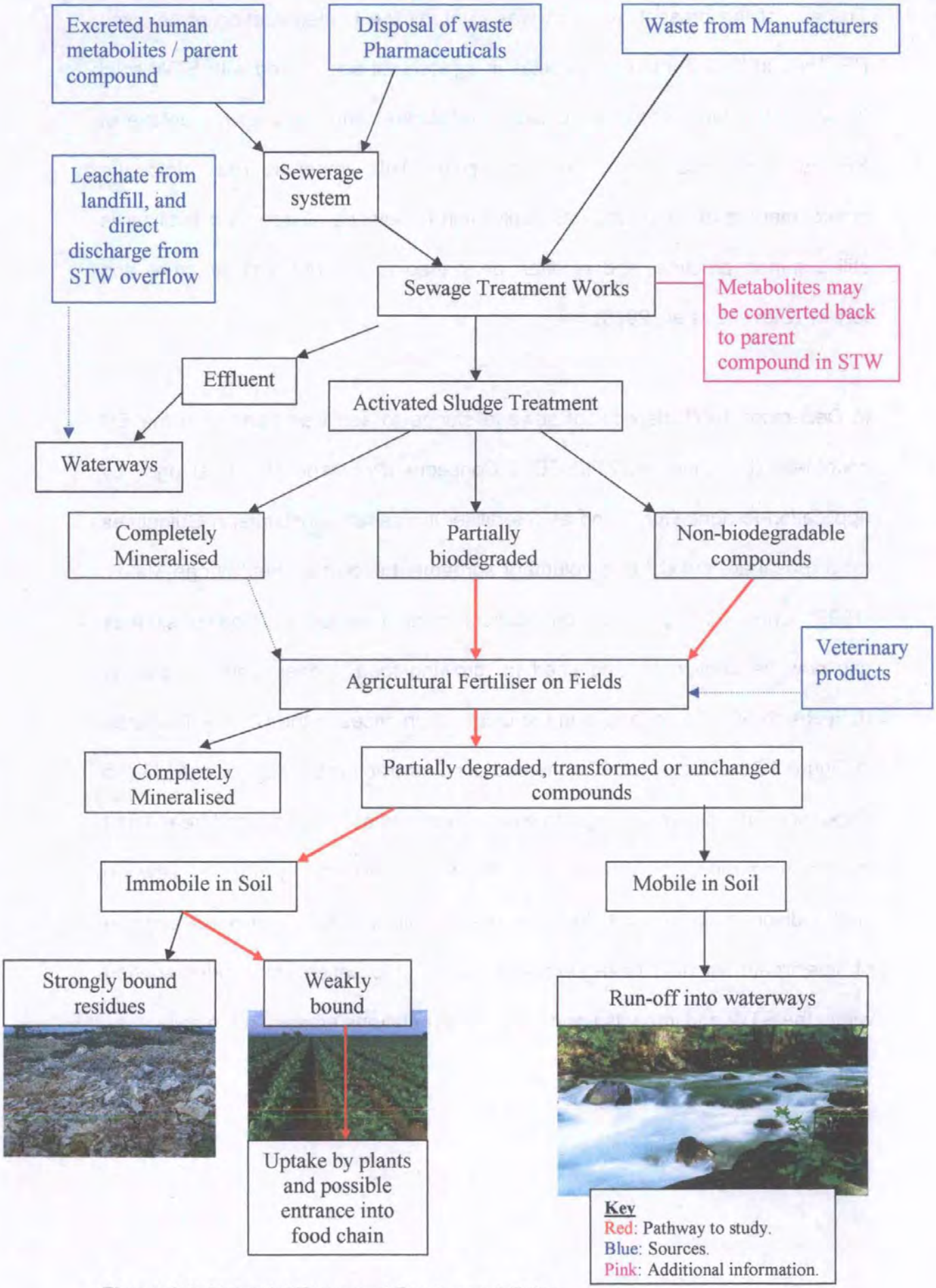


Figure 1.1. Potential Transport Routes and Fates

The aim of the present research was to study the biodegradation of selected PPCPs and their human metabolites in agricultural soil treated with STW solid faecal matter (also known as biosolids or sludge) and the possible uptake of PPCPs into crops grown on such soils. This transport route into the environment is of importance as application of sewage sludge as a fertilizer is still common practice, and is often performed to improve soil structure and fertility (Stevens *et al.*, 2003).

In December 1998 disposal of sewage sludge to sea was banned in the EU countries (Directive 91/271/EEC). Consequently disposal of sludge by application to agricultural land as a fertiliser increased substantially. Guidelines for disposal are set out in a voluntary agreement known as the 'Sludge Matrix' (1999). Under this agreement only conventional or 'enhanced- treated' sludges can now be applied to land used for growing food, fodder crops or grazing (DEFRA, 2005). The changes in the disposal practices in the UK are illustrated in Figure 1.2 to Figure 1.4. The method used for treating sewage sludge prior to disposal in UK, is also undergoing change. Lime treating ('enhanced treatment') is becoming more common in the UK, with approximately 40% of sewage sludge disposed in Devon being lime treated (Millns, 2004). The increasing use of lime treatment may have several important implications for biodegradation within the STW and impacts upon bacterial populations present in the soil.

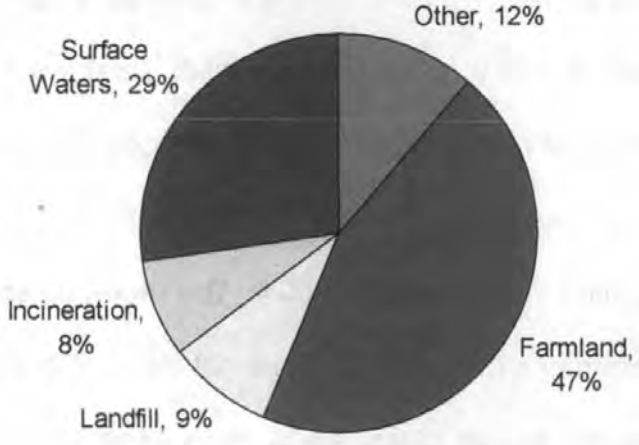


Figure 1.2. UK Sewage Sludge Disposal Outlets 1996/97

Source: Sewage Sludge Survey 1996 & 1997

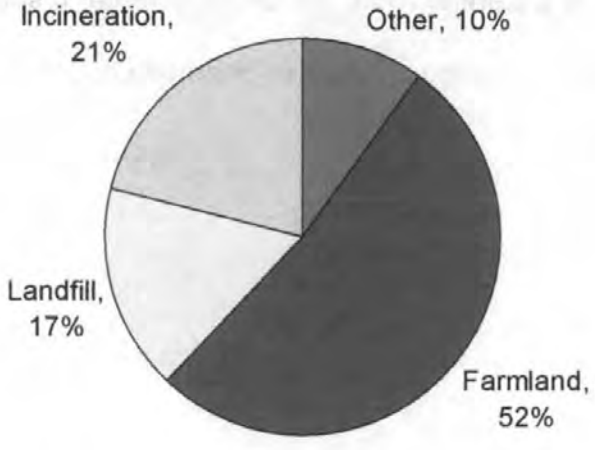


Figure 1.3. UK Sewage Sludge Disposal Outlets 1999/00

Source: OFWAT, Scottish Executive and DOE Northern Ireland (DEFRA, 2002a)

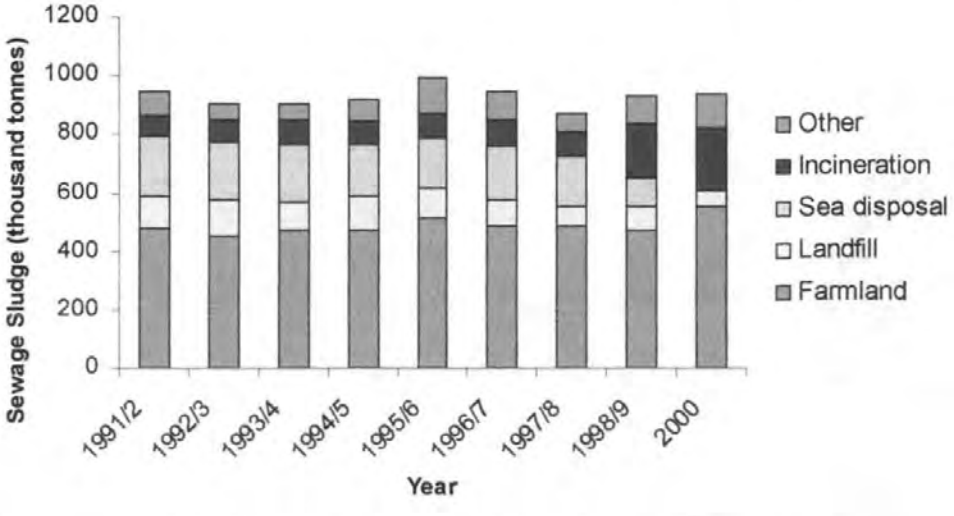


Figure 1.4. Change in UK Sludge production and disposal over time

Source: (Environment Agency and Water UK) (EA, 2002)

In the UK in 1999/00 around 1 million tonnes of sewage sludge were produced per annum, of which 610,200 tonnes of dry solids (tds) were disposed of to land, which equates to 54% of sewage sludge produced. The south west of the UK disposed of 5% of this sludge to land (ca 30,000 tds), of which about 40% (ca 12,000 tds) was lime treated (Millns, 2004). The receiving soils in Devon are typically derived from rocks of a slate or shale nature i.e. Denbeigh series (e.g. type 541, coarse loamy soil as classified by the Lawes Agricultural Trust Soil Survey (1983)). It has been estimated that 10 million tonnes of sludge were produced in the EC by 2005. Clearly disposal of such amounts of biosolid material represents a potential route for environmental dispersal of associated organic contaminants, including a range of pharmaceuticals.

1.2. Aims and Objectives

The objectives of this study were to determine the transport and fate of selected generic pharmaceuticals in sewage sludge-amended soils. This was to be achieved by first selecting the target compounds for study *via* an extensive literature search and review.

Methods for extraction and analysis of these compounds from water, soil and plant material were then to be developed; and these methods were then to be applied in a range of biodegradation and plant up-take experiments. As relatively little work had been performed on the fate and transport of pharmaceuticals in soils at the commencement of this study, it was necessary to consult literature on the fate of other polar organic chemicals such as pesticides, for background and method information.

For pesticide regulation there are four major issues that are considered. These are: transport of residues into crops, resultant effects on both aquatic and terrestrial biota and potential for transfer along the food chain; risk of groundwater contamination and long-term impacts upon soil quality (Gevao *et al.*, 2003). It therefore seemed logical to consider the same issues with regards to pharmaceuticals. This project aimed to answer some of these questions in respect of selected generic pharmaceuticals.

1.3. Selection Procedure

The first stage of the study was to complete an extensive review of the literature as a means to determine which pharmaceuticals (and human metabolites) should be selected as target analytes for these experiments. At the beginning of this study (2003) knowledge of the fate and transport of drug metabolites was particularly limited as they were not generally included within pharmaceutical studies even though many metabolites are known to be bioactive (Ayscough *et al.*, 2000). A list was compiled of all pharmaceuticals which were reported to have been found in the environment. Few quantitative data on pharmaceuticals in sewage sludge were found. However each one of the potential target pharmaceuticals was assessed for selection based on a number of criteria (Figure 1.5).

- Toxicity
- Usage (UK data where available)
- Biodegradation
- Photodegradation
- Metabolites (identity and bioactivity)
- Occurrence in the environment (location and concentration)
- Extent of removal in STW
- Other possible sources i.e. potential interference problems
- US EPA modelling suite results

Figure 1.5. Selection Criteria

There are over 3000 different pharmaceuticals licensed for use in the UK (Sebastine and Wakeman, 2003). Therefore it was important to prioritise research into those compounds that are most likely to be present within the UK environment. Sebastine and Wakeman (2003) state that the criteria to be considered should include those drugs that are most heavily used; hence they

began their research by collecting and manipulating data to generate a list of annual consumption by active ingredient mass. Kreuzig *et al.,* (2003) also used usage data along with positive findings of pharmaceuticals in the environment as their selection criteria, but also considered the availability of ^{14}C-labelled radiotracers as these may be required to trace their movement in soil.

Application of such choice criteria (Appendix; Tables A.1 to A.8) resulted in the selection herein of Fluoxetine Hydrochloride and Diazepam, (trade names include Prozac® and Valium®) (TOXNET, 2006b; TOXNET, 2006a). These compounds were selected as they have a high usage and their metabolites are known to be bioactive. Alongside this, information regarding toxicity to organisms was obtained and US Environmental Protection Agency (EPA) models predicted that the dominant removal process for these compounds in STW would be to adsorption to sludge, and that little or no biodegradation would be likely to occur (Appendix, Table A.1). Therefore it was felt that this risk of SS contamination by these compounds and subsequent transfer to the terrestrial environment was of significant concern and warranted further study. Subsequent press coverage received by these drugs further emphasised public concern over their fate and perhaps supports their selection (CBC, 2004; Townsend, 2004; Revill, 2003; Barron, 2004; Barnett, 2003; Ternes, 2004; EA, 2005a; EA, 2005b; EA, Environment Agency).

1.3.1. Fluoxetine HCl

The selective serotonin reuptake inhibitor (SSRI) Fluoxetine HCl (Figure 1.6), which is a racemate of two enantiomers, is commonly used for the treatment of depression, bulimia and obsessive-compulsive-disorders (Davis and Phil, 2001). SSRIs were first brought to the market in the 1980s and Fluoxetine HCl was first sold in 1986. Fluoxetine HCl is a very commonly used medication and has featured on the top 200 most prescribed drugs list for the USA since 1995, in the UK top 100 prescribed pharmaceuticals by mass, with 2.83 tonnes of Fluoxetine HCl dispensed in 2000 (Sebastine and Wakeman, 2003). It is estimated that over 34 million people have taken Fluoxetine HCl in over 100 countries (Fong, 2001; rxlist, 2006).

Human metabolism of Fluoxetine HCl by cytochrome P450 isozymes results in the formation of only one major bioactive metabolite; Norfluoxetine (also known as Desmethylfluoxetine). The majority is excreted in urine along with less than 10% of the parent compound or N-glucuronide (Figure 1.6) (Flaherty *et al.*, 2001; Hiemke and Hartter, 2000). Fluoxetine is also able to inhibit CYP IID6, CYP IA2 and CYP IIIA4 which are drug-metabolising enzymes and can cause complex drug-drug interactions. The potency of either S or R-Fluoxetine is equivalent to that of S-Norfluoxetine. The R-Norfluoxetine enantiomer is still bioactive although activity is significantly reduced (TOXNET, 2006b). Fluoxetine is known to have a higher volume of distribution that the other SSRIs, which implies extensive tissue accumulation, especially in the lungs, although brain accumulation is slower than with other SSRIs (Hiemke and Hartter, 2000).

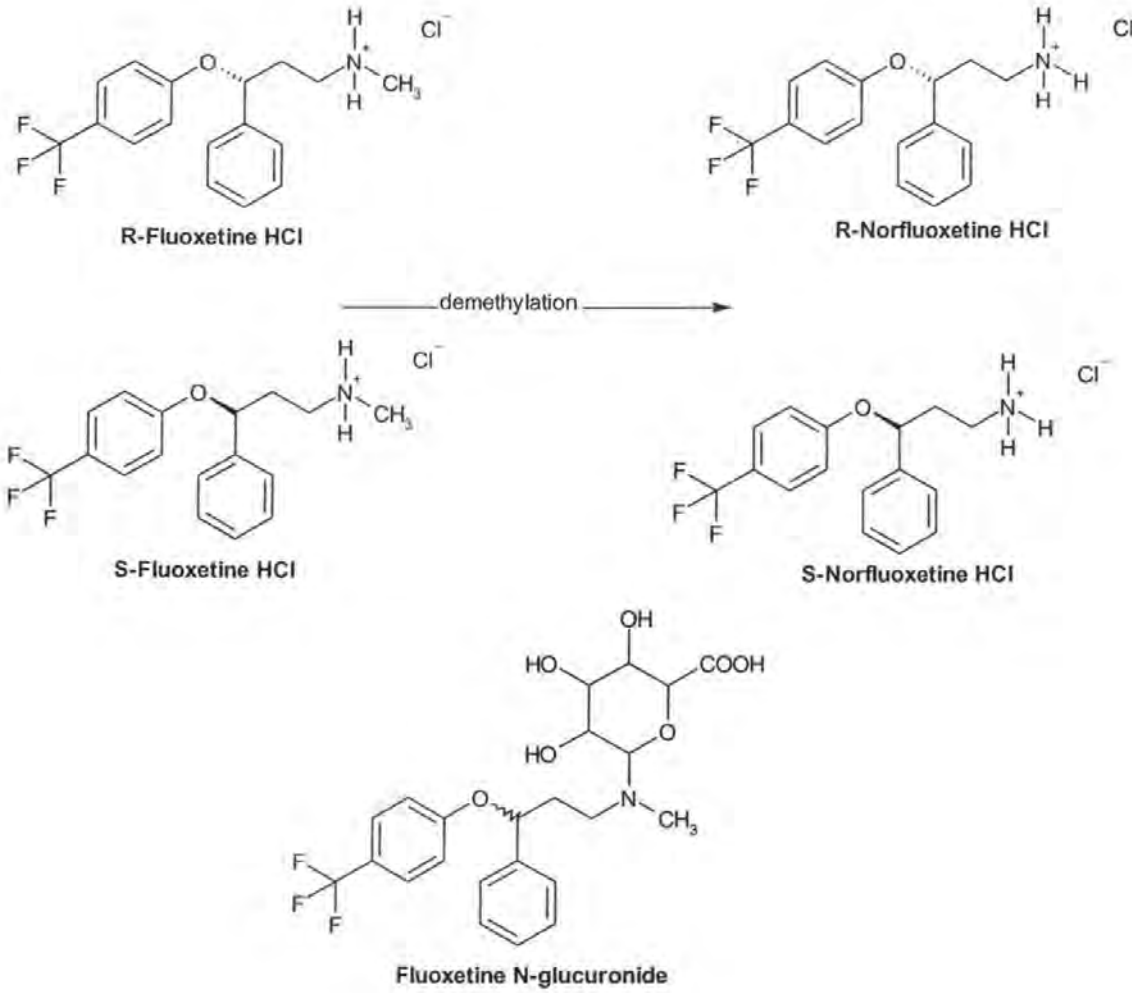


Figure 1.6. Human metabolism of Fluoxetine HCl; parent compound and metabolite structures

The mode of action of SSRIs such as Fluoxetine, involves blocking of reuptake of neurotransmitters such as serotonin, dopamine and norepinephrine. This blocking effectively increases the levels of 5-hydroxytryptophan (5-HT) available by preventing repackaging of this compound into synaptic vesicles (Fong, 2001).

Fong (2001) presents an extensive review of ecotoxicological data for Fluoxetine HCl and highlights the fact that serotonin plays a role in a variety of physical systems in a range of organisms. Therefore drugs, such as Fluoxetine,

that mimic this action could have deleterious effects on a large number of organisms if released into the environment. These effects, discussed in detail by Fong (2001) and summarised herein, range from vasoconstriction, effects on retinomotor activity, metamorphosis, reproduction and ciliary activity.

Serotonin has previously been shown be involved in the reproduction of the common estuarine fish, the Mummichog (*Fundulus heteroclitus*). It inhibits steroid-induced meiotic maturation of ovarian follicles. These fish spawn on a biweekly pattern, which appears to be triggered by the decrease of pituitary serotonin secretion and the increase of hypothalamic serotonin secretion, suggesting that the levels of serotonin in the brain play a key role in coordinating the specific spawning pattern. Serotonin is also known to increase gonadotropin levels in the Atlantic Croaker (Fong, 2001).

Various physiological systems in salmonids are regulated by serotonin, such as the control of vasoconstriction in trout. It also causes lowered blood pressure and decreases in arterial oxygen tension in trout. Salmon that have a tryptophan (a precursor to serotonin) deficient diet are often found with scoliosis, a spinal deformation. Teleosts and cartilaginous fish retinas contain serotonin, which appears to be involved in retinomotor movement (Fong, 2001).

Serotonin is also found in amphibians where its role is to disperse melanin in Red-spotted Newts (*Notophtalmus viridescens*). In the European Green Toad (*Bufo viridis)* and the African clawed Frog (*Xenopus laevis*) the role of serotonin is to prevent progesterone-induced oocyte maturation, and it is therefore serotonin antagonists that trigger maturation of the oocytes. However, in

Starfish, an opposite role is seen, where serotonin actually induces oocyte maturation (Fong, 2001).

There has been little research of this type involving mammals. However there is evidence that in mink, rats, mice and silver foxes, serotonin suppresses predatory aggression. This evidence points towards a serotonin-related dietary response that controls the predatory behaviour of these carnivores (Fong, 2001).

Serotonin also plays roles in the physiology of aquatic worms. It regulates egg-laying behaviour in *Caenorhabditis elegans* (nematode), and mediates swimming and feeding behaviour in leeches. In sea urchins serotonin has a number of roles, such as increasing swimming speed (as does 5-HT), regulation of cell division in early embryonic stages and in embryos serotonin antagonists elevate intracellular free calcium. Protozoans are also affected by serotonin. In *Tetrahymena thermophila*, cilia regeneration is down to the action of serotonin, as it stimulates phagocytosis.

The role of 5-HT in aquatic invertebrates is particularly variable. Its role includes involvement within the reproductive systems of clams and mussels, where it can induce spawning and oocyte maturation. It has also been linked to behaviour, as it induces aggression in crustaceans, especially lobsters and crayfish. Other roles include cilia regeneration (in protozoans), triggering of rhythmic contractions (in coelenterates), negative phototaxis (in bryozoan larvae), and metamorphosis (in hydrozoan larvae). The effect of Fluoxetine on aquatic invertebrates is thought to be due to its inhibition of serotonin reuptake

transporters e.g. in lobsters (effectively mimicking the action of 5-HT), or due to its ability to bind to 5-HT e.g. in frog embryos (therefore antagonising the 5-HT action) (Fong, 2001).

Fluoxetine has been shown to cause a serotonin-induced increase in gonadotropins in both male and female pre-spawn goldfish. Serotonin is also found in skate retina biopolar and amacrine cells. When Fluoxetine was applied to these fish serotonin uptake in these biopolar cells was not blocked, and was often elevated. In Zebra Mussels (*Dreissena polymorpha*) Fluoxetine triggers both sexes to spawn because in these organisms oocyte maturation, germinal vesicle breakdown and spawning is regulated by serotonin. Fluoxetine at a concentration of $5x10^{-6}$ M caused all male mussels to spawn. However, only $5x10^{-8}$ M of Fluoxetine was enough to cause statistically significant increases in male mussel spawning, and only $5x10^{-6}$ M for females. However, higher concentrations of Fluoxetine (10^{-3} to 10^{-4} M), resulted in decreased spawning. This suggests that higher concentrations of Fluoxetine may be toxic to these mussels. Fluoxetine has also induced spawning in both sexes of the marine bivalve, *Macoma balthica*. The reproductive system of fresh water Fingernail Clams (*Sphaerium striatiunum*) is also affected by Fluoxetine. By mimicking the action of 5-HT it causes juveniles to be released i.e. causing premature birth. In *Procambarus clarkii* (Crayfish) and *Uca pugilator* (Fiddler Crab) Fluoxetine exposure resulted in enlarged ovary and oocyte formation. It can also stimulate testicular development in the Fiddler Crab. Another effect of this pharmaceutical on the Fiddler Crab is enhanced pigment dispersion, which also occurs in *Macrobrachium ohione* (shrimp) (Fong, 2001).

The stimulation of ciliary beating in reptiles (frogs), echinoderms and molluscs are all affected by serotonin. In *Helisoma trivolis* (a freshwater gastropod) not only is ciliary beating affected by serotonin but so is embryo rotation, within egg masses. A greater degree of rotation has been measured when Fluoxetine was applied at 10^{-6} and 10^{-5} M. Although once again a toxic effect has been suggested, at higher concentrations (10^{-4} M) this rotation was decreased. Fluoxetine has also been shown to have an impact on marine gastropod larvae. Fluoxetine has caused metamorphosis in *Llyanassa obsoleta* (Mud Snail) larvae and stimulates out of season gamete release in *Macoma balthica* (bivalve) (Fong, 2001).

An interesting effect of Fluoxetine, that is highly relevant to the aims of the current project since the fate of selected pharmaceuticals in SS treated soil was to be investigated, occurs with *Lumbricus terrestris* (Earthworm). In the earthworm, Fluoxetine mimics the action of 5-HT and hence causes decreases in locomotor activity with circadian rhythm (Burns *et al.*, 1992).

Brooks *et al.*, (2003b) performed work into the toxicity of Fluoxetine to aquatic biota before producing a preliminary aquatic risk assessment for Fluoxetine (Brooks *et al.*, 2003a). Fluoxetine is known to act by blocking serotonin reuptake transporters. It is also known to work at other sites including norepinephrine uptake and Sigma receptors, as well as being active at neuronal and muscle nicotinic acetylcholine receptors. The binding of Fluoxetine to these other receptors is of concern as it could result in unknown and therefore unpredictable effects in organisms. Extracellular norephinephrine and dopamine has been shown to increase in rats exposed to Fluoxetine HCl. In fish such as

the Japanese Medaka (*Oryzias laptipes*) Fluoxetine has been shown to affect neuroendocrine function. Foran *et al.*, (2004) observed a low but significant number of developmental abnormalities in Japanese Medaka offspring, along with raised plasma estradiol levels in the maternal fish treated with Fluoxetine. Fluoxetine concentrations of only 116nM have been found to detrimentally affect *Daphnia Magna* (crustacean) reproduction (Brooks *et al.*, 2003b) .

Brooks *et al.*, (2003b) completed a study to evaluate the toxic environmental hazard of Fluoxetine HCl to benthic and pelagic organisms. *Hyalella azteca* (amphipod) and *Chironomus tentans* (insect) were used as test organisms for sediment toxicity experiments, and *Pseudokirchneriella subcapitata* (alga), *Ceriodaphnia dubia* (crustacean), *Daphnia magna* (crustacean), *Pimephales promelas* (fish) and *Oryias latipes* (fish) for waterborne toxicity experiments.

Low dosages (0.1-1 μM) have also been shown to impact embryonic *Physa elliptica* rotational behaviour (Uhler *et al.*, 2000), and even induce male mussel spawning (50nM) (Fong, 1998). Arcand-Hoy and Benson (2001) have shown serotonin to induce release of gonadotropins in some fish species, which in turn induces sex steroid synthesis, and controls development of oogenesis. Bodar *et al.*, (1988) and Ebert (1993) have shown that low-level exposure may increase fecundity but often results in decreased egg and body size. Hoonkoop, Luttikhuizen *et al.*, (1999) and Nation (2002) believe that future research into the impact of Fluoxetine on crustacean reproductive stimulation and invertebrate reproduction timing is essential.

In summary, the effects of SSRIs upon organisms in the environment have the potential to be wide reaching and very variable. Effects range from impacts upon the biochemical (blood system), cellular (reproduction), behavioural (mobility), and even physiological levels (metamorphosis). Such factors are important drivers for an investigation into the environmental fate of Fluoxetine.

The first evidence of Fluoxetine and Norfluoxetine bioaccumulation in three fish species, sampled from an effluent-dominated stream in Texas, USA, was published by Brooks *et al.*, (2005). The highest concentrations were found in the brain (Fluoxetine = 1.58 ng g^{-1}; Norfluoxetine = 8.86 ng g^{-1}), and liver (Fluoxetine = 1.34 ng g^{-1}; Norfluoxetine = 10.27 ng g^{-1}) followed by the muscle tissues (Fluoxetine = 0.11 ng g^{-1}; Norfluoxetine = 1.07 ng g^{-1}). Data were used to calculate predicted human exposure levels, based on a mean consumption rate of 0.286 kg $meal^{-1}$. For Catfish (*Ictalurus punctatus*) and Black Crappie (*Pomoxis nigromaculatus*) Fluoxetine exposure was calculated to be 34.3 ng $meal^{-1}$ and 37.2 ng $meal^{-1}$, and 22.9 ng $meal^{-1}$ for Bluegill (*Lepomis macrochirus*).

Sebastine and Wakeman (2003) identify Fluoxetine as a compound that may accumulate and cause problems in the aquatic compartment of the environment. They make this judgement based on usage data and PEC/PNEC (predicted environmental concentration / predicted no-effect concentration) ratios (sourced from Webb (2000)). Webb (2001) predicted that concentrations of Fluoxetine in UK effluents would be around 1.2 nM, from the UK European Medicines Evaluation Authority and the UK Medicines Control Agency data,

who also use PECs. In the EU PEC for the purposes of legislation is evaluated using the following equation.

$$PEC_{surface\ water}\ (g\ L^{-1}) = A\ (1\text{-}R \div 100) \div 365PVD$$

When; A = predicted usage per year, in the relevant geographic area (kg)

R = removal rate (in STW) (%)

P = population in geographic area

V = volume of wastewater per capita per day (m^3)

D = dilution factor (dilution of wastewater by surface flow)

When the calculated $PEC_{surface\ water}$ is <0.01 μg L^{-1} no further testing or evaluation is legally required. The PNEC is usually based on standardised acute toxicity studies on fish, algae or *Daphnia*. The lowest value achieved for either EC_{50} or LC_{50} is then used to calculate the PNEC. The formula for this calculation is:

$$PNEC = EC \div AF$$

When; EC = effective concentration (i.e. EC_{50})

AF = assessment factor (to account for a degree of uncertainty in the test data)

The PEC:PNEC ratio (or Hazard Quotient) can then be calculated. If this ratio is <1 the compound in question is not considered to be a problem. However if the

ratio is >1 then there is a risk of the compound accumulating in the environment.

The lowest observed effect seen by Brooks *et al.*, (2003b) was an order of magnitude higher than reported concentrations of Fluoxetine in the environment. Toxicity data generated in the study by Brooks *et al.*, resulted in a Hazard Quotient <<1, although they also stress the importance of chronic impacts, as opposed to acute toxicity, upon aquatic organisms. Fong (1998) believed that chronic responses to Fluoxetine may occur at nM or pM exposures.

The use of Hazard Quotients does not result in the classification of these target compounds as potential environmental accumulators. However there is criticism of these types of risk assessments. O'Brien and Dietrich (2004) point out that the dilution factor (1:10) typically used is not environmentally relevant. Most receiving surface waters in Europe actually have effluent-water dilution ratios between 1:1 and 1:5. Other criticisms arise from consideration of acute toxicity only and the disregard of potential toxicity arising from mixtures of compounds within the environment.

Johnson *et al.*, (2005) drew comparisons between EU PECs and US EICs (environmental introduction concentration, PEC equivalent) and undertook a water column dissipation study with a range of SSRIs, including Fluoxetine. Artifical ponds and microcosms were used to determine the aquatic half-lives in this 83 day study. Results indicated that the EU PEC system (Fluoxetine PEC = 0.220 μg L^{-1}) was more cautious than the US EIC system (0.23 μg L^{-1}), but this

is due to the assessment trigger values (EU = 0.01 μg L^{-1}, US = 1.0 μg L^{-1}). The dissipation study results pointed to Fluoxetine having a biphasic dissipation nature, with average first phase dissipation half-life of 3.8 days, and second phase of 76.7 days. Dissipation rates were researched at a range of concentrations, and at lower concentrations (23.5 μg L^{-1}) second phase half-lives were found to be close to infinity. Johnson *et* al., believe that this biphasic nature of dissipation in conjunction with Koc values (>4.3) acts as supporting evidence for the sequestration of Fluoxetine into sediments, and that concentration of Fluoxetine in sediments may be higher than in the water column.

For these reasons, alongside the lack of an equivalent terrestrial risk assessment, a PEC for sewage-sludge was calculated. This calculation took account of factors such as sewage sludge production rates, compound production rates and predicted partitioning behaviour and degradation within the STW. This resulted in the predicted concentration of 0.244 μg g_{ss}^{-1} for Fluoxetine (detailed calculations can be seen in Appendix; Figures A.3 and A.4) At the outset of this project virtually nothing was known about the behaviour of these target compounds on sewage sludge amended soils, including information on their persistence and hence potential accumulation. For this reason it was felt that the PEC_{ss} posed significant risks to the environment should accumulation within the soil occur.

1.3.2. Diazepam

Diazepam (Figure 1.7), a benzodiazepine, is a sedative and anti-anxiety drug. *Status epiepticus*, acute cocaine poisoning and a range of anxiety disorders are treated with Diazepam (Davis and Phil, 2001). It is a common and heavily prescribed drug featuring in the top 25 pharmaceuticals used in Denmark in 1997, with 20.7 million daily defined doses, the equivalent of 0.207 tonnes (Ayscough *et al.*, 2000). High levels of usage in the US lead to a ranking on the top 200 prescribed drugs from 1995 to current (rxlist, 2006).

Hepatic metabolism primarily using CYP2C19 and CYP3A4 followed by glucuronidation, causes demethylation and 3-hydroxylation of the parent compound to form the major metabolites Oxazepam, Temazepam and Nordiazepam (TOXNET, 2006a) which are all known to be bioactive, and two (Oxazepam and Temazepam) are also prescribed drugs in their own right (Figure 1.7).

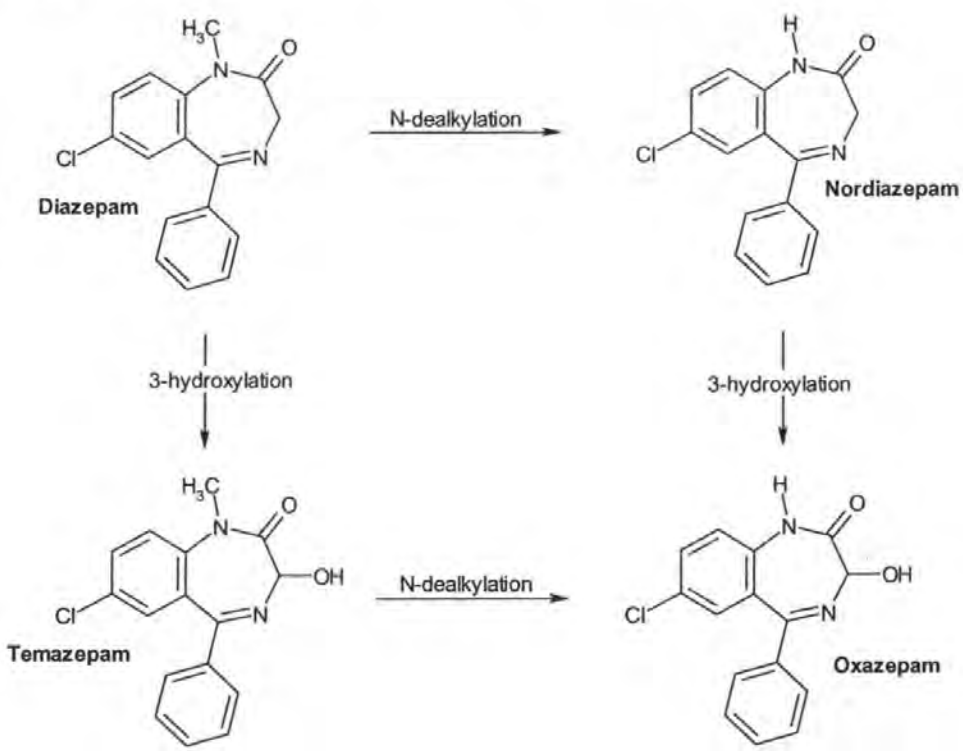


Figure 1.7. Human Metabolism of Diazepam; parent compound and metabolite structures

Diazepam's seizure suppression action comes from interaction with A-type aminobutyric acid ($GABA_A$) receptors. GABA is the central nervous system major inhibitor neurotransmitter. Normally GABA opens chlorine membrane channels when it interacts with this receptor. The presence of the chloride ions then causes an inhibitory potential. This lowers the capability of neurons to depolarize the threshold potential, which produces an action potential (i.e. chloride entrance inhibits neuron transmission). Seizures can be linked with excessive depolarisation of neurons. It is thought that the strength of the binding of GABA to the $GABA_A$ receptor is influenced by Diazepam and that Diazepam effectively increases GABA action (TOXNET, 2006a).

Diazepam was detected in eight out of twenty STWs tested by Ternes (1998) for a range of pharmaceuticals. The maximum concentration detected in STW was 0.04 ng L^{-1}. However when environmental samples, such as river and stream waters, were taken it could not be found at concentrations above the limit of detection (LOD = 0.03 ng L^{-1}). Other researchers such as Richardson and Bowron (1985), Ternes *et al.*, (2001) and Van der Ven *et al.*, (2004) have also found Diazepam in STW influent (0.59 – 1.18 µg L^{-1}) and effluents (< 1 µg L^{-1}, 0.053 µg L^{-1}, 0.66 µg L^{-1}). Detection of Diazepam in environmental samples such as river water (0.13 – 2.13 ng L^{-1}, ~ 10 ng L^{-1}, 0.033 µg L^{-1}, 0.5 – 1.2 ng L^{-1}) and drinking water (~ 10 ng L^{-1}, 0.2 – 23.5 ng L^{-1}) has been achieved by other authors (Calamari *et al.*, 2003; Richardson and Bowron, 1985; Ternes *et al.*, 2001; Zuccato *et al.*, 2000).

Aquatic studies completed in Sweden by Carlsson *et al.*, (2006) for Diazepam led to the generation of a Hazard Quotent < 1 and subsequent classification of

Diazepam as of no risk of accumulation within the aquatic environment. Equivalent data for SS could not be found in the literature, although a PECss for Diazepam of 0.063 μg g^{-1} SS was calculated herein (Appendix; Figure A.1 and A.2).

Calleja *et al.*, (1993) examined the predictive potential of ecotoxicological tests for calculating human acute toxicity. One of the compounds they selected to study was Diazepam which was assessed using the Microtox™ test with 100% assay protocol (a standardised marine bioluminescence bacterial toxicity test). The results of this were used in conjunction with a linear regression program to calculate EC_{50} values (data can be seen in appendix, Table A.7).

The POSEIDON project team (Ternes, (2004) discussed in section 1.1) which completed an extensive modelling study of the fate of various pharmaceuticals within WWTPs (Wastewater Treatment Plants), selected Diazepam as part of their study. They concluded that sorption was not a relevant process for the removal of Diazepam (Kd < 100 L kg^{-1} SS). A larger scale study using wastewater-irrigated land found no significant changes in concentration between the lysimeters and even post-treatment steps appeared to have no impact on the concentration of Diazepam. However the starting concentration of Diazepam was already close to the limit of quantification (LOQ).

Ternes (2004) concluded that Diazepam and other neutral compounds would not show any removal in either groundwater or post treatment steps. Oxidation of Diazepam however followed a second order rate constant (with ozone = 0.75 ± 0.15 M^{-1} s^{-1} at 20 °C, with OH radicals generated via UV / H_2O_2 γ-radiolysis =

7.2 ± 1 M^{-1} s^{-1} at 20 °C) and significant removal was seen, but the use of chlorine dioxide was ineffective (second-order rate constant for this reaction, at pH 7.4 < 0.025 M^{-1} s^{-1}). Nanofiltration and reverse osmosis membranes were effective for the removal of Diazepam (> 98%), as was the use of powdered activated carbon (< 0.2 mg L^{-1} activated carbon for 99% removal). Full scale waterworks studies showed comparable results when compared with Diazepam laboratory studies.

Although some results indicated that many compounds would undergo removal it was noted that most small waterworks would not have these technologies available to them. It was therefore concluded that contamination of drinking water with PPCPs including Diazepam, held appreciable risk.

A recent study designed to investigate the biodegradation of pharmaceuticals in sediment and water samples included Diazepam as a target compound. Samples were taken over 100 days and this data was then used to generate dissipation times of up to 1 year (Loffler *et al.*, 2005).

When radiolabelled Diazepam was incubated in a sediment-water system for 100 days, 95% of the radioactivity still remained as Diazepam which was undergoing continual partitioning onto the sediment. As the sediment pH was 7.7 Diazepam was found in its neutral, non-protonated form. This led to the conclusion that extensive occurrence of Diazepam in the sediment was predominantly due to non-ionic interactions. Diazepam underwent marginal degradation in surface waters, but was classified as highly persistent (DT_{90} (Dissipation Time) >>> 365 for sediment, 113 ± 17 for water). These results also

led to the conclusion that 60% of the loss was due to sediment sorption and less than 2% mineralisation occurred.

Oxazepam, one of the major metabolites, was also included in this study (Loffler *et al.*, 2005). It has a slightly higher polarity than Diazepam, but partitioned in the same manner (19 – 29%) and moderate degradation (DT_{90} = 179 ± 11 for sediment / water system, DT_{90} = 63 ± 6 for water) was seen. These results led to it's classification as moderately persistent with limited partitioning tendencies.

1.4. Sewage Sludge

There has been relatively extensive research into the fate of pharmaceuticals in the aquatic environment, e.g. (Heberer, 2002; Ternes, 2004). Aquatic studies have covered compounds ranging from analgesics and antibiotics to cardiovascular drugs and contraceptives. By comparison there is a substantial lack of work into the fate of the same compounds within the terrestrial environment. This lack of knowledge helped to focus the present project around the fate of pharmaceuticals in SS and SS-amended soil.

As sewage sludge production in the UK is currently in excess of 1.5×10^6 tonnes of dry solids (tds) per annum, treatment and disposal is essential (Ternes, 2004). The main purposes of sewage sludge treatments are to reduce volume, reduce health hazards, make it less offensive and to convert it into a form suitable for agricultural use, as approximately 50% of sewage sludge produced in the UK is land disposed (Ternes, 2004). This is most commonly done *via* the use of anaerobic digestion, although 'enhanced' treatments, such as lime treatment are becoming more common. Lime treated sludge, which is classified as 'enhanced', is produced by the addition of lime to liquid sludge until pH 12 is acquired and maintained for a minimum of 2 hours. This sludge can then be used directly on agricultural land. With all sewage sludges there is a risk of phytotoxic damage under acidic conditions. For this reason sludge cannot be used on soil with pH < 5 (DoE, 1996). Lime cake provides the greatest economic benefit (~ £110 fertiliser replacement value ha^{-1}) when compared to digested liquid, digested cake and thermally dried granules. These enhanced treated sludges are able to be applied directly onto more crops than conventionally treated sludges. There has therefore been significant investment

(£450 million) by the water industry to improve processing facilities, which is likely to increase the proportions of sludge undergoing enhanced treatment.

Between the years of 1998 to 2000 an average of 1,072,000 tds were produced per annum that needed to be disposed of in the UK (UK, 2001). Incineration and landfill are used but are significantly more expensive than recycling to land. Aside from this there are environmental and economic benefits of disposal to land, such as the improved soil structure and water holding capacity, higher yields, and saved fertiliser costs (estimated ~ £8 million saving per annum) (DEFRA, 2002b). Approximately 50% of the 1.1 million tonnes of sludge produced each year in the UK is disposed of to agricultural land, which equates to about 2% of organic materials used in land application (UK, 2001). Despite this, only a small proportion of farmers (~ 5%) use sewage sludge on less than 2% (80000 ha treated in 1996/7) of agricultural land (~ 60% arable, 40% pastural), so there is potential for expansion of this disposal route (DEFRA, 2002b). The areas to which this sewage sludge is disposed on in the UK are illustrated in Figure 1.8. A small number of treatment works in the UK (126) process the majority (60%) of the sludge, using a range of processes including; mesophilic anaerobic digestion (accounted for 31% of treatment in 1996/7), anaerobic digestion followed by dewatering and storage (23%), no treatment (26%), lime stabilisation (2%). In 1996/97 lime stabilisation accounted for 5% of sludge disposed of to agriculture in England and Wales, which equates to 480000 tds (1996/7 data) (Gendebien *et al.*, 1999).

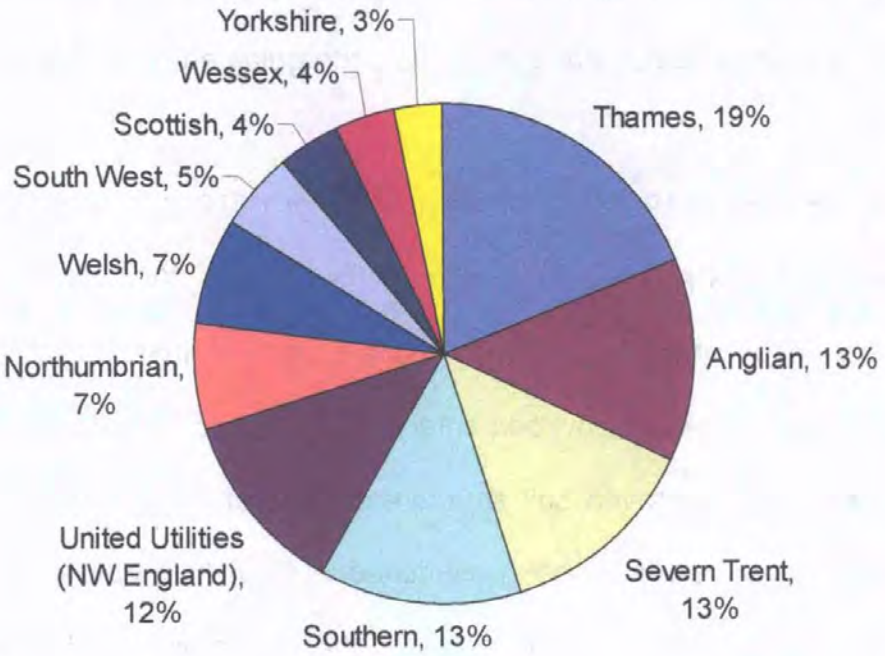


Figure 1.8. Regional percentage contribution to total land disposed SS (690000 tds in 2000).

Data sourced: (UK, 2001)

On a larger scale the EU produced 6.6 million tds in 1996, and an estimated 9.4 million tds by 2005. Alongside this increase in production, EU recycling of sewage sludge to land was predicted to increase by 73% in the same time period (Agency, 2001). Approximately 6.8 million tonnes of sewage sludge are produced per annum in the US, of which 54% is recycled to agricultural land (Velagaleti and Gill, 2001). These figures emphasize the potential wide reaching impacts of the problem of sewage sludge disposal in westernised nations.

1.4.1. Contaminants in Sewage Sludge

The physiochemical characteristics of an organic contaminant determines the extent to which it undergoes processes such as sorption onto solids, volatilisation and both biotic and abiotic degradation. This in turn determines the partitioning and concentration of these compounds within the different components of STW wastes. Regardless of the sewage sludge disposal method used (e.g. dumping at sea, use on agricultural land, incineration or landfill), the accumulation of contaminants within the sewage sludge poses a disposal problem which is potentially hazardous to the environment (Meakins *et al.*, 1994). Most published data on contaminants in sewage sludge have focused upon compounds such as polychlorinated biphenyls (PCBs), heavy metals and pesticides, and there is relatively little work on organic contaminants, including pharmaceuticals (Bright and Healey, 2003).

The POSEIDON program (Ternes, 2004) discussed and modelled in quite some detail the chemical processes involved during STW treatment. Volatilisation from STW settling tanks can be a major removal process for many compounds. However a Henry's coefficient > 3×10^{-3} atm m^3 $mole^{-1}$ is required for this to be an important removal mechanism (Ternes, 2004). Diazepam and Fluoxetine have Henry's coefficients of 6.5×10^{-10} and 2.7×10^{-7} atm m^3 $mole^{-1}$ respectively (US EPA modelling, HENRYWIN V3.10), and hence volatilisation, or stripping into the air (volatilisation during an aeration process in STW) is not considered to be an important process for these substances. As a general rule pharmaceuticals have a Henry's coefficient of < 10^{-5} atm m^3 $mole^{-1}$. Primary sludge is formed in settlement tanks where the predominant removal process is adsorption to solids, to which additional fats, oils and greases which are

skimmed from the tank surface, are added. Secondary sludge is generated through the use of microorganisms in activated sludge or trickling / percolating filters and a secondary sedimentation tank. It is the adsorption of organic contaminants onto cellular material (such as bacterial lipid or polysaccharide structures, nucleic acids or bacterial proteins), fats and greases that is the main mechanism by which these compounds become incorporated into sludge (Ternes, 2004).

Sorption onto biological material can also aid degradation as the entrance of a contaminant into a microbial cell is a requirement of intracellular enzyme induction and enzymes are then excreted into solution, or can are released at a later stage when the cell undergoes lysis. Generally cometabolic transformations cause the degradation of organic contaminants and mixed cultures are more successful at degradation of these compounds. The presence of fats, greases and surfactants in sewage sludge also encourages the partitioning of organic contaminants into the sewage sludge as these compounds are attracted to substances such as fats and hence are transported with them. The presence of dissolved salts or the absence of emulsifying agents decreases the solubility of these contaminants and therefore also increases their partitioning into the sludge *via* increased sorption (Ternes, 2004).

1.5. Transport and Fate

1.5.1. Sorption

The concept of pharmaceutical residues sorbed to soil is not an area that has been extensively researched, although more published work has appeared since the inception of the present study (2003). Since pharmaceuticals may be considered similar to pesticides, as stable bioactive compounds with similar transport routes onto fields and into the environment, it is not unreasonable to expect pharmaceutical residues to occur in soils as is well documented for pesticides. Soil is a complex matrix and sorption studies require a range of variables to be taken into consideration (Diaz-Cruz *et al.*, 2003; Bollag, 1991; Yeager and Halley, 1990; Khan and Ongerth, 2002).

Yeager and Halley (1990) define sorption as 'a general term that includes adsorption (surface binding) and partitioning', and desorption as 'the reverse process of sorption'. There are a variety of mechanisms involved in sorption of pharmaceuticals to solid matrices. Sorption to organic matter, surface adsorption to mineral components, ion exchange, hydrogen bonding and the formation of complexes with metal ions are considered to be the most important mechanisms (Diaz-Cruz *et al.*, 2003). Once compounds have been sorbed into the soil matrix in a manner in which they cannot easily be released they are often referred to as residues.

Pesticides are often transformed into aromatic amine or phenol intermediates which are thought to bind covalently to humics. Oxidoreductive enzymes or abiotic factors can catalyse this binding (oxidative coupling), and initiate covalent bonding between humics and xenobiotics; both of which can then be

suceeded by polymerisation and incorporation into the soil matrix. Biological and abiotic factors (such as microbial oxidoreductases, clay minerals and certain metal oxides) act as catalysts in the cross-linking of phenolic compounds to humics. Aromatic amines or anilines become bound to humics by mechanisms including hydrogen bonding, charge-transfer, and hydrophobic interactions (Bollag, 1991).

Bollag (1991) states there are two means by which xenobiotics can become integrated into soil organic matter. One is *via* incorporation into fulvic or humic acids during humification processes, in which case the xenobiotic becomes part of the structure of the humified residue. The other manner is *via* direct attachment to the surface reactive groups of the organic matter. Covalent bonds that are formed are usually fairly resistant to thermal and microbial degradation and to acid / base hydrolysis, and hence are classed as stable, persistent bonds.

However it has been questioned as to how stable these bonds are, and whether possible future release of xenobiotic residues could pose a risk to health. The majority of literature evidence suggests that the release of pesticide residues is extremely slow and once released further degradation of the pesticides can occur. These residues are therefore generally considered to be of low risk to the environment; although the mechanisms of release are not yet fully understood. It has been suggested that microbial activity can trigger the release of these residues. These released residues may then undergo further degradation / mineralization, or they may be taken up by plants, and hence could pose a risk to the environment (Bollag, 1991).

Bollag (1991) believes that there are three major environmentally relevant impacts due to residue formation. The first is reduced leaching of xenobiotics due to the formation of insoluble precipitates during the binding process. The second and third points relate to toxicity. Once the compound is bound, the bioavailability is reduced, as is the toxicity of a 'polymerised' compound compared to the parent compound. Work by Gevao *et al.*, (2003) confirms that the toxicity of pesticides is reduced; they become less bioavailable and progressively resistant to desorption as they age in the soil.

Yeager and Halley (1990) used a methodology set out by the US Food and Drugs Administration (FDA), for evaluating soil mobility of a veterinary pharmaceutical, Efrotomycin; an animal growth promoter. ^{14}C-Efrotomycin was used to generate sorption and desorption isotherms based on the Freundlich equation. Partial irreversibility of sorption occurred, highlighting that desorption is important in the avoidance of an overestimation of sorption.

Khan and Ongerth (2002) used a model to predict the concentrations of various pharmaceuticals in sewage sludge. They felt this work was necessary due to a review by The Environment Agency (UK) which stated that quantitative data was lacking for pharmaceuticals in sewage sludge. By acquiring pharmaceutical usage data and sewage generation rates for selected populations along with an adapted version of the Clarke *et al.*, model, they were able to predict steady-state concentrations and distribution of pharmaceuticals in aqueous, suspended solids and sludge.

Although Fluoxetine and Diazepam were not on the list of pharmaceuticals modelled, there were some interesting and relevant findings, which were comparable to laboratory generated data. Lipophilic compounds with a log K_{ow} < 2 were predicted to be found in primary sludge at concentrations greater than in the raw sewage. However the results predicted that concentrations in the digested sludge would be very low. It was therefore concluded that once the sludge had been digested it lost its lipophilic properties and compounds present began to partition into the aqueous phase.

Kinney *et al.*, (2006) investigated the accumulation of pharmaceuticals, including Fluoxetine, in soils on three sites across Colorado, USA. The use of wastewater effluent for land irrigation is becoming more common place to reduce treatment costs (2.4% of US wastewater). Effluent treatment before application to land consisted of coagulation, filtration and chlorine disinfection. Soil cores taken across the sites were found to contain from 366 to 14,400% of estimated Fluoxetine loading. This high recovery clearly shows the accumulation of Fluoxetine in soil from previous seasons irrigations. Fluoxetine was one of the four most detected compounds in this study, which the authors believed is due to an aqueous solubility of < 100 mg L^{-1} for Fluoxetine, as with Carbamazepine and Erythromycin.

A study by Bedner and MacCrehan (2006) indicated that Fluoxetine readily undergoes transformation into *N*-chlorofluoxetine during wastewater disinfection treatment. *N*-chlorofluoxetine also underwent dechlorination, a process often included in wastewater treatment, back to the parent compound. However this transformation did not occur in the dechlorination time period normally used in

wastewater treatment. Bedner and MacCrehan (2006) therefore believe that there is a risk of release of this chloramine into the environment. Chloroamines exhibit greater hydrophobicity than parent compounds and therefore are more likely to sorb to soils, sediments and biological membranes. Once sorption has occurred these chloroamines may transfer the active chlorine to a reductant compound, hence releasing the parent compound. The wastewater disinfection and dechlorination processes do not act as a means to remove Fluoxetine from water. This information regarding the formation of *N*-chlorofluoxetine may also help to explain the very high accumulation of Fluoxetine in soils seen in the study by Kinney *et al.*, (2006).

1.5.2. Degradation

There are several degradation processes to which pharmaceuticals may be exposed. The biodegradation of various pharmaceuticals has been investigated in a range of matrices (Marengo *et al.*, 1997; Zwiener *et al.*, 2002; Zwiener *et al.*, 2000; Mohle and Metzger, 2001; Ternes *et al.*, 2002). These degradation processes can be broadly classified into two groups; chemical and biological degradation, or biotic and abiotic degradation. Only biodegradation is considered in the scope of the present study and only examples of biotic degradation experiments are therefore presented herein. A very recent review on the impact of abiotic factors on the fate of 1,4-benzodiazepines has been made by West (2007).

Marengo *et al.*, (1997) performed a study into the biodegradation of an anti-bacterial agent in soil. The experiment was performed in soil incubation flasks spiked with ^{14}C-sarafloxacin HCl. Results indicated that the degradation of sarafloxacin may in fact be due to abiotic, rather than biotic factors.

A study into the biodegradation of Ibuprofen in batch experiments with activated sludge and in biofilm reactors resulted in the formation of different metabolites depending upon whether conditions were oxic or anoxic (Zwiener *et al.*, 2002).

The biodegradation of Ibuprofen, Diclofenac and the metabolite, Clofibric acid, was studied in a biofilm reactor experiment. During various biodegradation studies elimination rates increased with increasing time, leading to the conclusion that microorganisms gradually adapted to degrade the Ibuprofen (Zwiener *et al.*, 2000).

Mohle and Metzger (2001) studied the elimination of pharmaceuticals in activated sludge under aerobic conditions and developed a method using HPLC-MS-MS with an on-line batch reactor. This batch method allowed both adsorption and biodegradation / transformation to be taken into consideration simultaneously. Analysis of the compounds after only 15 minutes showed significant decreases in concentrations. The authors believed that this initial rapid concentration decrease was due to adsorption of the compounds to the sludge. Further slower decreases in concentration were seen later in the experiment, which the authors attributed to biodegradation.

Janusz *et al.*, (2003) used enrichment cultures to degrade target compounds in liquid media. Microbial populations in different soils (i.e. urban and agricultural), displayed different degradation rates (urban was more rapid) for phenyl-2-propanone and responded differently to additional carbon sources, such as glucose. In some cases the use of an additional carbon source increased the rate of degradation, and in others it slowed. The authors therefore drew the conclusion that the exact degradation profile and metabolites derived from a particular compound will vary between soils, depending upon the residual carbon concentrations and the microbial populations present.

An investigation into the removal of pharmaceuticals during drinking water treatment was performed by Ternes *et al.*, (2002). Batch experiments according to Organisation for Economic Cooperation and Development (OECD) guidelines were carried out to obtain a broad idea of the biodegradability of the target compounds. In this particular case no biodegradation or significant sorption of any target compounds occurred. However because esterase activity was

monitored it can be stated that the pharmaceuticals did not inhibit the microbial population and that biodegradation of DOC did occur.

Loffler *et al.*, (2005) studied the fate of pharmaceuticals in sediment - water systems. It was noted that pharmaceutical metabolites that are the result of phase I metabolism, such as Oxazepam, are more likely to undergo further transformation processes that the parent compounds. In the case of Oxazepam this was shown by its lower half life within the experimental test system. Transformation processes that occur within the human body are well known, but the use of pharmacacological data to predict the transformation behaviour of a compound within the environment is much more limited. Diazepam and carbamazepine provide good examples of this. Both drugs are easily metabolised by humans, but are both stable within the water - sediment test system. The sorption coefficients (K_{oc}s) generated by Loffler *et al.*, (2005) were found to be between 83 and 192 L kg^{-1} for Diazepam and Oxazepam, which correlates well with published K_{oc}s for sewage sludge, and for Diazepam in soil (Appendix, Table A.2). Loffler *et al.*, believe that for most of their target pharmaceuticals that biodegradation was unlikely as many of these compounds are stable under both aerobic and anaerobic conditions found in both STW and the human body.

Other authors (e.g. Joss *et al.*, 2006) have devised classification schemes for biodegradation of PPCPs during state-of-the-art wastewater treatment processes, based on results from batch experiments using sewage sludge. Numerical models, regression and estimation of degradation rate constants (K_{biol}) were then generated from these results using Matlab software. Pseudo

first order degradation was seen for all compounds that underwent transformation. Three categories were devised, into which all studied compounds could be classified:

90% +, significant removal. (K_{biol} > 10 L g_{ss}^{-1} d^{-1})

20 to 90%, partial removal. (K_{biol} 0.1 – 10 L g_{ss}^{-1} d^{-1})

<20%, unsubstantial removal (K_{biol} < 0.1 L g_{ss}^{-1} d^{-1})

Biological activity will vary been sludge and reactor types, but despite this Joss *et al.,* (2006) believed that compounds can be divided into classes with respect to their behaviour in these wastewater facilities.

The studies discussed highlight how complex the study of biodegradation in SS-amended soil might be. Biodegradation of a compound can be influenced by factors including; soil type and source or usage, abiotic factors such as redox conditions and adaptation of microorganisms to degradation of target compound. No Quantitative Structure Activity Relationships (QSARs) could be developed by the POSEIDON project (Ternes, 2004) and the importance of determining the biodegradation of each compound experimentally was stressed. For this reason the present study researched experimental biodegradation of the target compounds under environmentally relevant conditions.

1.5.3. Plant up-take

Smith *et al.*, (2001) reviewed the processes involved in the transport of persistent, semi-volatile and bioaccumulative organic contaminants into vegetation (most markedly atmospheric deposition and soil transfer), and the factors which cause dilution of the contaminant within the plant such as growth dilution, particle wash-off, volatilisation, photodegradation and metabolism. Most studies have shown that air-plant transfer predominates as the transfer mechanism to vegetation, as opposed to root uptake from soil which tends to be an inefficient process.

Smith *et al.*, (2001) also discuss in some detail the rationale and benefits behind the use of sewage sludge as an agricultural fertiliser, alongside the risks associated with the introduction of xenobiotics into soils. Various regulations have been put into place to limit the use of SS, but these regulations are predominantly based on heavy metal inputs. There is a lack of information on transport and fate of organic contaminants associated with SS. There is currently no consensus on whether limits for organic contaminants in SS are necessary and concerns arise that the cost of regulation would be prohibitive (McGrath, personal communication). Quantitative pathway analysis has shown that the risk of persistent compounds entering the food chain is greater for pastoral land due to the risk of ingestion by grazing animals. Legislation has dealt with this risk by laying down 'no grazing' ban periods (3 weeks in UK) or has banned addition of sludge to pasture land.

Despite the past concern and publicity over the transport of pesticides into food crops almost no research has been performed into the up-take of

pharmaceuticals into plants. This project aimed to provide initial background knowledge on the uptake of pharmaceuticals into plants, by first developing appropriate extraction methods and then by studying the uptake of Fluoxetine HCl into cauliflowers grown on gel media.

CHAPTER TWO

METHOD DEVELOPMENT OF ANALYSIS TECHNIQUES

2. Method Development of Analysis Techniques

2.1. Introduction

Published analytical techniques for the study of pharmaceuticals in soils, sediments and sludge, have been reviewed by Diaz-Cruz *et al.*, (2003) and Hao *et al.*, (2007). These findings will be summarised here along with reviews of subsequent literature.

Generally, analyte extraction from solid matrices has involved sonication or simple blending or stirring of polar organic solvents with the sample. More advanced techniques such as accelerated solvent extraction (ASE), pressurised liquid extraction (PLE), ultrasonic solvent extraction (USE) or microwave assisted extraction (MAE) have also been occasionally used (Hao *et al.*, 2007). These reviews concluded that use of advanced clean-up techniques along with advanced MS instrumentation are the most appropriate choices for the analysis of pharmaceuticals from solid matrices.

Solid phase extraction (SPE), liquid-liquid extraction (LLE), gel permeation chromatography and semi-preparative HPLC have been the most commonly used clean-up methods used for aqueous samples. SPE has been the most commonly used of these techniques for various reasons, mainly related to the ease of use, speed, low risk of contamination, use on-line and low volumes of organic solvent required (Diaz-Cruz *et al.*, 2003). Reversed phase adsorbents have often been used with SPE clean-up methodologies, as is the case with PPCPs where the readily available Oasis HLB cartridges have been commonly used (Hao *et al.*, 2007).

For analysis of the purified extracts, HPLC has been the preferred technique of choice, although GC-MS has also been used. This preference for HPLC is due to the polarity and / or thermolability of many pharmaceuticals. Generally reverse phase chromatography with so called C_{18} columns (octadecylsilane stationary phase) has been employed.

Detectors coupled to the HPLC instruments for analysis of pharmaceuticals have tended to be UV, MS or fluorescence detectors. In the past, UV detectors were most commonly used; use of MS has now overtaken this due to the higher sensitivity and selectivity of MS (Hao *et al.*, 2007). Fluorescence detectors have been used to a lesser extent, mainly due to the need for prior derivatisation of many analytes. Diaz-Cruz *et al.*, (2003), provide an approximate estimate of achievable limits of detection (LOD) with the different detection methods. When UV spectromphotometry was used, the LOD typically ranged from 10 to 200 ng g^{-1}. With MS, LODs ranged from between 0.2 to 40 ng g^{-1}, and a LOD of approximately 10 ng g^{-1} was achieved with fluorescence detection.

Within MS, the most favoured ionisation technique is now electrospray ionisation (ESI), although there are also reports of the use of fast atom bombardment and particle beam MS. The operating mode is usually selected ion monitoring (SIM) which allows greater sensitivity but has less rigorous confirmation of analyte identity.

Diaz-Cruz *et al.*, (2003) recommend greater used of advanced MS instrumentation, (e.g. LC-tandem MS), to lower detection limits and improve identification of analytes in complex matrices. Alongside this the use of more

advance purification/clean-up techniques is also recommended. Table 2.1 to Table 2.5 have been adapted and expanded from Diaz-Cruz *et al.*, (2003) and summarise techniques for the extraction and analysis of pharmaceuticals from a range of matrices.

	Sample	Compound	Extraction	Column	Mobile phase (Composition %)	Detection	Method limits	Ref
Soil Samples	Soil (waste water irrigated)	19 pharmaceuticals (including Fluoxetine)	ASE (ACN : water; 70 : 30)	Metasil Basic, 3 μm, 150 mm x 2.0 mm, C18 analytical column; coupled to Metasil Basic safeguard, 3μm, 2.0 mm guard column (or NewGuard RP-18, 7 μm, 15 mm x 3.2 mm guard column	Ammonium formate / formic acid buffer (10mM, pH 3.7)	HPLC, electrospray ionisation (+) mode / SIM	0.76 - 5.46 μg kg^{-1}	(Kinney *et al.*, 2006; Cahill *et al.*, 2004)
	Soil	Metronidazole & Olaquindox	Solvent extraction (MeOH)	Hypersil BDS 250x2.1 mm	Ammonium acetate (10mM); Methanol (80:20)	Electrospray MS (+) mode/SIM	not reported	(Rabolle and Spliid, 2000)
	Soil	Oxitetracycline	Solvent extraction (MeOH)	Hypersil BDS 250x2.1 mm	Sodium acetate (10mM) + calcium chloride (55mM) + Na_2EDTA (20mM).	Fluorescence, lexc 390nm, lem 512nm	not reported	(Rabolle and Spliid, 2000)
	Soil	Tylosin	Solvent extraction (MeOH)	Hypersil BDS 250x2.1 mm	Ammonium acetate (10mM); Methanol (10:90)	Electrospray MS (+) mode/SIM	not reported	(Rabolle and Spliid, 2000)
	Soil	Sulfadimethoxine	Solvent extraction (chloroform + acetone), followesd by SPE (SCX cartridge)	LiChrospher RP-C18 250x4 mm	Gradient. Methanol to phosphoric acid (10mM), pH 3	UV, l 275 nm	not reported	(Brambilla *et al.*, 1994)
	Soil	Flumequine	Solvent extraction (chloroform + acetone), followesd by SPE (SCX cartridge)	LiChrospher 100-RP18 125x4 mm	Phosphoric acid (50 mM), pH 3.5 + acetonitrile (90 + 10):phosphoric acid (50mM), pH 3.5 + acetonitrile (50 + 50), (70:30)	Fluorescence, lexc 327nm, lem 369nm	0.01 μg g^{-1}	(Brambilla *et al.*, 1994)
	Fertilised soil	Tetracycline & Chlortetracycline	Solvent extraction (1M citrate buffer + ethyl acetate)	Puresil C18 150x4.6mm	Gradient. Formic acid (0.5%) + ammonium acetate (1mM) + water (pH 2)	Electrospray (+) mode, MS/MS, MS/MS/MS	0.001 & 0.002 μg g^{-1} respectively	(Hamscher *et al.*, 2002)
	Fertilised soil	Oxitetracycline	Solvent extraction (1M citrate buffer + ethylacetate)	Puresil C18 150x4.6mm	Gradient. Formic acid (0.5%) + ammonium acetate (1mM) + water (pH 2)	Electrospray (+) mode, MS/MS, MS/MS/MS	0.001 μg g^{-1}	(Hamscher *et al.*, 2002)
	Fertilised soil	Tylosin	Solvent extraction (1M citrate buffer + ethyl acetate)	Puresil C18 150x4.6mm	Gradient. Formic acid (0.5%) + ammonium acetate (1mM) + water (pH 2)	Electrospray (+) mode, MS/MS, MS/MS/MS	0.001 μg g^{-1}	(Hamscher *et al.*, 2002)

Table 2.1. Literature examples of extraction and analysis methods of pharmaceuticals from soil matrices

	Sample	Compound	Extraction	Column	Mobile phase (Composition %)	Detection	Method limits	Ref
Natural Sediment Samples	Sediment	10 pharmaceuticals (including Diazepam & Oxazepam)	Sequential sediment extraction (MeOH & ethyl acetate) using ultrasonic treatment, and SPE (ICT RP-18ec) (for Benzodiazepines)	Lichrosphere RP_{18ec} 125 mm x 3 mm column	not reported	LC, electrospray (+) mode / MS^2 OR Radio-TLC	≤ 1% of initally applied concetration	(Loffler *et al.*, 2005)
	River sediment	18 pharmaceuticals (including Diazepam)	Ultrasonic extraction with MeOH	Intertsil ODS-2 column	not reported	HPLC-MS^2 / MRM	9 ng kg^{-1}	(Zuccato *et al.*, 2000)
	Natural marine sediment	Oxitetracycline	Solvent extraction (0.1 M Na_2EDTA + McIlvaine Buffer), followed by SPE (C_{18} cartridge)	RP-8 254x4 mm	Methanol:acetonitrile:oxalic acid (10mM), pH 2 (20:30:50)	UV, I 350nm	not reported	(Jacobsen and Berglind, 1988)
	Natural marine sediment	Oxitetracycline	Solvent extraction (0.1 M Na_2EDTA + McIlvaine Buffer), followed by SPE (C_{18} cartridge)	Microsorv-MV C8 25cm	Acetonitrile, pH 3.2:oxalic acid (10mM), pH2 (35:65)	UV, I 365nm	0.2 μg g^{-1}	(Capone *et al.*, 1996)
	Natural marine sediment	Sulfadimethoxine	Solvent extraction (0.1 M Na_2EDTA + McIlvaine Buffer), followed by SPE (C_{18} cartridge)	hypersil ODS 200x4.6mm. (50oC)	Acetonitrile:sodium phosphate (100mM) (25:75)	UV, I 270 nm	0.05 μg g^{-1}	(Capone *et al.*, 1996)
	Natural marine sediment	Ormethoprim	Solvent extraction (0.1 M Na_2EDTA + McIlvaine Buffer), followed by SPE (C_{18} cartridge)	Hypersil ODS C18 200x4.6 mm (50oC)	Acetonitrile:sodium phosphate (100mM) (25:75)	UV, I 270 nm	0.05 μg g^{-1}	(Capone *et al.*, 1996)

Table 2.2. Literature examples of extraction and analysis methods of pharmaceuticals from natural sediment matrices

Table 2.2 – continued...

	Sample	Compound	Extraction	Column	Mobile phase (Composition %)	Detection	Method limits	Ref
Natural Sediment Samples	Natural river sediment	Estriol & Estradiol & Ethynyl estradiol & Estrone & Diethylstilbestrol	Solvent extraction (MeOH + Acetone), followed by SPE (C_{18} cartridge)	LiChrospher 100-RP18 250x4 mm	Gradient. Acetonitrile:water (10:90) to acetonitrile	Electrospray MS (-) mode/SIM	0.05 - 1 $\mu g\ g^{-1}$	(Lopez de Alda and Barcelo, 2002)
	Natural river sediment	Estriol & Estradiol & Ethynyl estradiol & Estrone & Diethylstilbestrol	Solvent extraction (MeOH + Acetone), followed by SPE (C_{18} cartridge)	LiChrospher 100-RP18 250x4 mm	Gradient. Acetonitrile:water (10:90) to acetonitrile	Electrospray MS (-) mode/SIM	0.05 - 1 $\mu g\ g^{-1}$	(Lopez de Alda and Barcelo, 2002)
	Natural river sediment	Estriol & Estradiol & Ethynyl estradiol & Estrone & Diethylstilbestrol	Solvent extraction (MeOH + Acetone), followed by SPE (RAM cartridges; ADS C_4)	LiChrospher 100-RP18 250x4 mm	Gradient. Acetonitrile:water (30:70) to acetonitrile	Electrospray MS (-) mode/SIM	1 - 5 $\mu g\ g^{-1}$	(Petrovic *et al.*, 2002)
	Natural river sediment	Norethindrone & Levonorgestrel & Progesterone	Solvent extraction (MeOH + Acetone), followed by SPE (C_{18} cartridge)	LiChrospher 100-RP18 250x4 mm	Gradient. Acetonitrile:water (10:90) to acetonitrile	Electrospray MS (+) mode/SIM	0.04 $\mu g\ g^{-1}$	(Lopez de Alda and Barcelo, 2002)
	Natural river sediment	Norethindrone & Levonorgestrel & Progesterone	Solvent extraction (MeOH + Acetone), followed by SPE (RAM cartridges; ADS C_4)	LiChrospher 100-RP18 250xmm	Gradient. Methanol:water (30:70) to methanol	Electrospray MS (+) mode/SIM	0.5 $\mu g\ g^{-1}$	(Petrovic *et al.*, 2002)

Table 2.2. Literature examples of extraction and analysis methods of pharmaceuticals from natural sediment matrices

	Sample	Compound	Extraction	Column	Mobile phase (Composition %)	Detection	Method limits	Ref
Artificial sediment samples	Artificial and natural marine sediment	Oxitetracycline	Solvent extraction (0.1 M Na_2EDTA + McIlvaine Buffer)	Hypersil ODS 100x5 mm	Acetonitrile:water + Na2EDTA (1 mM) + potassium nitrate (100mM), pH 3.2 (20:30:50)	UV, I 365 nm	0.1 $\mu g\ g^{-1}$	(Samuelsen, 1989; Hansen *et al.*, 1993)
	Artificial marine sediment	Oxitetracycline	Solvent extraction (0.1M NaOH), followed by LLE (1M HCl + chloroform + ethylacetate)	LiChrospher 100-RP18 125x4.6 mm	Acetonitrile:orthophosphoric acid (20mM), pH 2.3 (24:76)	UV, I 355nm	0.01 $\mu g\ g^{-1}$	(Pouliken *et al.*, 1994)
	Artificial marine sediment	Oxitetracycline	Solvent extraction (0.1 M Na_2EDTA + McIlvaine Buffer), followed by SPE (C_{18} cartridge)	Spherisorb 53 ODS1 150x2.1mm	Graident. Oxalic acid (10mM):methanol (80:20) to acetonitril:methanol (80:20)	UV, I 355nm & Frit FAB (-) mode MS-MS & Particle Beam (-) mode/scan	none (UV). 0.01 $\mu g\ g^{-1}$ (FAB). 1 $\mu g\ g^{-1}$(PB MS)	(Delepee *et al.*, 2000)
	Artificial marine sediment	Tetracycline & Chlortetracycline	Solvent extraction (0.1 M Na_2EDTA + McIlvaine Buffer), followed by SPE (C_{18} cartridge)	Spherisorb 53 ODS1 150x2.1mm	Gradient. Oxalic acid (10mM):methanol (80:20) to acetonitrile:methanol (80:20)	UV I 355nm & Frit FAB MS & Particle Beam MS (-) mode /scan	none (UV); 0.01 $\mu g\ g^{-1}$(FAB). 1 $\mu g\ g^{-1}$ (PB MS)	(Delepee *et al.*, 2000)
	Artificial marine sediment	Sulfadimethoxine	Solvent extraction (0.1 M Na_2EDTA + McIlvaine Buffer)	Hypersil ODS C18 100x4.6 mm	Phosphoric acid (50 mM), pH 3.5 + acetonitrile (90 + 10):phosphoric acid (50mM), pH 3.5 + acetonitrile (50 + 50), (50:50)	UV, I 270 nm	not reported	(Samuelsen, 1989)
	Artificial marine sediment	Ormethoprim	Solvent extraction (0.1M NaOH & water & 0.1M HCl & 1M Na_2EDTA + McIlvaine Buffer)	Hypersil ODS C18 100x4.6 mm	Phosphoric acid (50 mM), pH 3.5 + acetonitrile (90 + 10):phosphoric acid (50mM), pH 3.5 + acetonitrile (50 + 50), (50:50)	UV, I 270 nm	not reported	(Samuelsen *et al.*, 1994)

Table 2.3. Literature examples of extraction and analysis methods of pharmaceuticals from artificial sediments matrices

Table 2.3 – continued...

	Sample	Compound	Extraction	Column	Mobile phase (Composition %)	Detection	Method limits	Ref
Artificial sediment samples	Artificial marine sediment	Oxolinic acid	Solvent extraction (0.1 M Na_2EDTA + McIlvaine Buffer)	Hypersil ODS 100x5 mm	Graident. Oxalic acid (25mM, pH 3.2):acetonitrile:methanol:tethrahydrofuran (80:2.5:15:2.5) to Oxalic acid (25mM), pH 3.2):acetonitrile:methanol:tethrahydrofuran (50:20:25:5)	UV, l 280 nm	not reported	(Hansen *et al.*, 1993)
	Artificial marine sediment	Oxolinic acid	Solvent extraction (0.1M NaOH), followed by LLE (1M HCl + chloroform + ethylacetate)	LiChrospher 100-RP18 125x4.6 mm	acetonitrile:orthophosphoric acid (20mM), pH 2.3 (24:76)	UV, l 262 nm	0.01, 0.04 $\mu g\ g^{-1}$	(Pouliken *et al.*, 1994)
	Artificial marine sediment	Flumequine	Solvent extraction (0.1 M Na_2EDTA + McIlvaine Buffer)	Hypersil ODS 100x5 mm	Gradient. Oxalic acid (25mM, pH 3.2):acetonitrile:methanol:tethrahydrofuran (80:2.5:15:2.5) to Oxalic acid (25mM, pH 3.2):acetonitrile:methanol:tethrahydrofuran (50:20:25:5)	UV, l 280 nm	not reported	(Hansen *et al.*, 1993)
	Artificial marine sediment	Sulfadiazine & Trimetroprim	Solvent extraction (0.1M NaOH & water & 0.1M HCl & 1M Na_2EDTA + McIlvaine Buffer)	Hypersil ODS C18 100x4.6 mm	Phosphoric acid (50 mM), pH 3.5 + acetonitrile (90 + 10):phosphoric acid (50mM), pH 3.5 + acetonitrile (50 + 50), (70:30)	UV, l 270 nm	not reported	(Samuelsen *et al.*, 1994)
	Artificial marine sediment	Sulfadiazine & Trimetroprim	Solvent extraction (0.1M NaOH & water & 0.1M HCl & 1M Na_2EDTA + McIlvaine Buffer)	Hypersil ODS C18 100x4.6 mm	Phosphoric acid (50 mM), pH 3.5 + acetonitrile (90 + 10):phosphoric acid (50mM), pH 3.5 + acetonitrile (50 + 50), (70:30)	UV, l 270 nm	not reported	(Samuelsen *et al.*, 1994)

Table 2.3. Literature examples of extraction and analysis methods of pharmaceuticals from artificial sediments matrices

	Sample	Compound	Extraction	Column	Mobile phase (Composition %)	Detection	Method limits	Ref
Sludge Samples	Sewage sludge	5 pharmaceuticals	Filtration followed by SPE (Isolute ENV+)	not reported	not reported	Derivistisation followed by GC-MS (Jones et al 2003 method)	4 ng L^{-1}	(Jones *et al.*, 2007)
	Primary & secondary sewage sludge	pharmaceuticals (including Diazepam)	Ultrasonic extraction (MeOH / acetone), followed by SPE.	not reported	not reported	LC tandem MS	LOQ 20 - 50 ng g^{-1}	(Ternes, 2004)
	Natural river sediment and sludge	Estriol & Ethynyl estradiol & Estrone & Mestranol	Solvent extraction (MeOH + acetone). SPE (C_{18} cartidges; silica gel). LLE (GPC with bio-beads SX-3; Semi-prep HPLC)	XTI-5 (30 m x 0.25 mm x 0.25 mm)	not reported	GC/MS/MS (EI)	0.2 - 4 µg g^{-1}	(Ternes *et al.*, 2002)

Table 2.4. Literature examples of extraction and analysis methods of pharmaceuticals from sludge matrices

	Sample	Compound	Extraction	Column	Mobile phase (Composition %)	Detection	Method limits	Ref
Biological Samples	Fish tissue	SSRIs	LLE followed by SPE (Bond Elute Certify)	Agilent HP-ULTRA-1 cross-linked methyl siolxane capillary column, length 12 m, inner diameter 0.2 mm, film thickness 0.33 μm	n/a	Derivitisation then GC-MS (-) mode / SIM	LOD = 0.01 ng g^{-1} LOQ ≈ 0.05 ng g^{-1}	(Brooks *et al.*, 2005)
	Human liver microsomes	Fluoxetine (& *p*-trifluoromethylphenol)	LLE followed by SPE (Bond Elute Certify)	HP-5 cross-linked 5% PH NE siloxane, 15 m x 0.53 mm, 1.5 μm film thickness	n/a	Derivaitisation then GC-ECD	LOD = 6.92 (1.62) ng ml^{-1}, LOQ = 34.6 (8.1) pg	(Liu *et al.*, 2002)
	Hair	Benzodiazepines (including Diazepam)	Soils combusted in sample oxidiser	not reported	not reported	Deriviitisation followed by GC-MS, or HPLC-UV, or HPLC-DAD, or GC-ECD	not reported	(Sachs and Kintz, 1998)
	Rat brain & liver tissues	*p*-Trifluoromethylphenol	LLE	Narrow bore fused silica capillary column, 25 m x 0.32 mm, 1.05 μm film of 5% phenylmethylsilicone	n/a	Derivistitsation followed by GC-ECD (15 mCi ^{63}Ni linear ECD)	LOD < 10 ng g^{-1} for brain tissue, < 25 ng g^{-1} for liver tissue	(Urichuk *et al.*, 1997)

Table 2.5. Literature examples of extraction and analysis methods of pharmaceuticals from biological tissue matrices

2.1.1. LC-MS^n

Historically the major limiting factor in the use of LC-MS was the issue of effective sample introduction. It was the advent of atmospheric pressure ionization (API) interfaces; such as electrospray ionisation (ESI) whose development began in the late 1960s, and atmospheric pressure chemical ionisation (APCI, early 1970s), that has resulted in significantly increased use of LC-MS (Niessen, 1998). LC-MS in this project was carried out using ESI with a Finnigan MAT LCQ™ quadrupole ion trap mass spectrometer. Thus the following brief explanation of instrumentation is specific to the ESI ion trap quadrupole instruments.

The process of ESI, which is a soft ionisation technique with little or no fragmentation, resulting in the formation of dominantly molecular or pseudomolecular ions, occurs in 3 stages; charged droplet formation, droplet shrinkage and disintegration, and the formation of gas phase ions. The sample solution flows through an electrospray capillary at a potential of ± 3 – 5 kV, towards the sampling orifice (heated capillary tube) at ± 0 – 50 V. The electrical field results in solution ions of similar polarity collecting at the capillary tip, which are then drawn out due to the potential gradient, creating a 'Taylor cone'. As solution ions collect at the liquid surface, electrostatic repulsion becomes stronger than surface tension, resulting in the emission of charged droplets. In most circumstances the loss of negative charge, *via* electrochemical discharge to the metal spray capillary wall, results in positively charged droplets. Direct removal of electrons from sample molecules with low ionization energy can however occur under certain conditions (Bruins, 1998; McCormack, 2003).

As these charged droplets travel down the potential gradient, solvent evaporation and droplet shrinkage occur, increasing charge density at the droplet surface. Surface tension is overcome by Coloumbic forces, and the droplet disintegrates to smaller droplets. This droplet shrinkage and disintegration continues until very small droplets (~ 10 nm radius) are formed which can release charged ions into the gas phase from the droplet surface. At high flow rates, pneumatically-assisted ESI, also known as ionspray, aids this process by increasing coaxial nitrogen flow (Bruins, 1998; McCormack, 2003). A diagram of the ESI process is shown in Figure 2.1. More detailed mechanistic information on ESI or other interfaces is given by Bier and Schwartz (1997), Niessen (1998), and Niessen and Tinke (1995).

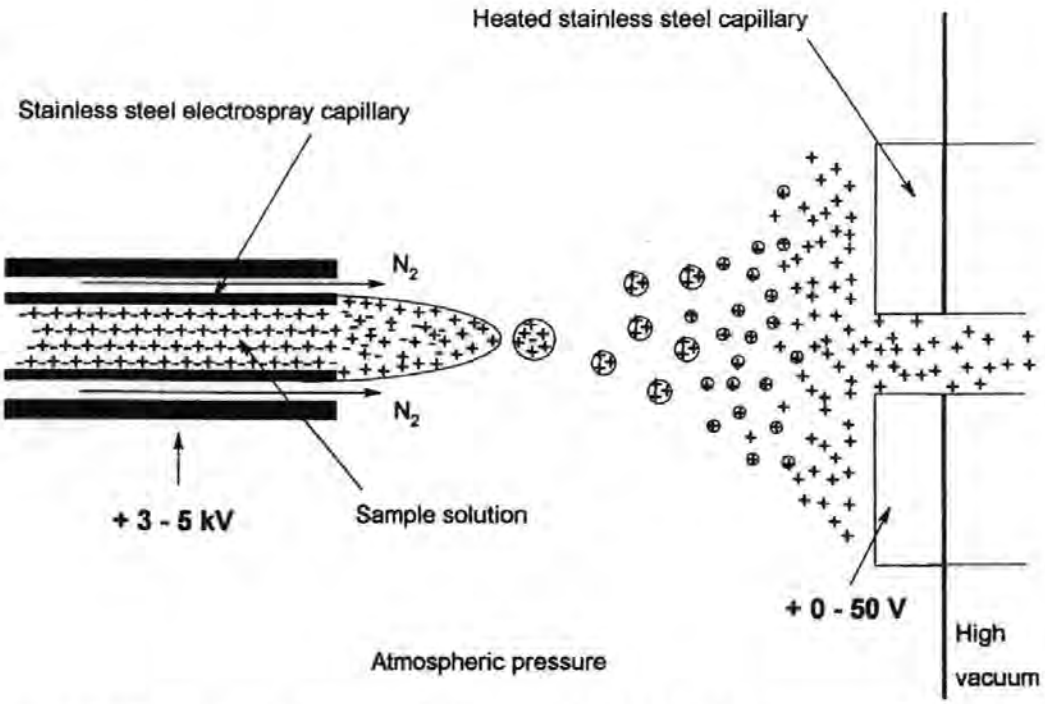


Figure 2.1. Simplified diagram of Finnigan MAT LCQ™ electrospray ionisation source with droplet and gas phase ion formation

Source: (McCormack, 2003)

Most types of mass analysers have at one point been interfaced with ESI. Single quadrupole MS instruments can provide structural information on drug metabolites using in-source fragmentation, but triple-quadrupole (QqQ) and ion trap (IT) mass analysers provide most selectivity because of their ability to perform collision induced dissociation (CID). IT instruments trap ions in a small volume with the use of electrodes, and alteration of electrode voltages causes ion ejection from the trap (Perez and Barcelo, 2007). Instruments which have the ability to perform in-source CID subject all ions to CID, whereas instruments with MS^n capabilities allow selection of a precursor ion prior to performing CID, therefore significantly improving signal to noise ratios (S:N) (Niessen, 1998). A distinctive feature of IT mass analysers is their ability to generate MS^n spectra (Hao *et al.*, 2007). This is due to their ability to trap, collect and perform operations e.g. CID on ions over a period of time within one analyser therefore increasing the signal to noise ratio (S:N) (Perez and Barcelo, 2007).

2.2. Chemicals

2.2.1. Target PPCPs

Structures and sources of the pharmaceuticals used in this project (Fluoxetine, Norfluoxetine, Diazepam, Temazepam, Oxazepam and Nordiazepam) along with deuterated analogues chosen as internal standards (d_5-Fluoxetine and d_5-Oxazepam) are presented in Figure 2.2 and Figure 2.3. Structures of related compounds are also presented to aid in understanding of the nomenclature and labelling system used with these drugs.

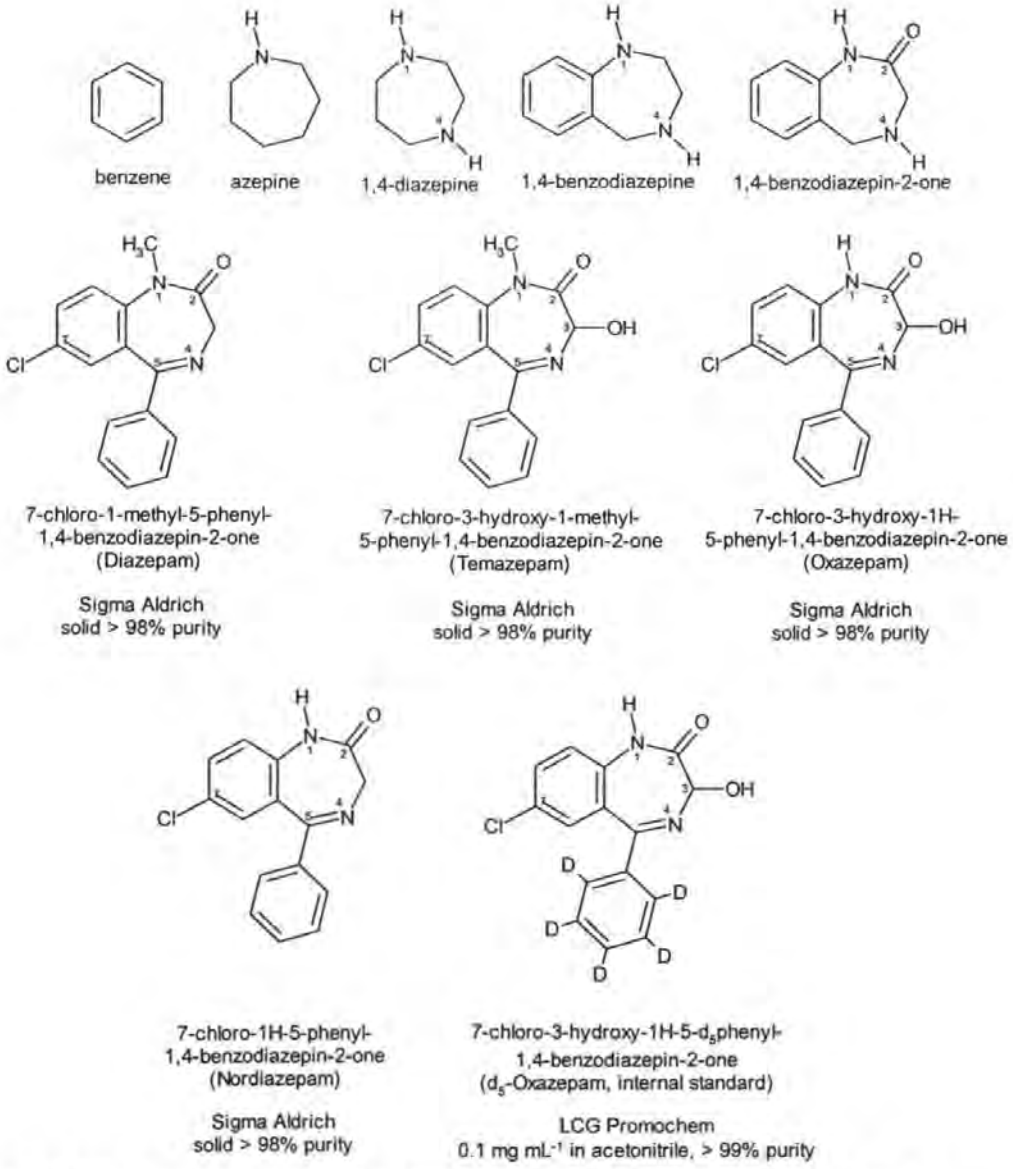
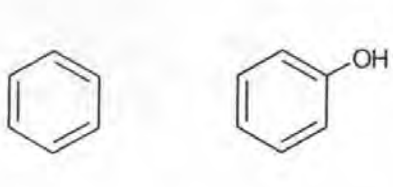
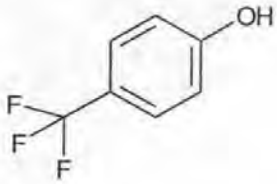


Figure 2.2. Structures and supply information for 1,4-benzodiazepines (Diazepam, Temazepam, Oxazepam, Nordiazepam and d_5-Oxazepam (IS)

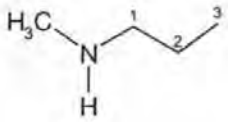


benzene

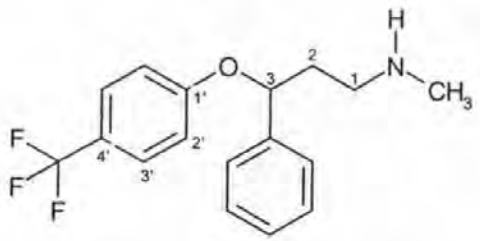
phenol



p-trifluoromethylphenol

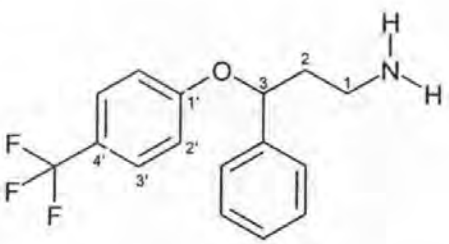


N-methyl-propan-1-amine



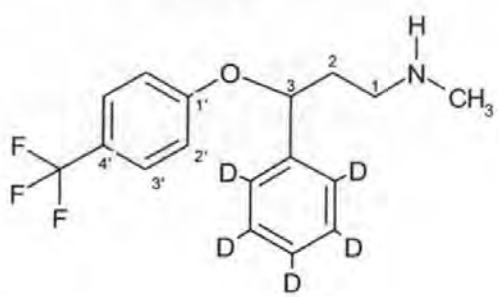
N-methyl-3-phenyl-3[4'-trifluoromethyl-phenoxy]-propan-1-amine
(Fluoxetine)

LCG Promochem
Purchased as hydrochloride
solid reference standard
> 99% purity



3-phenyl-3[4'-trifluoromethyl-phenoxy]-propan-1-amine
(Norfluoxetine)

Sigma Aldrich
solid > 97% purity



N-methyl-3-$d_5$phenyl-3[4'-trifluoromethyl-phenoxy]-propan-1-amine
(d_5-Fluoxetine, internal standard)

Sigma Aldrich
Purchased as hydrochloride
drug standard
1 mg mL^{-1} in MeOH
> 98% D

Figure 2.3. Structures and supply information for SSRIs (Fluoxetine, Norfluoxetine and d_5-Fluoxetine (IS)

2.2.2. Chemicals for SPE and HPLC

Eluents for HPLC-UV work were all of HPLC-UV grade and purchased from Fisher Scientific. Sigma-Aldrich was used as the source for acetonitrile (ACN) and methanol (MeOH) for LC-MS analysis (Chromasolv LC-MS grade, 99.9%). Formic acid (100% Aristar) was obtained from VWR, as was orthophosphoric acid (95% AnalaR BDH) used in SPE work with solid matrices.

2.3. HPLC-UV Method Development

HPLC and GC have been the most commonly used chromatographic techniques for the analysis of pharmaceuticals from environmental matrices. HPLC holds an advantage over the use of GC in the analysis of pharmaceuticals because PPCPs are often too thermolabile for GC and must be derivatised prior to analysis by GC. Another benefit of HPLC is that it is a common technique available in most laboratories and a range of detectors are available for use. HPLC has in the past been coupled to fluorescence or UV detectors for the analysis of veterinary antibiotics from soil and water matrices (Rabolle and Spliid, 2000; Blackwell *et al.*, 2004). Gonzalez-Barreiro *et al.*, (2003) used an on-line post-column photo-derivatisation procedure which allowed the analysis of pharmaceuticals in water matrices by HPLC-photochemically induced fluorimetry. By far the most commonly used detector with HPLC has been ESI-MS operated in a variety of modes for the analysis of pharmaceuticals from water, soil, sediment and even SS matrices (Kinney *et al.*, 2006; Cahill *et al.*, 2004; Zuccato *et al.*, 2000; Ternes, 2004).

The aim of the present experimental work was to develop a simple and practical chromatographic method for the separation of the four target analytes Fluoxetine, Diazepam, Temazepam and Oxazepam using HPLC-UV, for subsequent use with extraction method development samples from water and soil matrices. In development of this method is it was important to ensure that conditions were also amenable to ESI-MS for which this chromatographic method would be later modified.

2.3.1. HPLC-UV Method Development: Methodology and Stepwise Optimisation

A gradient pump (Dionex, GP40) coupled to a Ultraviolet diode array detector (UV-DAD) (Thermo separation products lamp: SPECTRASYSTEM UV 6000 LP) and Rheodyne injector valve (5µL sample loop) was used for chromatographic method development work to optimise LC conditions for later use with HPLC-ESI-MS^n. The detector was set to scan from 200 to 600nm, and with additional discrete scans at 214, 230 and 254nm. Chromquest software was used for data collection and interpretation. Throughout this method development work, eluent flow rates were 0.2 mL min^{-1} and a maximum run time of 45 minutes maintained.

Solutions of individual target compounds (Fluoxetine, Diazepam, Temazepam and Oxazepam; 0.01 mg mL^{-1}) were prepared alongside a mixed standard, to test compound separation using 3 different reverse phase columns. HPLC columns with different stationary phases (Discovery HS, Hypercarb and Gemini Hybrid) were used over a period of several weeks to optimise resolution of the four analytes. Step-wise testing and optimisation of conditions was carried out using mixed solutions. Solutions of individual pharmaceuticals were then analysed to allow peak identification based on retention time (Rt). The details of optimisation steps and results of each stage of method development are presented in Table 2.6, and example chromatograms can be found in the appendix (Figures A.9 to A.17). A chromatogram of the final optimised separation is shown in Figure 2.5.

	Aqueous Phase (A)	Organic Phase (B)	Gradient (with respect to B)	Standard Solution Matrix	Results / Comments	Chromatogram found in Appendix
Discovery HS C_{18}, 5µm, 10cm x 2.1mm i.d.	Milli-Q : MeOH : formic acid (95 : 5 : 0.1)	MeOH + 0.1% formic acid	50 - 100% over 20 min	MeOH : Milli-Q : formic acid (50 : 50 : 0.1)	Only 3 peaks of 4 expected, possibly due to co-elution of Fluoxetine with Oxazepam	Figure A.9.
	Milli-Q : MeOH : formic acid (95 : 5 : 0.1)	ACN + 0.1% formic acid	50 - 100% over 20 min	MeOH : Milli-Q : formic acid (50 : 50 : 0.1)	Fluoxetine response poor - solutions of higher concentration made (0.1 mg ml^{-1})	Figure A.10.
	Milli-Q : MeOH : formic acid (95 : 5 : 0.1)	ACN + 0.1% formic acid	isocratic (60:40, A:B)	MeOH : Milli-Q : formic acid (50 : 50 : 0.1)	Fluoxetine eluting within solvent front	Figure A.11.
	Milli-Q : MeOH : formic acid (95 : 5 : 0.1)	ACN + 0.1% formic acid	isocratic (A:B, 60:40); (65:35); (67:33)	MeOH : Milli-Q : formic acid (50 : 50 : 0.1)	Fluoxetine eluting too close to solvent front & contamination seen in solutions - solutions remade	Figure A.12.
	Milli-Q : MeOH : formic acid (95 : 5 : 0.1)	ACN + 0.1% formic acid	isocratic (A:B, 67:33)	MeOH : Milli-Q : formic acid (50 : 50 : 0.1)	Contamination no longer apparent. Concerns as to stability of target compounds in aqueous solutions	Figure A.13.
	Milli-Q : MeOH : formic acid (95 : 5 : 0.1)	ACN + 0.1% formic acid	isocratic (A:B, 67:33)	MeOH : ACN : formic acid (50 : 50 : 0.1)	Unable to obtain satisfactory separation between Fluoxetine and solvent front for later use of method with biodegradation samples which may contain more polar metabolites	

Table 2.6. Optimisation conditions and results for the chronological sequence of HPLC-UV method development using three columns with different stationary phases to optimise the chromatographic separation and resolution of four target compounds (Fluoxetine, Diazepam, Temazepam and Oxazepam)

Table 2.6 – continued...

	Aqueous Phase (A)	Organic Phase (B)	Gradient (with respect to B)	Standard Solution Matrix	Results / Comments	Chromatogram found in Appendix
Thermoquest, Hypercarb, 5µm, 10cm x 2.1mm i.d.	Milli-Q : MeOH : formic acid (95 : 5 : 0.1)	ACN + 0.1% formic acid	isocratic (A:B, 67:33)	MeOH : Milli-Q : formic acid (50 : 50 : 0.1)	Returning to aqueous solutions (~ 2 weeks old) saw reappearance of contamination peaks.	Figure A.14.
	Milli-Q : MeOH : formic acid (95 : 5 : 0.1)	ACN + 0.1% formic acid	isocratic (A:B, 67:33)	MeOH : ACN : formic acid (50 : 50 : 0.1)	All 4 peaks appear baseline resolved, however peaks are broad and run-time has significantly increased (~33 min)	Figure A.15.
Phenomenex, Gemini C_{18} hybrid, 5µm, 15cm x 2.1mm i.d.	Milli-Q + 0.1% formic acid	MeOH + 0.1% formic acid	5 - 100% over 30 min	MeOH : Milli-Q : formic acid (50 : 50 : 0.1)	Only 3 peaks, possible co-elution of Temazepam and Diazepam.	Figure A.16.
	Milli-Q + 0.1% formic acid	MeOH : ACN : formic acid (90 : 10 : 0.1)	5 - 100% over 30 min	MeOH : Milli-Q : formic acid (50 : 50 : 0.1)	Only 3 peaks, possible co-elution of Temazepam and Diazepam.	Figure A.17.
	Milli-Q + 0.1% formic acid	ACN + 0.1% formic acid	20 - 100% over 10 min	MeOH : Milli-Q : formic acid (50 : 50 : 0.1)	4 peaks baseline resolved, ~ 0.5 min broad, slight tailing seen on Fluoxetine peak.	Figure 2.5 Chapter 2

Table 2.6. Optimisation conditions and results for the chronological sequence of HPLC-UV method development using three columns with different stationary phases to optimise the chromatographic separation and resolution of four target compounds (Fluoxetine, Diazepam, Temazepam and Oxazepam)

2.3.2. HPLC Method Development: Discussion and Conclusions

After a series of experiments using three different stationary phases and numerous mobile phases the stepwise optimisation procedure resulted in the development of a method suitable for the analysis of the four target compounds by HPLC-UV. The final optimised method selected (Figure 2.4) resulted in chromatograms characterised by good Gaussian peak shapes, with baseline resolution between all the compounds in a practical, comparatively short analysis time. This method was subsequently used for analysis of mixtures during SPE method development from both water and soil matrices. An example chromatogram can be seen in Figure 2.5.

This work also highlighted the potential problem of compound stability within aqueous solutions. For HPLC-UV and later HPLC-ESI-MS^n analysis it was found that the inclusion of water within the injected sample aided chromatography by reducing peak broadening, improving peak shape, and in the case of ESI-MS^n also increasing compound electrospray capabilities. This knowledge allowed future experiments to be designed with this in mind. Therefore all subsequent samples were reconstituted on the day of analysis.

Column: Gemini C_{18}, 5µm, 15 cm x 2.1 mm i.d.
Phase A: Milli-Q + 0.1% formic acid
Phase B: ACN + 0.1% formic acid
Injection volume: 5 µl
Flow rate: 0.2 ml min^{-1}

Gradient:

min	A(%)	B(%)
0	80	20
10	0	100
20	0	100

Figure 2.4. Optimised HPLC-UV conditions for the chromatographic separation of a mixture of Fluoxetine, Diazepam, Temazepam and Oxazepam

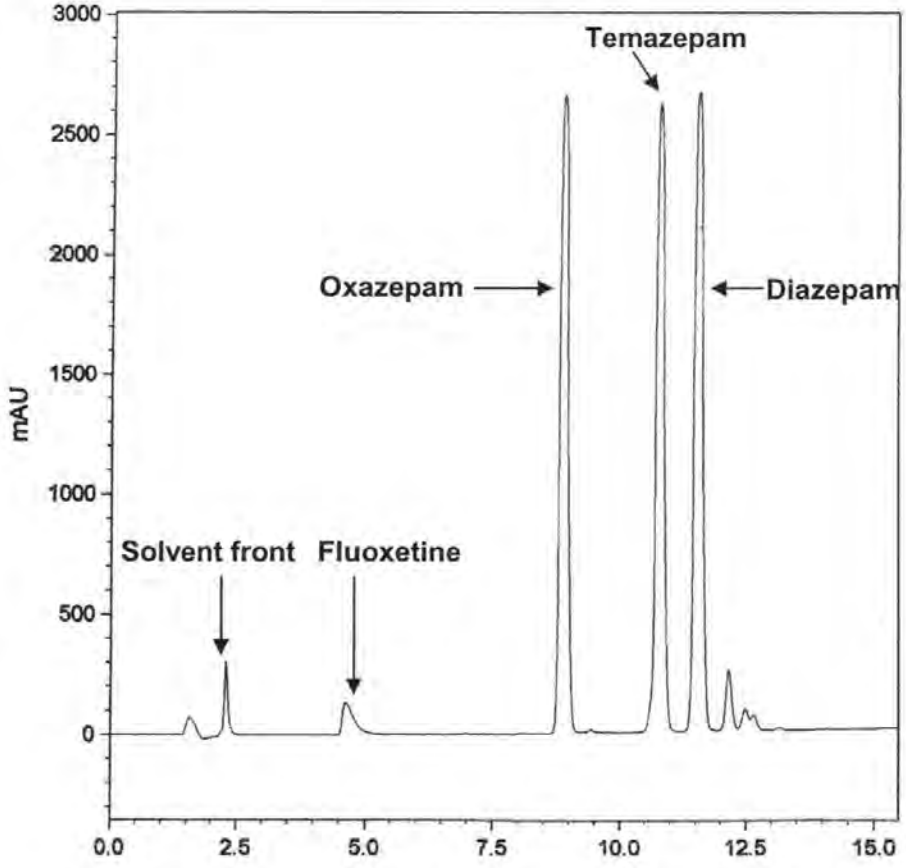


Figure 2.5. Example chromatogram of optimised reverse phase HPLC-UV analysis (@254 nm) of a mixture of Fluoxetine, Diazepam, Temazepam and Oxazepam

2.4. HPLC-ESI-MS^n Method Development

At the commencement of this project there were literature reports of the use of LC-MS for the analysis of many benzodiazepines and SSRIs, predominantly from biological matrices (Toyoka *et al.*, 2003; Lee *et al.*, 2003). Data on the ESI fragmentation of all target compounds was available from Sutherland *et al.*, (2001) and Smyth, *et al.*, (2000). The initial aim herein therefore was simply to confirm ESI-MS^n as a suitable analysis method for the detection of the target compounds. Optimisation of ESI-MS^n conditions for all six target compounds (Fluoxetine, Norfluoxetine, Diazepam, Temazepam, Oxazepam and Nordiazepam), and determination of the MS fragmentation pathways for each compound was then conducted. Optimised conditions were used to establish high flow MS^n methods for subsequent analysis of compounds from a variety of matrices. Determination of fragmentation pathways aided in peak identification in addition to retention time (Rt).

2.4.1. Low flow infusion

2.4.1.1. Methodology

All MS work carried out in this project used an electrospray interface fitted to a Finnigan MAT LCQ™ (ThermoFinnigan San Jose, CA, USA) quadrupole ion trap mass spectrometer. Instrument tuning and optimisation of mass calibration, was performed regularly throughout this project, using automatic calibration procedures and calibration solutions (caffeine, Sigma, St Louis, MO, USA; MRFA, Finnigan Mat, San Jose, CA, USA; Ultramark 1621, Lancaster Synthesis Inc, Widham, NH, USA; in MeOH : water : acetic acid (50:50:1 v/v/v)).

Low flow (3µL min^{-1}) infusion of standard solutions of each target compound (1 µg mL^{-1}; MeOH : MilliQ : formic acid, 50 : 50 : 0.1 v/v/v), were carried out using a syringe pump fitted with a 250µL syringe (Hamilton, Reno CA, USA). Infusion was performed under the following detector parameters: source voltage (±) 4.5 kV; capillary voltage (±) 0 – 50V (set by auto tune function); capillary temperature 200°C; nitrogen sheath gas flow rate, 40 arbitrary units.

Sequential product ion fragmentation and condition optimisation of each compound was performed in both positive and negative ionisation modes. The most abundant product ion at each stage was selected for sequential fragmentation, until no further MS^n transitions were obtained. This allowed generation of ESI fragmentation pathways, alongside development of optimised MS^n conditions for each compound. A full scan range of *m/z* 50 – 2000 was used. LCQ tune software was used for data acquisition and processing. Recording of spectral data for 1 minute time periods, was started once stable spectra were obtained.

2.4.1.2. Results and Discussion

The use of low flow infusion allowed optimisation of conditions for each target compound. These conditions were used throughout the duration of this project, specific to the compound of interest and are shown in Table 2.7.

		MS^2	MS^3	MS^4
FLUOXETINE (Auto tune *m/z* 310.0)	Isolation width (*m/z*)	1.5		
	Relative activation amplitude	20		
NORFLUOXETINE (Auto tune *m/z* 296.0)	Isolation width (*m/z*)	1.5		
	Relative activation amplitude	20		
DIAZEPAM (Auto tune *m/z* 285.3)	Isolation width (*m/z*)	1.5	2.0	1.5
	Relative activation amplitude	37	37	35
TEMAZEPAM (Auto tune *m/z* 301.1)	Isolation width (*m/z*)	1.5	1.5	
	Relative activation amplitude	35	22	
OXAZEPAM (Auto tune *m/z* 287.7)	Isolation width (*m/z*)	1.5	1.5	
	Relative activation amplitude	24	30	
NORDIAZEPAM (Auto tune *m/z* 271.2)	Isolation width (*m/z*)	1.5	1.5	1.5
	Relative activation amplitude	39	36	35

Table 2.7. Optimised MS^n analysis conditions

Note: activation Q & activation time were found to be optimum at 0.25 & 30 msec respectively, for all target compounds. Relative activation amplitude defines ion activation parameters and is expressed as a % of the maximum activation voltage.

Sequential fragmentation mass spectra and proposed ESI fragmentation pathways of each target analyte are shown in Figure 2.6 to Figure 2.11.

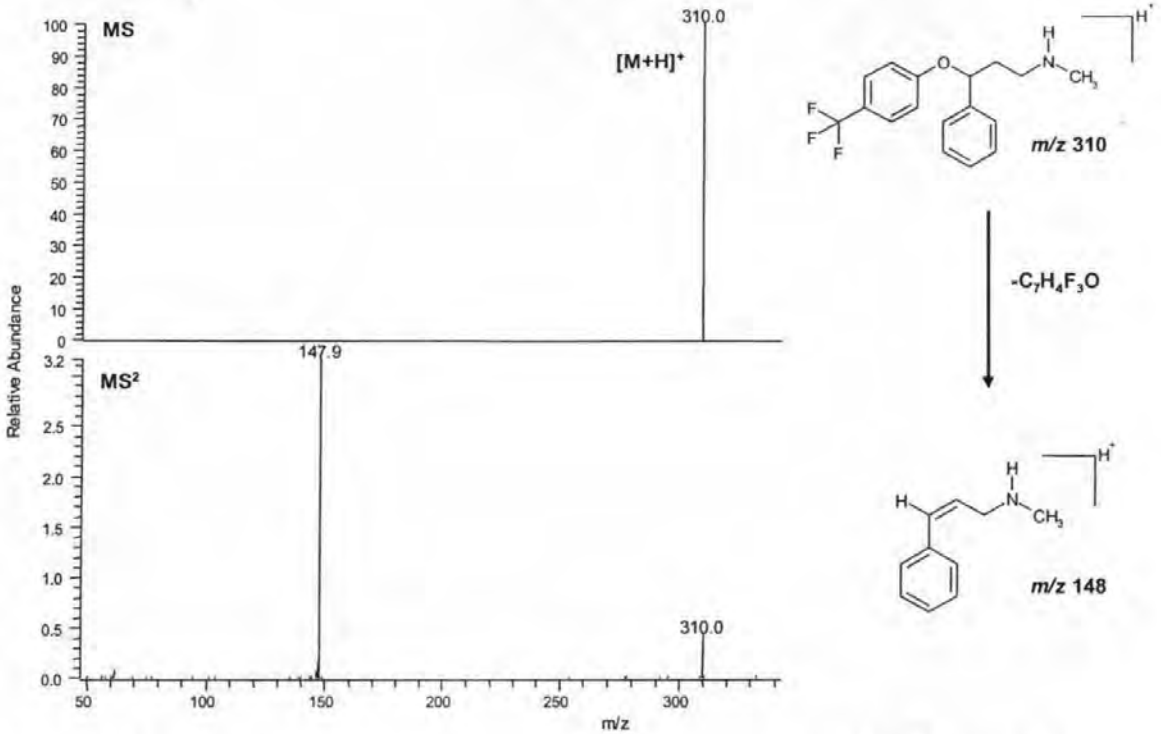


Figure 2.6. Proposed ESI-MS^n fragmentations for protonated Fluoxetine HCl

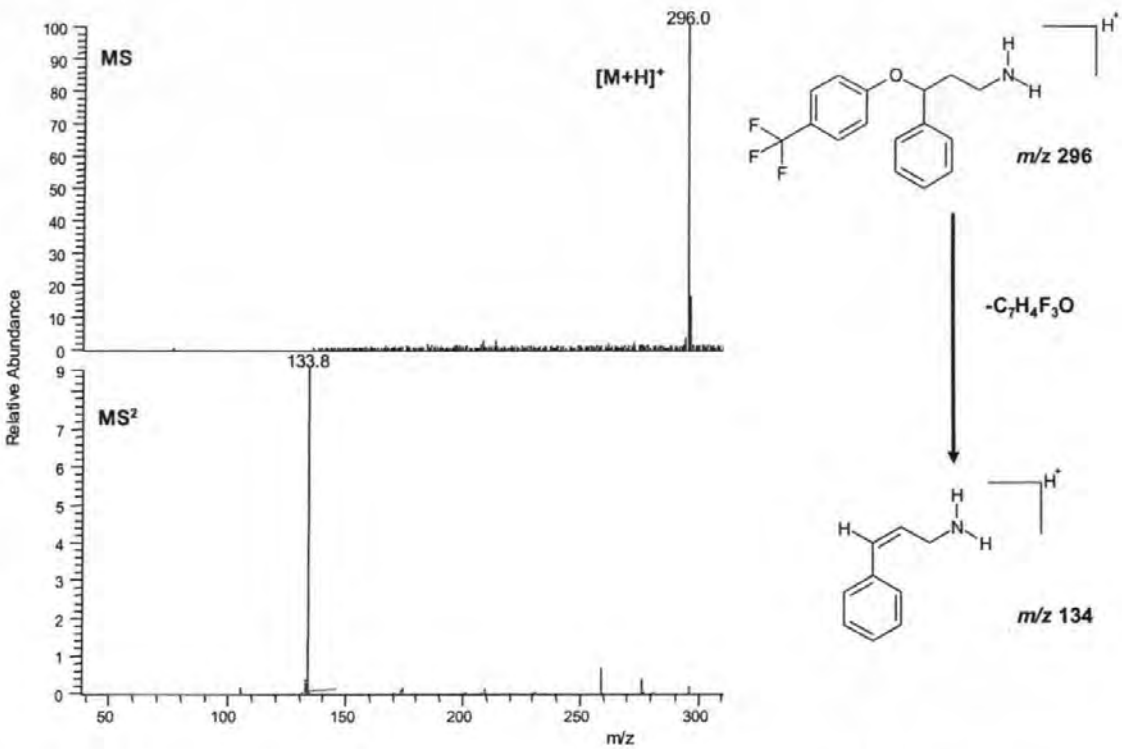


Figure 2.7. Proposed ESI-MS^n fragmentations for protonated Norfluoxetine

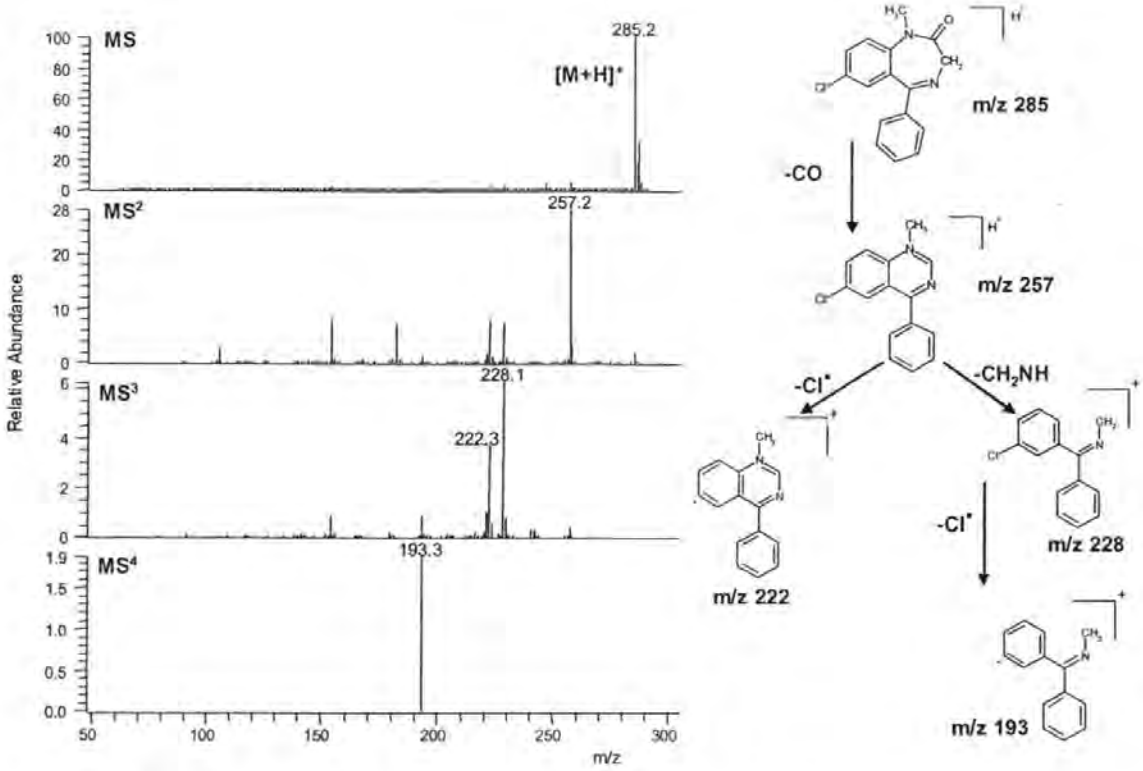


Figure 2.8. Proposed ESI-MS^n fragmentations for protonated Diazepam

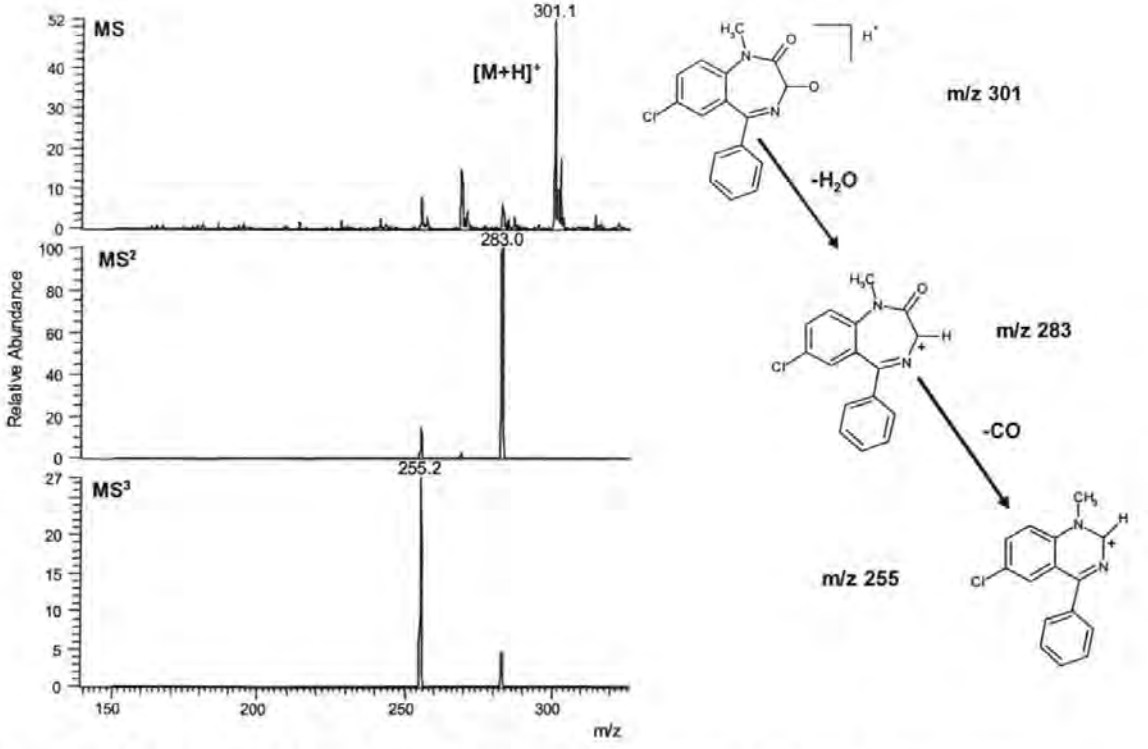


Figure 2.9. Proposed ESI-MS^n fragmentations for protonated Temazepam

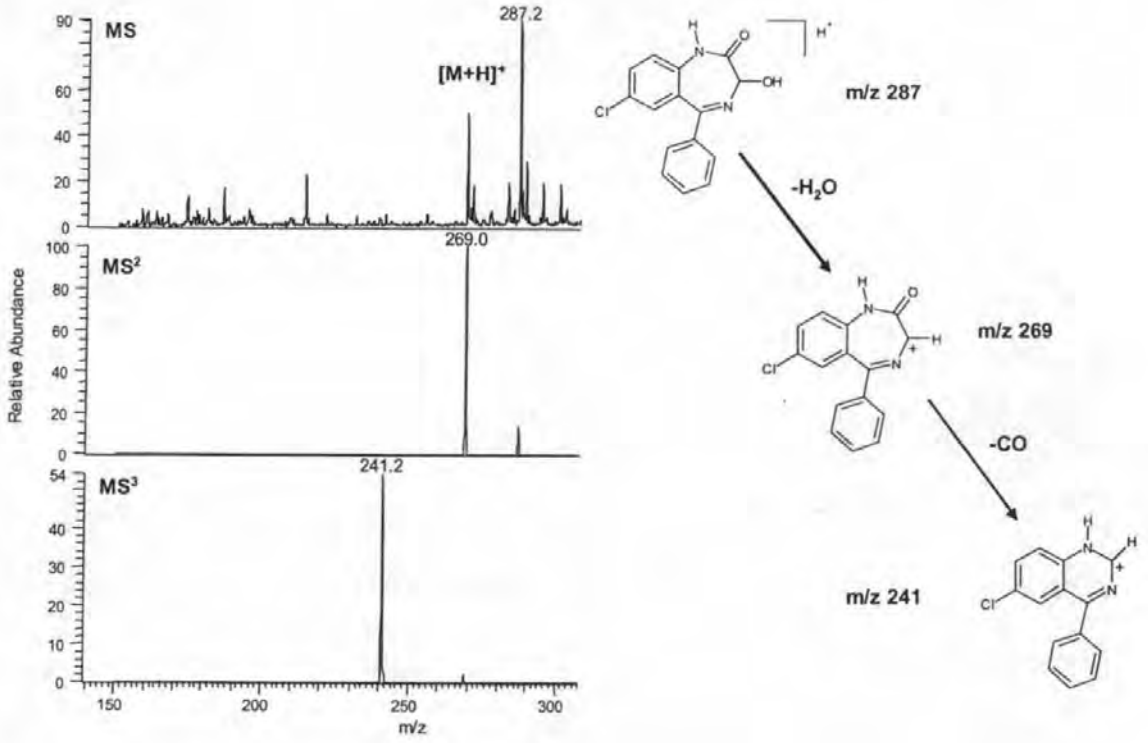


Figure 2.10. Proposed ESI-MS^n fragmentations for protonated Oxazepam

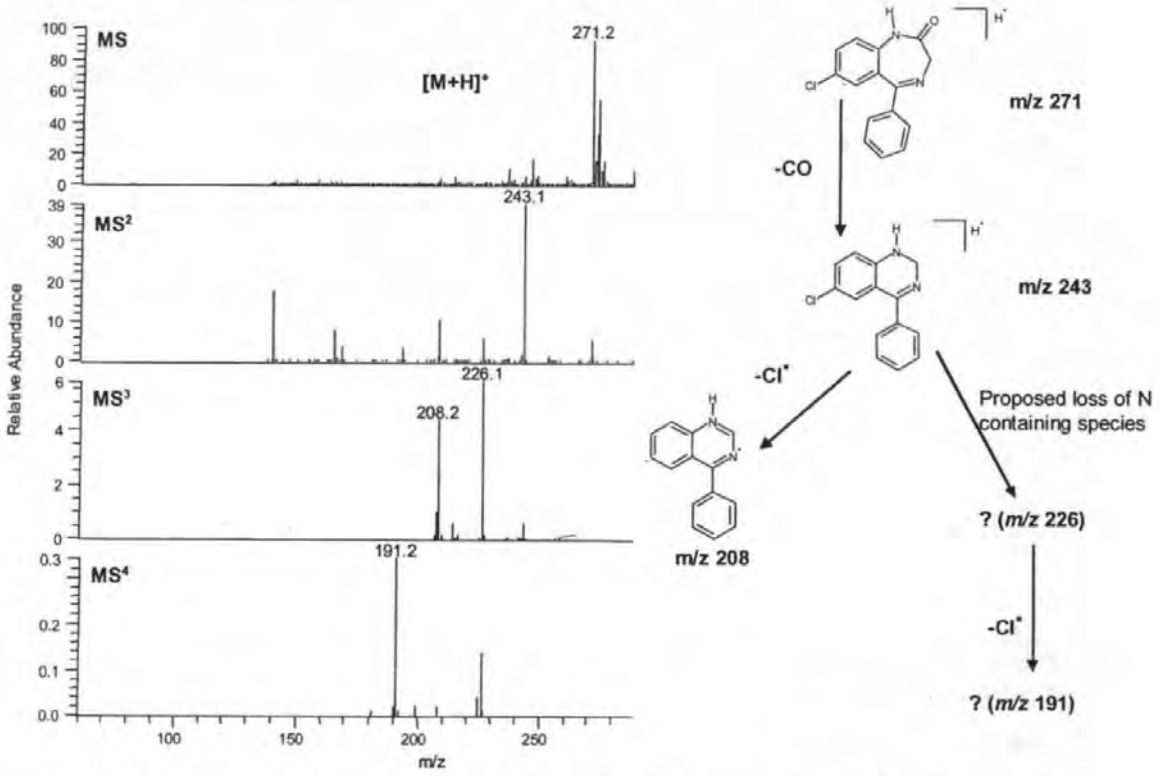


Figure 2.11. Proposed ESI-MS^n fragmentations for protonated Nordiazepam

ESI-MS^n appeared suitable for the analysis of each of the target compounds. Negative ion mode ionisation generated no useful data, so all future analysis was performed in positive ESI mode. It was possible to obtain stable MS^n mass spectra of each of the target compounds.

Fluoxetine and Norfluoxetine both underwent protonation under full MS conditions, producing $[M + H]^+$ protonated molecular ions, with *m/z* of 310 and 296 respectively (Figure 2.6 and Figure 2.7). Under secondary fragmentation (MS^2), a loss of 162 u, which corresponds to the loss of *p*-trifluoromethylphenol, occurred for both compounds (*m/z* transitions: 310 to 148, 296 to 134 respectively). The internal standard (IS; d_5-Fluoxetine HCl) underwent similar ESI fragmentations (*m/z* transition: 315 > 153). Further transitions, beyond MS^2 could not be obtained. Transitions seen here for both Fluoxetine and Norfluoxetine were consistent with literature data (Sutherland *et al.*, 2001; Vasskog *et al.*, 2006). However both the latter studies identified an additional fragment at MS^2 with an *m/z* of 44. This ion was identified by the later authors as due to the $[CH_2NHCH_3]^+$ fragment ion, but this was not identified in this work due to the mass range limitations (*m/z* 50 -2000) of the Finnigan MAT LCQ™ mass spectrometer.

All of the 1,4-benzodiazepines also underwent protonation, resulting in $[M + H]^+$ protonated molecular ions (Figure 2.8 to Figure 2.11). Further fragmentation revealed two major ESI fragmentation pathways. Under MS^2 conditions Temazepam (Figure 2.9) and Oxazepam (Figure 2.10) both lost H_2O (- 18u), followed by the loss of CO (- 28 u) with ring contraction at MS^3 to form a 6 membered resonance stabilised ring (*m/z* transitions: 301 to 283 to 255, 287 to

269 to 241 respectively). The IS, d_5-Oxazepam, also underwent a similar ESI fragmentation (*m/z* transitions: 292 to 274 to 246). It was not possible to maintain stable spectra for MS^4. Smyth *et al.*, (2000) found identical transitions for Temazepam and Oxazepam up to MS^3. They were however also able to obtain MS^4 fragmentation for Oxazepam, which resulted in the loss of the chlorine radical (*m/z* 206).

Diazepam and Nordiazepam fragment with proposed loss of CO (-28 u) and ring contraction, followed by a loss of a Cl radical at MS^2 (*m/z* transitions: 285 to 257 to 222, 271 to 243 to 208 respectively). These transitions confirm the findings of Smyth *et al.*, (2000). Two different product ions were seen at MS^3; the second most intense ion was the $[M - CO - Cl]^+$ (*m/z* 222 and 208) discussed above. The most intense ion at MS^3 for Diazepam had a *m/z* of 228 and was proposed by Smyth *et al*,. (2000) to be a result of the loss of a nitrogen containing species such as CH_2NH (-29 u). The loss of this nitrogen-containing species was then followed by a loss of a Cl radical at MS^4 (*m/z* 193). Results here indicate that Nordiazepam also has an alternative fragmentation at MS^3 (*m/z* 226), and like Diazepam this then underwent a loss of 35 u (-Cl) at MS^4 (*m/z* 191). The loss of 17 u at MS^3 (*m/z* 226) from the protonated MS^2 ion may also be due to the loss of a nitrogen containing species, such as NH_3, followed by contraction to a five membered ring. However, no literature evidence to support this proposed fragmentation pathway could be found.

These two sets of ESI fragmentation pathways for the target 1,4-benzodiazepines suggest that that the functional group on C3 (Figure 2.2) determines the initial fragmentation step. If an OH functional group is present

the MS transitions begin with the loss of H_2O, whereas if it is not, the first loss seen is that due to loss of CO.

In summary, all target compounds were amenable to ESI-MS^n and optimised parameters and ESI fragmentation pathways were ascertained for all of the target compounds. These optimised parameters were then used as the basis for high flow analysis methods, including full MS, MS^2, DDMS (data dependent mass spectrometry), SIM (selective ion monitoring) and SRM (selective reaction monitoring). During DDMS analysis the most intense ion from each scan, provided it had an ion count > 1 x 10^5, is promoted to undergo an MS^2 fragmentation. This is therefore an advantageous method to use with unknowns such as metabolites where interpretation of MS^2 fragmentation aids structural identification of the unknown. SIM and SRM are MS and MS^2 analysis methods designed for known transitions. This results in a much greater sensitivity and these are therefore valuable analysis techniques for samples with low concentrations of known analytes.

2.4.2. High flow injection

2.4.2.1. Methodology

High flow analysis required the coupling of an HPLC gradient pump (Dionex P580 quaternary pump) to the ESI-MS detector, alongside which a Rheodyne injector was used for sample introduction (5µL sample loop). LCQ tune software (ThermoFinnigan) was again used for data acquisition and processing. For more routine analysis work, once analysis methods were fully optimised, an autosampler was used (5µL injection volume; Dionex ASI-100 automated sample injector) and Xcalibur 1.0 spl software (ThermoFinnigan) was used for data acquisition and processing. All high-flow work was performed under the following parameters: source voltage (+) 4.5 kV; capillary voltage (+) 0 - 50 V (set by auto tune function); capillary temperature 220°C; nitrogen sheath gas flow rate 60 arbitrary units; auxiliary gas flow rate, 20 arbitrary units.

High flow full MS analysis of a mixture of all six target compounds (0.1 µg mL^{-1}; MeOH : Milli-Q : formic acid, 50:50:0.1 v/v/v), was performed under the HPLC conditions developed in section 2.3 (Figure 2.4). Small modifications were made to the gradient details between runs to optimise chromatographic separation. As a mixture of compounds was being analysed, optimum conditions for one analyte (Fluoxetine) were selected since Fluoxetine gave the weakest ionisation response.

2.4.2.2. Results and Discussion

Modification of the HPLC method developed using UV detection for use with ESI-MS^n for the analysis of samples from soil and plant tissue resulted in extension of the analysis time. When the HPLC-UV method was applied directly to LC-MS, separation between target peaks decreased. This was due to peak broadening and was suspected to be due to the use of silicone based tubing, and an overall greater tubing length and hence dead volume on the LC-MS system. For this reason it was necessary to slow the gradient to increase peak separation.

For MS analysis it is less essential to achieve baseline chromatographic resolution since MS provides the ability to identify compounds by specific ions. However this chromatographic method was to be used with samples that could contain significant quantities of potentially interfering and co-eluting components from soil and plant tissue matrices. If these components were to co-elute they could potentially cause matrix effects (i.e. ion suppression / enhancement). Considering the complex nature of matrices studied within this project, good chromatographic separation was considered an important objective, with maintenance of a practical run time, to reduce the risk of co-eluting interfering components (Figure 2.13).

The conditions of the final selected chromatographic method for use with LC-MS are summarised in Figure 2.12. This chromatographic method was subsequently used for the analysis of SPE method development samples (cress & cauliflower tissues), all biodegradation samples, cauliflower tissue culture samples, and samples from related experiments.

Column: Gemini C_{18}, 5µm, 15 cm x 2.1 mm i.d.
Phase A: Chromasolv LC-MS grade water with 0.1% formic acid
Phase B: Chromasolv LC-MS grade ACN with 0.1% formic acid
Injection volume: 5 µl
Flow rate: 0.2 ml min^{-1}

Gradient:	min	A(%)	B(%)
	0	80	20
	18	0	100
	23	0	100

Figure 2.12. HPLC-ESI-MS^n Chromatography Method Details for the Analysis of Target Compound and IS

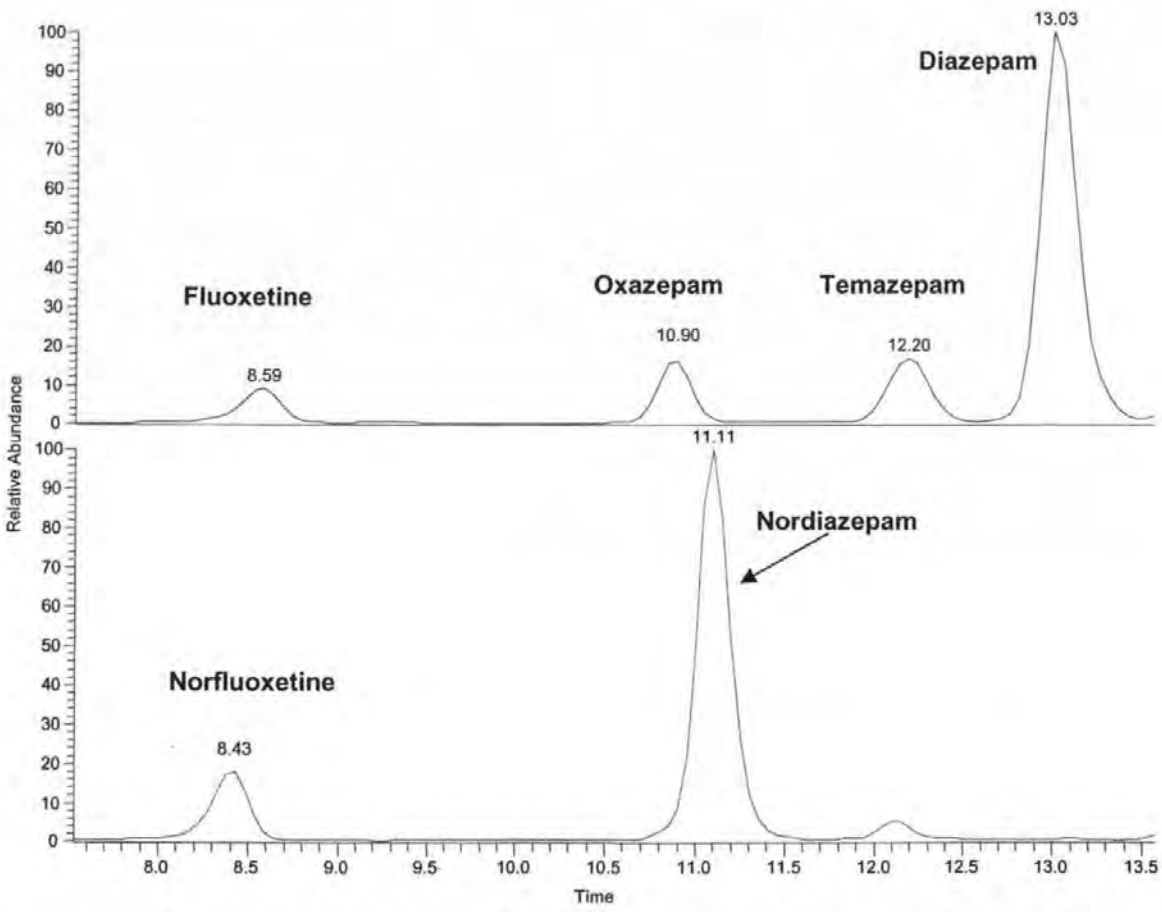


Figure 2.13. Example extracted ion chromatogram of reverse phase HPLC-ESI(+)-MS analysis of a mixture of Fluoxetine, Norfluoxetine, Diazepam, Temazepam, Oxazepam and Nordiazepam (*m/z* 310, 296, 285, 301, 287, 271 respectively) using HPLC conditions modified from a method using UV detection.

2.5. HPLC-ESI-MS^n Calibration

2.5.1. Ratio calibration

2.5.1.1. Methodology

It was felt it would be of benefit if both qualitative and quantitative data could be obtained from analysis using the Finnigan MAT™ LCQ mass spectrometer. Ion trap mass spectrometers are notoriously difficult to obtain quantitative data from, due to non-linear responses. This is thought to be due to a common ESI phenomenon whereby at high analyte concentrations, ESI droplets reach a saturation limit (Souverain *et al.*, 2004; Antignac *et al.*, 2005).

The issues over quantification and quality control have been reviewed by Hao *et al.*, (2007), who brings attention to the differences in presentation of data for pharmaceuticals and personal care products from environmental matrices. For example the threshold below which quantitative data cannot be obtained varies between published literature methods. Some authors chose to use LOD (limit of detection), others LOQ (limit of quantification), MDL (method detection limit), all of which are calculated differently and provide slightly different information. Hao *et al.*, (2007) reviewed LC-MS^n methods and found signal to noise ratio (S:N) approaches were most commonly used (23 from 30 studies). Due to the inconsistency in presentation of data, it was decided that herein the common S:N approach would be used, especially considering the known variable impact of matrix effects, even between samples. Xcalibur software was used to generate S:N for all samples analysed in this project. Samples with a S:N < 10 were rejected for quantitative work, and those with a S:N < 3 were rejected for qualitative work.

Published literature revealed several examples of the use of ratio based LC-MS calibration methods. A very simple ratio-calibration experiment was devised, whereby a series of solutions containing all 6 target compounds over a range of concentrations (0.05 – 5 μg mL^{-1}) and the two IS at a consistent concentration (1.25 μg mL^{-1}) were analysed by full MS. Normal linear calibration was carried out alongside this for the two IS (0.05 – 5 μg mL^{-1}), using full MS analysis, so that IS recoveries could be calculated in later experimental work. These solutions had a composition of ~ 50% ACN : 50% Milli-Q with 0.1% formic acid (v/v/v). This combination of eluents was selected as it was partially matched with HPLC eluents, and the inclusions of Milli-Q aid electrospray of the target compounds. Results in HPLC method development indicated some potential stability issues with the target compounds in water; therefore these solutions were made afresh every 3 days.

2.5.1.2. Results and Discussion

Appropriate protonated molecular ions were extracted from the full MS spectra and the peak areas integrated manually using Xcalibur, Qual Browser software. Integrated data allowed the generation of calibration graphs for each compound. Normal IS linear calibration (Figure 2.14 A and Figure 2.14 B) indicated a good linear response ($R^2 \geq 0.95$) with very little variation between replicates. These data indicate that IS concentration and hence recovery may be calculated from normal linear calibrations. The data also suggested that linear calibration may be appropriate for all target compounds, as structurally they are closely related to one of the ISs. However, consideration of the linear calibration data in Figure 2.14 C, which compares the response of Fluoxetine and d_5-Fluoxetine (IS), highlights just how much response variation was seen, even between compounds for which one would expect a very similar response.

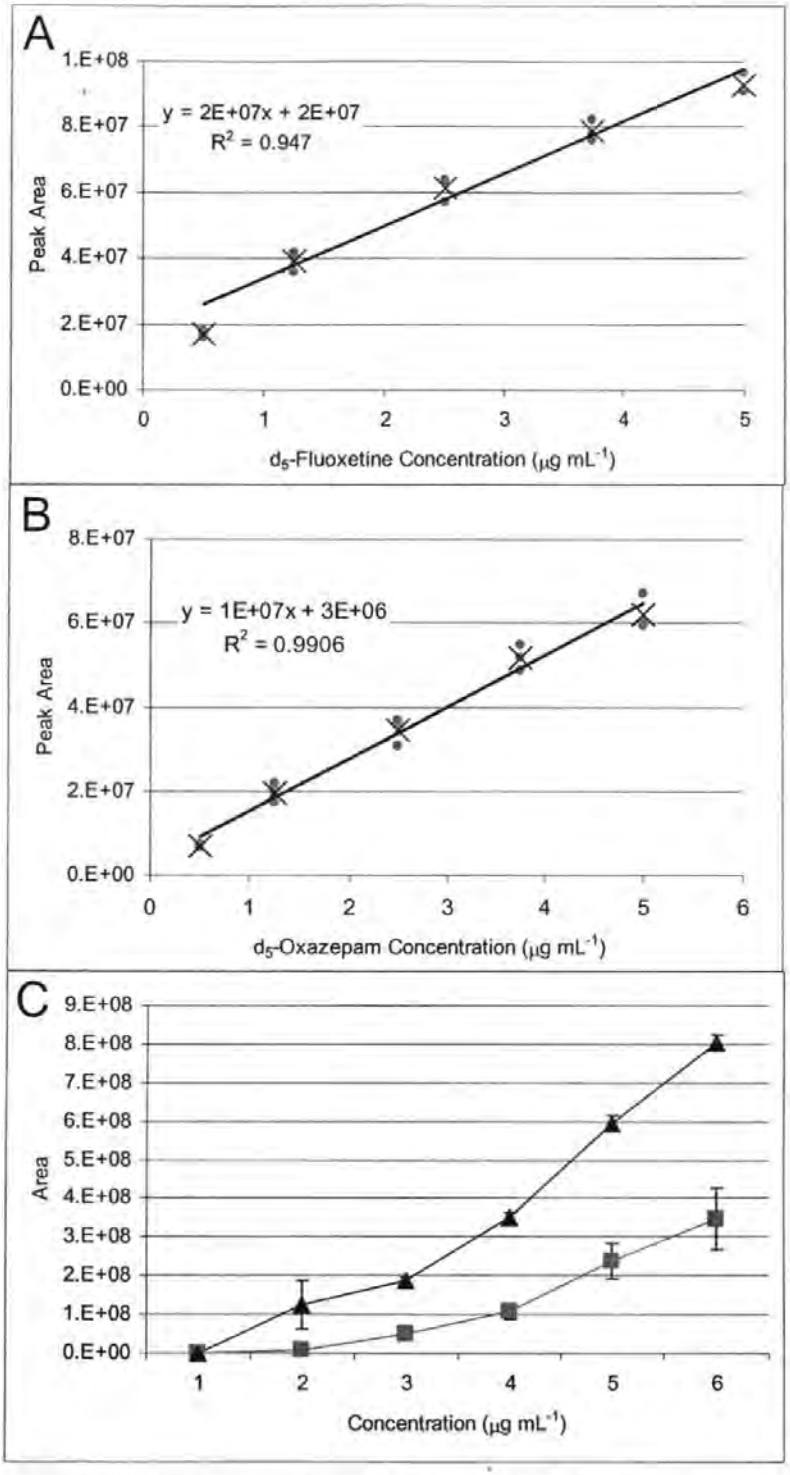


Figure 2.14. HPLC-ESI-MS^n normal linear external calibration curves: response against concentration.

A. d_5-Fluoxetine; B. d_5-Oxazepam. X = mean response (n = 3).
C. Comparison of Fluoxetine and d_5-Fluoxetine calibration curves, mean response (n = 3) ■ d_5-Fluoxetine, ▲ Fluoxetine. Error bars ± 1 standard deviation.

To generate ratio-calibration graphs for individual compounds the ratio of the target compound peak area to the IS peak area were plotted against target compound concentration. Use of the ratio calibration system (Figure 2.15) allowed variations in compound response to be accounted for and trend-lines with excellent R^2 obtained.

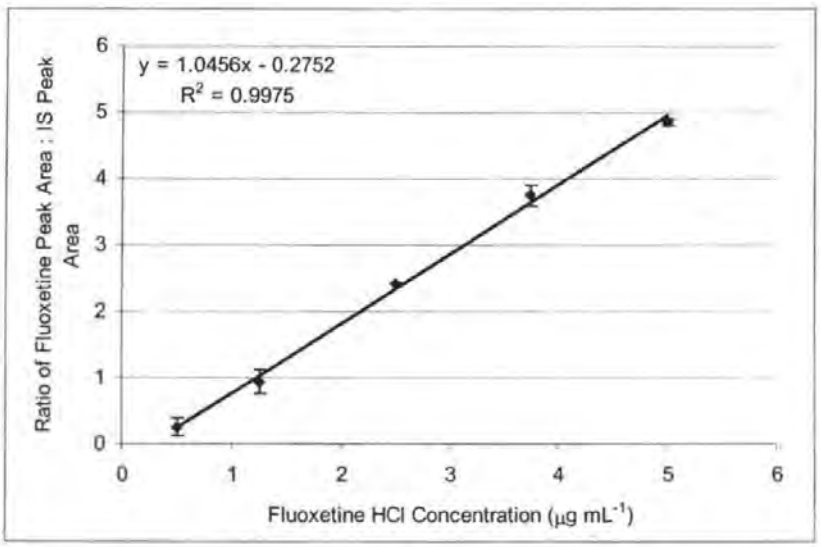


Figure 2.15. Example HPLC-ESI-MS ratio calibration trend line for the generation of quantitative data for Fluoxetine HCl liquid culture biodegradation studies.
Mean response ± 1 standard deviation shown (n = 3).

This process was repeated for each of the target compounds. In later experiments it was necessary to repeat the calibration using different IS concentrations specific to the particular experiment and samples in question. A summary of all equations of calibration trend-lines used to generate quantitative data is presented at the end of this chapter (Section 2.6).

2.5.2. Matrix Matched Cauliflower Calibration

2.5.2.1. Methodology

During cauliflower method development work (Chapter 3) it was noted that IS recoveries were significantly lower than expected. Ion suppression and enhancement due to matrix effects are a well known phenomenon in ESI-MS^n. These matrix effects are highly variable between samples and therefore have a significant impact upon reproducibility and reliability of quantitative data.

There are a range of theories as to why this phenomenon occurs in the presence of matrix components, including; impacts upon evaporation efficiency, increases in viscosity and droplet surface tension, co-precipitation with non-volatile components, all of which have an impact on the transfer of analytes to the gas phase (Antignac *et al.*, 2005). However the most commonly accepted mechanism is that competition between target analyte and co-eluting compounds, either endogenous or exogenous, for ionisation causes these effects (Souverain *et al.*, 2004). As matrix effects have been demonstrated in plant tissue matrices (Zrostlikova *et al.*, 2002), and as cauliflower tissue is a complex matrix, it was felt that the risk of ion suppression was significant enough of a risk to justify this work into ion suppression effects.

Cauliflower (Marks and Spencer Class 1; as sourced for T/C experiments) was extracted in the same manner as for T/C samples. Bulk extractions of curd, leaves and stems were performed using tandem SPE. Minor alterations were made to the extraction procedure which included; increasing vol of extraction solvent to 30 mL $20g^{-1}$ wet weight of plant material (250 mL^{-1} for media); following drying samples were made up to 200 mL (4 x 50 mL portions Milli-Q)

and re-filtered (Whatman No 1) before undergoing SPE extraction. Single replicates of 3 different concentrations of ratio calibration samples (0.5, 2.5 and 7.5 μg mL^{-1} Fluoxetine HCl with 5 μg mL^{-1} IS) were prepared using the extracted cauliflower stem, curd and leaf matrices.

Analysis was performed using SIM as in the final T/C experiment (Chapter 5). Data were then integrated and comparisons drawn between integrated data to ascertain whether matrix components were causing any interferences, such as ion suppression or enhancement.

2.5.2.2. Results and Discussion

The plot of Fluoxetine integrated area data for different tissue types and a non-matrix matched series (Figure 2.16 A), suggested that the curd matrix may be causing ion enhancement of Fluoxetine, relative to the non-matrix matched series, whereas in the stem and leaf series, Fluoxetine appeared to undergo some level of ion suppression relative to non-matrix matched series. Media samples apparently underwent substantial ion suppression due to matrix effects relative to non-matched series.

Figure 2.16 B shows d_5-Fluoxetine integrated data for different tissue types and a non-matrix matched series. All samples contained the same mass of IS (2 μg); therefore we would expect to see consistent integrated area data for all the samples. It is apparent that at different Fluoxetine concentrations the IS response differed. As a general rule, IS response decreased with increasing Fluoxetine concentration.

The non-matched series and the stem series gave similar responses (Figure 2.16 A), which suggested that little or no impact upon d_5-Fluoxetine ESI occurred with the stem samples. However evidence of ion suppression was seen on the IS in the curd and leaf series, and to an even greater extent in the media series (relative to the non-matrix matched series).

Considering either just the impact upon Fluoxetine or IS response due to matrix effects gives complex results for interpretation i.e. curd samples saw both ion enhancement and suppression, stem, leaf and media samples appeared to undergo ion suppression to differing extents. However it was the impact of these matrix effects upon the ratio calibration trend lines, and hence calculated Fluoxetine concentrations, within samples that were of most importance herein. Figure 2.16 C shows ratio-calibration trend lines for three plant tissue types, media and a non-matrix matched calibration series.

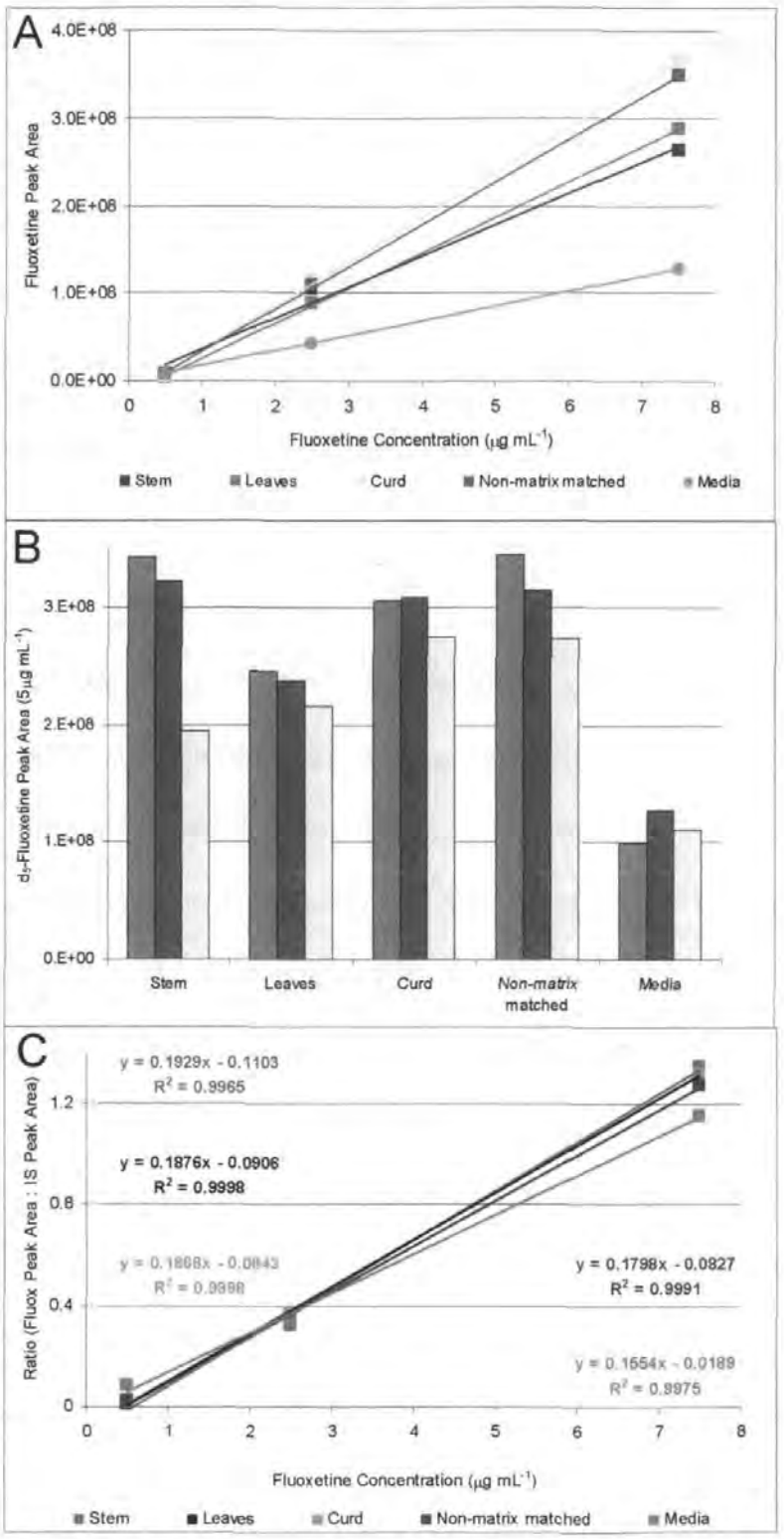


Figure 2.16. HPLC-ESI-MS^n matrix matched cauliflower tissue ratio calibration curves:

A. Comparison of Fluoxetine integrated area data with concentration for 3 cauliflower tissue matrices, one growth media matrix and a non-matrix matched series.
B. Comparison of IS area data Fluoxetine integrated area data at different concentrations for 3 cauliflower tissue matrices, one growth media matrix and a non-matrix matched series. Fluoxetine concentration; 0.5 (■), 2.5 (■) and 7.5 (■) µg mL^{-1}.
C. Comparison of matrix matched ratio calibration curves for Fluoxetine in 3 cauliflower tissue matrices, one growth media matrix and a non-matrix matched series.

Calibration Series	Equation of trend line	R^2
Stem	y = 0.1929 x - 0.1103	0.9965
Leaves	y = 0.1876 x - 0.0906	0.9998
Curd	y = 0.1868 x - 0.0843	0.9998
Media	y = 0.1554x - 0.0189	0.9975
Non-matched	y = 0.1798 x - 0.0827	0.9991

Table 2.8. Ratio calibration curve trend-line equations for Fluoxetine in cauliflower tissue matrices, one growth media matrix and a non-matrix matched series.

As can be seen from Table 2.8, all ratio calibration series gave R^2 values > 0.99. All trend lines had a y-axis intercept of ~ -0.1 ratio units. The steeper the gradient of the trend line the greater a ratio is required to achieve the same concentration of Fluoxetine, i.e. the steeper the trend line the greater the Fluoxetine response. All plant tissue specific series gave trend lines with steeper gradients, and lower y-axis intercepts, than the non-matrix matched series. This indicated that matrix components were having an ion enhancement impact upon all tissue specific ratio calibrations. The greatest ion enhancement was seen in stem > leaves > curd (> non-matrix matched series). However the gradient of the trend line for the media series is less steep than the non-matched series. This suggested that in media samples ion suppression, as opposed to enhancement, was occurring. Media area data for Fluoxetine and IS suggested more significant ion suppression was occurring in the media series than any of the other four calibration series, and it is likely that this suppression was due to the inference by matrix components such as salts. It is this relative reduction in response, for both the target compound and the IS that resulted in a shallower trend-line for ratio-calibration of media series, thus implying overall ion suppression.

To assess whether the differences seen in these trend lines were significantly different enough to warrant continuation of this calibration work an ANOVA statistical analysis was performed (Statsgraphics Version Xv.II) on ratio data. ANOVA generated an F-ratio of 0.0024 which equated to a P-value of 1.00 (> 0.05), indicating there were no statistically significant differences in the means of the five data sets, at the 95% confidence interval. These results indicate that the use of non-matrix matched calibration trend lines is acceptable for the generation of quantitative data in experiments with cauliflower tissues.

2.6. Analysis Method Development Summary

This aforementioned method development resulted in a reverse phase chromatographic method suitable for the analysis of four target compounds by HPLC-UV and all six target compounds by HPLC-ESI-MS^n. ESI was found to be suitable for the analysis of all target compounds and conditions were optimised for maximum sensitivity and selectivity. These conditions were then used as the basis for high flow analysis methods throughout this project. ESI(+) fragmentation pathways were ascertained along with the Rts of all target analytes and the ISs. Studies into ratio calibration and matrix matching to account for ion suppression revealed no statistically significant benefits for the use of matrix matched calibration series for cauliflower tissue samples. Details of calibrations that were developed for various experiments in this project are summarised in Table 2.9.

Experiment (compound)	MS analysis used for quantitative data generation	Equation of ratio calibration trend-line	R^2
Soil Biodegradation (Fluoxetine)	Full MS	y = 1.0456x - 0.2752	0.998
Liquid Culture Biodegradation (Fluoxetine)	Full MS	y = 1.0456x - 0.2752	0.998
Liquid Culture Biodegradation (Norfluoxetine)	Full MS	y = 0.8287x - 0.1662	0.993
Liquid Culture Biodegradation (Diazepam)	Full MS	y = 4.1064x + 0.0749	0.977
Liquid Culture Biodegradation (Oxazepam)	Full MS	y = 0.8094x + 0.0037	1.000
Liquid Culture Biodegradation (Temazepam)	Full MS	y = 1.5489x + 0.0509	0.997
Cauliflower T/C (Fluoxetine)	SIM	y = 0.1733x - 0.0543	0.996

Table 2.9. Ratio-calibration summary table for full scale experiments

CHAPTER THREE

METHOD DEVELOPMENT OF EXTRACTION TECHNIQUES

3. Method Development of Extraction Techniques

3.1. Introduction

Method development work (Chapter 2) resulted in the successful development of chromatographic methods suitable for the separation and analysis of four target compounds (Fluoxetine, Diazepam, Temazepam and Oxazepam) by HPLC-UV. This method was subsequently modified for use with HPLC-ESI-MS^n and optimised MS parameters were obtained for each of the six target compounds. Once analysis methods were established it was possible to begin method development work for the extraction of target analytes from a range of environmentally relevant matrices.

In the previous chapter, methods found in literature for the extraction and analysis of organic contaminants from solid matrices were briefly discussed and examples from the literature given (Tables 2.1 to 2.5). It was noted that SPE was the most commonly selected method for sample clean-up, due to the ease of use, speed, low contamination risk, the advantage of potential on-line use and that only low volumes of organic solvent are required (Diaz-Cruz *et al.*, 2003; Liska, 2000).

There are four major reasons for the use of SPE which are: removal of impurities such as endogenous compounds from the matrix; concentration of the target analyte; phase exchange for GC analysis and *in-situ* derivatization using specially coated cartridges, e.g. (Bouvier, 1995). For the present method development work SPE was selected because of its ability to remove interfering components and for concentration of the target analytes.

SPE has predominantly been used to prepare samples for subsequent analysis by HPLC because they both share the same fundamental basis of separation. Both techniques are based upon the differential migration of compounds as they are adsorbed and eluted while a mobile phase flow carries them through porous media. For this reason knowledge about compound retention behaviour under HPLC conditions can provide useful information in selection of appropriate loading and elution solvents for SPE, especially for reverse phase (RP) sorbents. HPLC using a column with the same stationary phase as the SPE cartridges to be used allows the generation of capacity factors (k') also known as retention factors or relative retention. These can, in turn, be used to optimise analyte elution from the stationary phase as a low capacity factor (typically 0 – 0.5) is required to elute the target analyte (Bouvier, 1995; Swadesh, 2001; Liska, 2000).

Most SPE procedures follow a series of steps known as conditioning, equilibration, loading, wash and elution steps. Conditioning with an organic solvent is used to activate the SPE cartridge by solvating the whole stationary phase which maximises available surface sites for adsorption. Equilibration is then used to prepare the sorbent surface for sample loading (Bouvier, 1995).

Frontal loading was used in initial method development work. During frontal loading, target analytes displace conditioning and equilibration solvent molecules bound to the solid sorbent surface and thus become bound to the sorbent, while unwanted components pass through the cartridge unretained (Henry, 2000).

The pH used during sample loading in SPE is important and is determined by the type of cartridge being used and the target analyte in question. For example although Si-based sorbents are mechanically very stable, they are prone to hydrolysis at pH extremes and are therefore only considered stable for use at pH ranges of 2 – 7, whereas polymeric sorbents can be used over a pH range of 1 – 14. Various modifiers are often added to the sample prior to loading to increase retention of the analyte. For example drug-protein interactions within a plasma matrix can be minimised by modification of the plasma with acid, salt or organic solvent, alongside which buffering the matrix so that the pH obtained ensures the analyte is in its neutral form and is a minimum of 2 pH units away from its pK will further increase analyte retention. When using ion exchange sorbents it is important to obtain a sample pH that will ensure that both the analytes and sorbent are charged. Premature breakthrough of target analytes is one of the most common problems in SPE, but can be easily overcome by the use of a weaker solvent or by diluting the sample. If breakthrough is still a problem then reduction of sample volume or the use of SPE cartridges with a greater mass of sorbent should solve the problem (Bouvier, 1995).

After loading the sample onto a cartridge the wash step is performed with a solvent which will displace weakly bound components but not the target analytes. Unwanted material can also be removed from the pore and interstices of the packed bed during washing (Henry, 2000).

For elution a stronger solvent that is able to displace adsorbed analytes on the sorbent surface is required. Hydrophobic, Coulombic, dipolar and electrostatic forces are the dominant forces involved in binding an analyte to the sorbent

surface, although this depends upon the cartridge type. In the present method development, two types of cartridge were used; C18 and strong anion exchange (SAX) cartridges. Van der Waals and hydrophobic interactions are the primary interaction mechanisms responsible for analyte retention in C18 cartridges, whereas primarily Coulombic anion exchange mechanisms occur in SAX cartridges (Henry, 2000). Of the RP Si-based sorbents available (C2, C4, C8 and C18) C18 sorbents generally provide the highest analyte retention, but this is superseded by the exceptional retention provided by polymeric sorbents (Bouvier, 1995).

3.2. Aims

At the commencement of this project there were no methods reported in the literature for the simultaneous extraction of the selected target analytes. The present method development work therefore aimed to develop methods appropriate for the simultaneous extraction and clean-up of four target analytes (Fluoxetine, Diazepam, Temazepam and Oxazepam). As a range of matrices were to be used in different experiments in this project, it was necessary to develop extraction methods for water, soil and plant matrices for later use in biodegradation and plant-up take studies. Literature searching showed that the most commonly used extraction and clean-up methods for pharmaceuticals from environmental matrices were usually based around LLE followed by SPE or column chromatography for clean-up as shown in Table 2.1. For this reason solvent extraction, where necessary, followed by SPE were the techniques selected for experimental work.

3.3. SPE Method Development for Extraction from Water

Water was selected as the first matrix to be used in method development work, as it is a relatively simple matrix, compared to the other matrices to be used herein.

3.3.1. Testing Compound behaviour on PRP-1

The commonly available Phenonmenex Strata-X SPE (500mg / 3ml) cartridges which are reverse phase cartridges containing polymeric sorbent, were selected for this method development work. To ascertain the behaviour of the target compounds (Fluoxetine, Diazepam, Temazepam and Oxazepam) on this stationary phase, a series of analyses using a PRP-1, 50 x 4.1mm, 5µm column with a stationary phase similar to that of Strata-X SPE cartridges, was performed.

An HPLC pump (HP 1050) with autosampler (90 µL injection volume) was coupled to a UV variable wavelength detector to examine individual standard solutions of Fluoxetine HCl, Diazepam, Temazepam, Oxazepam. Milli-Q and MeOH both modified with 0.1% formic acid, were used as the aqueous and organic eluents. Standard solutions of each target compound (0.1 mg mL^{-1} in eluent) underwent isocratic elution over 30 minutes at a range of organic phase concentrations (100, 80, 65 and 50%) and the Rt were noted.

The retention time for each of the target compounds at each concentration of organic eluent was then used to calculate capacity factors (k'; Table 3.1). The k' of a compound is a measure of the degree to which a compound will be retained under the given HPLC conditions relative to an unretained compound

(e.g. Uracil). The equation given below, was used to generate k' that are also presented as plots against organic concentration (Figure 3.1) (Swadesh, 2001).

$$k' = (t_R - t_0) \div t_0$$

Where; t_R – Rt of retained analyte

t_0 – Rt of unretained compound / solvent front

	Capacity Factor (k')			
% MeOH + formic (0.1%)	**Fluoxetine**	**Diazepam**	**Temazepam**	**Oxazepam**
100	0.1	1.4	0.8	0.4
90	0.1	3.5	1.8	1.0
80	1.9	9.0	4.1	2.5
65	36.5 (max)	36.5 (max)	20.6	36.5 (max)
50	36.5 (max)	36.5 (max)	36.5 (max)	36.5 (max)

Table 3.1. Capacity factors (k') for Fluoxetine, Diazepam, Temazepam and Oxazepam generated using PRP-1 column (50 x 4.1 mm, 5µm) with an organic phase of MeOH modified with formic acid (0.1%)

Max indicates the maximum possible value for k' as run time was restricted to 30 minutes (i.e. analyte did not elute within 30 minutes).

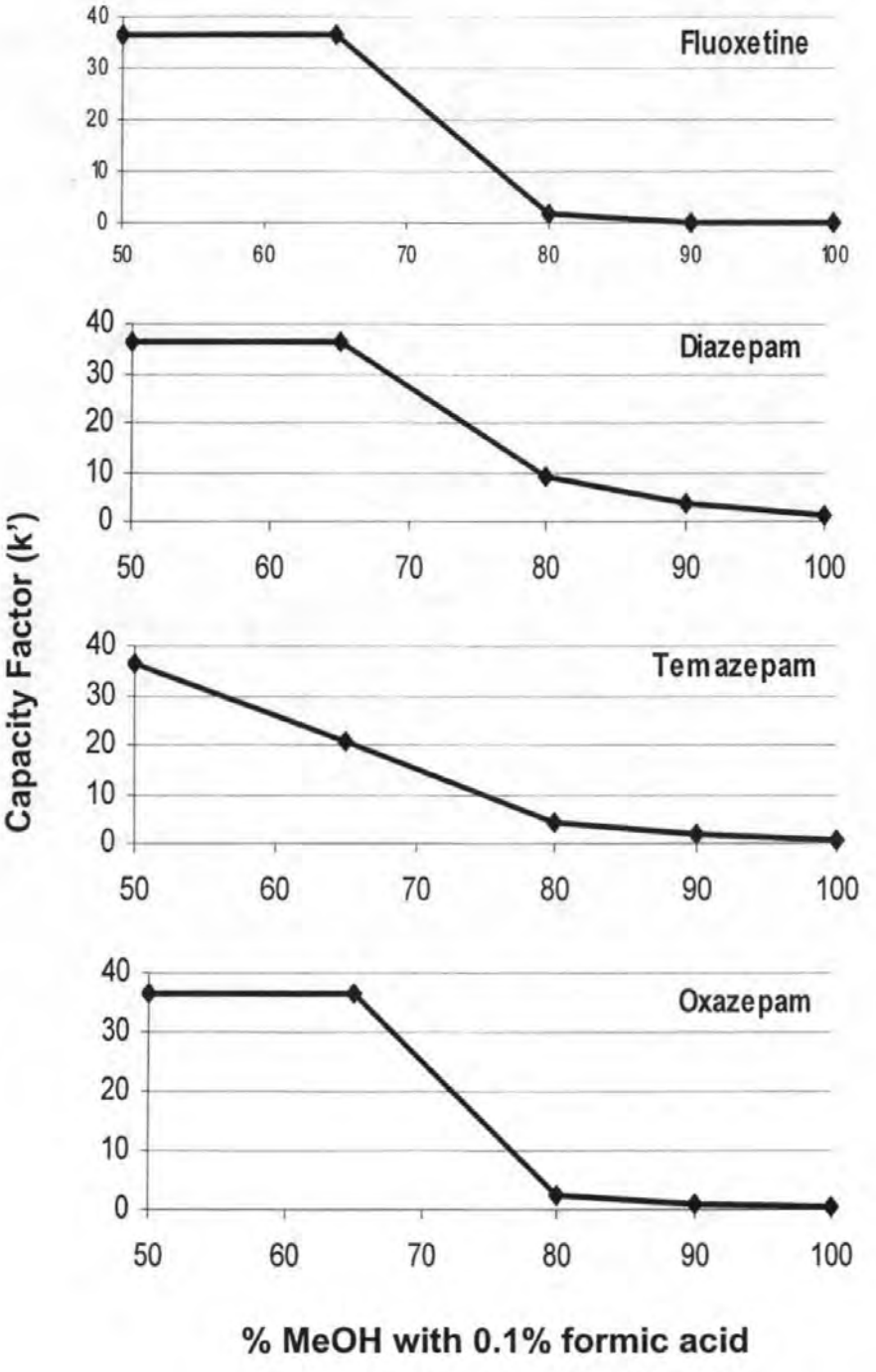


Figure 3.1. Capacity Factor (k') plots generated using PRP-1, 50 x 4.1mm, 5µm for Fluoxetine, Diazepam, Temazepam and Oxazepam

Generated k' plots (Figure 3.1) suggested that Strata-X SPE cartridges would be suitable for use with these drugs as it was possible to both retain and elute all target analytes from this phase. k' plots also aid in the optimisation of wash steps involved during SPE. The use of an organic solvent in combination with an aqueous solvent in the wash step maximises removal of interfering compounds. However if the organic content is too high this can result in the loss of target analytes. These plots indicated that washing Strata-X SPE cartridges with 50% MeOH and 50% Milli-Q would not cause the drugs to elute from the cartridges prematurely and hence be lost in the wash step. Table 3.1 shows that at 50%, MeOH k' were at a maximum for all target analytes (k' = 36.5). However at 65% MeOH, Temazepam (k' = 20.6) may elute from the SPE cartridges. Data in this table also indicates that 100% MeOH is required for the elution step in SPE for maximum recovery of all compounds to be obtained. Comparison of k' data for between 90 and 100% MeOH shows k' values to still be declining for all compounds, except Fluoxetine for which maximum elution was obtained at 90% MeOH (k' = 0.1).

3.3.2. SPE from Water: Method Development

A series of sequential optimisation process were carried out to maximise the extraction and hence recoveries of all four target compounds from water. Conditions which remained consistent throughout each step of the extraction process are summarised in Figure 3.2. The details of steps that were varied during this method development work are presented in Table 3.2 along with comments upon the results of each method development stage.

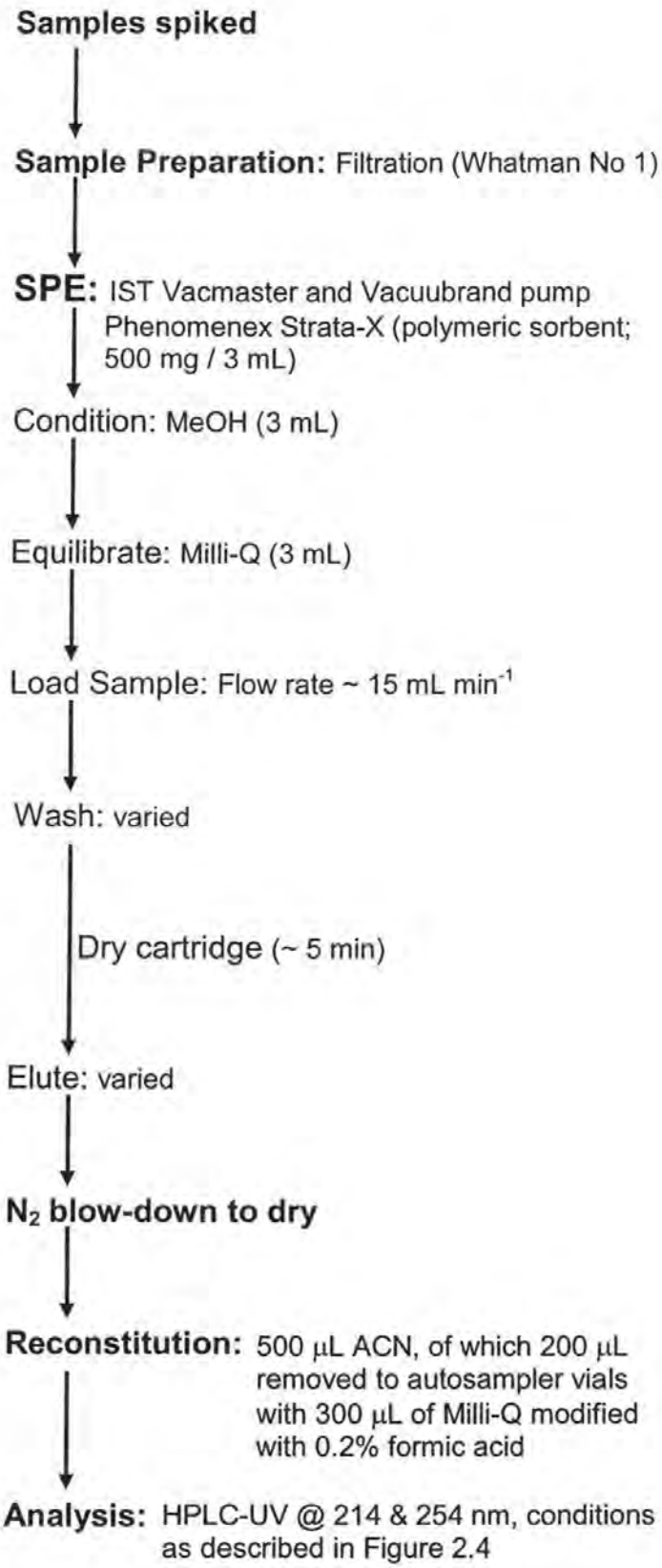


Figure 3.2. Flow diagram of generic experimental steps used during method development for the extraction of four target analytes from water

Method Development Stage	Sample Details	Wash	Elution	Percentage Recovery			
				Fluoxetine	Oxazepam	Temazepam	Diazepam
Optimisation of wash step	100µg each analyte in Milli-Q (500mL). Single replicate	Milli-Q (2 x 3 mL)	MeOH + 2% formic acid (3 x 10 mL; collected in 3 x 10 mL fractions)	118	81	63	31
		MeOH : Milli-Q (50 : 50; v/v) (2 x 3 mL)		111	151	90	70
Optimisation of elution step	100µg each analyte in Milli-Q (500mL). Single replicate	MeOH : Milli-Q (50 : 50; v/v) (2 x 3 mL)	MeOH + 0.5% formic acid (2 x 10 mL; collected in 3 x 10 mL fractions)	77	100	84	55
			MeOH + 1% formic acid (2 x 10 mL; collected in 3 x 10 mL fractions)	127	97	113	78
			MeOH + 2% formic acid (2 x 10 mL; collected in 3 x 10 mL fractions)	100	91	96	56
Confirmation of recoveries and reproducibility	100µg each analyte in Milli-Q (500mL). Triplicate	MeOH : Milli-Q (50 : 50; v/v) (2 x 3 mL)	MeOH + 1% formic acid (3 x 3 mL; collected in 3 x 10 mL fractions)	103 ± 24	104 ± 11	95 ± 16	58 ± 22 (71 ± 2)

Table 3.2. Sequential method development stages and results in the extraction of Fluoxetine, Diazepam, Temazepam and Oxazepam from water

Table 3.2 continued...

Method Development Stage	Comments & conclusions
Optimisation of wash step	Inclusion of 50% MeOH within wash step resulted in higher recoveries for all target compounds especially Temazepam due to lower losses of target compounds during the wash step as predicted by k'-plots. Collection & analysis of elution step in 3 fractions (10 mL) established that all compounds eluted within the first 20 mL.
Optimisation of elution step	Results suggest that the use of 1% formic acid within the elution solvent is optimum for maximum recoveries, although differences in recoveries do not appear to be significantly different. No target compounds were detected in the second fraction, therefore indicating that the elution volume could be reduced to 10 mL without a reduction in recovery.
Confirmation of recoveries and reproducibility	Recoveries obtained for all target compounds were good with acceptable standard deviations. The use of an internal standard may allow some of these minor variations to be accounted for. There was no detection of Diazepam in the first elution fraction for one of the replicates, although Diazepam was found in the second fraction. This anomalous result has therefore reduced the average recovery. If this replicate is discounted Diazepam recovery increases to 71% (data shown in brackets). No target compounds were detected in the third fraction, therefore indicating that the elution volume could be reduced to 6 mL without a reduction in recovery.

Table 3.2. Sequential method development stages and results in the extraction of Fluoxetine, Diazepam, Temazepam and Oxazepam from water

3.3.3. SPE from Water: Final Working Method

The step-wise method development procedure was successful in producing an extraction and pre-concentration method for the analysis of Fluoxetine, Diazepam, Temazepam and Oxazepam from water matrices with acceptable recoveries. Figure 3.3 shows that this procedure did not introduce any apparent contamination, nor affect the Gaussian peak shapes, nor Rt of the compounds in question. The final working sample preparation and SPE method from extraction from water matrices is presented in Figure 3.4.

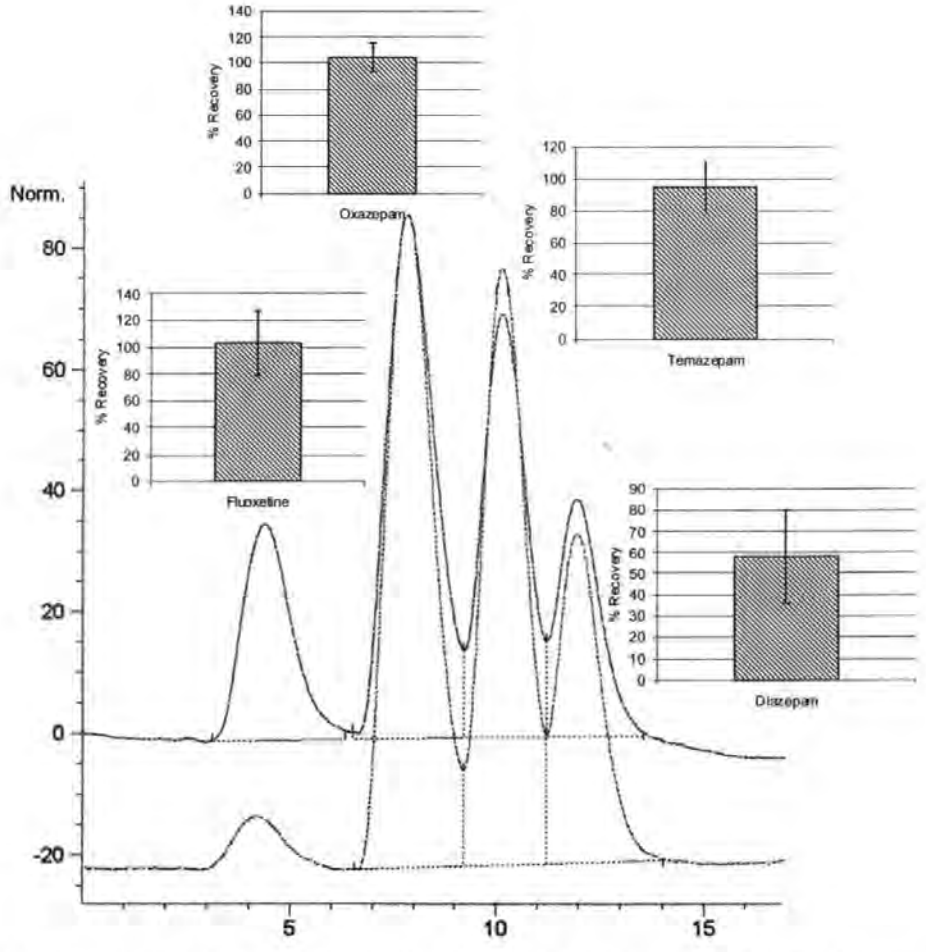


Figure 3.3. Example HPLC-UV chromatogram and recovery data for the extraction of Fluoxetine, Diazepam, Temazepam and Oxazepam from water using the final working SPE method

A: 214 nm. B: 254 nm
Error bars represent ± 1 standard deviation (n = 3)
Chromatogram shown is from 'Confirmation of recoveries and reproducibility' method development stage (Table 3.2); first elution fraction from one replicate.

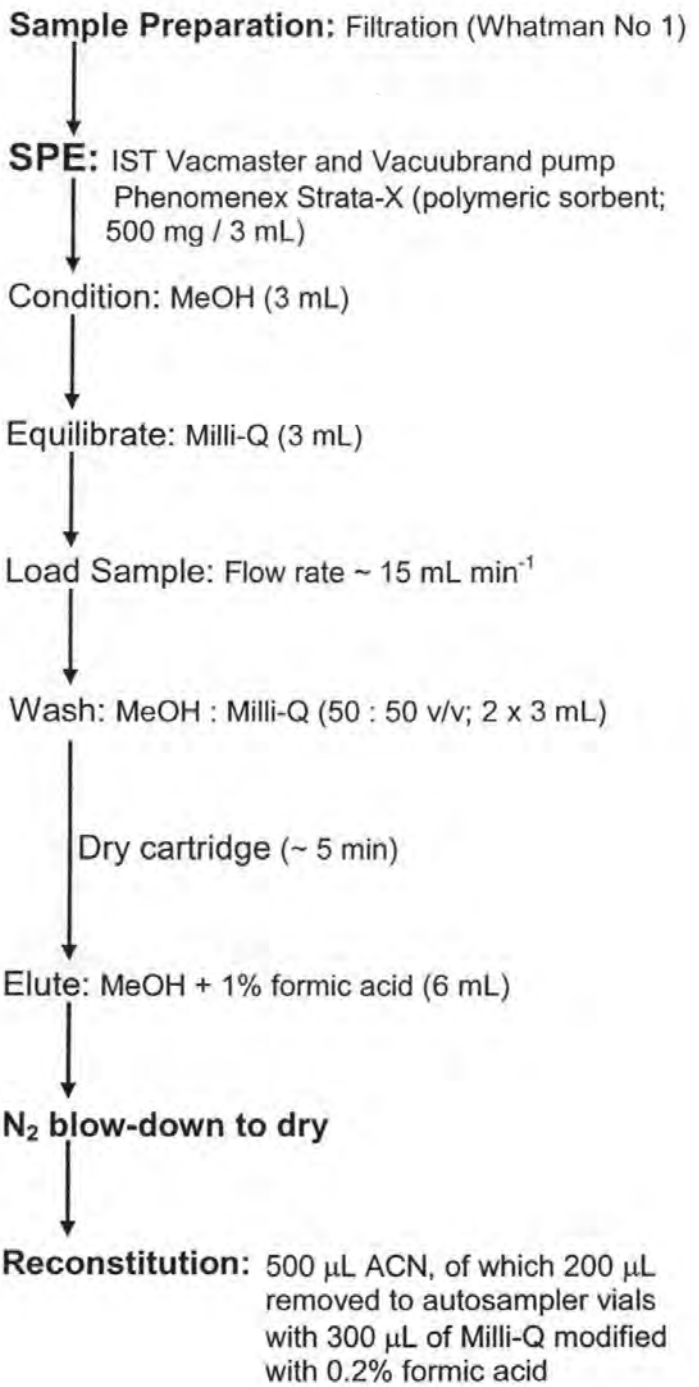


Figure 3.4. Flow diagram showing the final working method for sample preparation and SPE of Fluoxetine, Diazepam, Temazepam and Oxazepam from water

For the liquid culture biodegradation experiments reconstitution was as follows; 200 µL ACN, of which 50 µL was removed to an autosampler vial with 50 µL of Milli-Q modified with 0.12% formic acid (v/v).

3.3.4. SPE from Water: Summary and Method Limitations

The developed method is suitable for the extraction of Fluoxetine, Diazepam, Temazepam and Oxazepam from water. For use with water which may have high levels of humic or fulvic acids the use of the developed soil method (Section 3.4, Figure 3.8) is recommended as it was specifically designed to remove these interfering components. This SPE method is likely to require some modification for use with different compounds such as other 1,4-benzodiazepines or different water sources (e.g. marine or estuarine waters).

3.4. SPE Method Development for Extraction from Soil

The successful development of an SPE method for the extraction of target compounds from water provided the basis of an extraction methodology for soil matrices. As later studies were to involve biodegradation within soil it was necessary to develop an efficient extraction method with suitable clean-up that would allow a LOD good enough for quantification of target compounds at environmentally relevant concentrations in soils.

3.4.1. Extraction from Soil: Method Development

As with the method development for the extraction from water, a series of sequential method development steps was undertaken to optimise the extraction of Fluoxetine, Diazepam, Temazepam and Oxazepam from soil. The agricultural SS amended-soil used in this method development work was sourced from the same site as soil used in the subsequent biodegradation studies (Chapter 4). More details on the source of this soil can be found in Chapter 4, Section 4.3.1. Prior to the start of experimental work, soil was stored at 4°C in the absence of light. Large stones and any flora or fauna were removed from the soil but it was not altered in any other manner. A flow diagram showing generic experimental stages, including modifications to the water SPE method for use with soil is presented in Figure 3.5. In Table 3.3 details of altered extraction steps along with comments upon the results at each stage can be found.

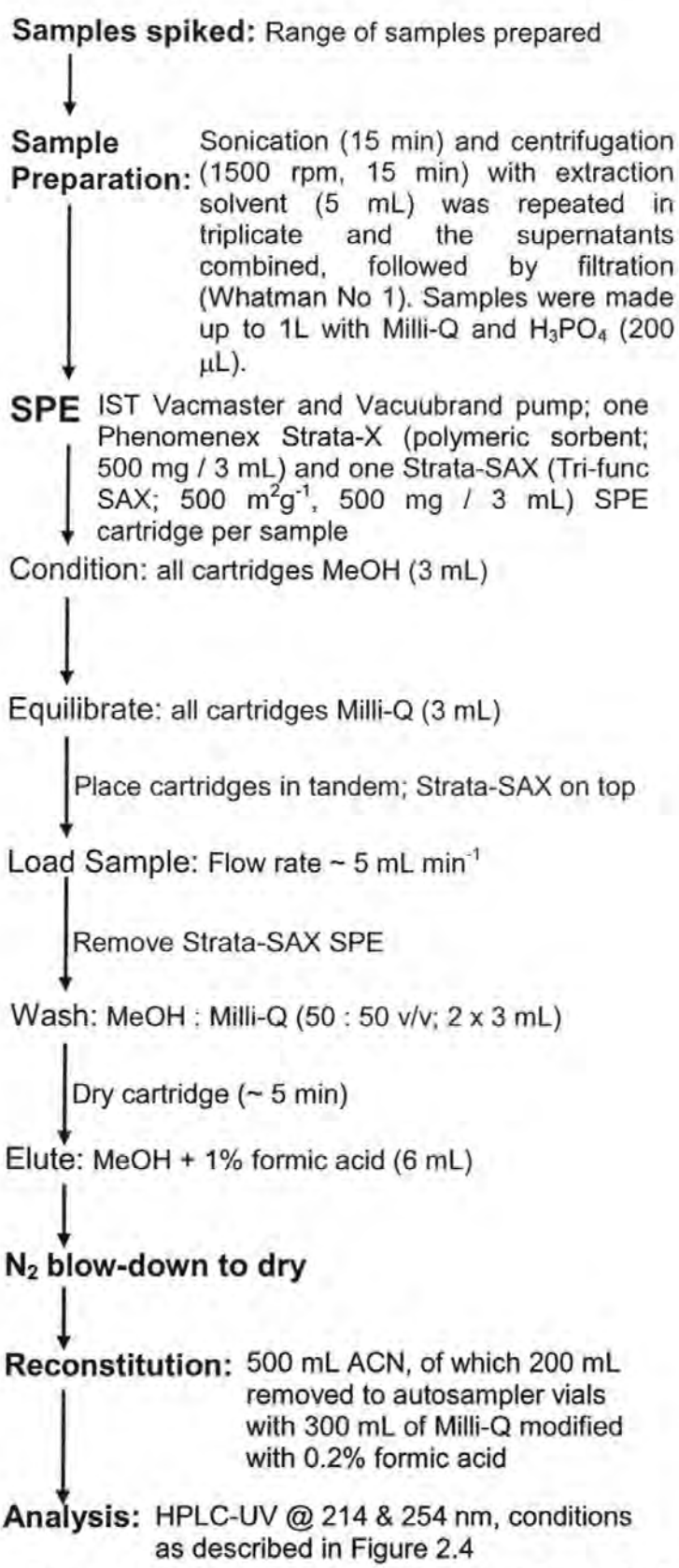


Figure 3.5. Flow diagram of generic experimental procedures, including details of modification of water SPE method, using during method development for the extraction of four target analytes from soil

Method Development Stage	Sample Details	Extraction Solvent	Percentage Recovery			
			Fluoxetine	Oxazepam	Temazepam	Diazepam
Selection of solvent for compound extraction from soil	100μg each analyte in soil (5 ± 0.1 g wet weight). Immediate extraction. Single replicate	MeOH + 1% formic acid	67	86	82	67
	100μg each analyte in soil (5 ± 0.1 g wet weight). Immediate extraction. Single replicate	ACN + 1% formic acid	85	84	85	69
	100μg each analyte in soil (5 ± 0.1 g wet weight). Immediate extraction. Single replicate	IPA + 1% formic acid	283	68	60	53
Confirmation of recoveries and reproducibility	100μg each analyte in soil (5 ± 0.1 g wet weight). Immediate extraction. Triplicate	ACN + 1% formic acid	79 ± 2	88 ± 2	83 ± 4	63 ± 5

Table 3.3. Sequential method development stages and results in the extraction of Fluoxetine, Diazepam, Temazepam and Oxazepam from soil

Table 3.3 continued...

Method Development Stage	Comments & Conclusions
Selection of solvent for compound extraction from soil	Use of ACN + 1% formic acid as the extraction solvent resulted in the highest set of recoveries for the target compounds, and so this solvent was selected for use in further soil extraction work. When IPA + 1% formic acid was used unrealistically high Fluoxetine recoveries were obtained, which was thought to be due to co-elution of extracted humic or fulvic materials from the soil.
Confirmation of recoveries and reproducibility	Recoveries obtained for all target compounds were good with acceptable standard deviations. The use of an internal standard may allow some of these minor variations to be accounted for.

Table 3.3. Sequential method development stages and results in the extraction of Fluoxetine, Diazepam, Temazepam and Oxazepam from soil

Table 3.3 continued...

Method Development Stage	Sample Details	Extraction Solvent	Percentage Recovery			
			Fluoxetine	Oxazepam	Temazepam	Diazepam
Testing impact of compound sequestration into soil matrix on recoveries	100μg each analyte in soil (5 ± 0.1 g wet weight). Spiked 7 days prior to extraction. Triplicate	ACN + 1% formic acid	70 ± 11	70 ± 4	73 ± 11	54 ± 2
Testing impact of sample size upon recoveries	50 g sample spiked at 1μg g^{-1} wet weight each analyte. Immediate extraction. Single replicate	ACN + 1% formic acid	59	48	37	28
	25 g sample spiked at 1μg g^{-1} wet weight each analyte. Immediate extraction. Single replicate	ACN + 1% formic acid	63	21	15	33
	15 g sample spiked at 1μg g^{-1} wet weight each analyte. Immediate extraction. Single replicate	ACN + 1% formic acid	75	86	61	81
Confirmation of recoveries and reproducibility using 15 g sample size	15 g sample spiked at 1.5μg g^{-1} wet weight each analyte. Immediate extraction. Triplicate	ACN + 1% formic acid	91 ± 21	48 ± 8	48 ± 3	69 ± 2

Table 3.3. Sequential method development stages and results in the extraction of Fluoxetine, Diazepam, Temazepam and Oxazepam from soil

Table 3.3 continued...

Method Development Stage	Comments & conclusions
Testing impact of compound sequestration into soil matrix on recoveries	Allowing 7 days for compounds to become incorporated into the soil matrix resulted in an expected reduction in recoveries. Fluoxetine and Oxazepam recoveries were reduced by ~ 15%, 10% for Temazepam and 18% for Diazepam.
Testing impact of sample size upon recoveries	Recovery data implied that the use of only 3 x 5 mL of extraction solvent to extract 15 g of soil did not result in any significant reduction in recoveries. However when the sample size was > 15g recovery data indicated significant losses of all target compounds and larger solvent volumes would be required to extract larger volumes of soil.
Confirmation of recoveries and reproducibility using 15 g sample size	Recoveries obtained for all target compounds were acceptable with appropriate standard deviations, although they were found on average to be slightly lower than when 5 g of soil were used. For this reason it is decided that this extraction and clean-up method should be used with as small a sample size as possible to obtain maximum recoveries.

Table 3.3. Sequential method development stages and results in the extraction of Fluoxetine, Diazepam, Temazepam and Oxazepam from soil

3.4.2. Extraction from Soil: Final Working Method

A simple extraction method using acidified ACN was developed for use with a modified version of the method developed for water. The use of a tandem SAX cartridge successfully allowed significant removal of interfering substances such as humic and fulvic acids from the soil samples. Comparison of HPLC-UV chromatograms from soil (Figure 3.7) and water extractions (Figure 3.3) indicated the removal of the majority of interfering components. Only one additional peak (~ 4 minutes, at 214 nm only) due to an unknown soil artefact was found.

During the sample preparation process the extracts from soil were heavily diluted to reduce the organic content to less than 2%, which ensured that the target compounds were retained upon the Strata-X cartridge and no premature breakthrough occurred. This acidification process involved reduction of the pH to below 2.9 so that the target compounds were neither positively nor neutrally charged and therefore not retained upon the Strata-SAX cartridge and were free to pass through to the Strata-X cartridge where they were retained (Blackwell *et al.*, 2004). The final working method from the extraction and clean-up of target compounds from SS amended-soil is summarised in Figure 3.8.

Figure 3.7 also shows a results summary for all ACN + 1% formic acid extractions that were performed in triplicate. It clearly shows the advantage of immediate extraction and the use of small soil sample masses. Statistical analysis to draw comparisons between the 5g sample sets extracted immediately or 7 days post spiking showed no difference in variation for any of the target compounds at the 95% confidence interval (F-test, P-value: Fluoxetine 0.090; Oxazepam 0.495; Temazepam 0.298; Diazepam 0.355).

Statistical analysis also indicated no differences in the mean recoveries of Fluoxetine or Temazepam (t-test, P-value: 0.228 and 0.233 respectively) when the spiked soil was left for a week before being extracted, as opposed to immediate extraction. However for Oxazepam and Diazepam, t-test P-values were found to be 0.003 and 0.035 respectively, indicating that the differences in Oxazepam and Diazepam recoveries seen in the two sets (immediate and 7 days) were statistically significantly different. Due to the commonality of functional groups between the 1,4-benzodiazepines studied it could be expected that their behaviour regarding sorption to soil would be similar. It is possible however that small differences in functional groups, such as the C3 hydroxyl in Oxazepam or the N1-methyl in Diazepam (Figure 3. 6), impacted the rate of sorption of these compounds into soil during this method development work. It would appear that if Temazepam undergoes sorption it is very rapid, whereas the sorption of Oxazepam and Diazepam was slower and hence appeared more progressive. It should however be noted that these analyses were only repeated in triplicate.

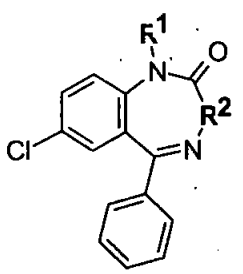


Figure 3. 6. Structures of target 1,4-benzodiazpines

Diazepam: $R^1 = CH_3$, $R^2 = CH_2$
Nordiazepam: $R^1 = H$, $R^2 = CH_2$
Temazepam: $R^1 = CH_3$, $R^2 = OH$
Oxazepam: $R^1 = H$, $R^2 = OH$

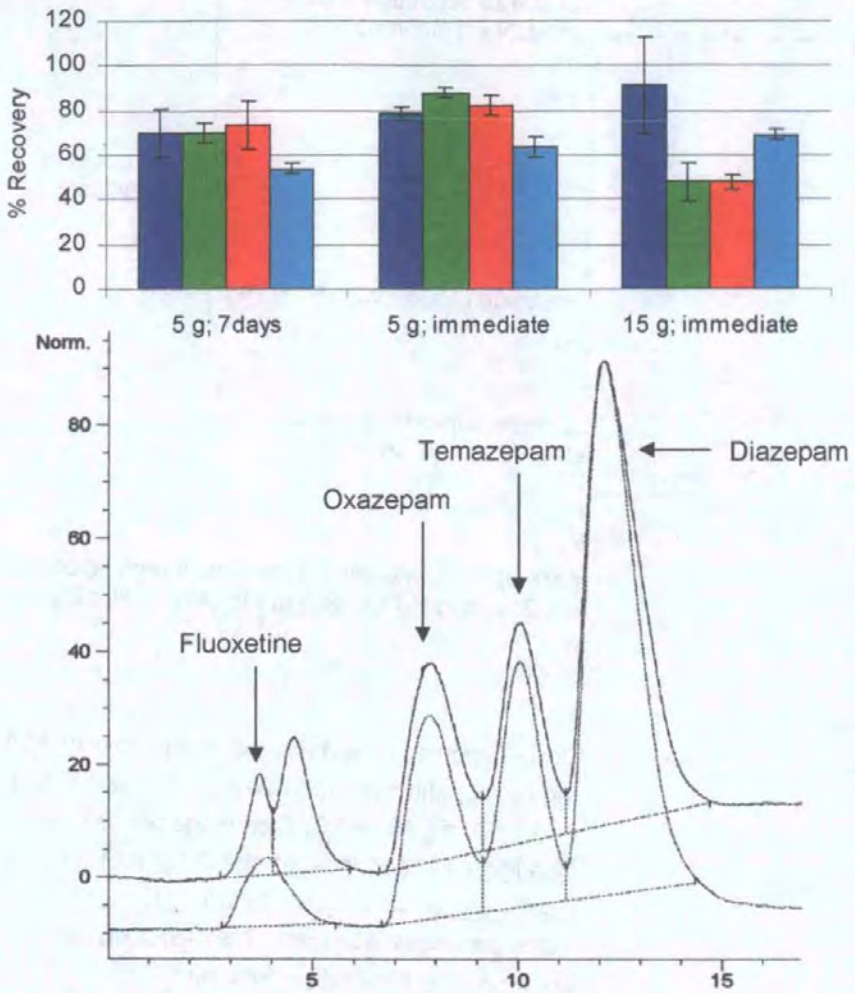


Figure 3.7. Example HPLC-UV chromatogram and recovery data for the extraction of Fluoxetine, Diazepam, Temazepam and Oxazepam from SS amended-soil spiked either immediately or 7 days prior to extraction using the final working soil extraction and SPE method

■ Fluoxetine; ■ Oxazepam; ■ Temazepam; ■ Diazepam
Error bars represent ± 1 standard deviation (n = 3)
Time reference in bar-chart refers to time between spiking and extraction
Chromatogram shown is from one replicate from 'Confirmation of recoveries and reproducibility using 15 g sample size' method development stage (Table 3.3).

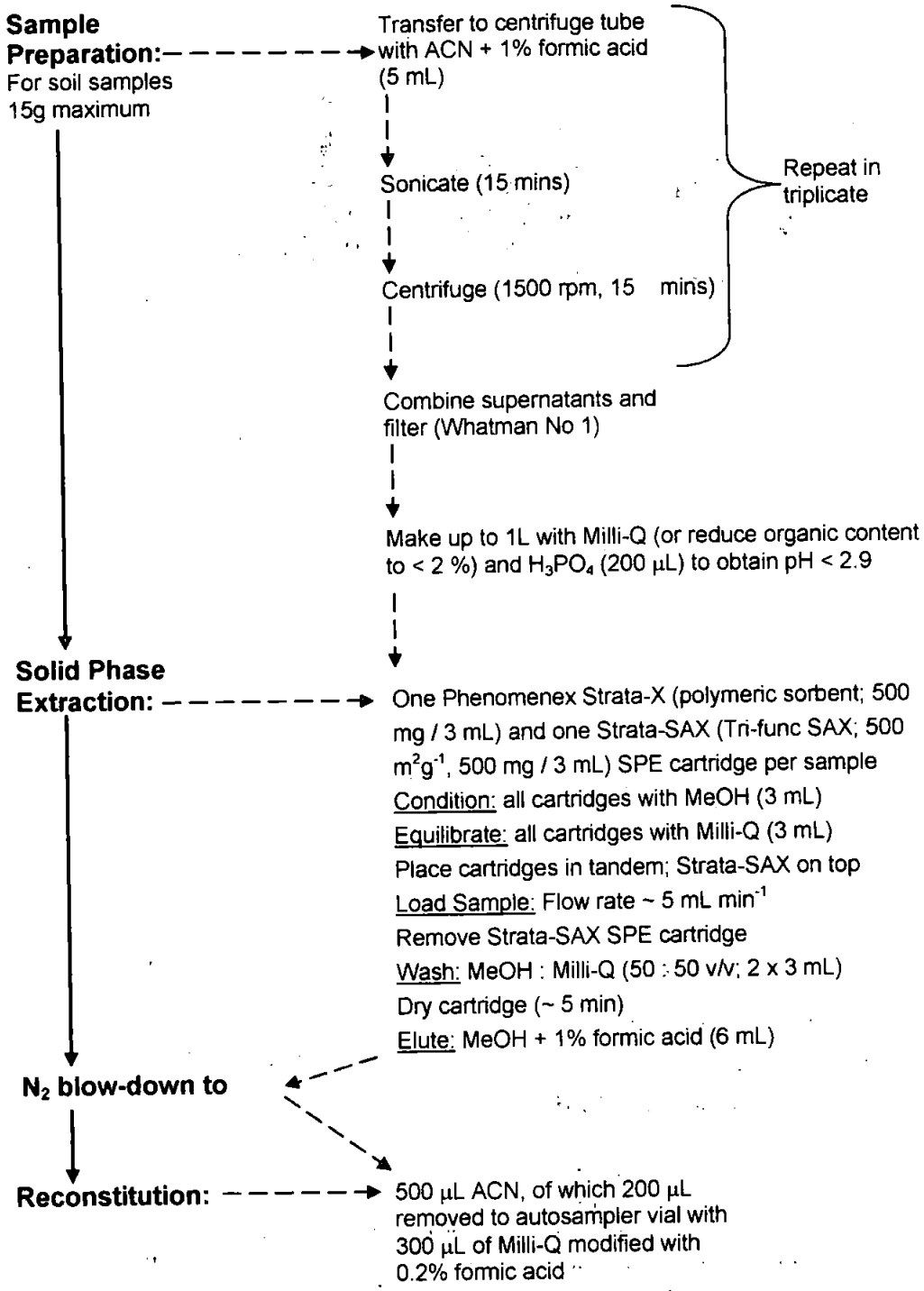


Figure 3.8. Flow diagram showing the final working method for sample extraction, preparation and SPE of Fluoxetine, Diazepam, Temazepam and Oxazepam from SS amended-soil

For the biodegradation of Fluoxetine HCl in SS amended soil experiment reconstitution was as follows; 200 µL ACN, of which 50 µL was removed to an autosampler vial with 200 µL of Milli-Q modified with 0.12% formic acid (v/v).
For the extraction of Rothamsted soils reconstitution was as follows; 200 µL ACN, of which 50 µL was removed to an autosampler vial with 50 µL of Milli-Q modified with 0.12% formic acid (v/v).

3.4.3. Extraction from Soil: Summary and Method Limitations

This study indicated that the soil extraction and clean-up method developed is suitable for the extraction of Fluoxetine, Diazepam, Temazepam and Oxazepam from SS amended-soils. However, as a final caveat, for use with soils that have unusually high SS application rates, or use with untreated sewage, some modifications may be required, since as part of this project agricultural soils that had been treated previously with SS for several years, as part of a study on heavy metal pollution of SS-amended soils, were obtained from Rothamsted Research, Harpenden, Hertfordshire, UK. These soils sourced from seven different sites (Woburn, Gleadthorpe, Watlington, Pwllpeiran, Rosemaund, Bridgets and Shirburn) across Britain were sampled annually and had undergone both short and long term treatments of annual SS application (1994 – 1997 and 1994 - 2005). The soils included control soils (no SS), soils treated with digested sludge cake (short and long term treatments) and soils treated with raw SS cake (short and long term treatments) all of which were sampled in 2005. More detailed information on the characteristics of these soils and sludges are presented by Gibbs *et al*,. (2006). The aim of obtaining these soils was to make an assessment of potential rates of accumulation and degradation of PPCPs in a range of soil types.

Initially soils treated with undigested sludge cake for 12 years from all seven sites were extracted and analysed. Samples (~ 40 g) were separated into four sub-samples (~10 g) and spiked with IS (d_5-Fluoxetine HCl (1 µg) and d_5-Oxazepam (1 µg) per sub-sample) before undergoing the developed soil extraction method (Figure 3.8), prior to tandem SPE supernatants were combined. HPLC-ESI-MS using conditions described in sections 2.4.1.2 and

2.4.2.2 was used for analysis in both full MS and SIM modes. Sub-samples of each of these soils treated with undigested sludge cake also underwent procedures to generate semi-quantitative data on the bacterial populations present as discussed in Section 4.5 and presented in the appendices (Figures A.21 to A.33).

Quantitative data generated from full MS extracted ion chromatograms indicated variable IS recoveries (d_5-Fluoxetine HCl 36 ± 19%; d_5-Oxazepam 44 ± 19%) that were lower than those obtained for Fluoxetine and Oxazepam in method development work (Figure 3.8; ~ 50 – 90%).The differing properties of the range of soils used may account for some of the variation of IS recovery seen in these samples. Neither full MS nor SIM analysis modes were able to detect any of the target compounds Fluoxetine, Norfluoxetine, Diazepam, Temazepam, Oxazepam or Nordiazepam. It was felt that to utilise these valuable samples for an accumulation and degradation study, further method development to clean the samples and reduce the LOD would be required. It should be noted however that the SS treatment rate used on Rothamsted soils was based upon heavy metal content, which resulted in biosolid application rates approximately 10 times greater than those normally used in agriculture. Also the particular treatment of these analysed sampled used undigested sludge cakes i.e. raw SS, which is a dirtier matrix than digested SS.

3.5. Extraction from Plant Tissues

It was necessary to develop or modify a method for the extraction and clean-up of Fluoxetine from plant tissues for later use in a plant-uptake study. Cauliflower was selected to be the final test plant but it was important to develop a method using a readily grown, inexpensive and available plant of similar nature ideally grown under similar conditions before pursuing the more elaborate cauliflower growth studies. Cress was selected as a test plant due to its fast growing nature, ability to grow without soil and because it is from the same *Brassica* family as the cauliflower.

3.5.1. Extraction from *Brassica* Tissues: Method Development

An important component of this method development work was to develop a method that was able to remove interfering components from the plant tissue with minimal loss of Fluoxetine. Humic and fulvic acids are thought to contain a large proportion of plant-derived materials. For this reason it was a logical first step to test the soil extraction method with plant material. It was also necessary to ensure that the extraction method developed was capable of extracting the drug from plant tissue when it has been naturally sequestered.

During this work two different of sources of plant tissue were used; laboratory grown cress and purchased 'mixed *Brassica*' (Sainsbury's salad cress: 85% rape; 10% cress; 5% mustard). Laboratory grown cress (fine curled cress, purchased from Wilkinson as seeds) was grown in circular drip trays (14cm radius, 2.5cm depth; 10% nitric acid washed) on a laboratory windowsill (20 + or – 5°C). Solvent blanks (100 µL MeOH) were set-up alongside spiked trays (0.1 mg Fluoxetine in MeOH) to which 35mL of sterile Milli-Q was added. Cardboard lids with a slit (10cm x 2cm) were fixed over the drip trays to reduce potential

photodegradation of the target compound (Figure 3. 9). Misshapen, cracked or discoloured cress seeds were disposed of. 150 cress seeds were sprinkled into each tub through the slits in the cardboard (i.e. so that the light slit cut in the cardboard was above the cress seeds). Trays were rotated daily to ensure even light distribution and watered with sterile Milli-Q until maturity (16 – 27 days). Upon harvesting, plants were washed in extraction solvent and the washings added to the tray sample.

A series of experiments was performed to test the suitability of the developed soil extraction and clean-up method for use with these *Brassica* samples. Figure 3.10 shows the generic experimental steps undertaken during this method development work, and the details of individual stages of method development are presented in Table 3.4 along with comments upon results of each stage.

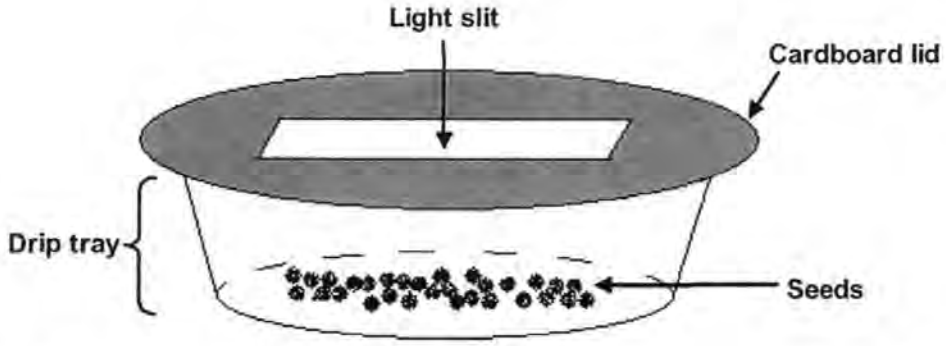


Figure 3. 9. Diagram of set-up for growth of Fluoxetine exposed Cress for use as samples in the development of an extraction method for Fluoxetine HCl from *Brassica* tissues

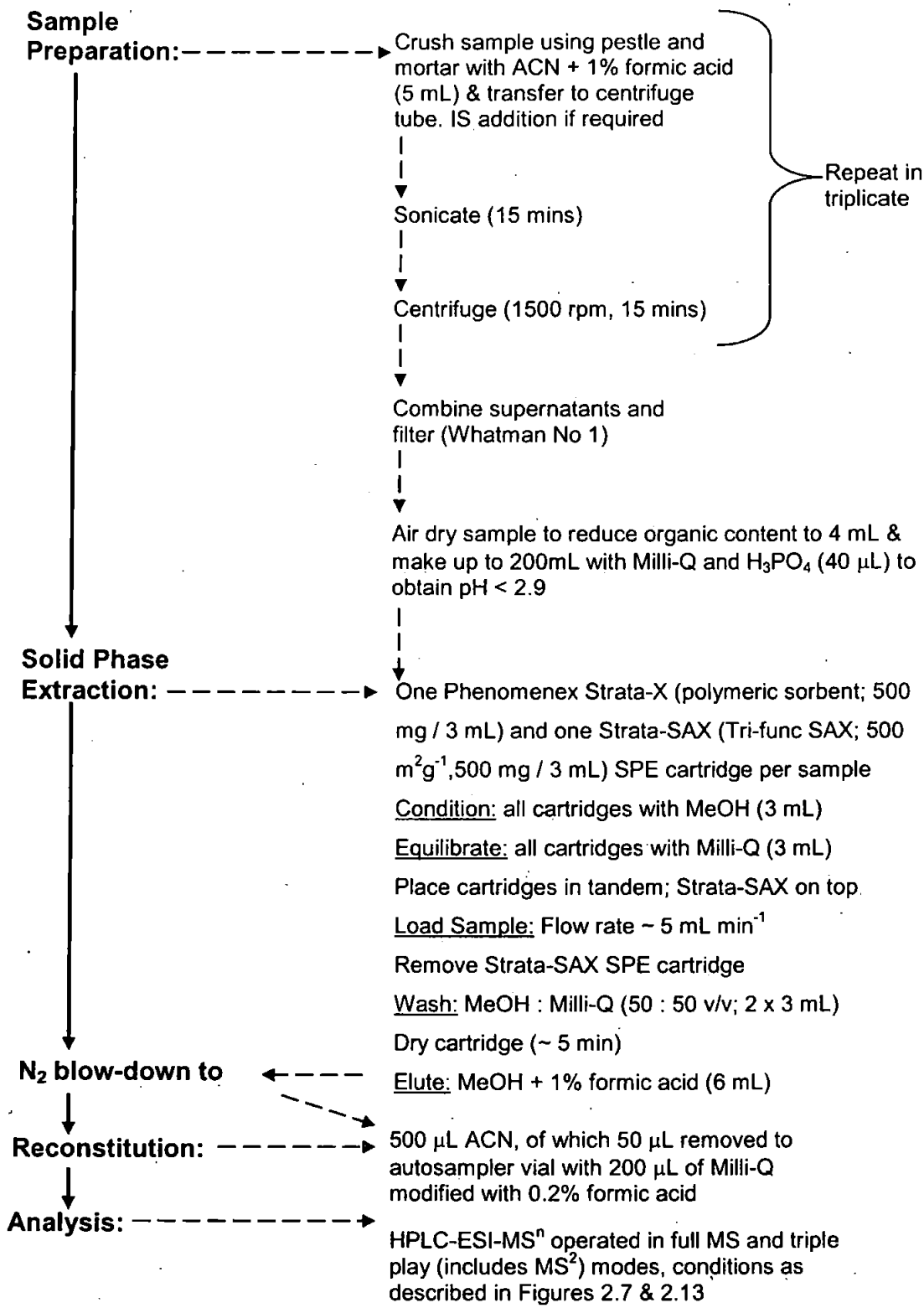


Figure 3.10. Flow diagram of generic experimental steps used during method development for the extraction of four target analytes from plant matrices

Method Development Stage	Sample Details	Extraction	Percentage Fluoxetine Recovery				
			Leaves	Roots & Stem	Seed-casing	Tray	Total
Testing developed soil extraction and clean-up method	Cress grown in 100 µg Fluoxetine HCl. Sampled as whole plants. Triplicate	ACN + 1% formic acid. IS 10 µg.	n/a	n/a		3 ± 0.3 (67 ± 19)	14 ± 1 (40 ± 12)
Adaptation of harvest process & confirmation of uptake	Cress grown in 100 µg Fluoxetine HCl. Sub-sampled. Triplicate	ACN + 1% formic acid. IS 10 µg.	6 ± 3 (IS; 13 ± 5)	6 ± 2 (IS; 26 ± 12)	18 ± 4 (IS; 19 ± 6)	8 ± 4 (IS; 32 ± 7)	39 ± 3
IS recoveries	*Brassicas* (Sainsbury's) spiked with 10 µg IS. Sub-sampled. Triplicate	ACN + 1% formic acid.	(IS; 28 ± 3)	(IS; 71 ± 4 stem only)	n/a	n/a	n/a
Confirmation of IS recoveries	Cress grown in laboratory spiked with 10 µg IS. Sub-sampled. Triplicate	ACN + 1% formic acid.	(IS; 11 ± 1)	(IS; 7 ± 0.4 stem only)	n/a	n/a	n/a
Use of alternative SAX cartridge	*Brassicas* (Sainsbury's) spiked with 10 µg IS. Sub-sampled. Single replicates	ACN + 1% formic acid. Alternative SAX SPE cartridge*	(IS; 6)	(IS; 5 stem only)	n/a	n/a	n/a

Table 3.4. Sequential method development stages and results in the extraction of Fluoxetine from *Brassica* plant tissues

Fluoxetine recovery data is presented as % of originally added amount. Equivalent IS (d_5-Fluoxetine HCl) recovery data are presented in brackets as % of IS added to each individual sub-sampled tissue

* Isolute SAX / PSA SPE cartridges (500 mg / 3 mL)

Table 3.4 continued...

Method Development Stage	Comments & conclusions
Testing developed soil extraction and clean-up method	Fluoxetine was positively identified in both the tray (3 ± 0.3% remaining) and cress sample (whole plant 11 ± 1% up-taken), but on average 86% of Fluoxetine could not be accounted for. Losses due to photodegradation / sorption or inefficiencies in extraction are likely explanations. No statistical differences were found in germination rates or harvest masses between the blank and spiked samples (t-test; P-value = 0.377 & 0.378 respectively) indicating no major phytotoxic impacts. Post harvest extraction efficiency (IS recovery) was 40 ± 12% for whole plant and 67 ± 19% for tray. As whole plants were sampled, including roots, it should be considered that these findings of apparent uptake could in fact be due to sorption and subsequent desorption from seed casings / roots.
Adaptation of harvest process & confirmation of uptake	Fluoxetine was positively identified in all tissues tested providing evidence of Fluoxetine uptake (6 to 18%) although the majority of the target compound (~60%) could not be accounted for. Again no significant differences were found in germination rates (t-test; P-value = 0.406) or harvest masses (t-test; P-value = 0.0794) between blank and spiked sample sets. IS recoveries (13 - 26% for the different plant tissues) were unacceptable and would need improvement to increase extraction and lower the LOD.

Table 3.4. Sequential method development stages and results in the extraction of Fluoxetine from *Brassica* plant tissues

Fluoxetine recovery data is presented as % of originally added amount. Equivalent IS (d_5-Fluoxetine HCl) data are presented in brackets as % of IS added to each individual sub-sampled tissue

* Isolute SAX / PSA SPE cartridges (500 mg / 3 mL)

Table 3.4 continued...

Method Development Stage	Comments & conclusions
IS recoveries	The use of *Brassicas* (Sainsbury's) as the plant tissue source resulted in good IS recovery from stem tissue (71 ± 4%) and improved, although still poor, recoveries from leaf tissue (28 ± 3%). The large increase in recoveries compared to the previous method development was unexpected as the only modification of experimental conditions was the source of plant tissue.
Confirmation of IS recoveries	IS recoveries from laboratory grown cress leaves (7 ± 0.4%) was a factor of 10 lower than with *Brassicas* (Sainsbury's), and was less than half for stem tissues (11 ± 1%).
Use of alternative SAX cartridge	An alternative SAX cartridge (SAX / PSA) was used in an attempt to improve sample clean-up and hence lower LOD. IS recoveries (6% for leaves; 5% for stem) were found to be lower than with the use of Strata-SAX cartridges. Comparison of TICs with SAX/PSA and Strata-SAX (Figure 3.12) showed Strata-SAX to be more efficient at the removal of interfering components.

Table 3.4. Sequential method development stages and results in the extraction of Fluoxetine from *Brassica* plant tissues

Fluoxetine recovery data is presented as % of originally added amount. Equivalent IS (d_5-Fluoxetine HCl) data are presented in brackets as % of IS added to each individual sub-sampled tissue

* Isolute SAX / PSA SPE cartridges (1 g / 6 mL)

This section of method development work which aimed to test the developed soil extraction method for use with *Brassica* samples had some successes. It was possible to extract Fluoxetine which had been naturally sequestered in cress tissues using the developed soil method and to generate MS^2 data (Figure 3.11; extracted ions *m/z* 310.0 (Fluoxetine HCl) and *m/z* 315.0 (d_5-Fluoxetine HCl) can be seen) to confirm the presence of Fluoxetine within the cress tissue samples. The target compound and the internal standard (d_5-Fluoxetine HCl) were initially identified based on their Rt and their full MS *m/z* values. Samples in which Fluoxetine was identified at full MS, were then subjected to triple play analysis which included an MS^2 fragmentation. MS^2 spectra generated were then compared to the known *m/z* transitions of Fluoxetine HCl.

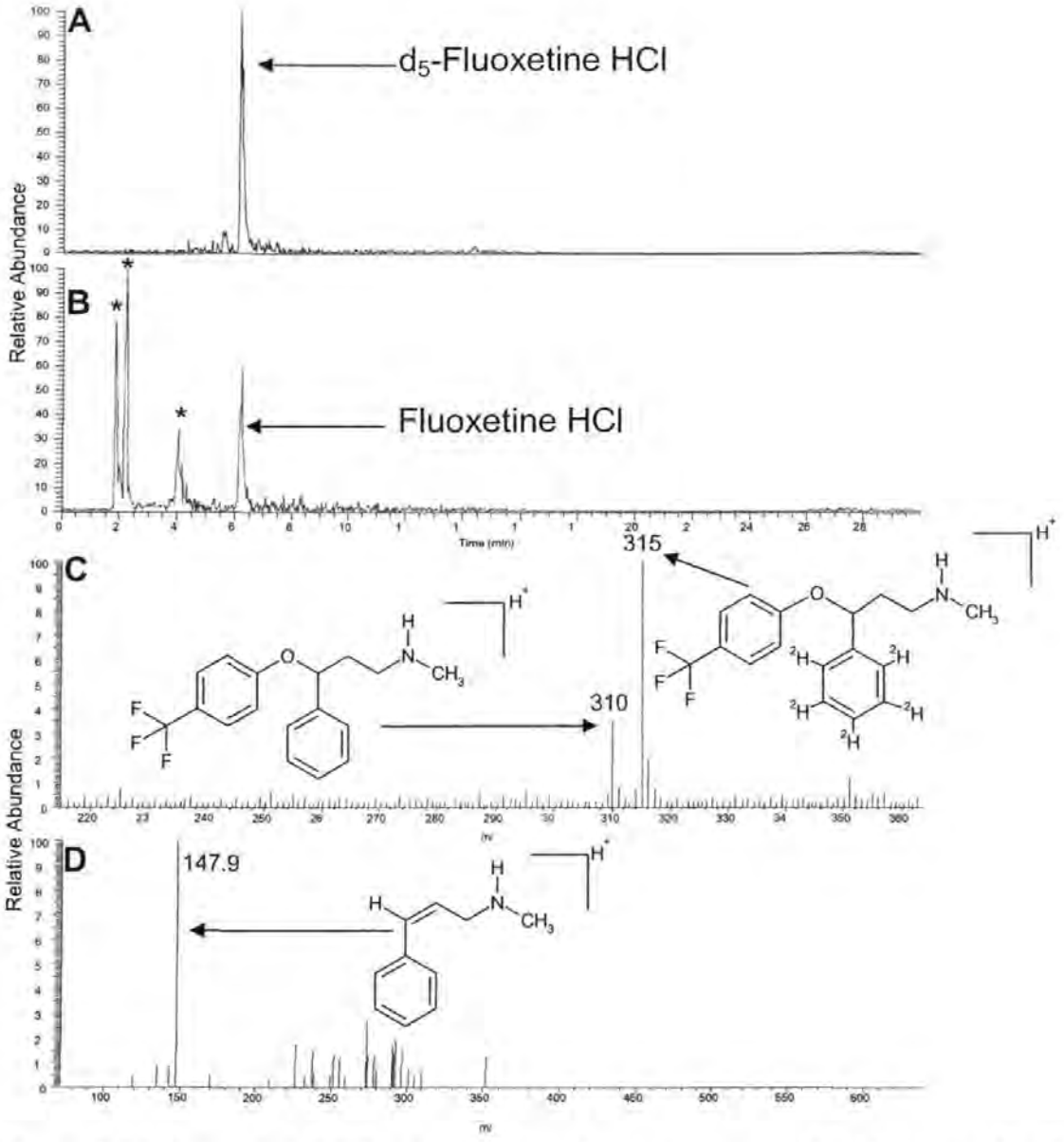


Figure 3.11. Example LC-MS extracted ion chromatogram and MS^n spectra for the extraction of Fluoxetine and IS from *Brassicas*

A: Extracted ion chromatogram *m/z* 315
B: Extracted ion chromatogram *m/z* 310
C: Full MS spectra of Fluoxetine and IS (d_5-Fluoxetine HCl)
D: MS^2 spectra of Fluoxetine
* artefacts with full MS *m/z* of 310 were confirmed not to be due to the presence of Fluoxetine by MS^2 transitions and their presence in non-exposed leaf samples (data not shown)
Chromatogram shown is from a leaf sub-sample from method development stage 'Adaptation of harvest process and confirmation of uptake' (Table 3.4).

An alternative SAX cartridge, an Isolute SAX / PSA (Tri-func SAX, 1 g / 6 mL) was used in tandem with a Strata-X SPE cartridge to test the potential for improved clean up using the SAX / PSA cartridge instead of a Phenomenex Strata-SAX (500 mg / 3 mL). This cartridge was selected as Isolute SAX / PSA

SPE cartridges have previously been shown to be effective for the purification of pesticide extracts from vegetables (Agronaut, 2003). In Figure 3.12 TIC are presented for stem and leaf samples which were extracted using tandem SPE with the different SAX cartridges.

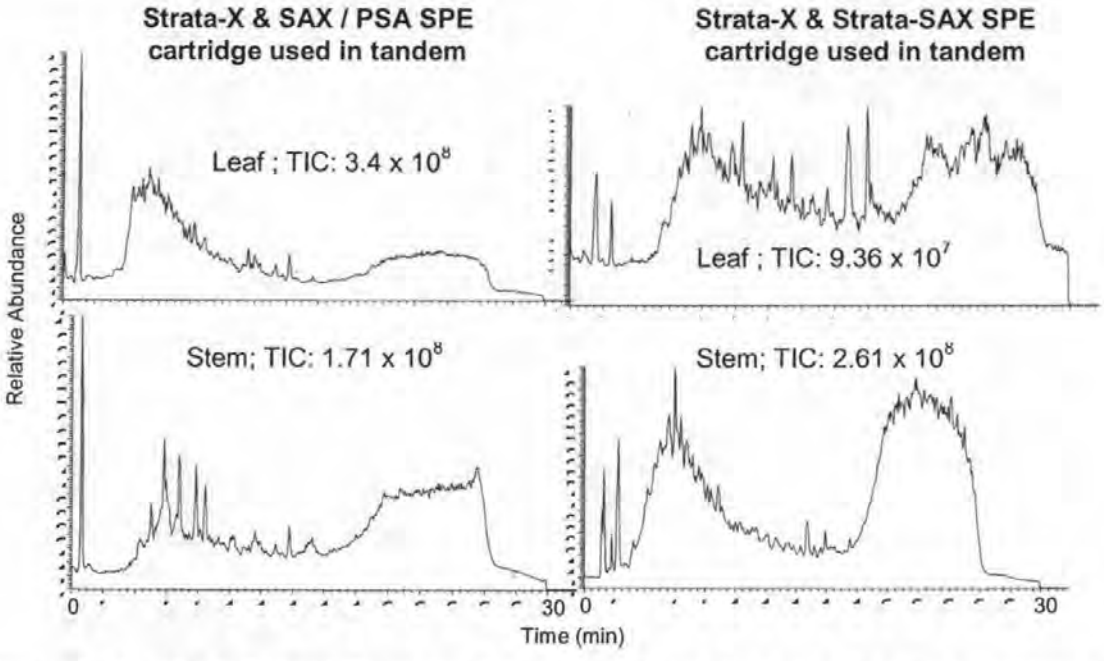


Figure 3.12. HPLC-ESI-MS Total Ion Chromatograms for leaf and stem samples extracted using tandem SPE with two types of SAX SPE cartridges

Phenomenex Strata-X (polymeric sorbent; 500 mg / 3 mL)
Phenomenex Strata-SAX (Tri-func SAX; 500 m^2g^{-1}, 500 mg / 3 mL)
Isolute SAX / PSA (1 g / 6 mL)

Figure 3.12 shows that the use of the SAX / PSA cartridge resulted in the extraction of more interfering components from the cress matrix, the majority of which eluted within the first 15 minutes of analysis. One particularly large unknown peak with an Rt of ~ 1min, which may be an artefact from the SPE cartridges themselves, resulted in much larger ion counts from the leaf sample when the SAX / PSA cartridge was used as opposed to the Strata-SAX cartridge. Ion counts for the stem samples were however within the same region. As recovery results (Table 3.4) were similar regardless of the cartridge

used, but Strata-SAX cartridges provided better sample clean-up, Strata-SAX cartridges were selected for future SPE method development work.

When cress was grown in the laboratory exposed to Fluoxetine HCl large losses (~60 – 80%) of the target compound occurred. It was suspected that these losses were most likely due to photodegradation. Attempts were made to minimise photodegradation during the growth period, but it could not be entirely be prevented. Cress growth periods ranged from 16 to 27 days, and the half-life ($t_{1/2}$) of Fluoxetine HCl is 62 hours (unpublished data from West; rate constant = 0.0111 h^{-1} ; Figure 3.13), which is consistent with the findings of Lam *et al.*, (2005) ($t_{1/2}$ 55 hours; rate constant = 0.0126 h^{-1}). Over 27 days (648 hrs) the Fluoxetine HCl would therefore have been through more than 10 half-lives. Thus potentially, only 0.049 µg of the total Fluoxetine HCl added in the cress experiment would remain after 27 days if full photodegradation has occurred. This would easily account for the losses seen. However photoproducts identified by Lam *et al* ., (2005) were not found to be present within the cress samples studied herein indicating perhaps complete degradation to CO_2 and H_2O.

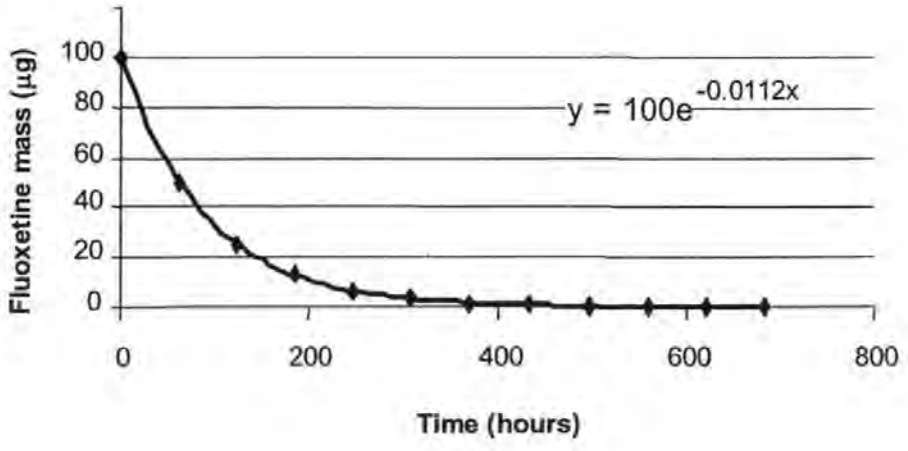


Figure 3.13. Exponential photodegradation of Fluoxetine HCl with time

Data Source: West, 2007; personal communication.

A window sill degradation study using a quartz tube containing Fluoxetine HCl (100 µg) in sterilised Milli-Q (35 mL) was carried out to confirm these losses. Samples indicated that after only 16 days of light exposure approximately 30% of the initial amount of Fluoxetine HCl added still remained. This equates to an approximate loss of 70% which helps to account for the Fluoxetine losses seen in the laboratory grown cress samples (~60 – 80% loss). Complete mineralization (either due to abiotic or biotic degradation), irreversible sorption or poor compound recovery using these methods may also play a role in the losses seen.

The use of two sources of plant tissue; laboratory grown cress and purchased Sainsbury's salad cress (containing three *Brassicas*; cress, rape and mustard) highlighted different recoveries from different plant types even though they were all *Brassicas*. For example, in the method stages 'improvement' and 'confirmation of IS recoveries' (Table 3.4) which used identical extraction methods but different plant tissue sources, IS recoveries differed by up to a factor of 10 between the Sainsbury's *Brassicas* (IS: stem 71 ± 4%; leaves 28 ± 3%) and the laboratory grown cress (IS: stem 7 ± 0.4%; leaves 11 ± 1%). It was this finding; that recoveries differed hugely depending upon the plant type used, that led to the closure of this section of method development work, as it was felt that pursuing the development of a method which eventually was to be used on a different Brassica (*viz* cauliflowers) may be futile.

3.5.2. Extraction from Cauliflower Tissue Cultures: Method Development

Method development work for the extraction of Fluoxetine from *Brassica* tissues including cress, rape and mustard highlighted the variability in IS recoveries depending upon the plant type used. For this reason it was felt important finally to use cauliflower tissues as target plant material in the development of a method for the extraction of Fluoxetine from cauliflower matrices.

Plant material used for this method development work consisted of cauliflower tissue cultures (T/Cs), grown and harvested for plant up-take studies as discussed in Chapter 5, Section 5.8.1. Due to the limited number of cauliflower T/Cs available, optimisation of extraction and analysis of Fluoxetine from T/Cs was limited to testing the developed soil method on single replicates. Generic extraction details, including SPE used during this method development work are presented in Figure 3.14. Flow diagram of generic experimental steps used during method development for the extraction of Fluoxetine from cauliflower matrices. Details of alterations to IS concentrations and MS operation modes are given in Table 3.5 along with recovery data and comments upon each method development stage.

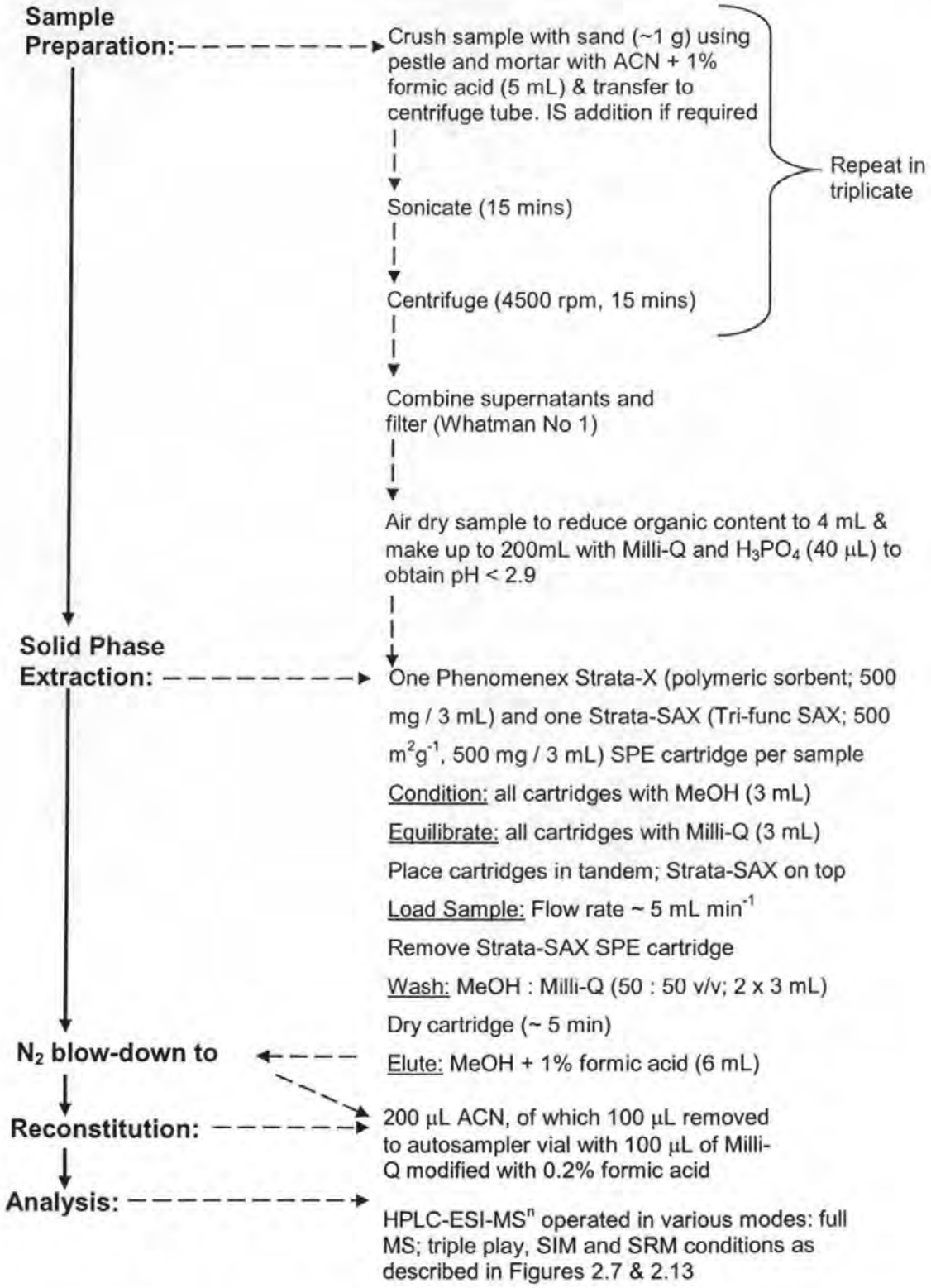


Figure 3.14. Flow diagram of generic experimental steps used during method development for the extraction of Fluoxetine from cauliflower matrices

For final working method: 2 µg IS used with MS operated in SIM and SRM modes

Method Development Stage	Sample Details	IS	MS analysis mode	Percentage Recovery					
				Media	Roots	Stems	Leaves	Curd	Total
Testing cress extraction method: non-exposed T/C sample	Cauliflower T/C; blank	IS (10 µg)	Full MS & triple play (includes MS^2)	(IS; 5)	IS; 5	IS; 5	IS; 4	IS; 4	n/a
Testing cress extraction method: exposed T/C sample	Cauliflower T/C; exposed	IS (10 µg)	Full MS & triple play (includes MS^2)	Fluoxetine; 44 (IS; 8)	(IS; 6)	(IS; 4)	(IS; 4)	(IS; 4)	44
Testing ion suppression due to IS	Cauliflower T/C; exposed	No IS	SIM & SRM	Fluoxetine; 10		Fluoxetine; 11			21
Optimisation of IS concentration	Cauliflower T/C; exposed	IS (2 µg)	SIM & SRM	Fluoxetine; 5 (IS; 77)	(IS; 16)	(IS; 40)	Fluoxetine; 7 (IS; 40)	(IS; 33)	12

Table 3.5. Sequential method development stages and results in the extraction of Fluoxetine from cauliflower tissue culture matrices

Fluoxetine recovery data are presented as % of originally added amount, equivalent IS (d_5-Fluoxetine HCl) data is presented in brackets but is a % of IS added to each individual sub-sampled tissue
Total refers to total % of Fluoxetine originally added that could be accounted for in the sub-samples
Only data for which Fluoxetine presence could be confirmed by acquiring known MS^2 transition are presented.

Table 3.5 continued...

Method Development Stage	Comments & conclusions
Testing cress extraction method: non-exposed T/C sample	IS recovery across all sub-sample types was found to be unacceptable (5 ± 0.7 %)
Testing cress extraction method: exposed T/C sample	Extracted ion chromatograms and full MS spectra indicated the presence of Fluoxetine (*m/z* 310) in the leaf sample, but due to low amounts of Fluoxetine within the sample this could not be confirmed by MS^2. Mean IS recovery was again found to be unacceptable (5 ± 1.7 %) although similar to recoveries achieved in the previous method development stage. This led to concerns that IS concentration may have been causing ion suppression.
Testing ion suppression due to IS	The use of no IS and changing the MS operation mode to SIM and SRM; which effectively increased S:N and hence lowered the limit of detection, allowed the presence and hence uptake of Fluoxetine in the stem sub-sample to be confirmed by MS^2 (*m/z* transitions: 310 to 147.9). 11% uptake of Fluoxetine to stem tissue was seen, with an overall loss of ~ 80% of originally added Fluoxetine (9.8 μg) which can be explained by potential photodegradation losses.
Optimisation of IS concentration	Substantial increases of IS recovery across all sub-sample types occurred with the use of less IS ($1/5^{th}$) in conjunction with use of SIM and SRM operating modes (IS recovery 16 - 77%), as opposed to full MS and triple play, (IS recovery 4 - 8%). More evidence to support the uptake of Fluoxetine was obtained with the confirmation of the presence of 7% of originally added Fluoxetine in the leaf sample.

Table 3.5. Sequential method development stages and results in the extraction of Fluoxetine from cauliflower tissue culture matrices

Fluoxetine recovery data are presented as % of originally added amount, equivalent IS (d_5-Fluoxetine HCl) data are presented in brackets but as % of IS added to each individual sub-sampled tissue. Total refers to total % of Fluoxetine originally added that could be accounted for in the sub-samples.
Only data for which Fluoxetine presence could be confirmed by acquiring known MS^2 transition is presented

3.5.3. Extraction from Cauliflower Tissue Cultures: Final Working Method

Initial testing of the developed extraction and tandem SPE method with cauliflower tissue cultures gave inadequate average IS recoveries of 5 % for all sub-sample types (Figure 3.15) and the presence of Fluoxetine in media samples only could be confirmed by MS^2. These exceptionally low recoveries led to concerns over potential ion suppression due to matrix effects and so further method development steps were then orientated towards finding an appropriate IS concentration alongside the use of SIM and SRM MS operating modes to lower the S:N and hence LOD. Alongside this an investigation into potential ion suppression or enhancement due to matrix effects and their impact upon the generation of quantitative data (Section 2.5.2) was carried out.

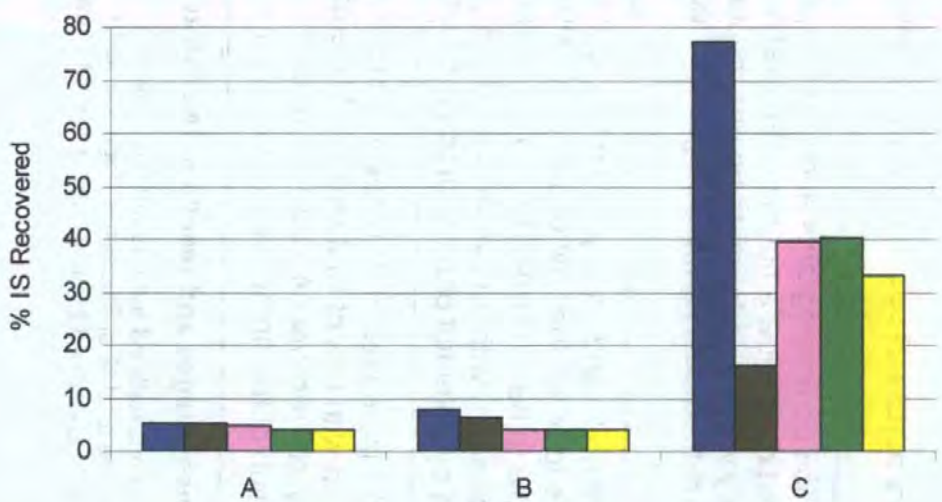


Figure **3.15. Internal standard (d_5-Fluoxetine HCl) recovery data from the extraction of Fluoxetine from cauliflower tissue cultures**

media roots stem leaves curd

A: Data from method development stage 'Testing cress extraction method: non-exposed T/C sample'
B: Data from method development stage 'Testing cress extraction method: exposed T/C sample'
C: Data from method development stage 'Optimisation of IS concentration'

The use of SIM and SRM operating modes in conjunction with a reduction in the IS concentration allowed for significantly more of the IS to be accounted for (16 – 77%) (Figure 3.15). Using these analysis modes it was also possible to generate MS^2 spectra for the leaf and stem sub-sample types and therefore confirm the presence and hence uptake of Fluoxetine. Example LC-MS results for the stem sample in which Fluoxetine was positively identified in presented in Figure 3.16.

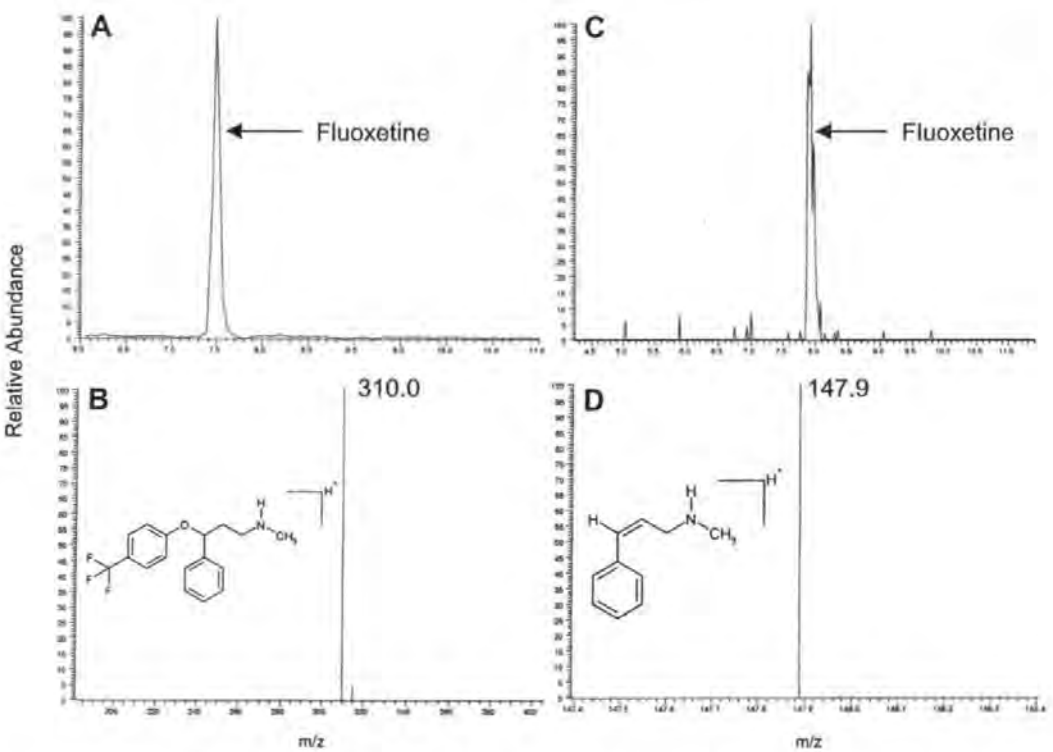


Figure 3.16. Example HPLC-ESI-MS^n data for the analysis of Fluoxetine extracted from cauliflower tissue culture matrices operated in SIM and SRM analysis modes

A: SIM TIC for *m/z* 310.0
B: SIM MS^2 spectra
C: SRM TIC for *m/z* 147.9
D: SRM MS^2 spectra
Data shown is from method development stage 'Optimisation of IS concentration', stem sub-sample

Although the extraction of Fluoxetine from cauliflower tissue cultures was not fully optimised as is indicated by IS recoveries (Figure 3.15) due to project time constraints further method development work had to be abandoned. It was felt

that sufficient improvements in IS recovery had been made with the use of more sensitive MS operating modes for this extraction method to be used in the preliminary cauliflower T/C plant uptake study.

3.5.4. Extraction from Cauliflower Tissue Cultures: Method Limitations

Method development for the extraction of Fluoxetine from cauliflower tissues had to be halted due to project time constraints. Further method development work would initially have involved testing the impact of the freeze drying samples prior to crushing, using MeOH as a component of the extraction solvent and the use of column chromatography for clean up, upon IS recoveries.

As the method stands, IS recoveries were found to be range from 16 – 40% for plant tissue sub-samples and 77% recovery was obtained for media. This method was deemed appropriate for the extraction and analysis of cauliflower T/C samples from a preliminary plant uptake experiment. However further use of this method would require further optimisation.

CHAPTER FOUR

BIODEGRADATION OF PPCPS IN SEWAGE SLUDGE-AMENDED SOIL

4. Biodegradation of PPCPs in Sewage Sludge-Amended Soil

4.1. Introduction

There are several degradation processes to which PPCPs may be exposed. These degradation processes can be broadly classified into two groups; chemical and biological degradation, (sometimes termed abiotic and biotic degradation respectively). Biotic degradation or biodegradation only will be considered herein as investigation of biodegradation was one of the major aims of the present research. A very recent review of into the abiotic degradation of pharmaceuticals, including a discussion of photodegradation, has been given by West (2007).

Biotic or biological degradation (including biotransformation whereby chemicals are incompletely degraded to carbon dioxide and water but rather are transformed to other intermediate organic chemicals) is caused by a range of organisms, of which microorganisms are the most important. Invertebrates, vertebrates and plants can nevertheless also play a role in degradation (Boucard *et al.*, 2005). The term 'microorganism' encompasses bacteria, fungi, archaea and actinomycetes and these are prevalent in most, if not all, compartments of the environment. Microbes are usually involved in at least one of three different biodegradation mechanisms; catabolism, co-metabolism and enzyme excretion (Ghosh and Philip, 2004). Catabolism is the term used when an organism utilises a compound as either a nutrient or energy source. Repeated exposure of an organism to a particular compound can result in acclimatisation of the organism, and hence result in efficient degradation; often referred to as enhanced microbial degradation and often encountered in bacterial catabolism (Berg and Nyholm, 1996). The susceptibility of a compound

to enhanced microbial degradation will depend on several factors. The toxicity of the compound to the microbes is of major importance, as is the ease of hydrolysis, and whether the hydrolysis products have a high nutritional value (Ghosh and Philip, 2004).

Co-metabolism is the term used when a compound is degraded, but not used as a sole carbon source i.e. a compound is degraded, but not for the purpose of growth, reproduction or dispersal.

Enzyme excretion into the soil is performed to enable substrate digestion. These enzymes can persist in the soil for significant periods, even beyond the organisms death; therefore providing soil with a biochemical catalytic ability e.g. phosphatases and amidases (Coats, 1991). Enzymatic hydrolysis or oxidation typically results in the formation of metabolites with higher polarities that the parent compound (Kreuzig *et al.*, 2003).

4.1.1. Environmental Biodegradation Fate Models

An alternative to experiments designed to measure the biodegradability of organic compounds, is the use computer-modelling programmes to estimate biodegradation using QSARs. One of these tools readily available is the Environmental Fate Database EPI Suite™ (www.epa.gov/opptintr/exposure/pubs/episuite.htm), which allows prediction of a range of parameters such as K_{ow}, BCF, K_{oc}, etc, as well as likely behaviour regarding biodegradation, based upon the structure of the compound in question. The EPI Suite™ is considered as a 'screening level tool and should not be used if representative measured values are available' (www.epa.gov/opptintr/exposure/pubs/episuite.htm). A detailed discussion of the operation of this model has been presented by Howard *et al.*, (1986) and Tunkel *et al.*, (2000) who also discuss the accuracy of MITI models.

Yu *et al.*, (2006) performed a study into pharmaceutical fate during waste water soil aquifer treatment. The use of reclaimed wastewater for irrigation is as high as 80% in some states in the US. They then compared their results to those generated by the Environmental Fate models. The Environmental Fate BIOWIN model was used to generate biodegradation predictions using the BIODEG linear and non-linear programmes, and the MITI linear and non-linear programmes. Aerobic batch biodegradation results showed that 13 of the target 18 PPCPs underwent more than 80% biotransformation in 50 days. Yu *et al.*, (2006) found that their experimental data correlated best with the non-linear BIODEG program, although inconsistencies could be found between the modelled and experimental data. Equivalent results generated by the biodegradation programs in EPI Suite™ from the Environmental Fate Database

model (www.epa.gov/opptintr/exposure/pubs/episuite.htm) for the pharmaceutical compounds studied herein are summarised in Table 4.1.

It is for reasons such as these that predicted data should be treated with some level of caution, as regards environmental fate. The results generated under experimental conditions can be highly variable. A number of these contributing factors such as temperature, adapted microbial population, additional nutrient sources, can be better controlled under laboratory conditions via careful experimental design. Reviewing literature on both pharmaceutical and pesticide biodegradation studies aided in the development of experimental procedures which would take into consideration a number of these factors.

	Linear model prediction	Non-linear model prediction	Ultimate biodegradation timeframe	Primary biodegradation timeframe	MITI linear model prediction	MITI non-linear model prediction	Anaerobic model prediction	Ready biodegradability prediction
Diazepam	biodegrades fast	biodegrades fast	weeks - months	days - weeks	does not biodegrade fast	does not biodegrade fast	does not biodegrade fast	No
Desmethyldiazepam	biodegrades fast	biodegrades fast	weeks - months	days - weeks	does not biodegrade fast	does not biodegrade fast	does not biodegrade fast	No
Oxazepam	biodegrades fast	biodegrades fast	weeks - months	days - weeks	does not biodegrade fast	does not biodegrade fast	does not biodegrade fast	No
Temazepam	biodegrades fast	biodegrades fast	weeks - months	days - weeks	does not biodegrade fast	does not biodegrade fast	does not biodegrade fast	No
Fluoxetine HCl	does not biodegrade fast	does not biodegrade fast	months	days - weeks	does not biodegrade fast	does not biodegrade fast	biodegrades fast	No
Norfluoxetine HCl	biodegrades fast	does not biodegrade fast	months	days - weeks	does not biodegrade fast	does not biodegrade fast	biodegrades fast	No

Table 4.1. Predicted biodegradation behaviour of pharmaceutical target compounds in present study using US EPA modelling suite (EPI Suite V3.20

4.1.2. Standardised Tests

Law requires the biodegradation behaviour of many potential environmental contaminants to be ascertained following standardised test guidelines. In the UK many of the tests used are from the OECD. The OECD holds a three tier testing system for target compounds; ready biodegradability, inherent biodegradability and activated sludge simulation test. The first two tests are considered as screening tests and the final test as a simulation (of behaviour in STP) type test. These tests are rigorous, and are considered by Nyholm (1996) to only allow the most readily degradable compounds to pass.

Other studies, such as the inter-laboratory comparison by the European Economic Community and OECD on standardised tests for aquatic environments (Nyholm *et al.*, 1984), have shown variable biodegradation results from supposedly 'standardised' tests. Nyholm, *et al.*, (1984) selected *p*-nitrophenol, which has previously been shown to give variable biodegradation results, as their target compound for the identification of the source of this variability. The Zahn-Wellens test (ZWT) and the modified OECD screening test (MOST) were selected as test models, as they differ greatly regarding both microbial biomass and target compound concentration.

ZWT is a sludge batch testing method that uses DOC or COD analysis to monitor biodegradation rates. The OECD classifies this as a test for 'inherent' biodegradability whereas MOST is classified as a test of whether a compound is 'readily' biodegradable, and is a shake flask die-away method. STW secondary effluent, surface water, soil or a composite of all three is used to obtain inoculum. Nyholm, *et al.*, (1984) also ran these tests with both adapted inocula

(i.e. pre-exposed to target compound) and non-adapted inocula. Their findings indicated that the variation in the degradation of *p*-nitrophenol occurred in the population growth lag phase, which showed differential biodegradation rates depending on whether or not adapted incocula had been used. They proposed than an additional classification; 'readily biodegradable after adaptation' should be considered.

Another commonly used standardised test by which a compound is classified as 'readily' biodegradable, is the closed bottle test (CBT). This was used in research into the biodegradation and toxicity of four antineoplastics in the aquatic environment carried out by Al-Ahmad and Kummerer (2001). For biodegradation studies they used both the closed bottle test (CBT) and the ZWT. The CBT uses low bacterial density and low compound concentrations, whereas ZWT uses high bacterial density and compound concentrations. The concentration of DO (dissolved O_2) was measured as a method of monitoring the biodegradation in the CBT and DOC was measured in the ZWT. None of the target compounds were found to biodegrade under either set of test conditions.

Gerike and Fischer (1981) compared the biodegradation of 44 compounds under a range of standardised tests; Coupled Units Test, ZWT, Japanese MITI test, French AFNOR T 90-302 test, carbon dioxide test according to Strum, OECD screening test, EPA activated sludge and CBT. Compounds which gave variable results under screening type tests (e.g. CBT), all underwent extensive degradation in the activated sludge test. The authors concluded that the AFNOR T 90-302 was an appropriate test, but only for compounds that did not express inhibitory or toxic properties whereas the EPA activated sludge test is

highly resistant to toxic or inhibitory effects, but therefore tends to overestimate biodegradation which is likely to occur in the environment. Three tests of those selected were considered to provide environmentally relevant results; MITI test, CBT and modified OECD test.

Purely due to the number of PPCPs in use, it would not be possible to assess each individual compound for environmental fate due to biodegradation. It is for this reason that Ingerslev and Halling-Sorensen (2000) chose to research a group of structurally related drugs, the sulfonamides, which have a range of therapeutic uses including as diuretics, tuberculostatics and for oral hypoglyemic medication. None of the twelve sulfonamides were classifiable as readily biodegradable, as none underwent degradation in the screening test. However, in the activated sludge simulation test using non-adapted sludge, lag phases of 7 to 10 days were seen at 20°C, after which biodegradation occurred. However, lag phases and degradation rates were 3 to 4 times longer at 6°C, highlighting the importance of environmental conditions within biodegradation studies. Interestingly, adapted bacterial cultures were able to degrade the same compounds (mix of four), or any of the other combinations of four sulfonamides, rapidly and consistently ($t_{1/2}$ = 0.2 to 3 days). Therefore these compounds were classified as 'inherently' biodegradable under a simulation test, as opposed to a screening test.

Nyholm *et al.*, (1984) also found differential biodegradation trends depending upon whether pre-adapted inocula were used. Other potential variables they also took into account included test duration and target compound concentration. They concluded that more consistent results could be obtained

by using higher inoculum concentration and a pre-adapted inoculum (e.g. Sturm test and CBT). Both inoculum and compound concentrations had an impact on the lag phases seen; lag phase increased with increasing compound concentration and decreased with increasing inoculum concentration. A range of inoculum sources was also tested, and unsurprisingly had an impact upon degradation rates.

Research into the biodegradation and toxicity of four antineoplastics in the aquatic environment carried out by Al-Ahmad and Kummerer (2001) also found variability in biodegradation results regarding the bacterial density used. For biodegradation studies they used both the closed bottle test (CBT) and the Zahn-Wellens test (ZWT). They stressed that it is important in biodegradation studies to first ensure that the target compounds are not toxic to, or have an inhibitory effects upon, the bacteria themselves. For this Al-Ahmad and Kummer used a growth inhibition test with *Pseudomonas putida* ATCC 50026. A series of concentrations of the target compounds was tested and the biomass of the *P. putida* was recorded both before and after exposure by measuring the optical density (at 436 nm) and protein concentration.

4.1.3. Simulation Tests

Many of the standard screening tests have been criticised for their lack of environmental relevancy. Simulation tests are seen as a better model for the environment. Despite this there have still been criticisms upon standardised simulation tests such as the OECD activated sludge biodegradability test. This test is criticised over the guideline dosages to be used (20 – 40 mg L^{-1}), which are significantly higher than those likely to occur in a STW. Bergand and Nyholm (1996) tested the impact of dosage upon both the degradation kinetics and adaptation behaviour by using low (10 µg) and high (10 mg) dose semi-continuous reactors. Results indicated substantial differences in biodegradation trends between the two concentrations used.

Working with soil in biodegradation studies can add additional problems, such as sequestration, also known as ageing, which refers to the decreased bioavailability of a compound in soil with time. Pesticide data have generally shown a decline in the rate of compound degradation in soil as it ages. The extent of sequestration is dependent upon a range of factors including soil type and the target compound itself. Nam and Alexander (2001) have shown through a phenanthrene biodegradation study, that biodegradative activity and incubation temperature had an impact upon biodegradation trends. Soils with high levels of total bacterial activity expressed rapid biodegradation of phenanthrene, whereas in those with low activity expressed slow initial biodegradation and therefore higher levels of compound sequestration occurred. Initial biodegradation rates are one of the factors that determine the extent to which a compound will undergo sequestration in soil.

The importance of using consortia in biodegradation studies, as opposed to pure cultures, was highlighted by Van Ginkel, (1996) in a study on the biodegradation of surfactants in sludge, during STW processes. Total biodegradation of surfactants was only achieved through the use of consortia, as initially bacteria degraded the alkyl chain and the remaining hydrophilic moiety was degraded by other bacteria. In the natural environment and in STW, complete biodegradation is dependent upon a mixed microbial population. Incomplete degradation can lead to the formation of potentially toxic intermediates, which in a pure culture biodegradation study may impact upon the population present. Aside from this, pure cultures are arguably not environmentally realistic.

Ghosh and Philip (2004) also presented evidence for increased atrazine biodegradation rates (1^{st} order) when a mixed culture was used. Variability in the extent of atrazine biodegradation and rate has in the past also been attributed to variations in nitrogen sources, additional carbon sources, C:N ratio, moisture level and pH.

Ingerslev *et al.*, (2000), investigating the rationale behind the use of pure cultures and while appreciating the need to minimise variation in experimental procedures, reported that these single culture procedures lack environmental relevance and cannot therefore be used to predict environmental behaviour of a chemical. When inocula sourced from the natural environment (e.g. STW, soil) are used in biodegradation studies, results tend to be variable. It is thought that this difference may be due to the variation in biomass used for inoculation (i.e. microbial density). Ingerslev *et al.*, (2000) tested the impact of test volume (i.e.

culture volume), used in shake flask experiments on the lag phase, in experiments on 2,4-dichlorophenoxyacetic acid (2,4-D) and *p*-nitrophenol. They found decreased lag times when the test volume was increased, and they also noted that when volumes were below 10 mL, biodegradation appeared to fail randomly. They hypothesised that not only must the concentration of relevant microorganisms present be high enough for *initial* biodegradation to occur, but the total biomass must also be sufficient. Smaller test volumes that do not allow this biomass to be achieved, therefore showed random occurrences of no biodegradation occurring.

Another factor to consider in experimental design is test length. Many authors, such as Fenyvesi *et al.*, (2005) in their study into biodegradation of cyclodextrins as bioremediation additives in hydrocarbon contaminated soil, use reference material for test validation. They selected cellulose as their reference material, which determines the test validity once 60% degradation is achieved (at the plateau phase or by the end of the test). By day 178 in their experiment, cellulose had been totally degraded. However they chose to extend their experiment to 280 days, and achieved total degradation of all cyclodextrins tested. This included randomly methylated β-cyclodextrin, which has previously been shown to be non-biodegradable using standardised tests with uncontaminated soil. The difference as to whether random methylated β-cyclodextrin undergoes biodegradation is dependent upon previous contamination to the soil, and hence microbiological adaptation to the target compounds.

Some PPCP research has also included veterinary pharmaceuticals. Ingerslev *et al.*, (2001) studied the veterinary antibiotics Olaquindox, Metronidazole, Tylosin and Oxytetracycline, and performed both aerobic and anaerobic shake flask biodegradation experiments. Olaquindox and oxytetracycline exhibited no lag phase prior to degradation, with half lives of 4 – 8 days and 42 – 46 days respectively. Metonidazole had a lag phase of 2 to 34 days, and a half life of 14 – 104 days; tylosin had respective timings of 31 to 40 days and 9.5 – 40 days. In the absence of oxygen the biodegradation trend was significantly slower. These data again highlight the variability that can be seen in data generated using inocula sourced from the natural environment.

Kummer *et al.*, (1997) assessed the biodegradation potential of ifosafamide, an anti-tumour agent, using a screening test (ZWT) and a simulation test (biological sewage treatment). Ifosafamide did not undergo biodegradation in either test, but the authors indicated the importance of also studying the metabolites for complete risk assessment to be achieved. Alongside this the abiotic fate of ifosafamide must also be determined before definitive conclusions can be drawn regarding environmental impact. Since then Kummer *et al.*, (2000) have continued their research into antineoplastics. Research has focused around isophosphoramidmustard, which is the active metabolite of both cyclophosphamide and ifosafamide. Isophosphoramidmustard is too reactive for medicinal use, but has now been synthesised as β-D-glycosylisophosphoramidmustard (active against tumours) and β-L-glycosylisophosphoramidmustard (has no antineoplastic effect). β-D-glycosylisophosphoramidmustard was found to be inherently biodegradable in contrast to most other antineoplastics researched. It is important to note that

only one of the enantiomers was biodegraded, and that not only does stereochemistry play an important role in therapeutic capabilities, but also in biodegradation.

In summary, there are a range of tools available to scientists to assess whether a potential target compound is biodegradable, including modelling tools and standardised screening tests. However, to better simulate environmental conditions a huge range of potential variables have to be taken into consideration and incorporated into a simulation type test. For this reason herein it was decided that a simple shake flask screening type test would be performed on all target compounds, alongside a simulated agricultural field soil test on one target compound. As there are such a large number of factors that could be varied within these biodegradation experiments, the choice was made to keep experimental design as close to true environmental conditions (i.e. moisture content, temperature, additional nutrient sources) for the target geographical area: SW England, while giving consideration to optimum conditions for biodegradation such as the use of specific pre-adapted inocula.

4.2. Aims

The aims of this study were to ascertain whether Fluoxetine, Diazepam and their major human metabolites Norfluoxetine, Oxazepam and Temazepam were susceptible to biodegradation in a simple shake flask screening type test. A simulated agricultural field soil test on Fluoxetine HCl was also to be conducted under as environmentally relevant conditions as possible. If biodegradation occurred and metabolites were formed, quantitative and qualitative data on both metabolites and the parent compounds would be obtained, therefore allowing calculation of biodegradation rates and metabolite formation rates. Qualitative data on the bacterial population present in this specific environment were also required.

As the target compounds are pharmaceuticals they are designed to be stable so that they are able to perform their function within the body, and hence they have potential to be persistent within the environment. At the commencement of this project (2003) there was no literature regarding Fluoxetine or Norfluoxetine degradation under environmentally relevant conditions. However evidence for the persistence of Fluoxetine in the environment had been found in streams and STW effluent and concerns about the presence of Fluoxetine in the environment have been expressed (Webb, 2000; Brooks *et al.*, 2003; Kolpin *et al.*, 2002; Metcalfe *et al.*, 2003). In the case of the 1,4-benzodiazepines, the hydrolysis products for Diazepam and Oxazepam were known from early studies (Han *et al.*, 1977), and fungal degradation of Diazepam was demonstrated over 30 years ago (Ambrus *et al.*, 1975). The presence of Diazepam in rivers, STW, lakes and drinking water, had nevertheless only been reported more recently (Richardson and Bowron, 1985; Halling-Sorensen *et al.*, 1998; Ternes *et al.*,

2001; Zuccato *et al.*, 2000; Stuer-Lauridsen *et al.*, 2000; Snyder *et al.*, 2001). There appeared to be no literature available on the biodegradation of these compounds under environmentally relevant conditions. *Computer-modelled* data (Table 4.1) gave variable results for each compound, ranging from qualitative descriptions such as 'biodegrades fast' to a time frame of months. *Due to their presence in certain environmental* compartments, it was hypothesised that the compounds would be resistant to total biodegradation and complete mineralisation, although they may undergo partial degradation (biotransformation) to intermediates. It was also possible that some losses might be seen due to abiotic factors (e.g. hydrolysis; (Han *et al.*, 1977).

4.3. Soil

4.3.1. Soil Source and Excavation

Soil was sourced from a field that had been treated with known quantities (Table 4.2) of sewage sludge (total ~ 400 m^3 SS) for two years in East Cornwall (grid reference withheld; South West Water farm code 2138, field code O896).

This field was selected as a typical agricultural field to which sewage sludge has been and is, applied. The soil was classified as a well drained coarse loamy soil (type 541B as classified by Lawes Agricultural Trust Soil Survey). Such slate and shale type soils (i.e. Denbeigh series), are the most common soil types to which sewage sludge is applied within this geographical area (ADAS 2004 personal communication; Millns, 2004).

When SS is disposed of to agricultural land it is the responsibility of the local water company to ensure that the receiving land is suitable as regards its soil chemistry. All soils used in this project were sampled from the same site, to which the safe disposal of SS is regulated by SWW. Soil characterisation results from SWW can be seen in Table 4.2.

Parameter measured	Result
Soil Density (by weight)	0.80 kg L^{-1}
pH (electrode)	7.4
N total (combustion)	3100 mg kg^{-1}
P (ICP-OES)	1430 mg kg^{-1}
Cu (ICP-OES)	23 mg kg^{-1}
Zn (ICP-OES)	78 mg kg^{-1}
Pb (ICP-OES)	41 mg kg^{-1}
Cd (ICP-OES)	0.14 mg kg^{-1}
Cr (ICP-OES)	40 mg kg^{-1}
Ni (ICP-OES)	24 mg kg^{-1}
Mo (ICP-OES)	<0.5 mg kg^{-1}
As (ICP-OES)	31 mg kg^{-1}
Se (ICP-OES)	1.1 mg kg^{-1}
Hg (ICP-OES)	0.13 mg kg^{-1}

Table 4.2. Soil characterisation results generated by South West Water plc

Sampling date: 23rd May 2002 (final SWW soil characterisation sampling time point, prior to soil sampling for this experimental work at 4pm).
Sampling point: Location witheld, Cornwall, UK – parcel 0896 (F2138-0896)
Biosolid type: Limed SS cake
Biosolid amount applied to working area (12.356 acres): 220 m^3 on 21.09.2003, 191 m^3 on 29.09.04.
Note: units above refer to soil dry weight

Two tubs (acid-washed with 10% nitric acid; polypropylene bases; linear low density polyethylene lids (Whitefurze)) of soil were sampled from the A-horizon (top 20cm) using a spade (Decon washed). Lids were left loose on live samples to allow airflow, but tightly closed on samples to be sterilised. Within the field a bund of lime treated sewage sludge stored prior to application provided two tubs of sewage sludge sampled as described above. Lids were left tightly closed for health and safety reasons.

Once back at the laboratory all soil samples were weighed and homogenised. This weight was taken as the field capacity moisture content to which the samples were maintained (by the addition of sterile artificial rainwater (0.01 M $CaCl_2$ (analysis grade >99%; Fisher Scientific) in Milli-Q, autoclaved at 125°C

for 40 mins) until the experiment began. One tub of soil and one tub of sewage sludge were then sent for sterilisation by γ-irradiation (25kGy, ^{60}Co irradiation source) at Becton Dickenson & Co, Roborough, Plymouth, Devon.

Both the sterile γ-irradated (500g) and live soils (4kg) were then sieved (4.75 mm) to remove large stones, macrofauna and microfauna. All samples were then stored in a cold room (7 °C) in the absence of light as a pre-incubation period of 2 to 28 days is recommended to allow equilibrium of microbial metabolism and to allow any seeds to germinate and hence be removed (OECD, 2002a).

4.4. Liquid Culture Biodegradation Experiments

4.4.1. Enrichment Cultures

An initial culture procedure was carried out to generate enriched cultures for use in the full scale experiment and as a means to check extraction and analysis efficacy of added ISs.

4.4.1.1. Experimental Procedure

Soil was used to generate enhanced cultures (sourced from the same site as the afore mentioned experiment; Table 4.2) by growing bacteria (dark conditions, shaking incubator, 27°C, 30 days) on individual target compounds (Fluoxetine HCl, Norfluoxetine HCl, Diazepam, Temazepam and Oxazepam; 2 µg) in minimal salts media (MSM; 100 mL; Appendix, Figure A.18). Cultures were then sub-sampled (1 mL culture in 100 mL MSM, with 2 µg target compound, 25°C) to create enriched cultures. All work was performed using aseptic techniques.

4.4.1.2. Sample Preparation

Sub-samples (30 mL) of cultures were taken on days 0 and 30. These sub-samples were transferred to sterile centrifuge tubes and the appropriate IS added (1µg, d_5-Fluoxetine HCl or d_5-Oxazepam). Samples were stored at -20°C until extraction. The remaining 20 mL of culture was returned to the incubator for subculturing.

Samples were rapidly defrosted by placing in warm water and centrifuged (4500 rpm, 5 minutes). All samples were then subjected to the developed SPE method and reconstitution processes described in Section 3.3.3 (Figure 3.4).

4.4.1.3. Analysis

HPLC-ESI-MS was used for the analysis of ISs in the enrichment culture samples. External linear calibrations allowed the quantification of IS (d_5-Fluoxetine HCl and d_5-Oxazepam) concentrations, and hence calculation of extraction efficiencies. Conditions and parameters used for LC-MS are given in detail in Section 2.4.2.

4.4.1.4. Results and Discussion

Single replicates from the start (day 0) and the end (~ day 30) of the growth period for each target compound were extracted an analysed. Extraction efficiencies were then calculated based upon the IS recoveries. As two different IS were used depending upon the target compound in question, the data in Table 4.3 are presented for the respective IS.

For d_5-Fluoxetine and d_5-Oxazepam the mean extraction efficiencies obtained were 70.4% (n = 4) and 91.0% (n = 8) respectively, with relative standard deviations of 41% and 20%. Extraction efficiencies were rather variable for d_5-Fluoxetine but somewhat better for d_5-Oxazepam. It is likely that these lower recoveries for the day 30 samples were due to the fact that samples from day 30 contained more residual soil than those from day 0, due to the sub-sampling process i.e. more soil was present within this samples for the IS to sorb to; hence the lower recoveries. Despite the variability in extraction efficiencies these experiments provided enriched cultures for use in the full scale study.

	Enrichment Culture Number	Sampling Day	IS Extraction Efficiency (%)
d_5-Fluoxetine HCl	1	0	72.9
		29	45.1
	2	0	110.2
		31	53.5
	Mean		70.4
	Standard Deviation		28.9
	% RSD		41.1
d_5-Oxazepam	3	0	100.4
		30	98.9
	4	0	92.7
		30	90.0
	5	0	110.2
		30	102.5
	6	0	82.3
		31	50.8
	Mean		91.0
	Standard Deviation		18.3
	% RSD		20.1

Table 4.3. Internal standard recoveries in liquid culture biodegradation study enrichment cultures

d_5-Fluoxetine HCl was used as IS for SSRIs
d_5-Oxazepam was used as IS for 1,4-benzodiazepines

4.4.2. Full Scale Liquid Culture Biodegradation Study

The preceding enrichment culturing generated environmentally relevant pre-adapted inocula for all six target compounds (Fluoxetine, Norfluoxetine, Diazepam, Temazepam, Oxazepam and Nordiazepam) and provided data for the efficacy of the designed extraction and analysis methods. Unfortunately there were supply problems with one of the target compounds, Nordiazepam, and therefore it had to be excluded from this full scale biodegradation experiment.

4.4.2.1. Experimental Procedure

For the full scale study the appropriate target compound (5µg), along with the corresponding enrichment culture (1 mL) was added to sterilised MSM (25 mL; autoclaved at 121°C, 15 minutes) in culture flasks. Blanks (containing no target compound) and abiotic sterile controls containing no culture were also prepared for each experiment. Caps with Teflon septa were used to seal the flasks which were placed at 25 °C in the dark. Samples and blanks were sacrificed in triplicate on days 0, 35, 45 and 60. Abiotic controls were sampled in triplicate on days 0 and 60. Appropriate IS (2.5µg; d_5-Fluoxetine or d_5-Oxazepam) was added to each flask before being frozen at -20°C.

4.4.2.2. Sample Preparation

Samples were prepared, extracted and reconstituted in the same manner as for the enrichment cultures (Section 4.4.1.2) except that whole samples were sacrificed as opposed to sub-sampling. Samples were reconstituted just prior to analysis as in Figure 3.4.

4.4.2.3. Analysis

HPLC-ESI(+)-MS^n was used for determination of the concentration of the target compounds versus known concentration of ISs. Full MS analysis was performed on all samples for the generation of quantitative data. DDMS, which involves MS^2 fragmentation, was then performed on a selection of samples, controls and blanks, for the confirmation of peak identity, *via* the generation of daughter ions (*m/z* transitions: Fluoxetine 310.0 to 147.9; Norfluoxetine 296.0 to 133.9; Diazepam 285.2 to 257.2; Temazepam 301.1 to 283.0; Oxazepam 287.2 to 269.0; d_5-Fluoxetine 315.0 to 152.9; d_5-Oxazepam 292.2 to 274.0). Ratio based calibration (Section 2.5.1) was used to generate quantitative data. Complete details of analytical conditions can be found in Sections 2.5 and 2.6.

4.4.2.4. Results and Discussion

The use of HPLC-ESI-MS^n for analysis allowed identification of all target compounds and ISs based upon *m/z* transitions and Rt, along with the generation of quantitative data.

Figures 4.1 to 4.5 show example extracted ion chromatograms from four sampling timepoints, full MS spectra and MS^2 spectra for each target compound, along with the extracted ion chromatogram and full MS spectra of the internal standard.

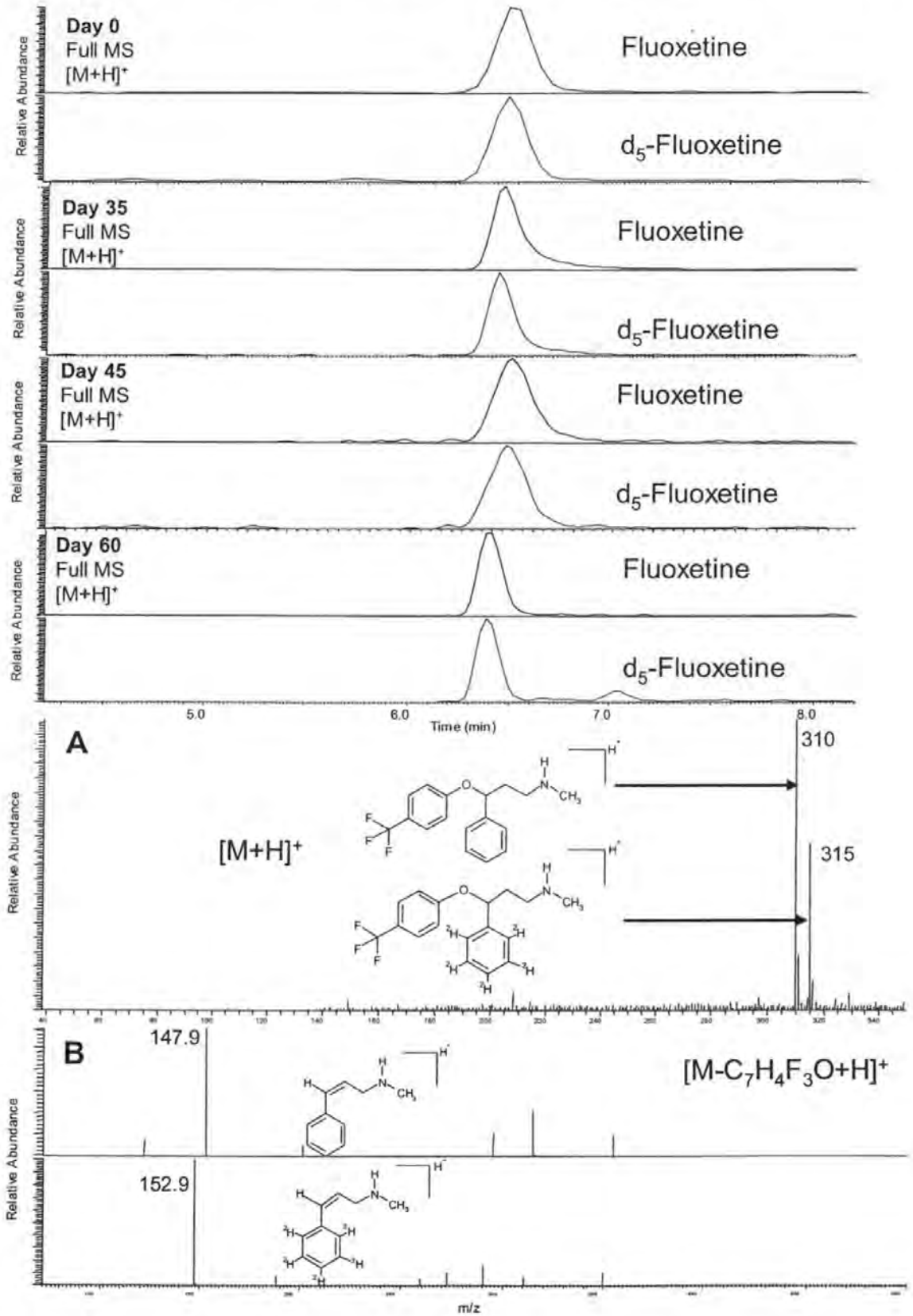


Figure 4.1. HPLC-ESI-MS^n extracted ion chromatograms and spectra for Fluoxetine and d_5-Fluoxetine in full scale liquid culture biodegradation study

Extracted ion chromatograms for Fluoxetine (*m/z* 310) and d_5-Fluoxetine (*m/z* 315) shown are from individual sample replicates from four sampling time points (day 0, 35, 45 and 60). HPLC and MS conditions as developed in Sections 2.4 and 2.5.
A. Full mass spectra of Fluoxetine and IS.
B. MS^2 spectra generated by DDMS analysis showing known fragments for Fluoxetine and IS.

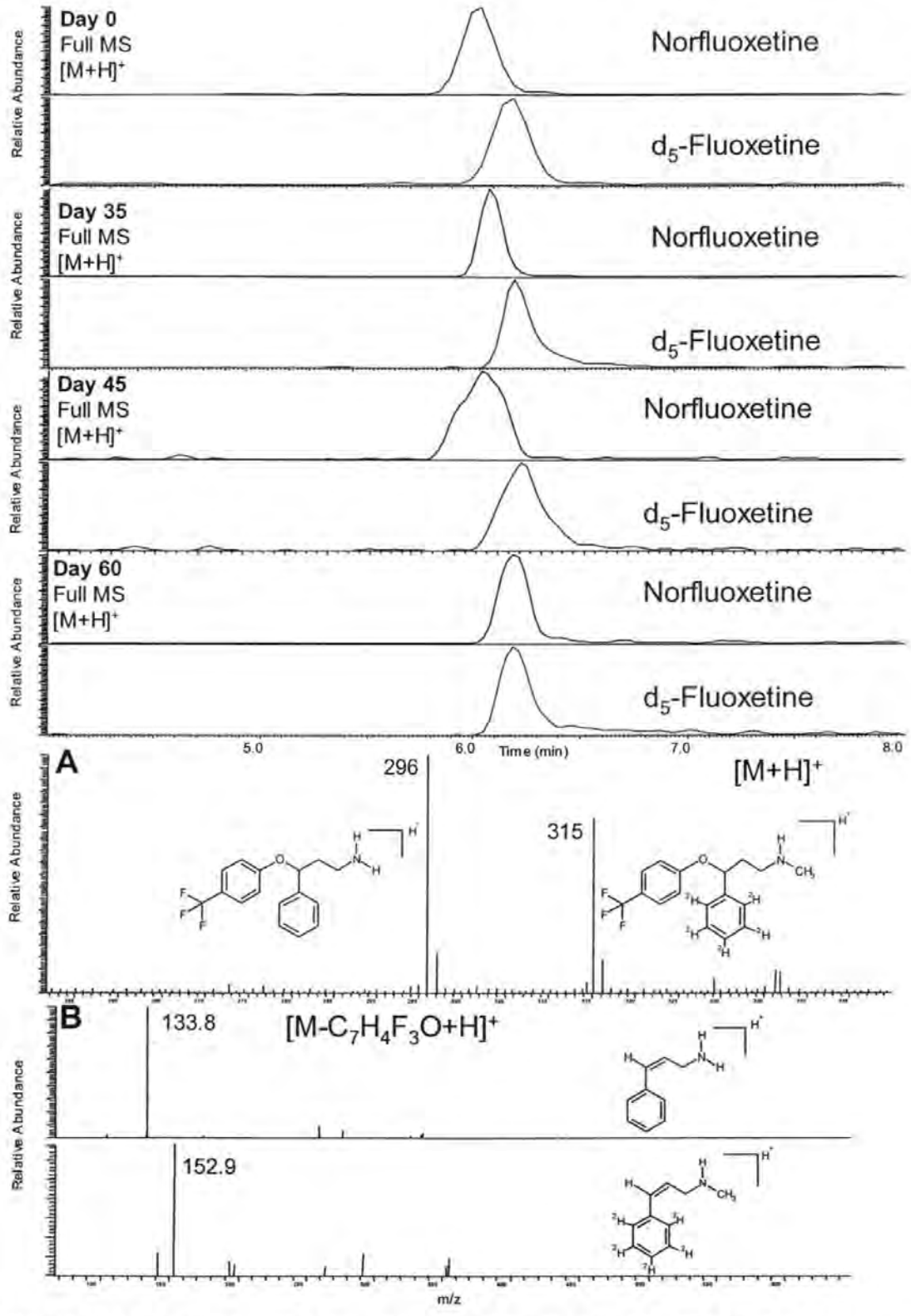


Figure 4. 2. HPLC-ESI-MS^n extracted ion chromatograms and spectra for Norfluoxetine and d_5-Fluoxetine in full scale liquid culture biodegradation study

Extracted ion chromatograms for Norfluoxetine (*m/z* 296) and d_5-Fluoxetine (*m/z* 315) shown are from individual sample replicates from four sampling time points (day 0, 35, 45 and 60). HPLC and MS conditions as developed in Sections 2.4 and 2.5.
A. Full mass spectra of Norfluoxetine and IS.
B. MS^2 spectra generated by DDMS analysis shows known fragments for Norfluoxetine and IS.

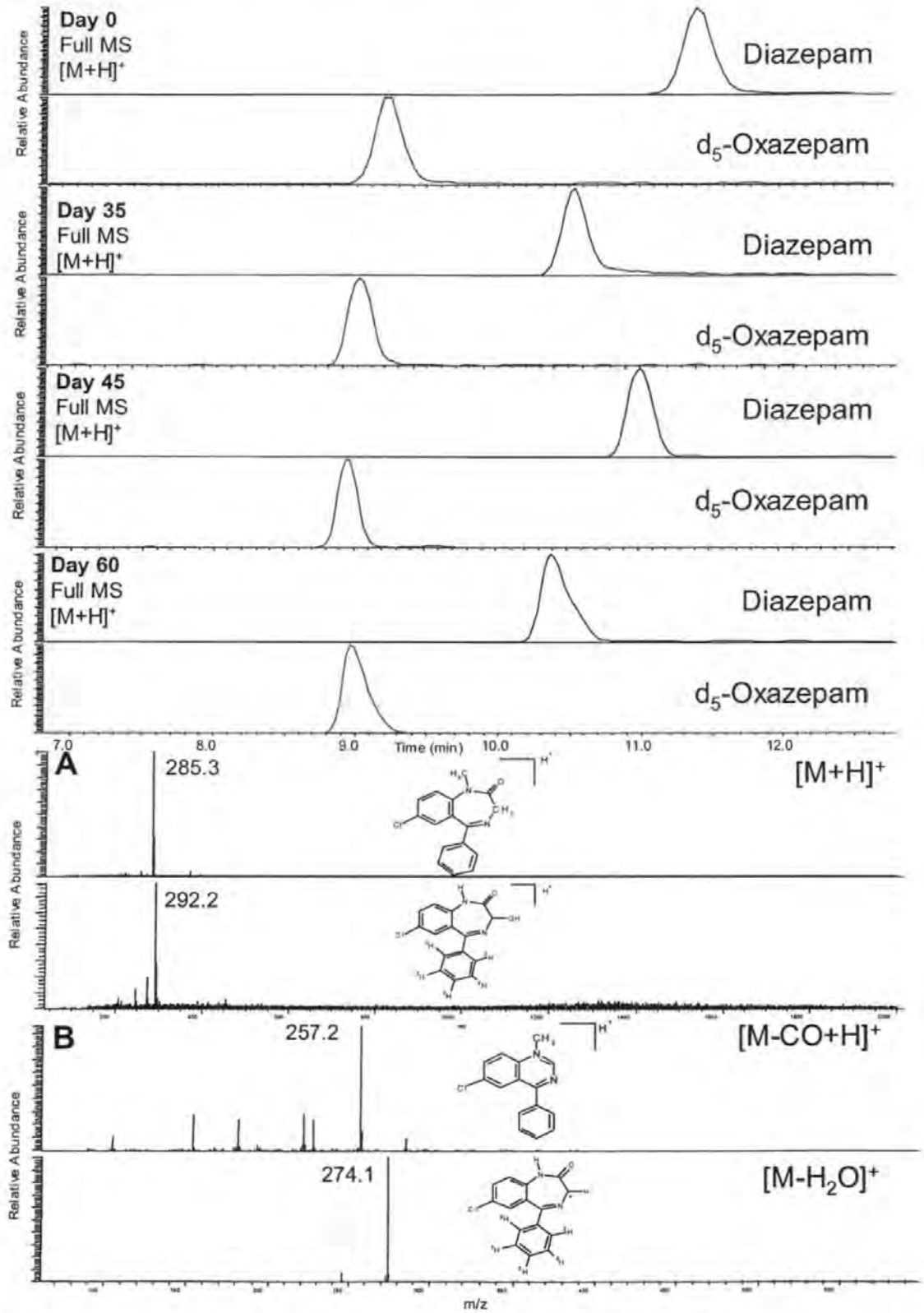


Figure 4. 3. HPLC-ESI-MS^n extracted ion chromatograms and spectra for Diazepam and d_5-Oxazepam in full scale liquid culture biodegradation study

Extracted ion chromatograms for Diazepam (*m/z* 285) and d_5-Oxazepam (*m/z* 292) shown are from individual sample replicates from four sampling time points (day 0, 35, 45 and 60). HPLC and MS conditions as developed in Sections 2.4 and 2.5.

A. Full mass spectra of Diazepam and IS.

B. MS^2 spectra generated by DDMS analysis shows known fragments for Diazepam and IS.

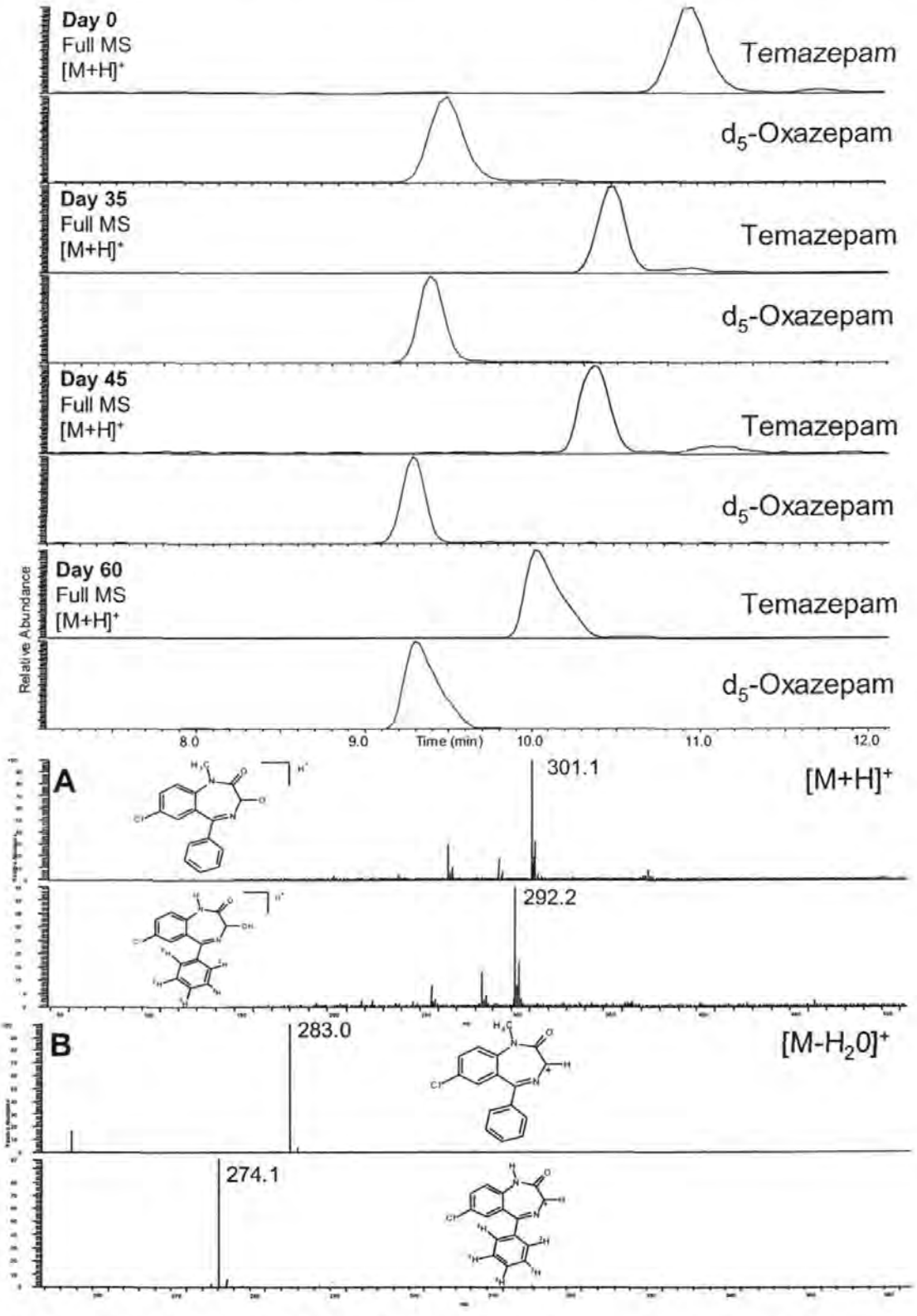


Figure 4. 4. HPLC-ESI-MS^n extracted ion chromatograms and spectra for Temazepam and d_5-Oxazepam in full scale liquid culture biodegradation study

Extracted ion chromatograms for Temazepam (*m/z* 301) and d_5-Oxazepam (*m/z* 292) shown are from individual sample replicates from four sampling time points (day 0, 35, 45 and 60). HPLC and MS conditions as developed in Sections 2.4 and 2.5.
A. Full mass spectra of Temazepam and IS.
B. MS^2 spectra generated by DDMS analysis shows known fragments for Temazepam and IS.

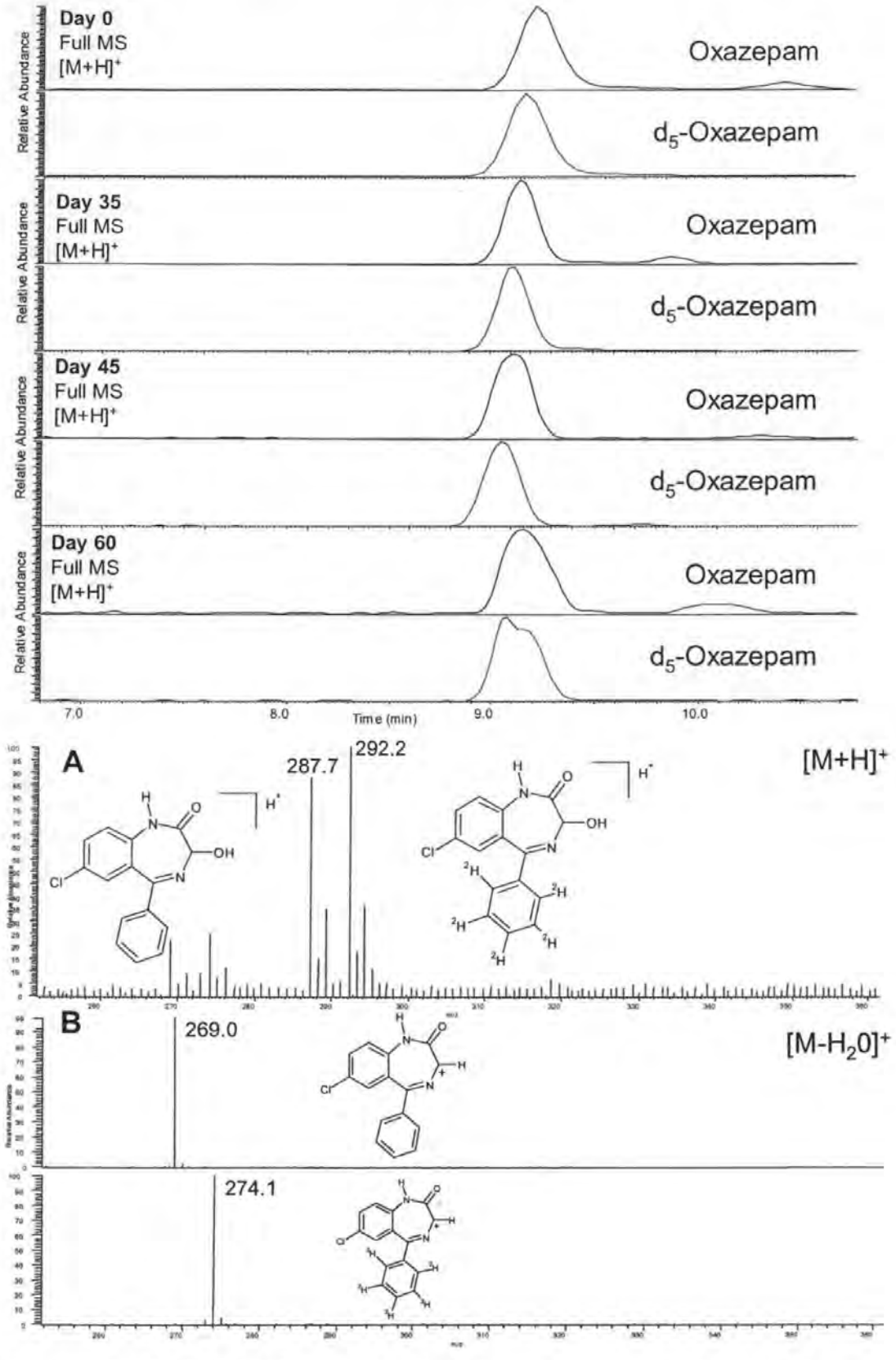


Figure 4. 5. HPLC-ESI-MS^n extracted ion chromatograms and spectra for Oxazepam and d_5-Oxazepam in full scale liquid culture biodegradation study

Extracted ion chromatograms for Oxazepam (*m/z* 287) and d_5-Oxazepam (*m/z* 292) shown are from individual sample replicates from four sampling time points (day 0, 35, 45 and 60). HPLC and MS conditions as developed in Sections 2.4 and 2.5.

A. Full mass spectra of Oxazepam and IS.

B. MS^2 spectra generated by DDMS analysis shows known fragments for Oxazepam and IS.

Extracted peak areas for relevant $[M+H]^+$ ions for each PPCP were integrated and these data used in conjunction with ratio calibration data to calculate the concentrations of target compounds. These data were then manipulated to obtain normalised % recovery of the target compounds. Data for each target compound were normalised against the mean day 0 percentage recovery for the corresponding sample or control. It was necessary to normalise the data in this way to account for differences in starting concentrations of the controls and samples due to the sub-culturing process involved during inoculum introduction. The normalised % recovery data were used as input for statistical analysis. DDMS spectra were obtained to confirm the identity of each compound by comparison of fragmentation pathways and Rt with those of the known analytes.

Figures 4.6 to 4.10 show the normalised percentage recovery of PPCPs at the specified sampling points (day 0 – 60) from both sample and control flasks. None of the blank samples contained the target compounds (as expected). Sample and control data points from the start (day 0) and end (day 60) of the study were subjected to statistical analysis; F-tests were first performed so that the appropriate t-test, assuming either equal or unequal variance could be selected. All statistical analyses were performed at the 95% confidence interval. P-values and their significance were generated by statistical analysis for all target compounds, and these results are presented in the Appendix (Table A.9).

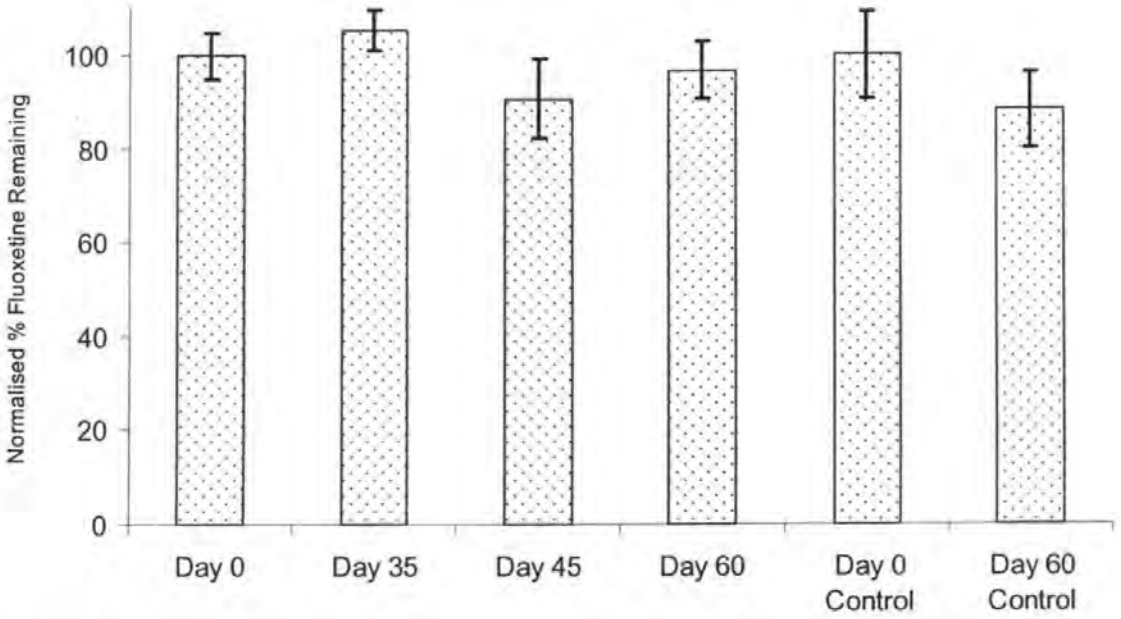


Figure 4. 6. Percentage of Fluoxetine remaining in full scale liquid culture biodegradation study over 60 days

Error bars ± 1 standard deviation. n = 3.

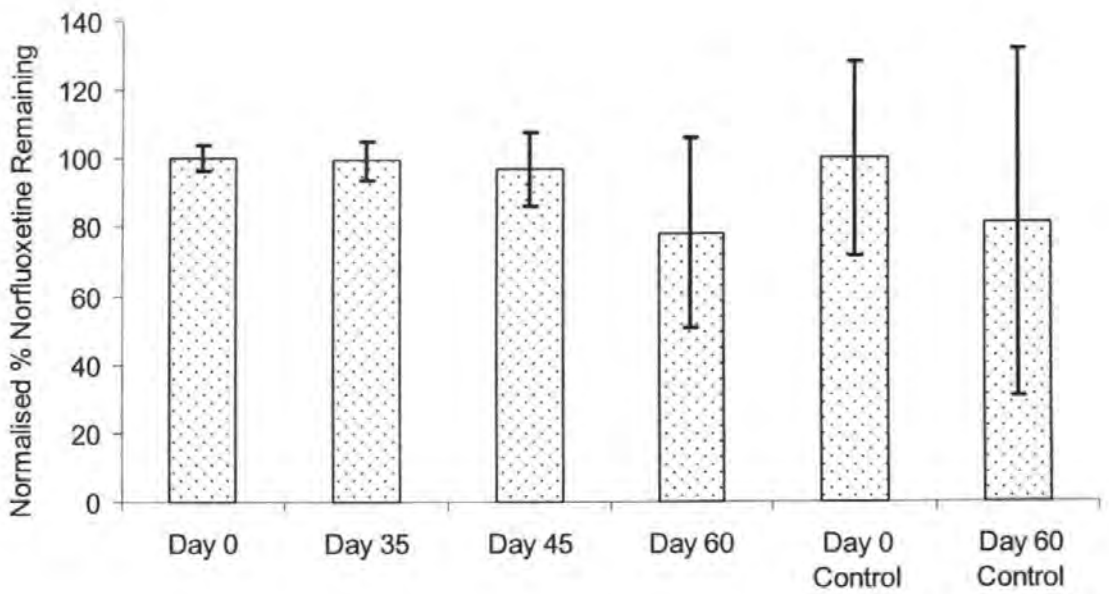


Figure 4. 7. Percentage of Norfluoxetine remaining in full scale liquid culture biodegradation study over 60 days

Error bars ± 1 standard deviation. n = 3.

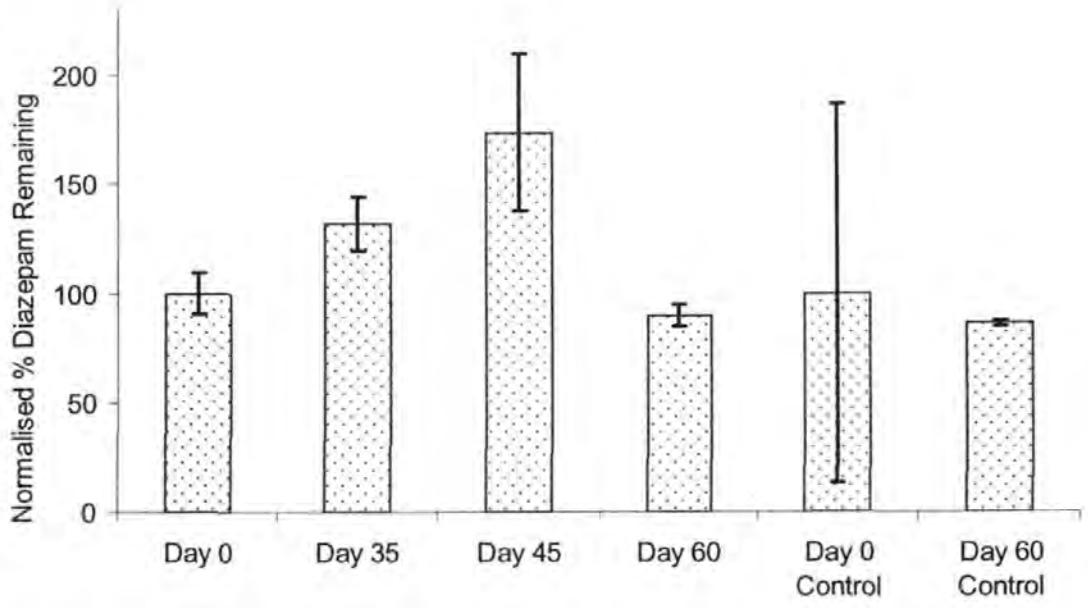


Figure 4. 8. Percentage of Diazepam remaining in full scale liquid culture biodegradation study over 60 days

Error bars ± 1 standard deviation. n =3.

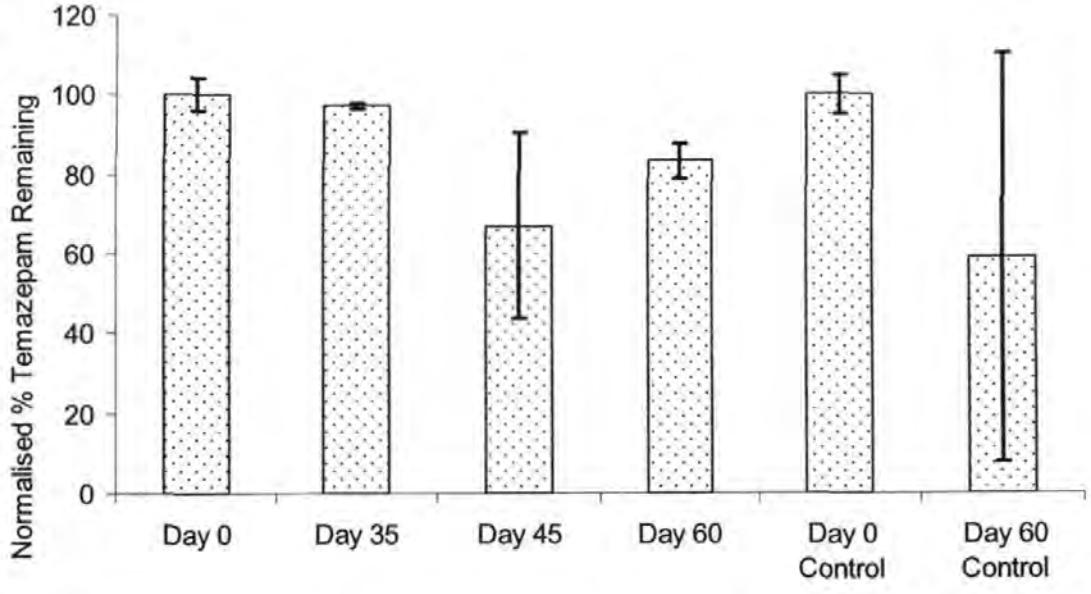


Figure 4. 9. Percentage of Temazepam remaining in full scale liquid culture biodegradation study over 60 days

Error bars ± 1 standard deviation. n =3.

As is apparent from the data represented in Figure 4. 6 and Figure 4. 7, no degradation of Fluoxetine or Norfluoxetine occurred over the 60 day period. This was also supported by the t-test results from statistical analysis which compared day 0 and day 60 concentrations ($P = 0.507$ and $P = 0.304$ for Fluoxetine and Norfluoxetine respectively). Error bars on the day 0 and day 60 control samples for both Fluoxetine and Norfluoxetine overlap, also indicating no statistically significant abiotic degradation ($P = 0.170$ and $P = 0.604$ respectively). There were also no statistically significant differences between the control and sample mean end concentrations ($P = 0.234$ and $P = 0.938$). Neither Fluoxetine or Norfluoxetine underwent any losses due to either biotic or abiotic factors under these experimental conditions.

In the case of Diazepam the results were rather variable (Figure 4. 8). Overall there was no statistically significant difference between the concentration of Diazepam measured in the samples at the start (day 0) and end (day 60) of the experiment ($P = 0.180$). No biodegradation had apparently occurred. Neither was there any statistically significant difference ($P = 0.807$, > 0.05) between the mean values for the controls from day 0 and day 60, thereby indicating that no abiotic degradation had occurred. However the concentrations of Diazepam seen in day 0 controls were very variable and it is therefore difficult to definitively draw conclusions as regards abiotic degradation. Also no differences were found in the mean concentrations of the day 60 samples and controls ($P = 0.278$). No losses, due to either abiotic or biotic factors, of Diazepam were apparently seen.

Statistical comparison of Temazepam concentrations (Figure 4. 9) in day 0 and day 60 samples (t-test; P = 0.009, < 0.05) suggests that degradation of some form has occurred. To identify whether this degradation is abiotic or biotic control data must be considered. Statistical analysis on control samples showed no degradation (P = 0.300), thereby leading to the conclusion that degradation seen was due to biotic factors. As expected, due to the normalisation process, comparison of day 0 control and sample data sets showed no statistical difference (P = 1.000). However comparison between day 60 samples and controls also showed no significant difference (P = 0.497). If Temazepam in sample chambers was undergoing biodegradation, but not in the control chambers, then a significant difference between these two data sets would be expected. It is possible that the losses seen in the sample chambers were due to sorption to dead biomass; this loss would not have occurred in the control chambers due to the lack of presence of microbial biomass. However due to the significant variability in Temazepam concentrations from day 60 samples no definitive conclusions can be drawn.

Data from the control day 60 data set were highly variable; significant differences found in variance, when comparing data sets, were only seen when one of these data sets was the control day 60 set. Both the F-test and t-test assume a normal distribution, with such a variable data set (control day 60) it is possible than a non-normal distribution affected the output of these statistical tests, especially the t-test which is particularly sensitive to this assumption. For these reasons it was not possible to conclude whether Temazepam underwent degradation (either biotic or abiotic) under these experimental conditions. Other researchers have also had problems concluding whether other compounds from the 1,4-benzodiazepine group undergo partial or no removal. Ternes (2004)

was unable to discriminate between partial or no removal (0 – 60%) for Oxazepam in liquid cultures, and under anaerobic sludge digestion conditions it was also not possible to distinguish whether Diazepam underwent no or partial removal.

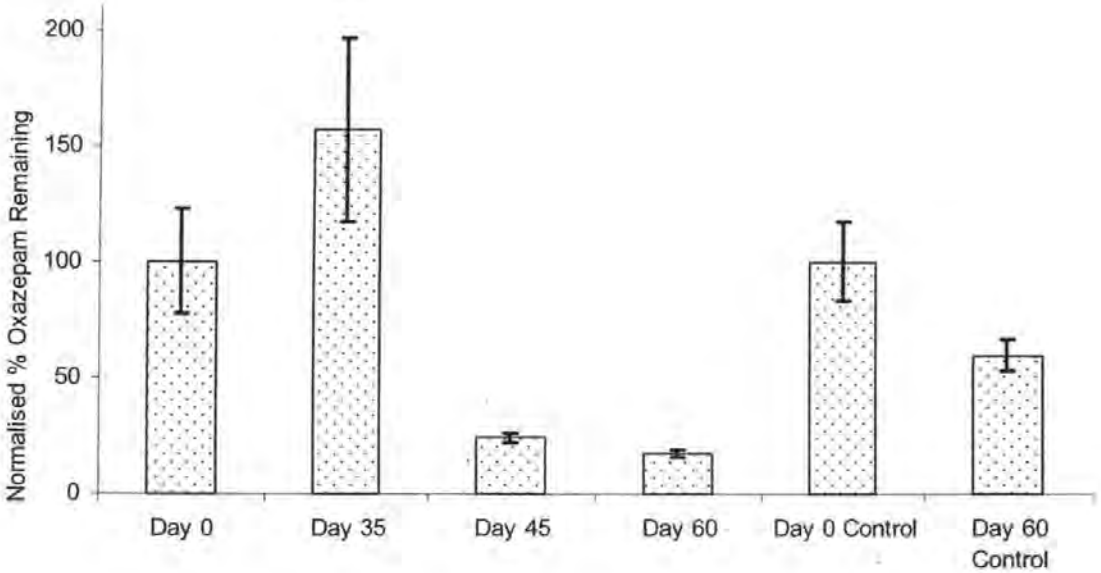


Figure 4. 10. Percentage of Oxazepam remaining in full scale liquid culture biodegradation study over 60 days

Error bars ± 1 standard deviation. n =3.

In the case of Oxazepam the t-test data ($P = 0.024$, < 0.05) for comparison of day 0 and day 60 samples shows a statistically significant change in concentration, suggesting degradation has occurred. This significant change in concentration over the duration of the experiment was also seen in controls ($P = 0.019$, < 0.05), therefore signifying that abiotic factors played a role in the concentration decrease seen in these control chambers. Comparison of the controls and samples from day 60 allow assessment of whether the losses seen in the sample chambers were due to abiotic or biotic factors. Statistical analysis between these two data sets shows a significant difference in the means ($P = 0.0004$), with live samples containing much lower concentrations of Oxazepam at days 45 and 60 than in the day 60 control samples (Figure 4. 10) . This leads

to the tentative conclusion that whilst both samples and controls were undergoing abiotic degradation or irreversible sorption, in live sample chambers, biotic degradation was also occurring.

Comparison of base peak spectra for sample, control and blank chambers (Figure 4. 11) clearly showed an unknown peak in all samples from day 60, that was not present in the corresponding blank or control samples.

The mass spectrum of the unknown suspected metabolite had a base peak ion at *m/z* 271. Assuming this is due to a protonated molecular ion, which is typical of the 1,4-benzodiazepines (Section 2.4.1), this is attributed to a molecular weight of 270. This is also the molecular weight of Nordiazepam (Figure 2.11). Electrospray MS conditions were therefore optimised to allow investigation of *m/z* 271 to 243 fragmentation shown previously to be characteristic of Nordiazepam. The day 60 biodegradation samples containing the unknown were then re-examined by ESI-MS. An authentic sample of Nordiazepam (2 μg mL^{-1}, also containing 2 μg mL^{-1} d_5-Oxazepam) was also examined for direct comparison purposes. Full MS analysis and MS^2 for *m/z* 271 were performed with HPLC conditions as discussed previously (Section 2.3, Figure 2.4). Figure 4.12 shows the mass chromatograms for extracted ions *m/z* 271 and 292, alongside MS spectra.

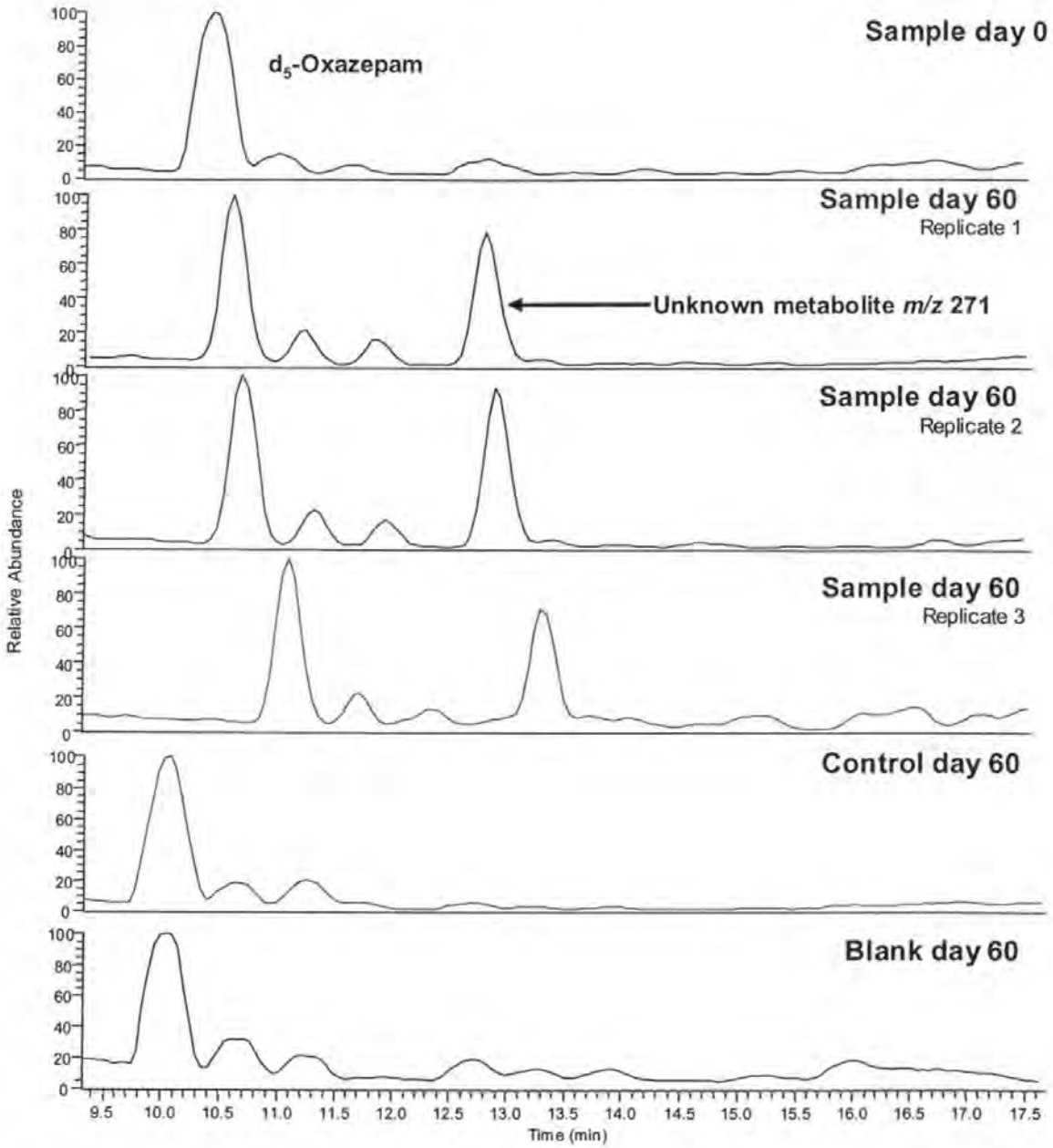


Figure 4. 11. HPLC-ESI-MS base peak chromatogram for Oxazepam liquid culture full scale biodegradation experiment: sample, control and blanks for day 60

Base peak chromatograms shown in each cells are from single replicates from the beginning (Day 0) and end (Day 60) of the experiment..
IS, d_5-Oxazepam (*m/z* 292) at Rt ~ 10 minutes.
Unknown (*m/z* 271) at Rt ~ 13 minutes present in all three replicates from Day 60.
HPLC and MS conditions as developed in Sections 2.4 and 2.5.

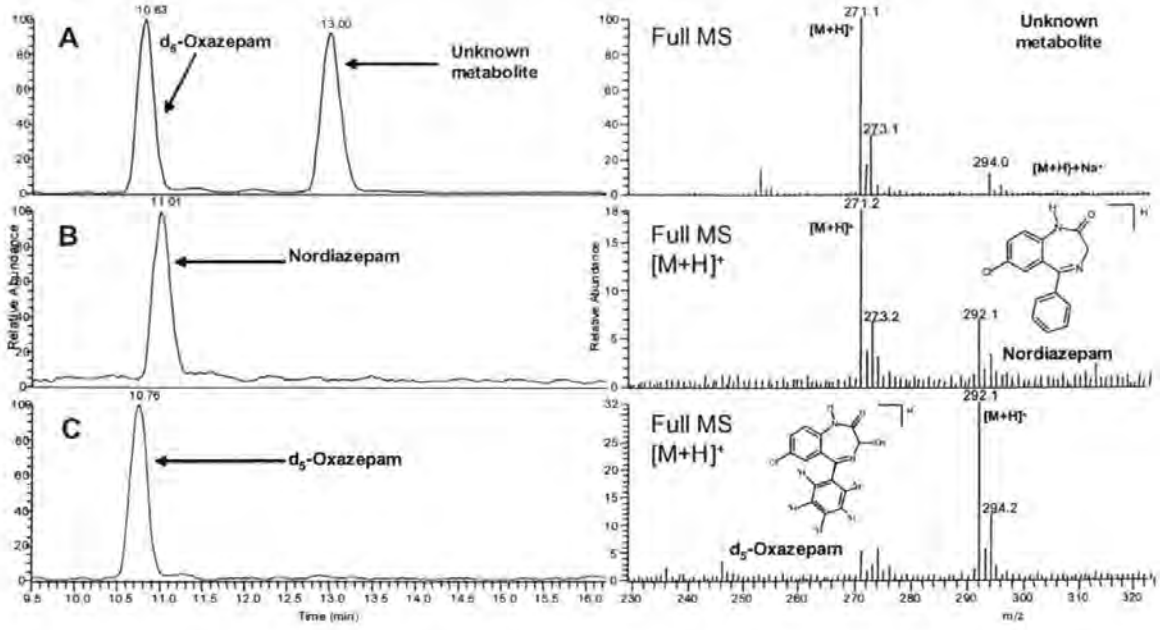


Figure 4.12. Example full MS extracted ion chromatograms and mass spectra for Oxazepam liquid culture full biodegradation study: sample day 60, authentic Nordiazepam and authentic d_5-Oxazepam

A – Extracted ion chromatogram of IS (d_5-Oxazepam *m/z* 292) and unknown product (*m/z* 271) and full mass spectrum of unknown product
B – Extracted ion chromatogram and full mass spectra of Nordiazepam
C – Extracted ion chromatogram and full mass spectra of d_5-Oxazepam (IS)

The data in Figure 4.12 show that although the unknown metabolite (Figure 4.12 A) and Nordiazepam (Figure 4.12 B) have very similar MS spectra, the Rt of the two compounds differ by approximately 2 minutes (unknown 13 mins; Nordiazepam 11 mins), with the unknown metabolite eluting later.

For Nordiazepam, MS^2 fragmentation generated abundant ions at *m/z* 243 (Figure 4.15), 208 and 140, among others. The formation of the ion *m/z* 243 is attributed to loss of CO from the protonated parent ion (*viz m/z* 271 to 243, $\Delta m/z$ = 28; CO). This contrasted sharply with the MS^2 fragment ions produced by fragmentation of the protonated parent ion of the ukknown (Figure 4.16) in which an ion *m/z* 253 was abundant. The latter is attributed to loss of water from the parent ion (*viz m/z* 271 to 253; $\Delta m/z$ = 18; H_20). Clearly the difference in Rt

and MS^2 spectra (Figures 4.12, 4.15 and 4.16) show that the unknown metabolite is not Nordiazepam.

Other possibilities were thus examined for the identity of the unknown. Compounds such as the 1,4-benzodiazepines undergo rapid keto-enol tautomerism (Figure 4. 13) .

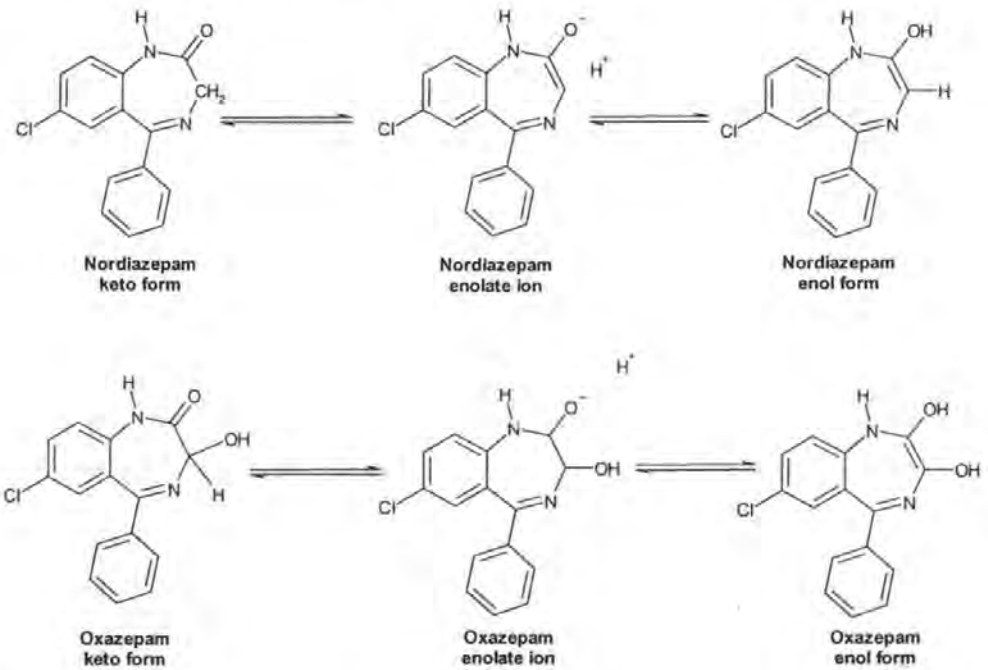


Figure 4. 13. Strutures of Nordiazepam and Oxazepam keto-enol tautomerism

Often the enol tautomers are more unstable and tend to revert to keto tautomers, as is the case with 1,4-benzodiazepines (Yang *et al.*, 1995; Yang, 1994). In other words many ketones are dominated by the lower energy keto form at equilibrium. This dominance of the keto form was confirmed herein for all target compounds and was confirmed by NMR spectroscopy analysis (Oxazepam NMR spectra are presented Appendix, Figures A.19 and A.20). Given the similarity of the mass spectra of Nordiazepam and the unknown metabolite, it was postulated that the unknown metabolite might be the enol tautomer of Nordiazepam (Figure 4. 13). However, given that the precursor of

the unknown metabolites was Oxazepam, which has a keto group at C2 and a hydroxyl group at C3, a further possibility might be the keto or enol tautomer of C3 Nordiazepam (Figure 4.14).

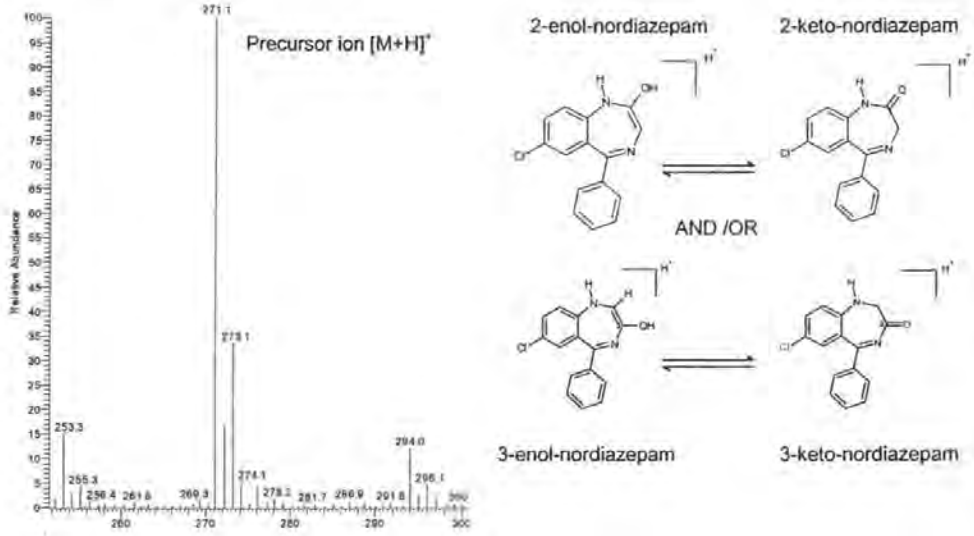


Figure 4.14. Full mass spectra of unknown product from Oxazepam liquid culture biodegradation sample day 60: all possible precursor ion structures shown

Data shown is from day 60 Oxazepam biodegradation sample, first replicate.
Unknown metabolite (Figure 4.12 A) molecular ion $[M+H]^+$ (*m/z* 271) and MS^2 fragment (*m/z* 253) proposed to be 2- or 3- keto or enol Nordiazepam.
HPLC and MS conditions as developed in Sections 2.4 and 2.5.

HPLC-ESI(+)-MS^2 of fragments ions *m/z* 271 of the unknown metabolites in the Oxazepam day 60 biodegradation samples and of an authentic sample of Nordiazepam generated the MS^2 spectra shown in Figure 4.15 and Figure 4.16.

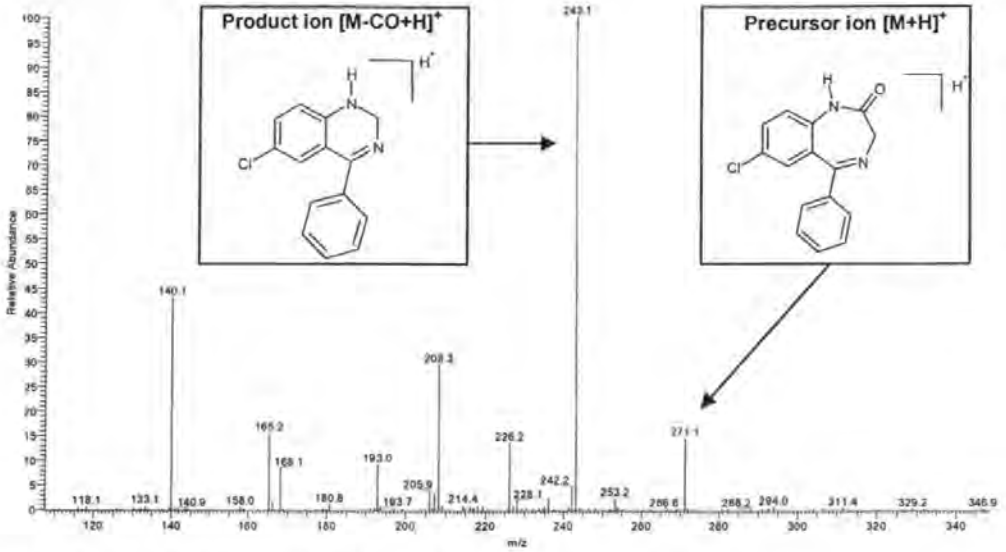


Figure 4.15. HPLC-ESI(+)-MS^2 of m/z 271 of authentic Nordiazepam (2-keto-nordiazepam)

Nordiazepam authentic standard (Figure 4.12 B) molecular ion $[M+H]^+$ (*m/z* 271) and MS^2 fragment attributed to $[M-CO+H]^+$ (*m/z* 243) shown.
HPLC and MS conditions as developed in Sections 2.4 and 2.5.

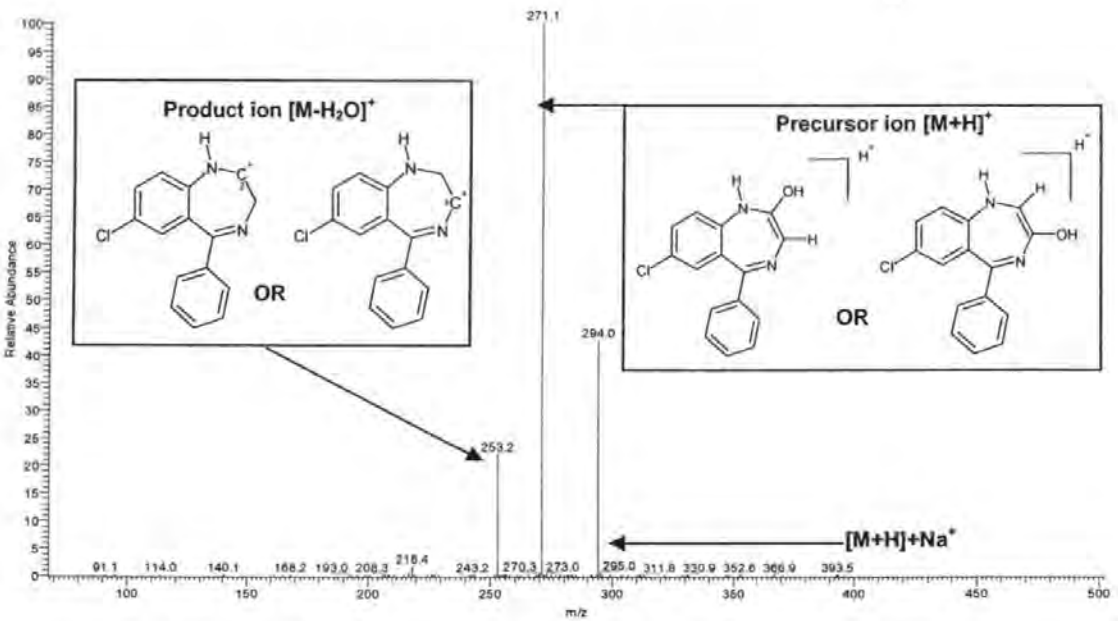


Figure 4.16. Example HPLC-ESI(+)-MS^2 of m/z 271 unknown product (suspected to be enol-nordiazepam) from Oxazepam liquid culture biodegradation experiment: Sample day 60

Data shown is from day 60 Oxazepam biodegradation sample, first replicate.
Unknown metabolite (Figure 4.12 A) molecular ion $[M+H]^+$ (*m/z* 271), molecular ion adduct $[M+H]+Na^+$ (*m/z* 294) and MS^2 fragment attributed to $[M-H_20]^+$ (*m/z* 253) proposed to be 2- or 3-keto or enol Nordiazepam. HPLC and MS conditions as developed in Sections 2.4 and 2.5.

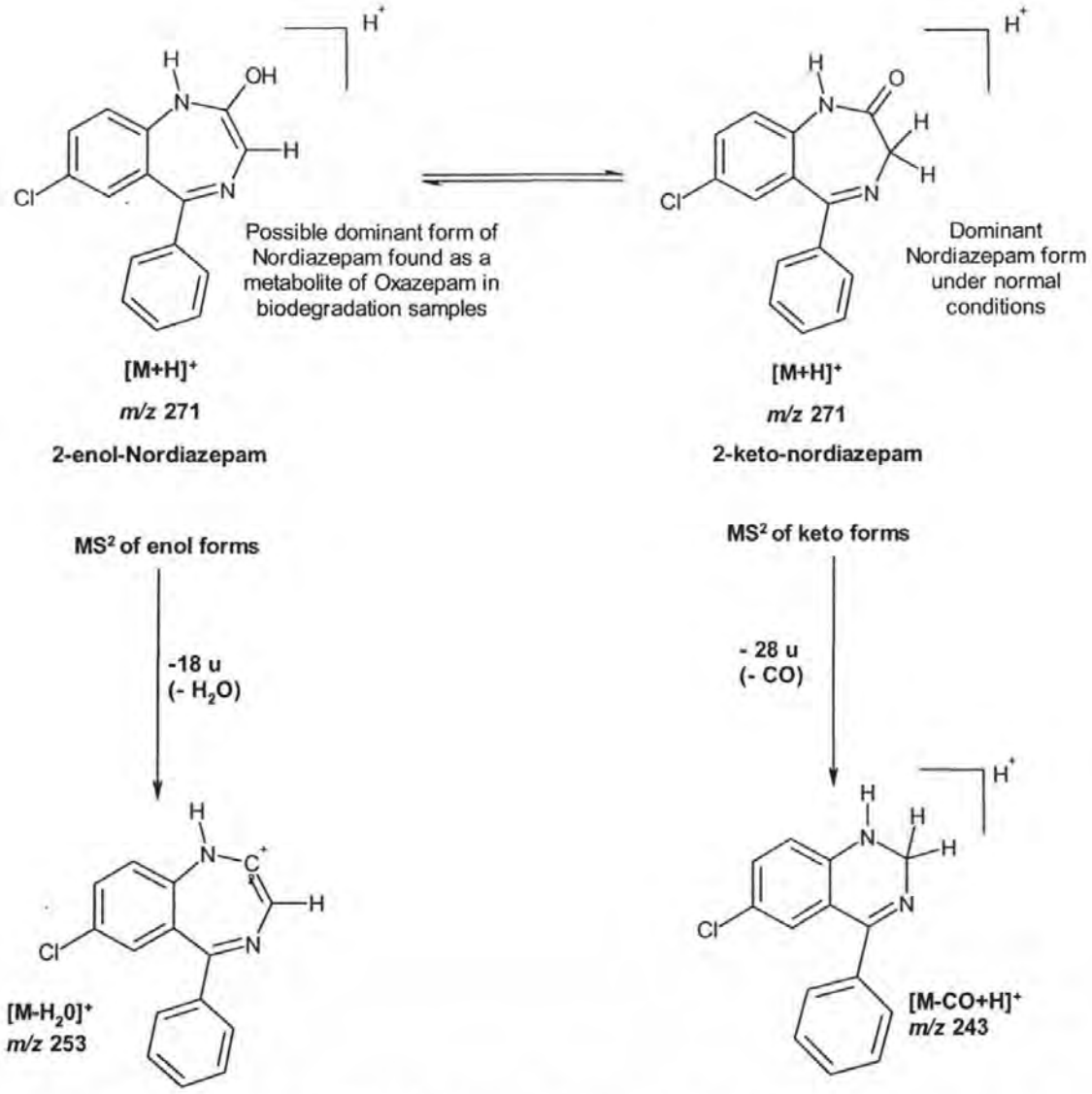


Figure 4. 17. Proposed ESI(+) fragmentation pathways of Nordiazepam Tautomers up to MS^2

Only structures for C2 enol and keto forms are shown, although C3 equivalents are equally possible.

To confirm suspicions that the unknown metabolite was an enol form of Nordiazepam, a tautomerism experiment was conducted to transform keto tautomers of Oxazepam and Nordiazepam to their enol forms under basic conditions (Yang *et al.*, 1995). This was done by shaking (5 mins) Oxazepam and Nordiazepam (1 mg each) respectively with MeOH (0.02 M NaOH), followed by heating (50°C, 20 h). The experiments were also repeated using CD_3OH (0.02 M NaOH) in place of MeOH. An aliquot (100µL) of a solution (1 mg mL^{-1}) of each of the products was then mixed with Milli-Q and 0.2% formic acid (100µL) prior to HPLC-ESI-MS^n. Full MS and MS^2 analysis was performed on all samples. Analysis conditions and methods for the analysis were as previously used for Oxazepam liquid biodegradation samples (Sections 2.3 and 2.4)

Figure 4.18 to Figure 4.27 show the resultant HPLC-ESI-MS^n chromatograms and spectra of each product of basification of Oxazepam and Nordiazepam with MeOH, and the proposed fragmentation pathways. Information gained from deuterated analogs (*m/z* transitions included in fragmentation pathway figures in brackets) was used in conjunction with the MS^n spectra generated to elucidate proposed structures and ESI fragmentation pathways.

Two peaks (A and B) were identified in the HPLC-ESI total ion current chromatogram from the basified Noridazepam sample (Figure 4.18). The retention time of the first peak corresponded to that of 2-keto-Nordiazepam (Figure 4.12 B). The second compound eluted approximately 2.5 minutes later (Rt 13.4 mins). This is close to the Rt of the unknown biodegradation metabolite. Unfortunately due to the low signal response of peak B it was not possible to obtain MS^2 spectra of this component for further confirmation.

However mass spectra of the corresponding components to A and B produced when basification in CD_3OH was conducted, both contained protonated molecular ions consistent with bis-deuteration (Figure 4.19 and Figure 4.20). This is also indicative that both peaks A and B are tautomeric forms of Nordiazepam. The full MS of this component (Figure 4.18, peak B) indicated the presence of $[M+H]^+$ ion with an *m/z* of 271 (Figure 4.19), as would be expected for Nordiazepam and consistent with the identification of this component as the 2-enol form (Figure 4.16).

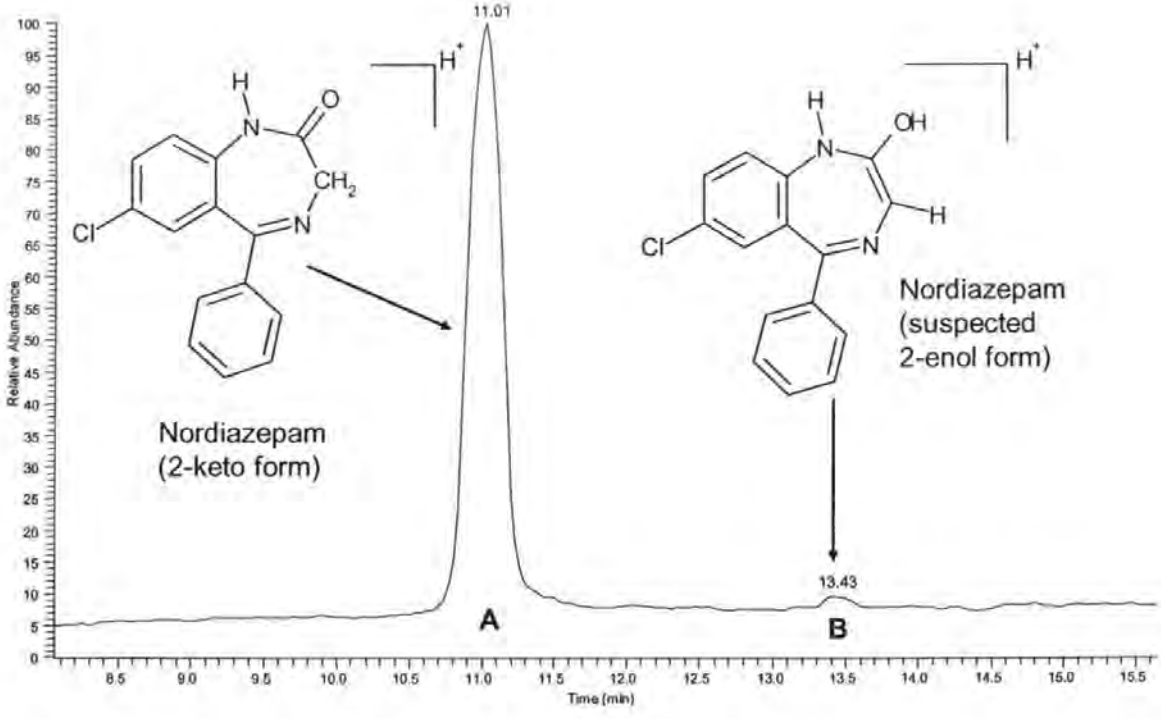


Figure 4.18. Tautomerism experiment: HPLC-ESI-MS total ion current for Nordiazepam tautomer sample

TIC shown is for basified Nordiazepam in MeOH. Equivalent TIC was obtained using CD_3OH.
A. 2-keto-Nordiazepam (*m/z* 271) at Rt ~ 11 minutes.
B. Unknown (*m/z* 271) at Rt ~ 13.5 minutes suspected to be 2-enol form of Nordiazepam.
HPLC and MS conditions as developed in Sections 2.4 and 2.5.

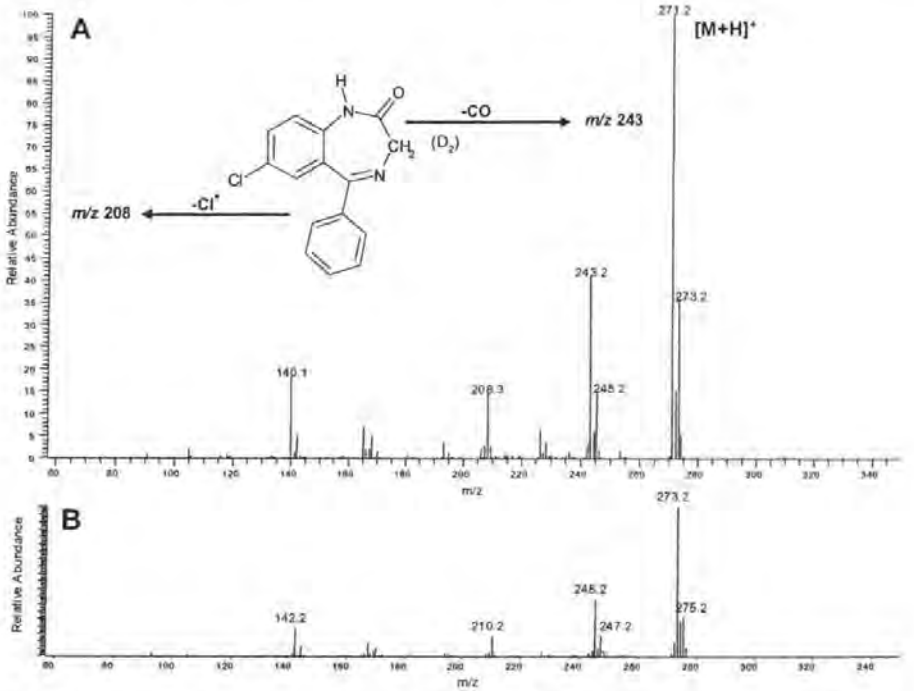


Figure 4.19. Tautomerism experiment: HPLC-ESI-MS mass spectra for Nordiazepam tautomer sample, peak A

A. Full mass spectrum shown is for basified Nordiazepam in MeOH, peak A (Figure 4.18).
B. Full mass spectrum shown is for basified Nordiazepam in CD_3OH, peak A (Figure 4.18).
Molecular ion $[M+H]^+$ (*m/z* 271), MS^2 fragment attributed to $[M-CO]^+$ (*m/z* 243), MS^3 fragment attributed to $[M-CO-Cl]^+$ (*m/z* 208) identified as 2-keto-Nordiazepam.
HPLC and MS conditions as developed in Sections 2.4 and 2.5

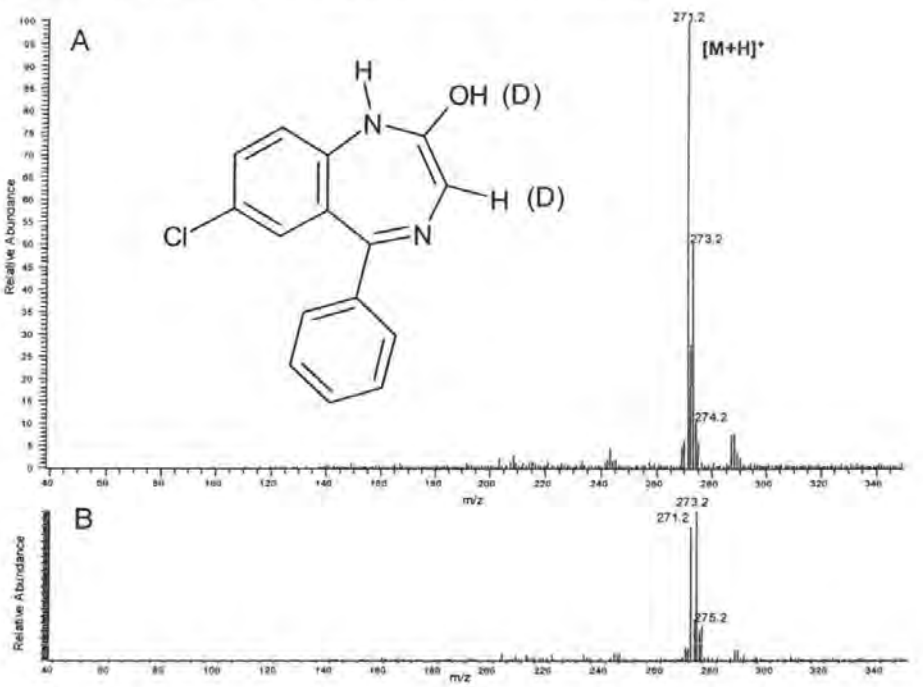
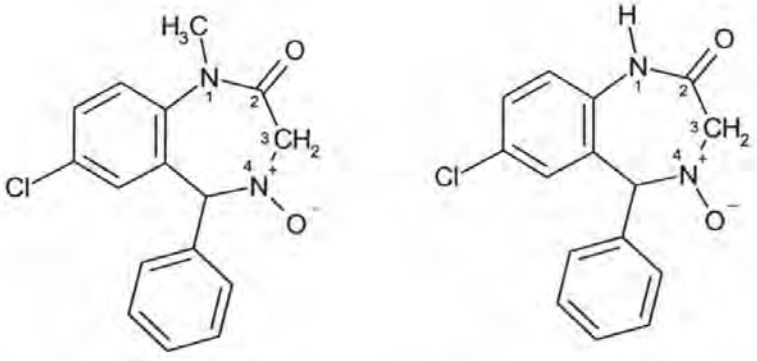


Figure 4.20. Tautomerism experiment: HPLC-ESI-MS mass spectra for Nordiazepam tautomer sample, peak B

A. Full mass spectrum shown is for basified Nordiazepam in MeOH, peak B (Figure 4.18).
B. Full mass spectrum shown is for basified Nordiazepam in CD_3OH, peak B (Figure 4.18).
Molecular ion $[M+H]^+$ (*m/z* 271) Rt ~ 13.5 minutes, proposed to be 2-enol form of Nordiazepam. HPLC and MS conditions as developed in Sections 2.4 and 2.5

The spectra presented in Figure 4.19 show the *m/z* transitions 271 to 243 to 208, which indicates the loss of CO with ring closure to a 6 membered resonance stabilised ring, followed by the loss of the Cl radical. The same losses were seen for the deuterated analalog (*m/z* transitions 273 to 245 to 210) indicating that the two deuterium ions on either C2 or C3 were not lost during ESI fragmentation. Yang *et al.*, (1996) also found bis-deuteration occurred at C3 under similar experimental conditions using either Diazepam-4-oxide and Nordiazepam-4-oxide as starting materials (Figure 4.21). The presence of an O on the N4 position, or a methyl at the N1 position, had no impact upon the susceptibility of C3 hydrogens to undergo exchange with deuterium ions from CD_3OH. This confirms the identity of peak A as the 2-keto form of Nordiazepam. The ESI fragmentation pathway of keto-Nordiazepam can be seen in Figure 4.22.



Diazepam-4-oxide **Nordiazepam-4-oxide**

Figure 4.21. Structures of Diazepam-4-oxide and Nordiazepam-4-oxide

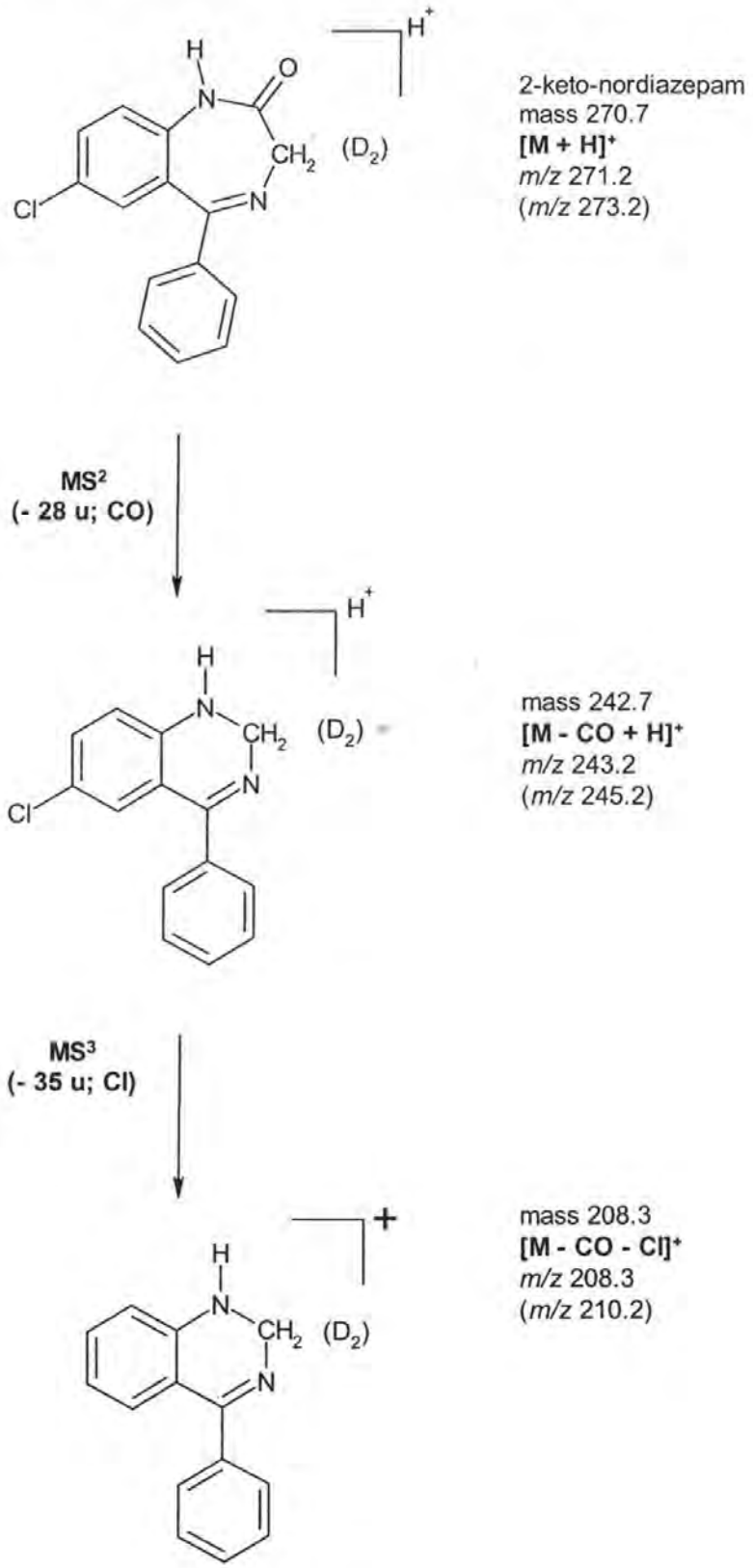


Figure 4.22. Tautomerism experiment: Proposed ESI fragmentation pathway of peak A, Figure 4.18, proposed to be 2-keto Nordiazepam

Position of deuterated ions and *m/z* for equivalent deuterated analogs are shown in brackets.

Further evidence for the formation of 1,4-benzodiazepine tautomers was produced by the products of basification of Oxazepam. When Oxazepam was basified in MeOH, five peaks were identified in the HPLC-ESI total ion current chromatogram (Figure 4.23). However only two of the peaks (A and B) relevant to the identification of the biodegradation metabolite formed in the liquid culture biodegradation of Oxazepam will be discussed.

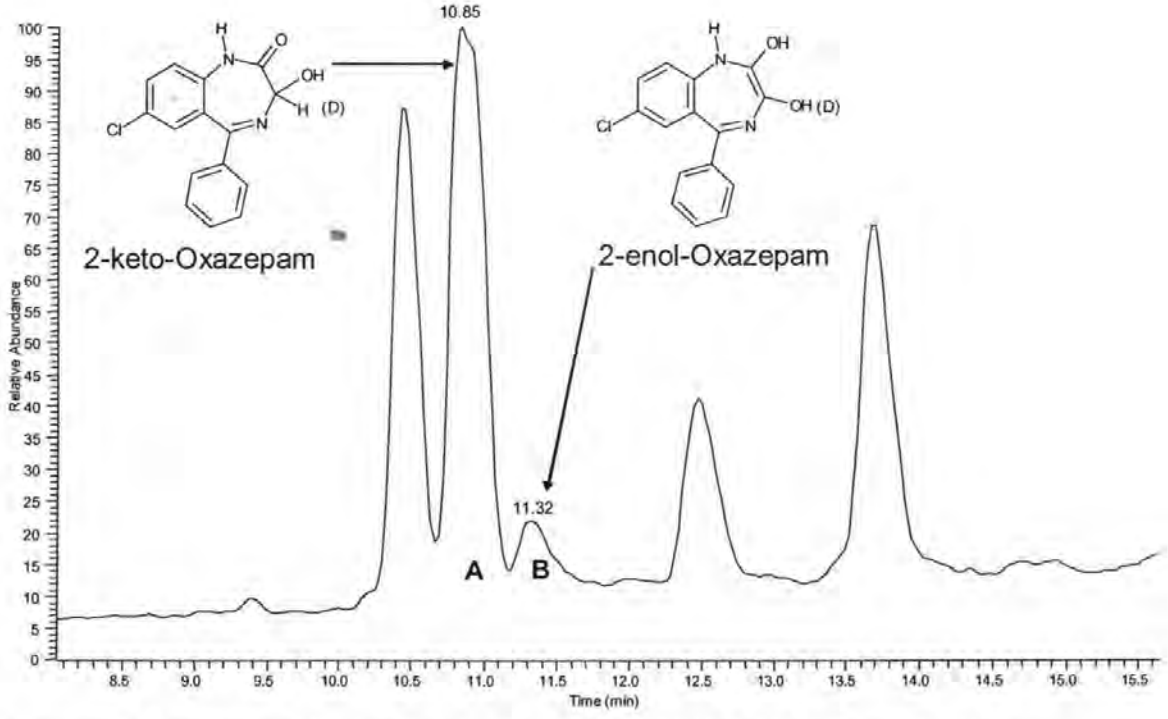


Figure 4.23. Tautomerism experiment: HPLC-ESI-MS total ion current for Oxazepam tautomer sample

TIC shown is for basified Oxazepam in MeOH. Equivalent TIC was obtained using CD_3OH.
A. 2-keto-Oxazepam (*m/z* 287) at Rt ~ 10.8 minutes.
B. Unknown (*m/z* 287) at Rt ~ 11.3 minutes suspected to be 2-enol form of Oxazepam.
HPLC and MS conditions as developed in Sections 2.4 and 2.5.

Figure 4.24 shows the mass spectral product ions resulting from MS^2 fragmentation of the protonated molecular ion of peak A from basified Oxazepam (*m/z* 287) which had a Rt of 10.85 minutes (Figure 4.23). The ion *m/z* 269 is attributed to the loss of water ($\Delta m/z$ = 18). The MS^3 fragment (*m/z* 241) can also be seen and is attributed to loss of CO from ion *m/z* 269 ($\Delta m/z$ =

28). The mass spectrum of the corresponding product obtained by basification in CD_3OH (Figure 4.24) show analogous *m/z* transitions (288 to 270 to 242) indicating mono deuteration at C3, as expected (Yang and Bao, 1994; Yang, 1994; Yang *et al.*, 1995). These data support the identification of peak A as 2-keto-Oxazepam. The ESI fragmentation pathway of this compound can be seen in Figure 4.26.

The mass spectrum (Figure 4.25) derived from peak B (Figure 4.23) was qualitatively similar to that assigned to 2-keto-Oxazepam (Figure 4.24). However, the relative intensities of the ions differed. MS^2 fragmentation of the parent ion (*m/z 287)* produced an ion due to loss of water (*m/z* 287 to 269). The structure of this ion (Figure 4.25) is proposed to be due to that of an analogous rearrangement product, Oxazepam quinalozine carboxyaldehyde, previously identified as a theromolysis product (Sadee and Van der Kleijn, 1971; Forgione *et al.*, 1971; Yang *et al.*, 1995). MS^3 fragmentation of *m/z* 269 produced an ion due to further loss of 28 u, which corresponds to the loss of CO from the carboxyaldehyde (Figure 4.25). Corresponding fragmentations were seen for the mono-deuterated analog (*viz m/z* transitions 288 to 270 to 242; Figure 4.25). These transitions, in conjunction with the later Rt of component B relative to 2-keto-Oxazepam, suggest that component B is the enol form of Oxazepam (Figure 4.25). The postulated ESI fragmentation pathway for component B is shown Figure 4.27.

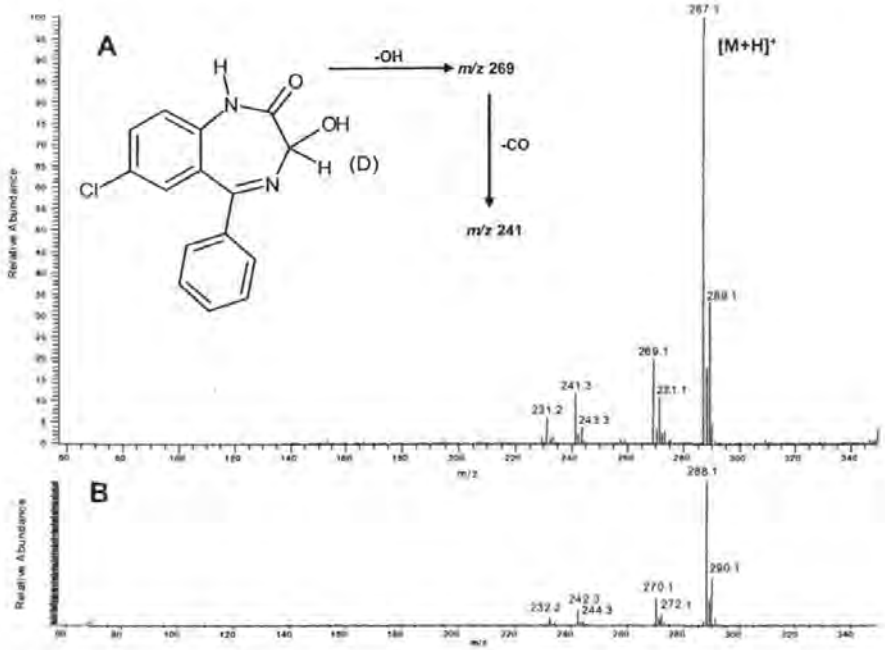


Figure 4.24. Tautomerism experiment: HPLC-ESI-MS^n spectra for Oxazepam tautomer sample, peak A

A. Full mass spectra shown is for basified Oxazepam in MeOH, peak A (Figure 4.23).
B. Full mass spectra shown is for basified Oxazepam in CD_3OH.
Molecular ion $[M+H]^+$ (*m/z* 287), MS^2 fragment attributed to $[M-CO]^+$ (*m/z* 241), MS^3 fragment attributed to $[M-CO-H_2O]^+$ identified as 2-keto-Oxazepam.
HPLC and MS conditions as developed in Sections 2.4 and 2.5

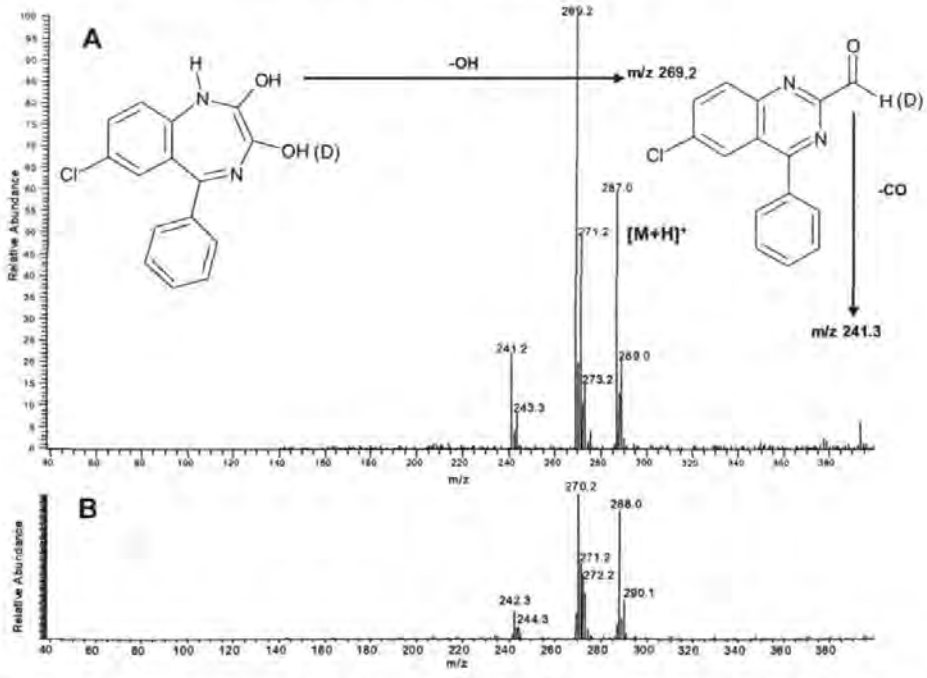


Figure 4.25. Tautomerism experiment: HPLC-ESI-MS^n spectra for Oxazepam tautomer sample, peak B

A. Full mass spectra shown is for basified Oxazepam in MeOH, peak B (Figure 4.23).
B. Full mass spectra shown is for basified Oxazepam in CD_3OH.
Molecular ion $[M+H]^+$ (*m/z* 287), MS^2 fragment attributed to $[M-H_2O]^+$ (*m/z* 269), MS^3 fragment attributed to $[M-H_2O-CO]^+$ proposed to be 2-enol form of Oxazepam.
HPLC and MS conditions as developed in Sections 2.4 and 2.5

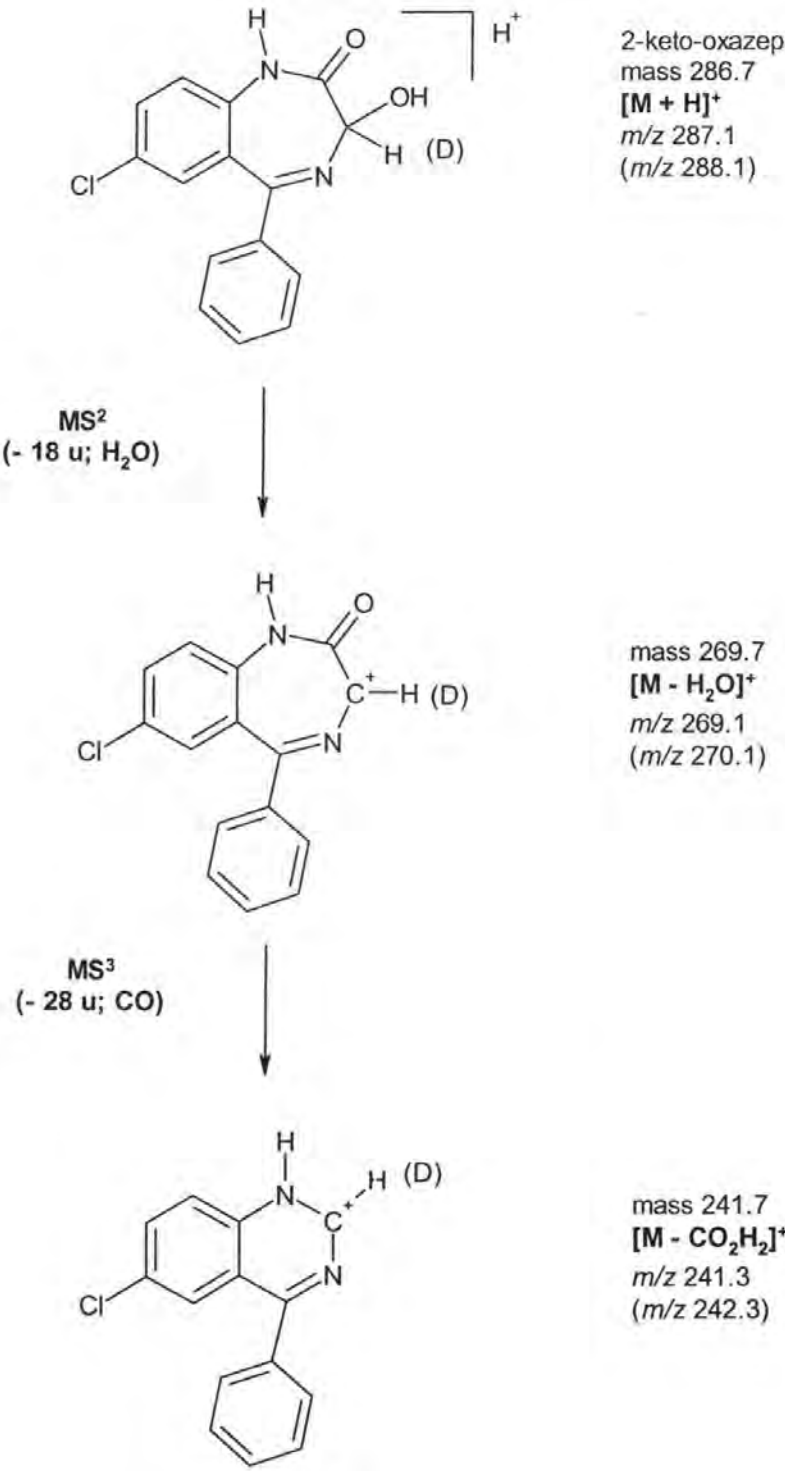


Figure 4.26. Tautomerism experiment: Proposed ESI fragmentation pathway of peak A, Figure 4.23, proposed to be 2-keto Oxazepam

Position of deuterated ions and *m/z* for equivalent deuterated analogs are shown in brackets.

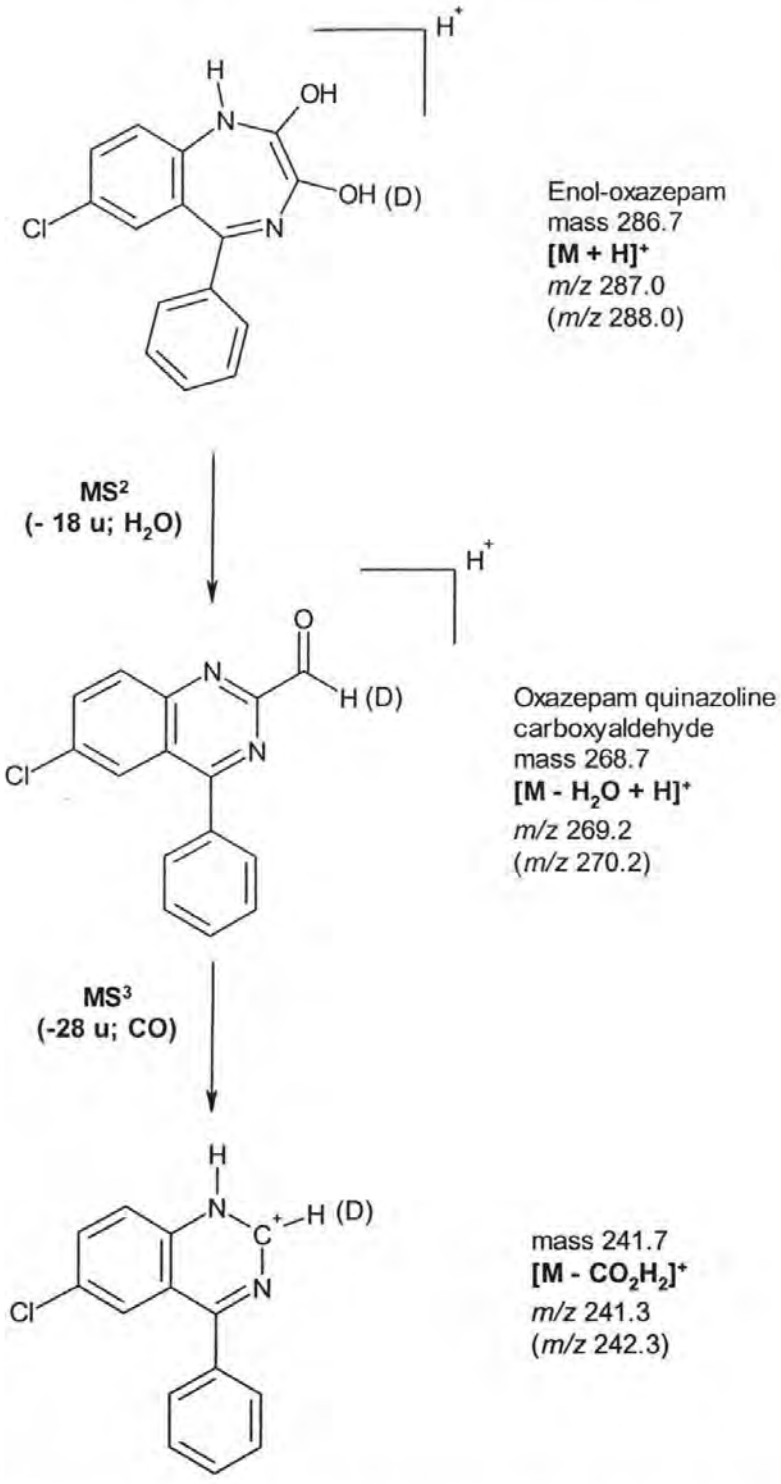


Figure 4.27. Tautomerism experiment: Proposed ESI fragmentation pathway of peak B, Figure 4.23, proposed to be 2-enol Oxazepam

Position of deuterated ions and *m/z* for equivalent deuterated analogs are shown in brackets.

4.4.2.5. Summary

The aim of the series of tautomerism experiments was to confirm suspicions that the unknown metabolite formed in the biodegradation experiment with Oxazepam was an enol form of Nordiazepam. Unfortunately the conversion of 2-keto-Nordiazepam to 2-enol-Nordiazepam in significant enough quantities to gain MS^2 data was not achieved. However the Rt of the product of basification of Nordiazepam was close to that of the unknown and the molecular ion produced was the same. Clearly the keto – enol Nordiazepam equilibrium lies further to the left for Nordiazepam than that of Oxazepam, where evidence for the existence of the enol form was more clearly demonstrated (Figure 4.25). Additional experimental work such as NMR, is required to confirm these findings further but this would require milligrams quantities of pure materials. It is equally possible that the unknown biodegradation product corresponds to 3-keto or 3-enol forms of Nordiazepam (Figure 4.16), although the mass spectral fragmentation loss of water favours the latter.

Although some data for some controls were poorly reproducible, it was clear that neither Fluoxetine, Norfluoxetine or any of the 1,4-benzodiazepines except Oxazepam, were biodegraded in 60 days in liquid cultures from SS-amended soil, pre-acclimatised to drug degradation (Figure 4.28). Oxazepam was however subject to both biotic and abiotic processes under the experimental conditions. Incubation for 60 days could result in the loss of approximately 80% of the initial amounts of target compound, half of which could be attributed to biotic factors. Degradation within sample chambers resulted in the formation a metabolite, proposed to be 2- or 3- enol-Nordiazepam. Figure 4.29 summarises this proposed transformation. It was not possible to decipher whether

enolisation was occurring prior or post transformation as there was no evidence of either the enol-Oxazepam nor the keto-Nordiazepam, nor whether this enolisation was occurring on C2 or C3.

The lack of biodegradation of all SSRIs and 1,4-benzodiazepines, except Oxazepam, under these experimental conditions could have implications for persistence within the environment. Liquid culture experiments such as those performed herein can be classified as screening experiments. It was therefore deemed important to perform a simulation type experiment using just one target compound (Fluoxetine HCl) in SS-amended soil under more environmentally relevant conditions (i.e. moisture content, temperature regime, presence of fungal communities).

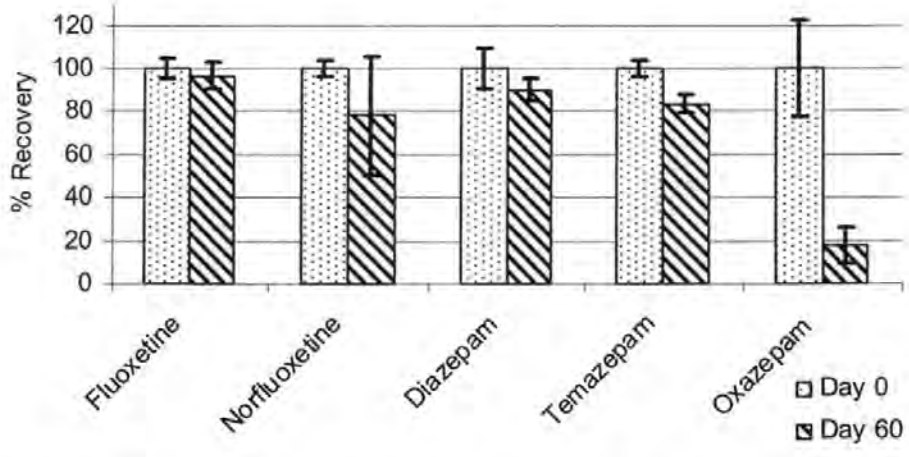


Figure 4.28. Summary of recoveries from liquid culture biodegradation experiments from the beginning (day 0) and end of the experiment (day 60) for Fluoxetine, Norfluoxetine, Diazepam, Temazepam and Oxazepam

Error bars ± 1 standard deviation (n = 3).

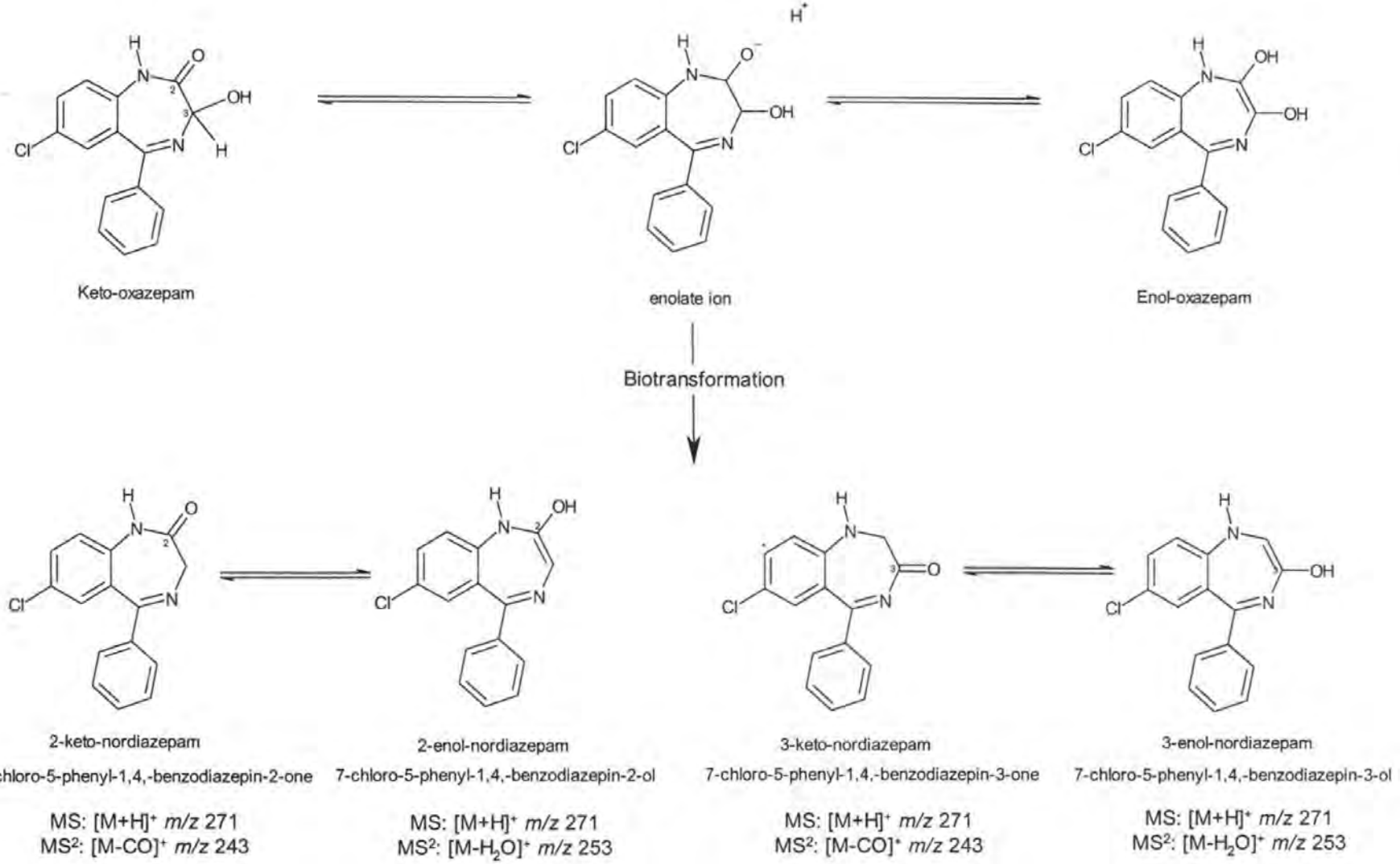


Figure 4.29. Proposed bacterial mediated transformation of Oxazepam to Nordiazepam

4.5. Biodegradation of Fluoxetine HCl in Sewage-sludge amended-soil

Previous biodegradation experiments with five target PPCPs in liquid cultures derived from SS-amended soil found all PPCPs, except Oxazepam, to be resistant to biodegradation over 60 days, thus indicating their potential persistent nature within the environment. A more rigorous biodegradation experiment over 270 days with more environmentally relevant conditions and just one target compound, Fluoxetine HCl was therefore performed.

4.5.1. Experimental Procedure

The basis of the proposed methodology outlined below was based upon OECD guideline 307 (OECD, 2002b). Soil was sourced from a typical agricultural field to which SS had been applied on a regular basis (Table 4.2; ~ 400 m^3 over 2 years). Soil (15g ± 0.5g) was placed into each incubation chamber, which was protected from light to minimise photodegradation and fitted with a filter cap. The spiking concentration was determined by calculating the predicted concentration of target compounds in SS and hence in amended soil (Appendix, Figures A.3 to A.7). Samples, sterile controls (γ-irradiated) and viability test chambers were spiked with 1.5 µg Fluoxetine HCl in MeOH and mixed by hand shaking. Solvent blanks were also prepared, so that any impacts of the solvent upon the microbiological populations within the soil would remain consistent throughout all chambers. Throughout the duration of the experiment the flasks were weighed once a fortnight and sterile artificial rainwater was added (0.01 M $CaCl_2$ in Milli-Q, autoclaved at 125°C for 40 mins) when necessary, to keep the moisture content at field capacity. Table 4.4 summarises the details of the different incubation chambers.

Incubation Type	Microbiological Status	Fluoxetine HCl Spiked	Number of Flasks	Labelling Code
Sample	Live	Yes	95	S
Sterile Control	Sterile	Yes	20	C
Solvent Blank	Live	No (MeOH only)	20	B
Viability	Live	Yes	10	V

Table 4.4. Details of incubation chambers used to investigate the biodegradation of Fluoxetine in sewage-sludge amended-soil

Three incubation chambers were sacrificed on each sampling day. Sampling took place on days 0, 1, 2, 4, 6, 9, 12, 15, and then weekly for 2 months and fortnightly for the remaining duration. Control (sterile and blank) chambers were sampled and viability tested to ensure continued sterility on days 0, 90, 180 and 270. Viability chambers were sub-sampled fortnightly, diluted (0.1g of soil shaken with 10 mL phosphate buffered saline) and plated onto Tryptone Soya Agar (TSA; for bacterial growth) and Malt Agar (MA; for fungal growth). Details of the constituents used to make the buffer and both types of agar can be found in the appendix (Table A.10). Plates were placed into an incubator (25°C for 10 days) and photographed. SS in the UK is usually spread in September and February (SWW, 2004 personal communication). As this experiment was designed to mimic field conditions as closely as possible, average South West February temperatures were selected as a starting condition for all the samples. Temperatures were selected by using data kindly supplied on request by the UK Meteorological Office (Mount Batten average monthly mid-range air temperatures for 1971 – 2000). All chambers were stored in the dark (to prevent photodegradation) at 7°C for 2 months. Temperature was then increased to 9-12°C (average SW April & May; 2 months) and then 14-16°C (average SW June

& July; 3 months) and then finally returned to 9 – 12°C (average SW September & November; 3 months).

4.5.2. Sample Preparation

Just prior to extraction 1.5 µg d_5-Fluoxetine HCl in MeOH was added to each incubation chamber as an IS. Each chamber then underwent extraction, clean up and reconstitution using the developed soil extraction and tandem SPE method presented in Section 3.4.2, Figure 3.7.

4.5.3. Analysis

Analysis was performed using HPLC-ESI-MS^n in positive ion mode. During the first set of analyses a calibration series for Fluoxetine HCl and d_5-Fluoxetine HCl was analysed for quantification purposes (further details of calibration can be found in Sections 2.5 and 2.6). Full MS analysis was used for preliminary identification (from parent *m/z* 310.0, and Rt) and quantification of Fluoxetine HCl remaining within the samples. DDMS was used for further confirmation of peak identity, *via* generation of the principal daughter ion (*m/z* 147.9). Under DDMS conditions the largest ion for each scan undergoes MS^2 fragmentation (provided that the ion count is > 10^5). DDMS was selected as the preferred analysis technique, as the use of DDMS can also result in MS^2 fragmentation of any biodegradation products formed thereby providing some structural information regarding the products. Details of HPLC are given in Section 2.3, Figure 2.4. Analysis was performed on a Finnigan MAT LCQ™ quadrupole ion trap mass spectrometer. ESI in positive mode was used for ionisation. This chromatograph was interfaced with a Dionex P580 (gradient) pump and a rheodyne injector valve was used for sample introduction. Xcalibur™ software was used for data collection and manipulation.

4.5.4. Results and Discussion

4.5.4.1. Soil Microbe Viability

HPLC grade water (Fisher Scientific), Milli-Q and sterilised Milli-Q water (autoclaved 125°C for 40 mins) were plated onto TSA (for bacterial growth) or malt agar (for fungal growth) plates prior to the start of the experiment, so that appropriate sterile water could be selected for use as artificial rain (to maintain field moisture capacity) without introducing additional microbes. No growth was seen on the sterilised Milli-Q; hence its use as the base for artificial rainwater.

Viability chambers, containing live soil and target compound (i.e. identical to sample chambers), were sub-sampled fortnightly and plated, so that major changes in the microbial populations present could be monitored. Photographs of a selection of these plates are presented in Plate 4.1. These show that over the duration of this experiment both the bacterial and fungal populations present within the samples remained relatively stable, i.e. the bacterial and fungal colonies grown at different time points contained a similar range of microbes. However the rate of growth appeared to have declined by the end of the experiment.

More sophisticated analyses of the bacterial DNA composition using Denaturing Gradient Gel Electrophoresis (DGGE) were planned and samples sent to the University of Exeter Microbiology Department for analysis but unfortunately these were not performed. However, bacteriohopanepolyol analysis (Section 4.6) provided additional complementary data on microbe populations in this soil.

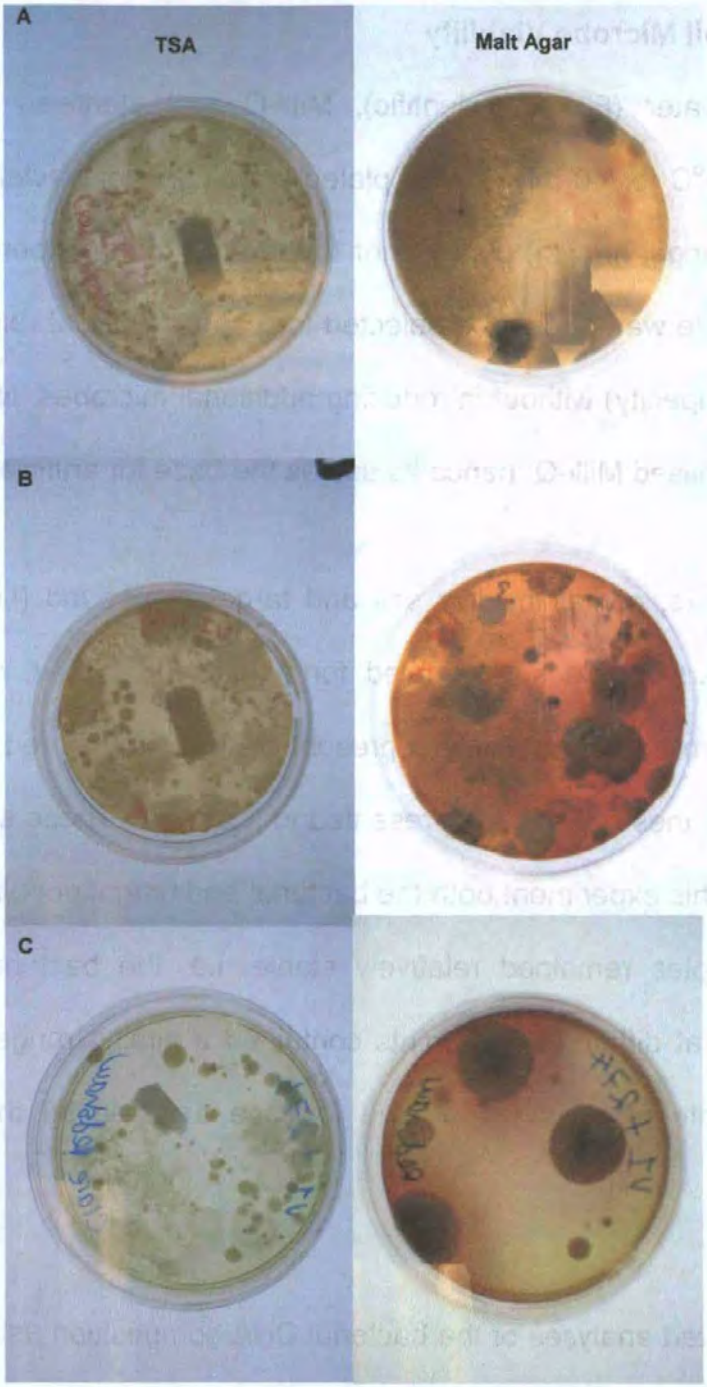


Plate 4.1. Microbial growth on TSA and Malt Agar plates to test respective bacterial and fungal fungal population viability for soil biodegradation experiment: Viability chambers

A – Day 0; B – Day 90; C – Day 274

Blank chambers, containing live soil but no Fluoxetine, also underwent regular viability checks, and a selection of these plates can be seen in Plate 4.2. This was performed so that comparison of these plates with those from viability chambers would allow the identification of any toxic effects of Fluoxetine HCl upon the soil microbes. No obvious changes in the microbial populations were seen over the duration of the experiment. Comparison of these plates with those from the viability incubation chambers (Plate 4.2) showed a comparable range of microbes present in the samples suggesting that Fluoxetine HCl did not have any substantial toxic impact upon populations present.

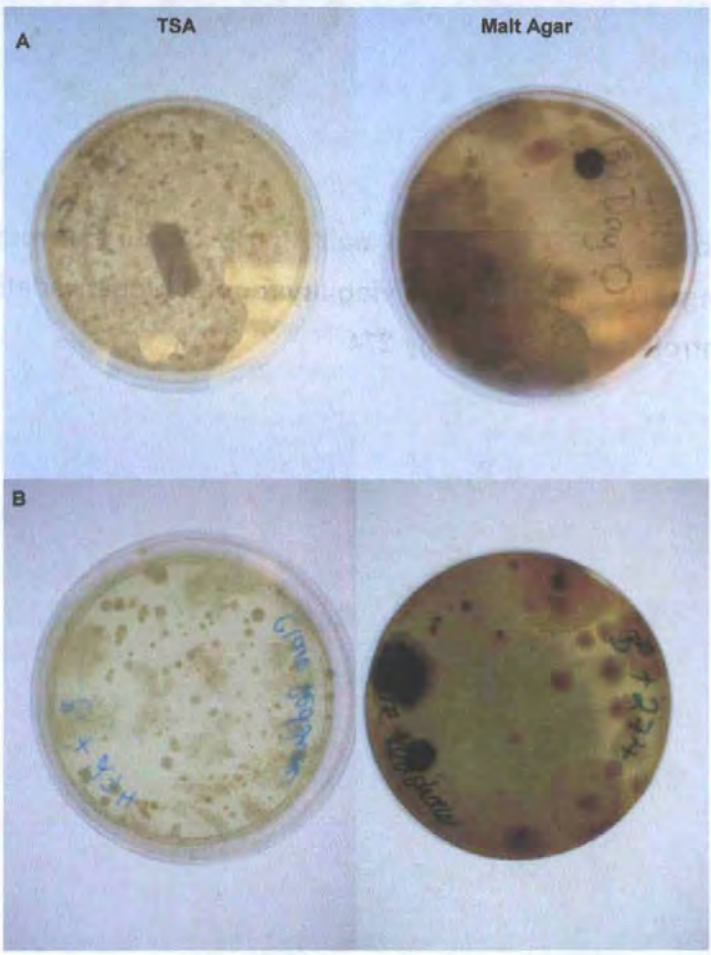


Plate 4.2. Microbial growth on TSA and Malt Agar plates to test respective bacterial and fungal fungal population viability for soil biodegradation experiment: Blank chambers

A – Day 0; B – Day 274

Sterile control (γ-irradiated) chambers were viability checked regularly to ensure continued sterility. Plate 4.3 from day 274 showed the first signs of loss of sterility from these chambers; for this reason the experiment was brought to a close on day 270.

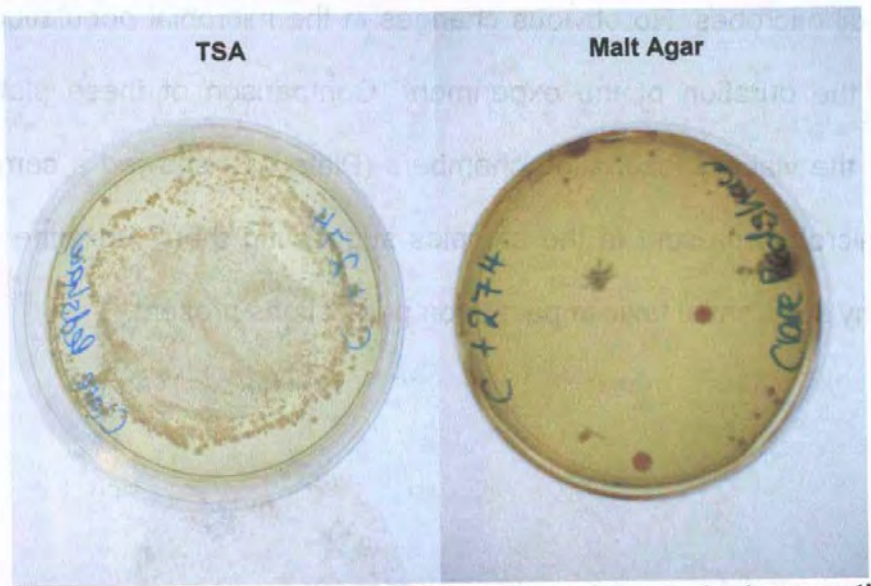


Plate 4.3. Microbial growth on TSA and Malt Agar plates to test respective bacterial and fungal fungal population viability for soil biodegradation experiment: Control chambers for day 274

4.5.4.2. Biodegradation of Fluoxetine HCl in SS amended soil

Soils from a selection of sampling points (days 0, 18, 32, 109, 125, 165, 180, 238 and 270) were chosen for initial analysis to give an overview of degradation rates. Quantitative data were obtained using ratio calibration. Blank and control samples (days 0, 180 and 270) were also analysed. No Fluoxetine was identified in any of the blank samples (data not presented). Figure 4.30 shows a typical example extracted ion chromatogram, full MS spectra and MS^2 spectra of Fluoxetine for a soil biodegradation sample (day 0), along with the extracted ion chromatogram and full MS spectra of the internal standard. Other representative chromatograms are shown in Figure 4.31.

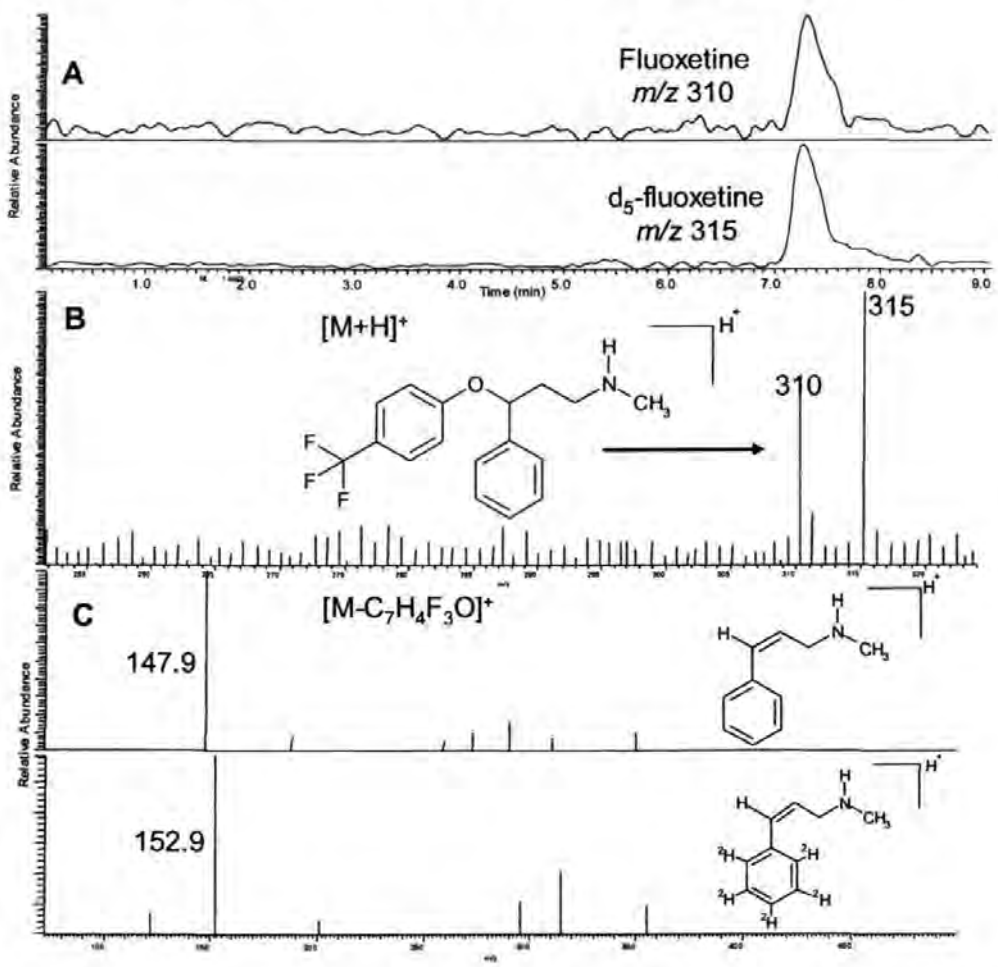


Figure 4.30. Example chromatogram and spectra for Fluoxetine and IS in sewage-sludge amended soil biodegradation sample (day 0)

A – Extracted ion chromatograms for Fluoxetine (*m/z* 310) & d_5-Fluoxetine (*m/z* 315)
B – Full MS spectra for Fluoxetine (*m/z* 310) & d_5-Fluoxetine (*m/z* 315)
C – MS^2 spectra for Fluoxetine & d_5-Fluoxetine (*m/z* transitions: 310 to 147.9 and 315 to 152.9 respectively). HPLC and MS conditions as developed in Sections 2.4 and 2.5

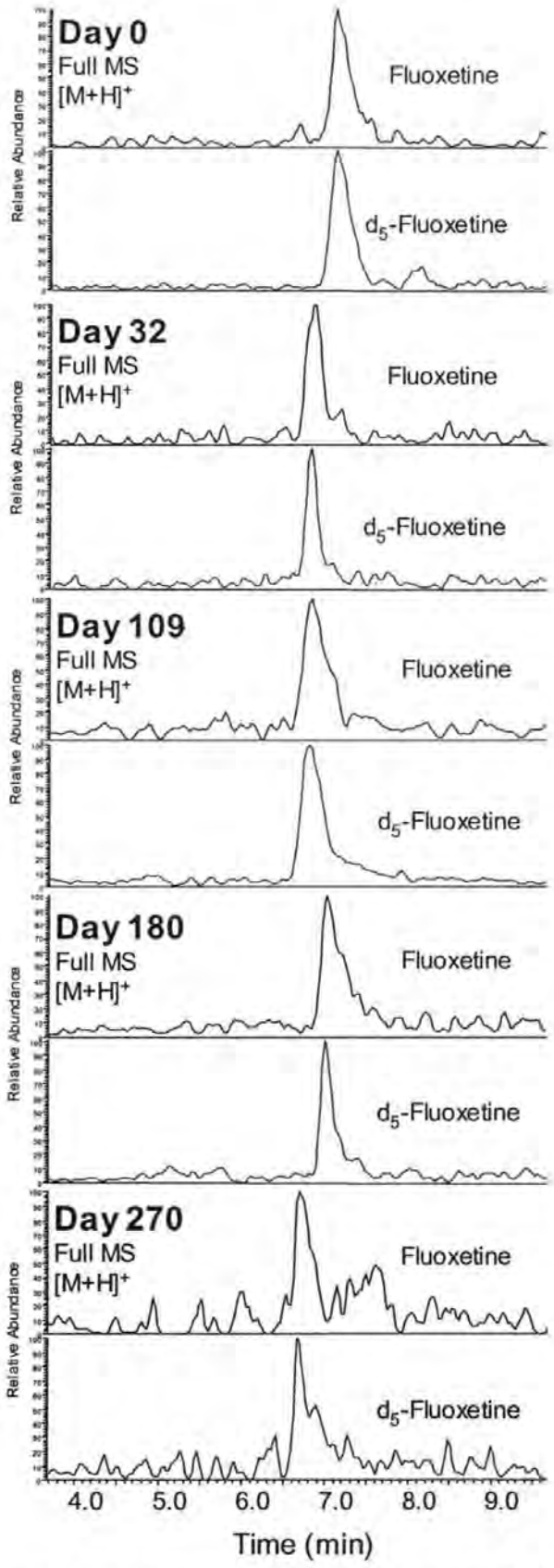


Figure 4.31. Example chromatograms for Fluoxetine and IS in sewage-sludge amended soil samples from long term soil biodegradation study over 270 days

Data shown is from individual sample replicates for a selection of sampling time points (days 0, 32, 109, 180 and 270). For each time point extracted ion chromatograms for Fluoxetine (*m/z* 310) & d_5-Fluoxetine (*m/z* 315) are shown

Visual appraisal of the quantitative data (Figure 4.32) reveals a slight decline in Fluoxetine HCl concentrations with time. It is apparent however that there is considerable variability between the data sets. It can also be seen that the sterile control data points obtained were very close to the mean Fluoxetine concentration found at the equivalent time points, indicating variations in day-to-day instrument performance.

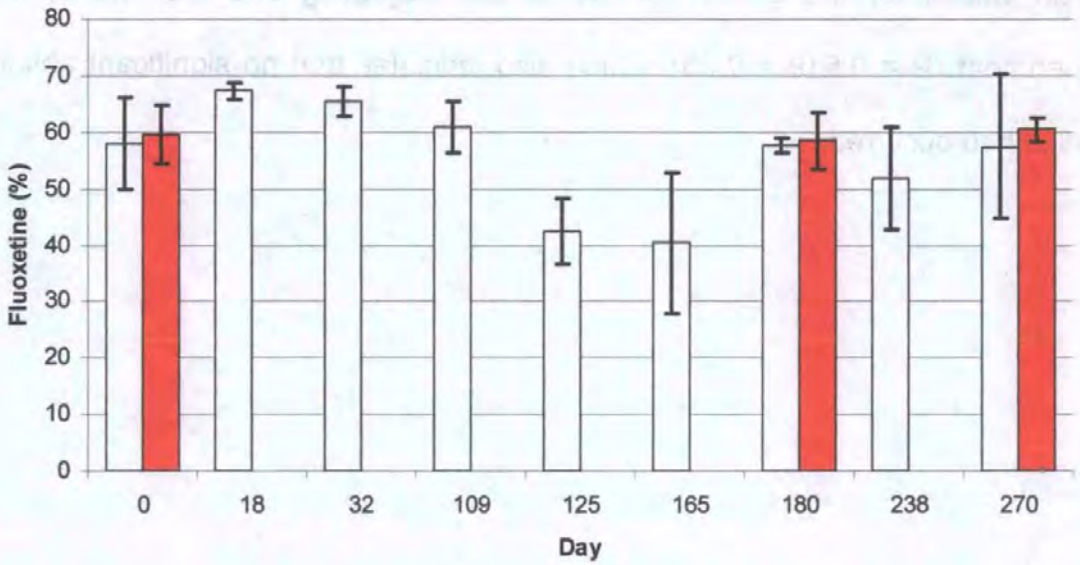


Figure 4.32. Percentage recovery of Fluoxetine from sewage-sludge amended soil biodegradation samples over 270 days

□ Average percentage Fluoxetine recovery from sample chambers; ■ average percentage Fluoxetine recovery from sterile control chambers
Error bars represent ± 1 standard deviation (n = 3)

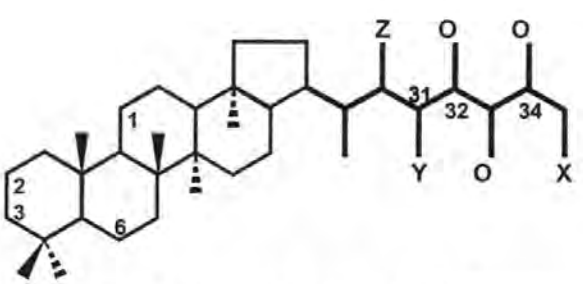
The similarity in values between the sterile controls and the live samples at the same time points, suggests that no biodegradation had occurred. An F-test followed by a t-test was used to statistically confirm these findings (Statsgraphics V.5.1). At the 95% confidence interval the P-value obtained for the F-test was 0.582, thus the differences between data sets are deemed to be not statistically significant different. This confirms that the variance seen between samples taken on day 0 and those taken on day 270 data sets is

equal. Results from the t-test ($P = 0.946$, > 0.05) comparing Fluoxetine concentrations in sample chambers from the beginning and end of the experiment were also not statistically significantly different thereby indicating that no biodegradation occurred. The most likely explanation for the very slight apparent downward trend is the progressive slight irreversible sorption of the target compound the soil with time. However no statistically significant difference was found between either the variance ($P = 0.282$; > 0.05) or the mean values for the sterile controls at the beginning and the end of the experiment ($P = 0.519 > 0.05$), which also indicates that no significant abiotic losses had occurred.

4.6. BHP Analysis

As the source of inocula for both the soil and liquid culture biodegradation experiments was lime treated sewage-sludge (pH 12+) amended soil (Table 4.2), it was considered necessary to try to characterise the microbial populations present. Data from planned collaborative DGGE experiments was not forthcoming so an alternative molecular method of bacterial population characterisation was sought.

Bacteriohopanepolyols (BHPs) are compounds found in the bacterial membrane and are involved in membrane stabilisation; they are comparable to eukaryote steroids. BHPs have been identified in more than 50% of bacterial taxa studied, and it thought that certain side chain functional groups may be unique to specific bacterial taxa and species (Bednarczyk *et al.*, 2005). Tetra-functionalised alcohol side chains are predominant, although also common are the penta- and hexa-functionalised side chains (Figure 4. 33).



Tetrafunctionalised
Z=H, Y=H, X=OH or NH_2 or composite group*

Pentafunctionalised
Z=H, Y=OH, X=OH or NH_2 or cyclitol ether
Z=OH, Y=H, X=OH

Hexafunctionalised
Z=OH, Y=OH, X=NH_2

Figure 4. 33. Generic structure of Bacteriohopanepolyols

Side chain variants include a methyl group at C-31, a ketone group at C-32, -$OCONH_2$ at at C-34, and structures with the side chain condensed in a cyclic ether form.
Variants in the ring system include methylation at either C-2 or C-3, and a double bond at C-6 and / or C-11.
*Composite groups typically comprise of sugar or amino acid derivatives.

The use of BHPs as bacterial markers is still a technique in its infancy and will be improved by extension of sampling locations to establish a larger data base

of BHPs (H.Talbot, University of Newcaste; personal communication). A selection of seven SS amended soils (Gibbs *et al.*, 2006b; Gibbs *et al.*, 2006a) sourced from Rothamsted soil research centre (discussed in more detail in Section 3.4.3) underwent this analysis alongside the soil used as an inoculum source in all biodegradation studies so as to provide a comparative BHP data set for SS-amended soils. Current correlation between bacteria and BHP is limited to 25 BHPs, which is sufficient to test the hypothesis that BHP structures can be used to identify soil bacterial processes (Talbot *et al.*, 2003). It is not however yet known what impacts of stress and laboratory growth conditions have upon BHP production, and this must be taken into account.

4.6.1. Experimental Procedure

The extraction and analysis procedures were performed by M.Cooke at Newcastle University. The methodology will only be briefly summarised herein. Ground freeze dried soil (~3g) was sieved (2mm) and suspended in a monophasic solvent mixture (50mL; MeOH : chloroform : MilliQ; 2:1:0.8), sonicated (1 hour at 40 ^{o}C) and shaken (rotary shaker, 180 rpm, 4 hours). This was followed by centrifugation (12000rpm, 15 mins) and removal of supernatant. This extraction was performed in triplicate and supernatants labelled A, B and C. Gentle shaking with chloroform and Milli-Q (5mL each) broke the monophasic solvents which were then centrifuged again (12000 rpm, 2 mins). The bottom chloroform layer from fraction B was removed and added to fraction A. This was centrifuged again (12000 rpm, 2 mins) and the chloroform layer transferred to a round bottom flask. This was then repeated by moving the chloroform layer from fraction C to B, and then to A, before combining the supernatants in the round bottom flask. Rotary evaporation and nitrogen blow-down was the used to dry the chloroform extracts.

4.6.2. Sample Preparation

It was necessary to acetylate the sample prior to analysis to improve HPLC separation of the BHPs. This was achieved by heating (50°C, 1 hour) the extract with pyridine and acetic anhydride (2 mL each) in a closed vial, which was then left to stand overnight before drying with rotary evaporation. Extracts were reconstituted in MeOH : IPA (60:40, 0.5 mL) prior to analysis.

4.6.3. Analysis

Analysis was performed on a HPLC ion trap MS in positive ionisation mode. Explanation of the MS fragmentation pathways seen including serial loss of acetylated hydroxyl functional groups and ring system fragmentation, and identification of BHPs are given in the appendix (Figures A.21 and A.22). Areas of the characteristic base peak ions and the IS peak area (acetylated 5α-pregnane-3β,20 β-diol; *m/z* 345; $[M+H-CH_3COOH]^+$) were used for the generation of semi-quantitative data. The use of acetylated authentic BHP standards for both nitrogen and non-nitrogen-containing BHPs generated mean relative response factors. Nitrogen-containing compounds gave an average response ~ 12 times greater than the standard, whereas those not containing nitrogen give a response ~ 8 times greater.

4.6.4. Results and Discussion

BHP analysis was completed upon the soil used to generate inocula for biodegradation studies and SS-amended soils sourced from seven different UK sites (Woburn, Gleadthorpe, Watlington, Pwllpeiran, Rosemaund, Bridgets and Shirburn). Table 4.5 and Figure 4.34 show only the bacterial sources identified in the soil used to generate the inocula. Additional information including example chromatograms and spectra along with data for the other seven soil samples can be found in the appendix (Figures A.23 to A.33). Results for all eight soils will be briefly discussed.

Living bacteria, dormant cells in soil, recently dead cells and free relic BHPs are thought to be the sources of BHPs extracted using this methodology. It is thought that the majority of BHPs are produced by living cells and that the quantities of BHPs reflect the dominance of living bacteria within the soil, and therefore allows conclusions to be drawn about the dominant bacterial processes occurring within the soil.

Soils studied in the past (Talbot *et al.*, 2001) have contained total BHPs at concentrations of several hundred μg g^{-1} dry soil. All of the SS-amended soils tested herein contained much lower total BHPs than this (mean of 5.7 μg g^{-1}; max 9.8 μg g^{-1}), with the soil used for biodegradation experiments lower still (3.7 μg g^{-1}; Table 4.5). This implies low numbers of bacteria and a high dominance of soil processes by fungal populations. These concentrations of BHPs are more similar to garden soils and soils from below a depth of 10 cm where bacterial activity is usually limited (the soils tested were sampled up to 20 cm).

m/z $[M+H]^+$ or $[M+H-CH_3COOH]^+$	Bacterial source	Concentration ($\mu g\ g^{-1}$ dry wt)
746	Purple non-sulphur, nitrogen fixing, ammonia oxidising	1.1
714	Various	0.8
1002 (cyclitol ether)	Methylotrophs, Cyanobacteria, Purple non-sulphur, acetic acid, *Burkholderia*	0.4
655	Various	0.4
760	Nitrogen fixing bacteria	0.2
1086	Facultative Methylotrophs	0.1
1060	Various	0.1
669	Cyanobacteria	0.1
761	Purple non-sulphur	0.1
772	Methanotrophs (Types I & II)	0.1
1016	Cyanobacteria	0.1
728	Unknown species (possible in cyanobacteria as 2-methyl)	0.1
1074	Cyanobacteria	0.03
	Total BHP	3.7

Table 4.5. Summary table of BHP analysis results of sewage-sludge amended soil from Cornwall, UK; used as a source of inoculum for biodegradation experiments in liquid (Section 4.4) and soil (Section 4.5) media

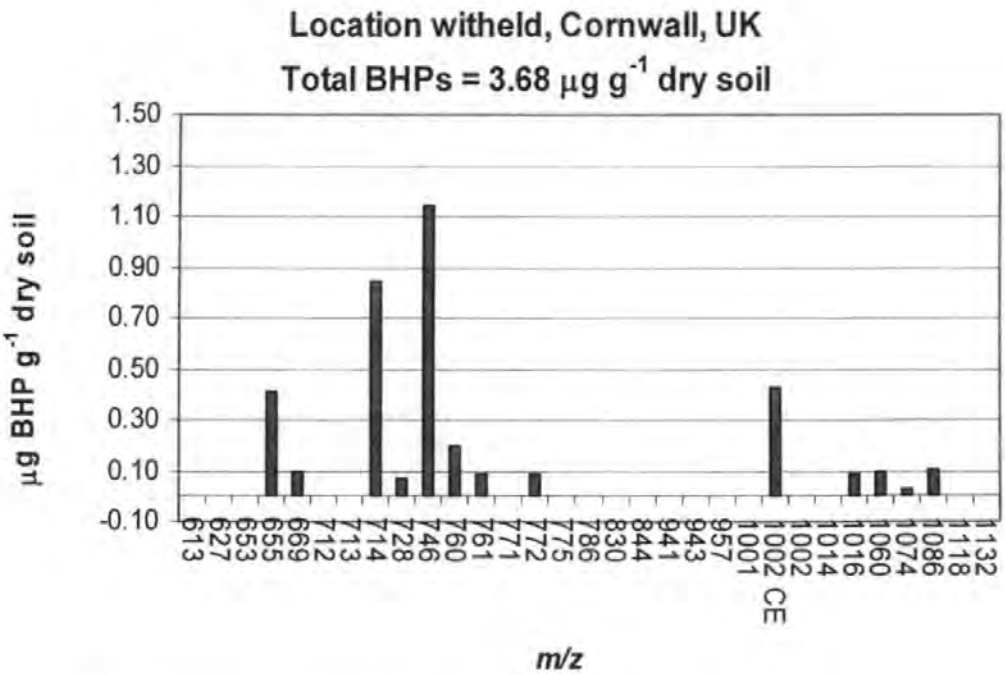


Figure 4.34. Bacteriohopanepolyols Analysis: Identification of Bacteriohopanepolyols in sewage sludge amended-soil from Cornwall, UK

Base peak m/z = $[M+H]^+$ or $[M+H-CH_3COOH]^+$
Data sourced from Cooke (2007).
This soil was used in extraction method development and as inocula for all biodegradation experiments.

Similar BHP profiles for all eight soils were dominated by four BHPs (characterised by mass spectral ions *m/z* 655, 714, 746 & 1002; Table 4.5). A wide range of bacteria are associated with these BHPs (Table 4.5) and their presence tends to give an indication of overall bacterial population. Variations between relative proportions of these compounds was apparent in all the soils and may be due to differences in the bacterial populations, or due to differential response of bacterial to environmental conditions (e.g. soil moisture, temperature).

In most soils with low total BHPs a limited bacterial diversity is postulated. For example, previously analysed garden soil contained only eight BHPs (M.Cooke, Univeristy of Newcastle; personal communication). However in the present eight soils much wider diversity was seen, ranging from 14 to 19 BHPs per sample with 13 BHPs in the soil used for biodegradation experiments (Figure 4.34). Bacterophopaneaminopentol (*m/z* 830) is thought to be uniquely produced by methanotrophs, whose presence is generally rare (Neunlist and Rohmer, 1985), but this was found in 6 of the 8 soils tested, including the soil used herein for biodegradation experiments. Its presence has only been detected in two other locations; from a paddy field and agricultural soil treated with manure for 100 years (Palace Leas Plot 2–http://www.staff.ncl.ac.uk/r.s.shiel/Place_ Leas/index). Even samples from adjacent field plots examined previously showed no evidence of Bacterophopaneaminopentol. This leads to the conclusion that methane-oxidising bacteria can be found in both manure and SS treated soils. It cannot however be concluded whether the source of the methanotrophs is the soil itself, or the applied manure or SS.

In all eight soils, including the soil used for biodegradation experiments herein, another rare BHP (*m/z* 1086) was also found to be present. This has also been associated with manuring and is thought to be produced only by facultative methylotrophs, therefore suggesting access to a source of readily metabolisable C_1 compounds. This BHP was found in the same manured source mentioned earlier (Palace Leas Plot 2), but has occasionally been identified in other non-manured sites.

In summary, these soils would not be classified as typical agricultural soils due to their low total BHP concentration, which indicates the domination of soil processes by fungal populations. It is however possible that the sampling depth (up to 20 cm) may have influenced this result. These soils can also not be classified as typical low total BHP soils, due the wide diversity of BHPs seen. Two rare BHPs, which have previously been related to manured soils, indicate the unusual presence of methanotrophs and methylotrophs. However, though this BHP method is in its infancy for microbial population characterisation it is clear that the soil used herein for the biodegradation studies has a microbial (bacterial and fungal) population typical of SS-amended soils. This justifies the use of this environmentally relevant soil for the biodegradation experiments.

4.7. Concluding Remarks

The selective serotonin re-uptake inhibitors (SSRIs) Fluoxetine and Norfluoxetine did not undergo statistically significant degradation due to either biotic or abiotic processes in either soil or liquid cultures containing microbial populations representative of SS-amended soils.

Literature data, such as those of Ternes (2004) and the present results, show that biodegradation behaviour of 1,4-benzodiazepines is more variable and likely to be very sensitive to specific environmental conditions. Diazepam was the most persistent of the compounds studied, with no losses due to either biotic or abiotic factors. In the case of Temazepam it was not possible to classify whether no or partial (0 - 20%) loss was occurring, and whether this possible loss was caused by abiotic or biotic factors due to the variability seen in control samples. However, Oxazepam underwent statistically significant losses (~80% total) within 60 days due to both abiotic and biotic factors. Approximately half of this loss was due to the biotransformation of keto-Oxazepam to a metabolite proposed to be the 2- or 3- enol tautomer of Nordiazepam on the basis of basification experiments and detailed multistage MS analysis.

The variability seen in the behaviours of the different 1,4-benzodiazepines maybe partly due to differences in functional groups (Figure 4.35). The results suggest that the presence of a C3 hydroxyl group, present in both Temazepam and Oxazepam, resulted in abiotic losses possibly due to increased polarity compared to Diazepam (Log P: Diazepam = 2.988, Temazepam = 2.479, Oxazepam = 2.34, as predicted by US EPA modeling suite Bcfwin V2.15) and

therefore more sorption. This functionality was recently related to the rate of photodegradation of Temazepam and Oxazepam due to sorption to aquatic humic substances (West, 2007). The presence of this hydroxyl group may also aid enolisation, therefore allowing formation of more polar compounds and increasing sorption further.

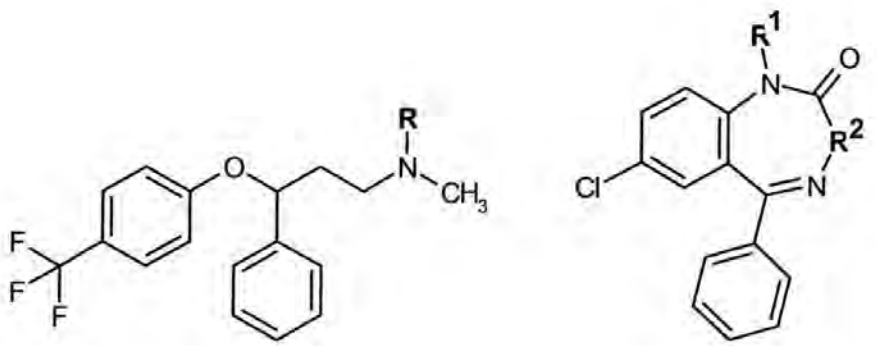


Figure 4.35. Chemical Structures of Target Compounds: Fluoxetine, Diazepam and their major Human Metabolites

Fluoxetine (R = H), Norfluoxetine (R = CH_3), Diazepam (R1 = CH_3, R2 = CH_2), Temazepam (R1 = CH_3, R2 = OH), Oxazepam (R1 = H, R2 = OH) and Nordiazepam (R1 = H, R2 = CH_2).

Since the commencement of this project this research field has developed significantly and more evidence of the presence of these compounds in the environment has been published. There have been further findings of Diazepam (Carlsson *et al.*, 2006), Fluoxetine and Norfluoxetine (Furlong, 2007) in the environment including in soil (Kinney *et al.*, 2006) and bioaccumulating in fish (Brooks *et al.*, 2005). There have also been a few findings regarding losses of these compounds under environmentally relevant conditions. Fluoxetine has been shown to photodegrade forming three products (Lam *et al.*, 2005) and has been shown to have a biphasic nature of dissipation within the water column (Loffler *et al.*, 2005). No environmentally relevant literature on the biodegradation of Fluoxetine or Norfluoxetine under field conditions was found. For the 1,4-benzodiazepines, literature on the dissipation nature of Diazepam

and Oxazepam in the water column has been published. Diazepam was found to be highly persistent and Oxazepam as moderately persistent with limited sediment sorption tendencies (Loffler *et al.*, 2005). There is still a substantial lack of knowledge about the fate of many pharmaceuticals within the environment, especially under conditions found in the disposal of sewage sludge and the data presented herein help to bridge this deficiency somewhat.

Considering the lack of biodegradation and the biotransformation of only one of these compounds to another suspected bioactive agent (Nordiazepam tautomer) under environmentally relevant conditions, alongside the possible risk of benzodiazepine sorption to soil and biannual SS application, there is potential for accumulation of these target compounds within agricultural soils. Future research into the fate and transport of these compounds should therefore focus around potential terrestrial ecotoxicity and bioaccumulation. The potential for plant uptake may lead to entrance into the food chain. Sorption data for pharmaceuticals in the environment have become more common place (Ternes, 2004; Kreuzig *et al.*, 2003; Kinney *et al.*, 2006; Brooks *et al.*, 2003; Jjemba, 2006), but there is a lack of terrestrial ecotoxicology data for Diazepam, and only limited research has been performed on Fluoxetine (Fong, 2001). The possibility of uptake of pharmaceuticals into agricultural crops and subsequence entrance into the food chain has not featured in the literature to date, and an attempt was made herein to address this issue (Chapter 5).

CHAPTER FIVE

UPTAKE OF PHARMACEUTICALS BY CROP PLANTS

5. Uptake of Pharmaceuticals by Crop Plants

5.1. Introduction

The study of pharmaceuticals as environmental contaminants is still an emerging field and such research as has been conducted in this area has been focussed mainly around monitoring, biodegradation and sorption studies. There appear to be no publications regarding the exposure of terrestrial plants to pharmaceuticals, although one study of the phytotoxicity of Fluoxetine to *Lemna gibba*, (aquatic duck weed), has been reported (Brain *et al.*, 2004). That study showed no evidence of phytotoxic impacts of Fluoxetine upon the measured endpoints.

However, some relevant information regarding the fate of xenobiotics other than pharmaceuticals can be found in studies of pesticides, herbicides and other organic compounds on plants.

Organic compounds can enter plants *via* a range of pathways (Hellstrom, 2004). Only uptake from soil is considered herein.

5.2. Plant uptake of xenobiotics from soil

Plant uptake of a compound depends upon both the specific properties of the compound and on the prevailing environmental conditions; factors such as the plant species, soil organic carbon content, temperature and many others. Compounds that are taken up into plants and followed by metabolism tend to form polar carbohydrate or amino acid conjugates. These conjugates, along with their parent compounds, are commonly stored *via* binding to cell wall components (Harms, 1996).

Uptake and translocation of xenobiotics within plants involves a complex series of processes, potentially involving compound-specific passive and active processes. Prior to uptake from soil, compounds will partition between the soil particles, interstitial water and interstitial air, depending upon *their individual* physio-chemical properties, which in turn determine their further transport into the plant. The extent of compound sorption to the soil is often the major limiting factor in availability for uptake. Sorption effects have been classified as: strongly sorbed $K_d > 10$ if $K_{ow} > 4$ or moderately sorbed K_d 1 – 10 if K_{ow} 2 – 4, for soils with OC 1 – 5% (Hellstrom, 2004). Generally, passive diffuse transport processes are responsible for the movement of organic chemicals from the soil into root systems. In the case of non-ionised compounds this process consists of two stages; the first is equilibration of the aqueous phase in the root with the surrounding soil water, which is then followed by sorption onto lipophilic root solids (e.g. lipids in cell walls (*Collins et al.*, 2006)).

Once compounds have entered the root system three potential pathways may be followed; either through the cell walls (apoplastic), *via* the plasmodesmata (symplastic) or from vacuole to vacuole (transcellular). Apoplastic transport allows compounds to move without entering cells until they reach the endodermis, where active transport is required for hydrophobic compounds. Active transport of anthropogenic contaminants has only been seen for hormone-like chemicals (Hellstrom, 2004). The relative solubility of the compound in water and the lipid-rich cell membranes, as well as diffusion, then determine the subsequent fate in passive transport from root to shoot. Compound hydrophobicity controls further transport and determines partitioning to solid structures, thereby limiting long distance transport. This also determines

the ability of a compound to move across the membranes. For non-ionised compounds, partitioning to the stem has been shown to have a linear relationship with K_{ow} (Collins *et al.*, 2006).

Most studies into the uptake of hydrophobic organic compounds from soil indicate that such compounds (e.g. PCB, DDT), tend not to translocate any further than the root system. This is thought to be due to binding to lipid-type components within the root. These compounds are therefore more at risk of entering the human food chain when edible roots and tubers are contaminated (e.g. carrots (Hellstrom, 2004)).

One of the major differences between plant and animal metabolism is that animals tend to excrete metabolites, whereas in plants they are stored. In plants oxidation is the most commonly seen metabolic process, although reduction and hydrolytic processes are also known. Conjugation in plants will either form soluble conjugates (e.g. glucoside, glutathion, amino acid, malonyl) or insoluble or bound conjugates by incorporation into biomolecules. For example, hydroxyl, carboxyl, amino or sulfhydroxyl aromatics tend to be incorporated into lignin or other cell wall components (Hellstrom, 2004). Lipophilic compounds tend to partition into leaves, where they are stored in two different compartments; a large reservoir with relatively slow deposition and a small surface compartment with faster uptake and clearance rates (Collins *et al.*, 2006).

5.3. Experimental design of uptake studies

O'Connor (1996) discusses and reviews the range of different experimental designs used to study the uptake of organic compounds from sludge-amended soils, including hydroponic studies and field experiments. Each of these different experiment types are designed to provide data for specific interpretations such as for mechanistic studies, compound structure behaviour models, worst case scenario exposures and solute transport models. It is necessary to design plant uptake studies with specific aims because of the wide range of factors which can influence both compound and plant behaviour. For example, Harms (1996) discusses experiments designed to study metabolism pathways of anthropogenic chemicals. A study of 4-chloroaniline, at two concentrations (0.5 and 1 ppm), allowed the elucidation of the system of storage of these metabolites. In this case 4-chloroaniline was first transformed to sugar conjugates which were stored in leaf vacuoles, and once this capacity was exceeded excess 4-chloroaniline became incorporated into the cell wall structures. In contrast, anthracene metabolites from soybean cell suspensions underwent various degrees of association with cell wall components and transferred back to the nutrient solution. Studies of phenanthrene metabolism showed formation of up to three metabolites depending upon the plant culture; barley, wheat, soybean and carrot. The extraction and clean-up procedures for organic compounds in plant tissues tend to involve the use of LLE or ASE, followed by column adsorption chromatography. Extensive reviews regarding both extraction and analysis have been written by Motohashi *et al.*, (1996), Chen and Wang (1996) and Tekel and Hatrik (1996), and so will not be discussed in any further detail here. Table 5.1 provides a summary of methods for some example experiments from the literature.

Plant(s)	Compound	Exposure procedure	Extraction	Clean-up	Analysis	Results	Ref
Wheat	DDT	Transplanted into contaminated soil	ASE (*n*-hexane : acetone)	Sulfonication & Florisil column	GC-ECD	Concentration in aerial parts < roots	(Tao *et al.*, 2004b)
Zucchini	Chlordane	Transplanted into contaminated soil	LLE (petroleum ether : IPA)	Florisil column	GC-MS	Concentration highest in roots & declines up xylem. Enantiomer ratios also altered	(White *et al.*, 2002)
Soybean, wheat, corn, alfalfa, bromegrass, cucumber	DDT, Dieldrin, Endrin & Heptachlor	Transplanted into 5 different contaminated soils	Soxhlet (chloroform : MeOH, 12 hrs), transfer to ACN and then petroleum ether	Florisil column	TLC radioautograph & GLC	Concentration of all 4 compounds; wheat > soybean seedlings. Endrin concentration in bromegrass & alfalfa > soil	(Beall and Nash, 1969)
Various vegetables including a range of *Brassicas*	16 PAHs	Environmental / agricultural samples	ASE (*n*-hexane : conc sulphuric acid with 2% $NaSO_4$	Sulfonication & Silica gel column	GC-MS	Concentration highest in cauliflowers. Predominantly foliar uptake, therefore suggesting aerial transport pathway	(Tao *et al.*, 2004a)
Carrots, potatoes, peas, cucumbers, tomatoes, cabbage, beets, radishes, ratabaga	DDT, Aldrin & Dieldrin	Grown in soils 1, 2 or 3 years after insecticide application	LLE (IPA for DDT; IPA + hexane for Aldrin & Dieldrin)	For DDT; Aluminium oxide column, carrot samples required additional clean with Nuchar & Celite mix). For Aldrin & Dieldrin; Florisil column, Aldrin exposed carrot samples require additional Altasol Celite column	No details given	Concentration of all compounds was highest in carrots, and lindane was found at concentrations > soil	(Lichtenstein, 1959)

Table 5.1. Methodologies from literature for studies into the uptake of xenobiotic compounds by plants

5.4. Selection of pharmaceuticals for uptake studies

Xenobiotic compounds with log K_{ow} 2 - 5 within the soil system are most likely to undergo plant uptake and moderate sorption. The risk with highly water soluble compounds (log K_{ow} < 2) is that they will leach out of the soil system and into surrounding waterways, whereas hydrophobic compounds (log K_{ow} ≥ 5) are more likely to be strongly retained on soil particles (O'Connor, 1996).

Although, as stated above, few pharmaceuticals have been studied in this respect, based on a pH-dependent Kow of between 1 – 4.7, (Table A.2). Fluoxetine HCl might be predicted to undergo plant uptake, which is of high significance in the SS disposal route to land and subsequent compound fate, particularly since the compound appears to be resistant to biodegradation in soils (Chapter 4). For a compound to undergo plant uptake it must be stable within the soil system for a sufficiently long period ($t_{1/2}$ > 14 days; (O'Connor, 1996)). The results of the long term soil biodegradation study performed herein (Chapter 4,) showed that Fluoxetine HCl was stable in soil for at least 270 days.

5.5. Selection of plants for uptake studies

As can been seen from Table 5.1, a wide range of plants has been used to study the uptake of organic compounds from soils. Selection of an appropriate test plant species is essential for results to be applicable to real world environments. The current project was orientated towards environmental conditions prevailing from the disposal of pharmaceuticals via SS disposal in the SW of England. Therefore it was felt appropriate to select a common crop grown locally in the SW as a test species. Cauliflower (*Brassica oleracea*) is an important economic vegetable crop in the UK. The main growing regions of UK

cauliflowers, production rates and income are shown in Figure 5.1 and Figure 5.2. In 2005 more than 130,000 tonnes of the worldwide 11,000,000 tonnes of cauliflower was produced in the UK with a commercial value of around £50,000.

Figure 5.1. UK cauliflower growing regions

Grey areas (■) show major cauliflower growth regions
Source: Living Countryside (www.ukagriculture.com; accessed 2007)

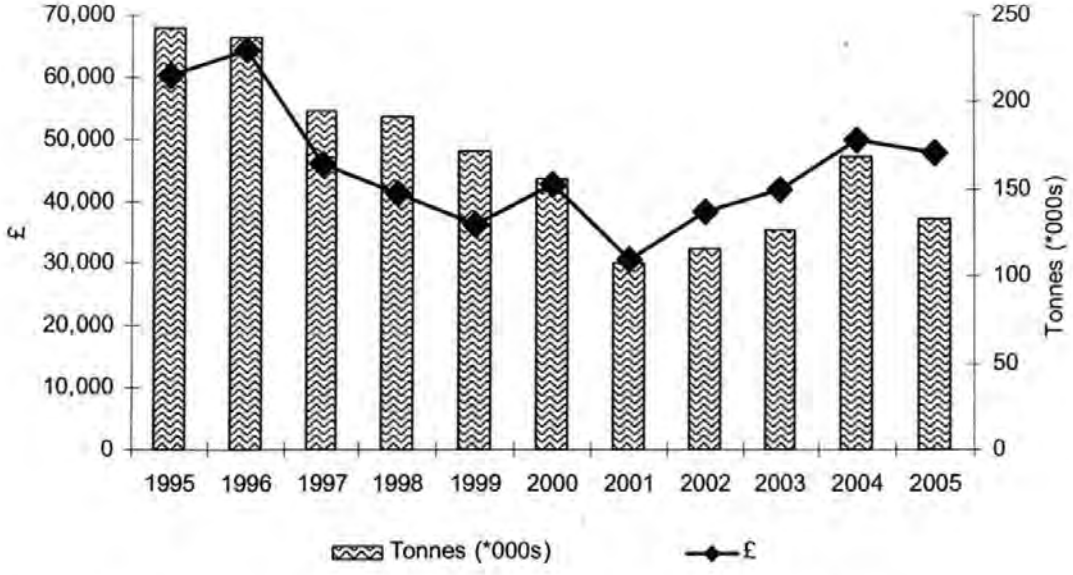


Figure 5.2. UK annual cauliflower production and market value

Data source: Defra (http://statistics.defra.gov.uk; accessed 2007)

5.6. Plant Uptake Models

A range of plant uptake models can be found in literature, such as the simple three compartment fugacity model for herbaceous plants developed by Hung and Mackay (1997). Many models are able to generate concentration factors for different plant tissues, which have been related to chemical constants of the target analyte in question. Zebrowski *et al.*, (2004) found that the maximum transpiration stream concentration factor, which demonstrates compound translocation from root to shoot, occurred at log K_{ow} 1.78 for pesticides.

5.7. Aims of current study

Literature searches did not reveal any studies or equivalent models for the uptake of pharmaceuticals, and therefore few predictions regarding Fluoxetine behaviour could be made. Xenobiotic uptake has in the past been shown to involve a complex system of interlinking processes and factors, as discussed previously. Therefore for this preliminary study it was felt important to limit many of the environmental factors which may influence uptake and interpretation of results. Since the current work is believed to be the first example of a study into the plant uptake of pharmaceuticals it was felt that a simple preliminary laboratory study was sufficient.

The primary aim of this experimental work was to ascertain whether cauliflower tissue cultures (T/Cs) were able to uptake Fluoxetine under laboratory conditions. Qualitative and qualitative data regarding the transport of Fluoxetine within the T/C were to be generated if uptake occurred.

5.8. Cauliflower Tissue Culture exposure to Fluoxetine HCl

5.8.1. Experimental Procedure

5.8.1.1. Lipid Extraction and Quantification

The established Bligh and Dyer (1959) method for the extraction, purification and quantification of lipids was modified for use with cauliflower T/C stem and leaf samples. The Bligh and Dyer method is designed for use with 100 g samples containing ~ 80 ± 1% water and ~ 1% lipid. However cauliflower T/C sub-samples are significantly lower in mass than this, and the cauliflower florets have a mean moisture content of ~ 91% and a lipid content of ~ 0.4% wet weight (Baardseth, 1977). Solvent volumes were therefore altered to take account of the different tissue masses and average water content.

Cauliflower T/C as described in Section 5.8.1, were used as the sample source for this lipid work. The following method applies to a tissue mass of 1 g, therefore solvent volumes were altered to take account of the different masses of each of the 5 stem and 6 leaf samples. After sectioning the T/C into leaf and stem samples, they were homogenised using a pestle and mortar, transferred to a vial and the wet weight recorded.

Chloroform and MeOH (1.1375 and 2.275 mL g^{-1}) were then added to vials, to give a chloroform : MeOH : H_2O of 1 : 2 : 0.8, and shaken for two minutes. Additional chloroform was then added (1.1375 ml g^{-1}) and shaken for 30 seconds, followed by Milli-Q (1.1375 ml g^{-1}) and an additional 30 seconds shaking (chloroform : MeOH : H_2O of 2 : 2 : 1.8). A Büchner funnel lined with filter paper (Whatman No 1) operated with slight suction was used to filter and transfer extracts to clean graduated vials. The volume of the chloroform layer,

which contained the lipids, was then recorded after complete separation and clarification. An aliquot (typically 500 µL) of the chloroform layer was then transferred to pre-weighed vial, and dried using a gentle stream of nitrogen at 40°C, prior to re-weighing. To confirm that all non-lipid material had been removed chloroform (~ 300 µL) was added to each dry vial to ensure that no insoluble material remained. The lipid content of each sample was then calculated (Total lipid mass = (weight of lipid in aliquot x volume of chloroform layer) ÷ volume of aliquot). This was then used to determine average lipid contents for leaves and stems which was in turn used to calculate lipid quantities in exposed T/C samples.

5.8.1.2. Preparation of Cauliflower Growth Medium

The totipotent capacity of many plant cells lies at the basis of cell culture work. To take advantage of this capacity and to create clones from apical meristems, the growth medium for tissue culture must contain all the nutrients required, a carbon source, agar to solidify the medium and growth regulators, such as the plant hormone indole-3-acetic acid (IAA) and kinetin which promotes cell division. Murashige and Skoog (1962) medium which was adapted for use with cauliflower floral meristems (50% strength with 8 g L^{-1} agar, 20 g L^{-1} sucrose, IAA 0.1 mg L^{-1}, kinetin 3.8 mg L^{-1}, adjusted to pH 5.8 with KOH) was used for the present work. Details of macronutrients that constitute Murashige and Skoog medium are given in Table A.12.

Media were sterilised by autoclaving (120°C, 20 min) and then placed in a steamer (110°C for 2 hours) to liquefy. Prior to pouring, the medium was spiked with Fluoxetine HCl in MeOH (140 µg 500 mL^{-1} of medium) and stirred with a magnetic flea. Medium (35 mL) was then poured into plastic pots with lids and

allowed to set in a laminar flow hood, resulting in a final concentration of 9.8 µg pot^{-1}. This spiking concentration was selected based upon the expected concentration in an equivalent volume of SS amended soil (justification of spiking calculations is given in Figure A.8). Equivalent blank pots, spiked with only MeOH, were also prepared.

5.8.1.3. Tissue Culturing

A cauliflower curd (Organic, Marks and Spencer Class 1) was aseptically dissected into approximately 1g curd meristem sections (~ 4 – 5 mm diameter at base). Each explant was then weighed and the weight recorded. Explants were washed in 100% ethanol before being placed into beakers containing bleach solution (10% Marks and Spencer thick bleach with limescale control in deionised water) and a few drops of Tween (wetting agent) for sterilisation and shaken on a rotary table (5 mins).

Tissue culturing was performed in a laminar flow hood where explants were removed from the bleach using forceps (heat sterilised), rinsed with deionised water and dried on non-fibrous tissue paper. These were then transplanted to Murashige and Skoog medium in airtight plastic pots. All pots were then placed into a growth cabinet at 20°C ± 2°C with a 12 hour light / dark cycle (cool-white fluorescent light source; photosynthetic photon flux 130 ± 10 µmol m^{-2} s^{-1}). During the growth period the cauliflower plantlets were checked regularly for contamination or death and any infected samples were removed from the growth cabinet immediately. After 12 weeks growth all samples were stored at -80°C.

5.8.1.4. Harvest

Samples for harvest were selected at random (5 blank and 5 spiked) from the freezer. The frozen plants were then cut into sections using a scalpel. Meanwhile media and roots were allowed to defrost at room temperature before separating the roots from the medium. The T/C were sectioned to create sub-samples; curd, leaves, stem (including internode, node, stem and petiole), roots and media (Figure 5.3). All sub-samples were then washed in extraction solvent, and the washings added to the corresponding medium sample before weighing each sub-sample.

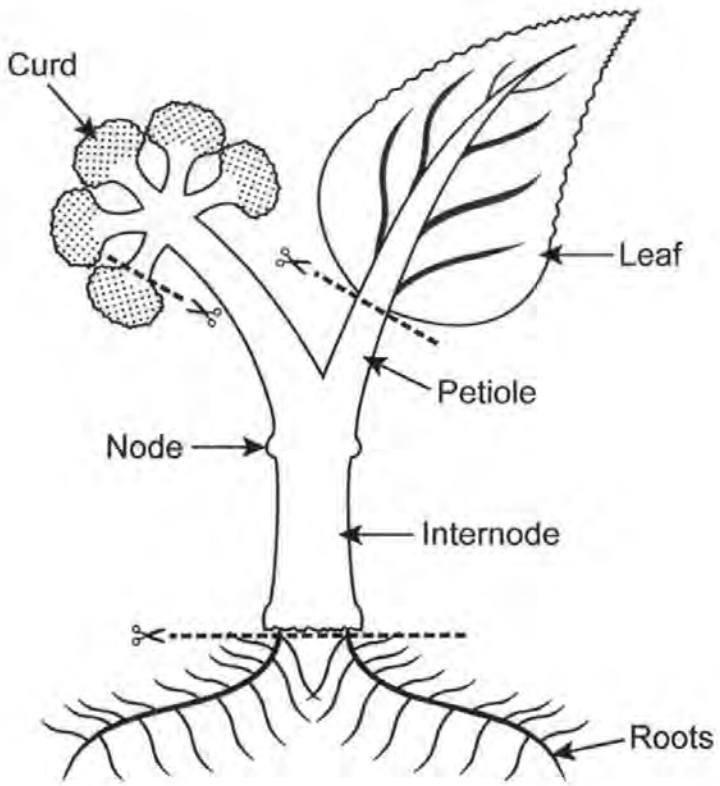


Figure 5.3. Sub-sample sectioning of cauliflower tissue cultures

5.8.2. Sample Preparation

For the extraction of Fluoxetine from cauliflower T/C the method presented in Section 3.5.3, which includes details on sample preparation, extraction, tandem SPE clean-up and reconstitution, was used.

5.8.3. Analysis

Analysis was performed using HPLC-ESI(+)MS^n. Samples were initially analysed using SIM for Fluoxetine (*m/z* 310.0) and d_5-Fluoxetine (*m/z* 315.0), for the generation of quantitative data. SRM was then used to confirm the identity of peaks with full MS *m/z* 310.0 as Fluoxetine (*m/z* transition: 310.0 to 147.9). A ratio calibration series was also analysed using SIM to allow the subsequent development of quantitative data. Full details of MS analysis, HPLC conditions and calibration are given in Chapter 2, Sections 2.4.1.2, 2.4.2.2 and 2.6.

5.8.4. Results and Discussion

5.8.4.1. Growth Rates

Cauliflower tissue masses were recorded prior to tissue culture and at harvest so that growth masses (Table 5.2) could be determined and any potential phytotoxic effects identified. Results of statistical analyses using F-test and t-tests, showed no significant differences in either the means or variance, between spiked and blank sample sets for the whole plant or individual sub-samples at the 95% confidence interval. Thus there was no evidence of phytotoxic impacts upon the growth masses of cauliflower T/C exposed to Fluoxetine HCl.

		Harvest Mass (g)						
	Sample	Root	Stem	Leaf	Curd	Total	Original T/C section mass (g)	Growth mass (g)
Exposed Samples	**Mean**	**0.23**	**1.29**	**2.08**	**1.21**	**4.39**	**1.07**	**3.32**
	Standard Deviation	0.34	0.48	1.59	0.45	2.62	0.11	2.57
	% RSD	152	37	76	37	60	11	77
Blank Samples	**Mean**	**0.81**	**1.19**	**1.79**	**1.07**	**4.69**	**1.07**	**3.61**
	Standard Deviation	0.17	0.52	0.72	0.29	1.65	0.20	1.60
	% RSD	22	44	40	27	35	18	44

Table 5.2. Harvest and calculated growth data for Fluoxetine exposed and non-exposed cauliflower tissue cultures

5.8.4.2. Lipid Data

A modified version of the Bligh and Dyer (1959) method for the extraction, purification and quantification of lipids was used to generate quantitative data on the amounts of lipid in cauliflower T/C stem and leaf samples. Average lipid contents were found to be higher in leaves than stems (Table 5.3).

		Lipid concentration (mg g^{-1} wet weight)	Lipid concentration (% wet weight)
Stem (n = 5)	**Mean**	**1.69**	**0.17**
	Standard Deviation	0.92	0.09
	% RSD	54.6	54.6
Leaves (n = 6)	**Mean**	**4.15**	**0.42**
	Standard Deviation	1.63	0.16
	% RSD	39.2	39.2

5.3. Bligh and Dyer extracted lipid concentrations for cauliflower tissue culture leaf and stem sub-samples

5.8.4.3. HPLC-ESI-MS^n Qualitative and Quantitative Data

Extracts of cauliflower T/C obtained by the method presented in Section 3.5.3, which included tandem SPE clean-up followed by reconstitution, were examined by HPLC-ESI-MS in positive ion mode. Example extracted ion chromatograms and mass spectra can be seen in Figures 5.4 to 5.8. Peaks in the extracted ion chromatograms that were positively identified as Fluoxetine by SRM (*m/z* transition 310 to 147.9) were integrated and these data used in ratio calibration calculations to obtain quantitative data on uptake. Details of the calibration method are given in Chapter 2, Section 2.5.2.

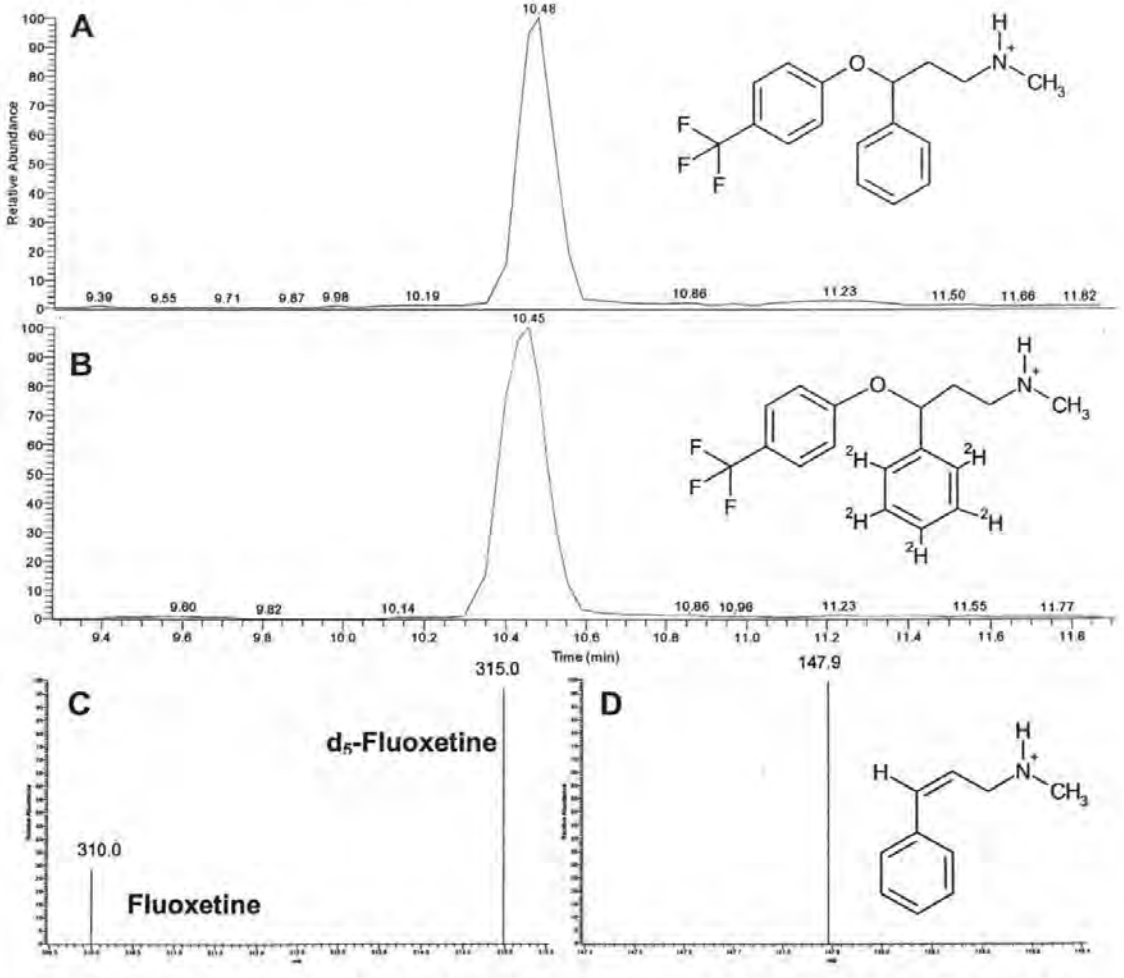


Figure 5.4. Example HPLC-ESI-MS^n extracted ion chromatograms and mass spectra for Fluoxetine-exposed cauliflower tissue culture (Sample 28, leaf sub-sample)

A – SIM *m/z* 310 (Fluoxetine full MS $[M+H]^+$)
B – SIM *m/z* 315 (d_5-Fluoxetine full MS $[M+H]^+$)
C – Full MS spectra of Fluoxetine and IS (from SIM analysis)
D – MS^2 spectra of Fluoxetine (*m/z* transition 310 < 148; from SRM analysis)

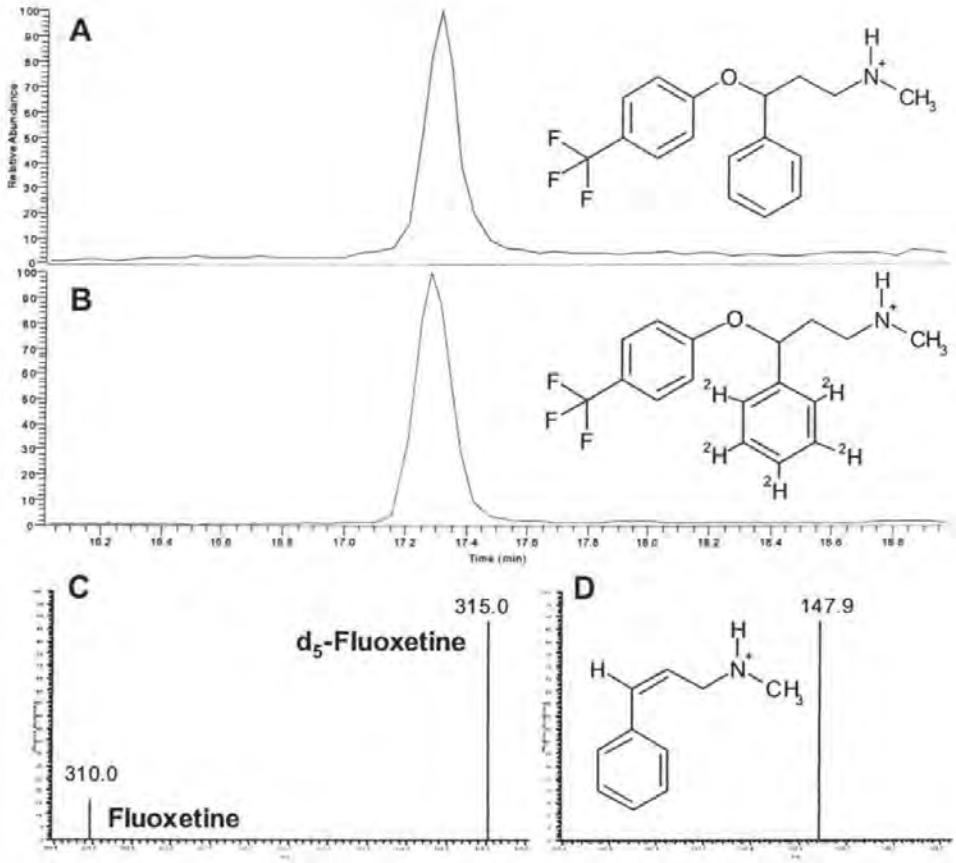


Figure 5.5. Example HPLC-ESI-MS^n extracted ion chromatograms and mass spectra for Fluoxetine-exposed cauliflower tissue culture (Sample 28, media sub-sample)

A – SIM *m/z* 310 (Fluoxetine full MS $[M+H]^+$)
B – SIM *m/z* 315 (d_5-Fluoxetine full MS $[M+H]^+$)
C – Full MS spectra of Fluoxetine and IS (from SIM analysis)
D – MS^2 spectra of Fluoxetine (*m/z* transition 310 < 148; from SRM analysis)

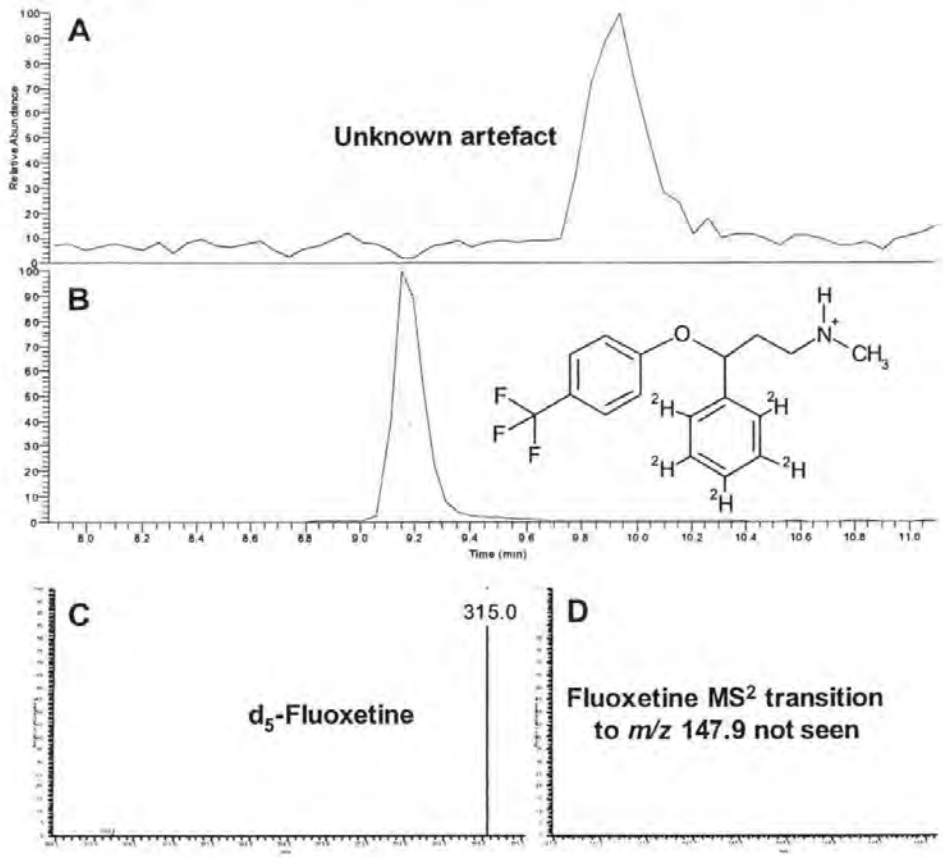


Figure 5.6. Example HPLC-ESI-MS^n extracted ion chromatograms and mass spectra for Fluoxetine-exposed cauliflower tissue culture (Sample 28, root sub-sample)

A – SIM *m/z* 310; Fluoxetine not seen
B – SIM *m/z* 315 (d_5-Fluoxetine full MS $[M+H]^+$)
C – Full MS spectra of IS; Fluoxetine not seen (from SIM analysis)
D – Fluoxetine MS^2 transition spectra (*m/z* transition 310 < 148; from SRM analysis)

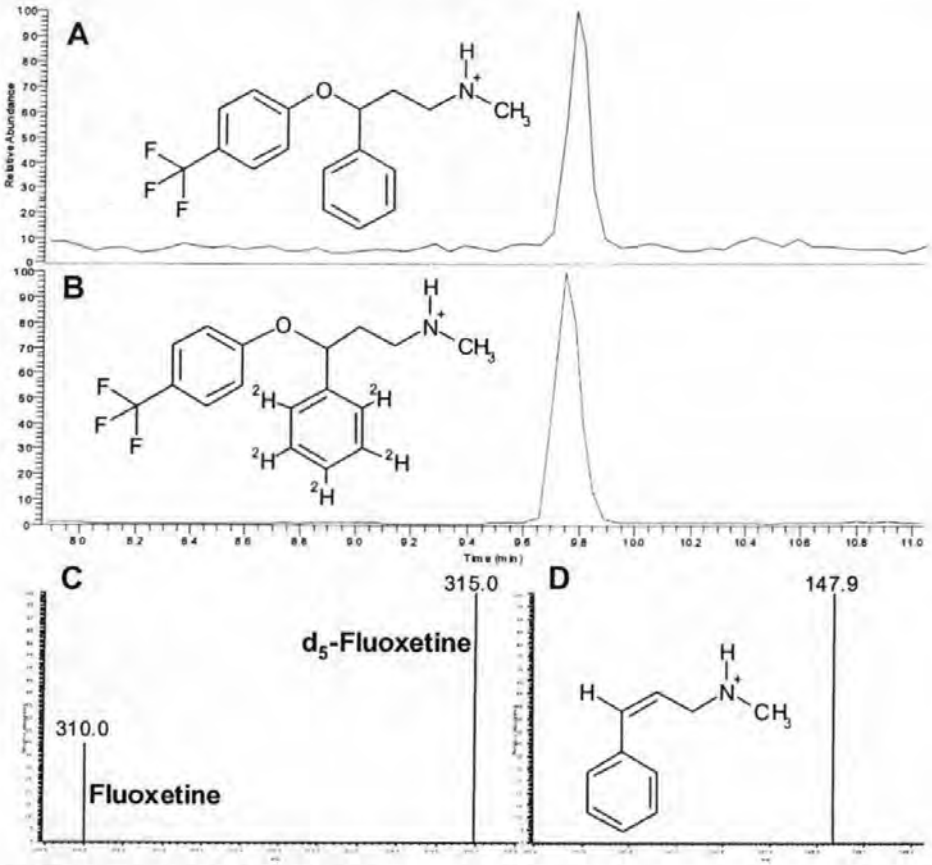


Figure 5.7. Example HPLC-ESI-MS^n extracted ion chromatograms and mass spectra for Fluoxetine-exposed cauliflower tissue culture (Sample 28, stem sub-sample)

A – SIM *m/z* 310 (Fluoxetine full MS $[M+H]^+$)
B – SIM *m/z* 315 (d_5-Fluoxetine full MS $[M+H]^+$)
C – Full MS spectra of Fluoxetine and IS (from SIM analysis)
D – MS^2 spectra of Fluoxetine (*m/z* transition 310 < 148; from SRM analysis)

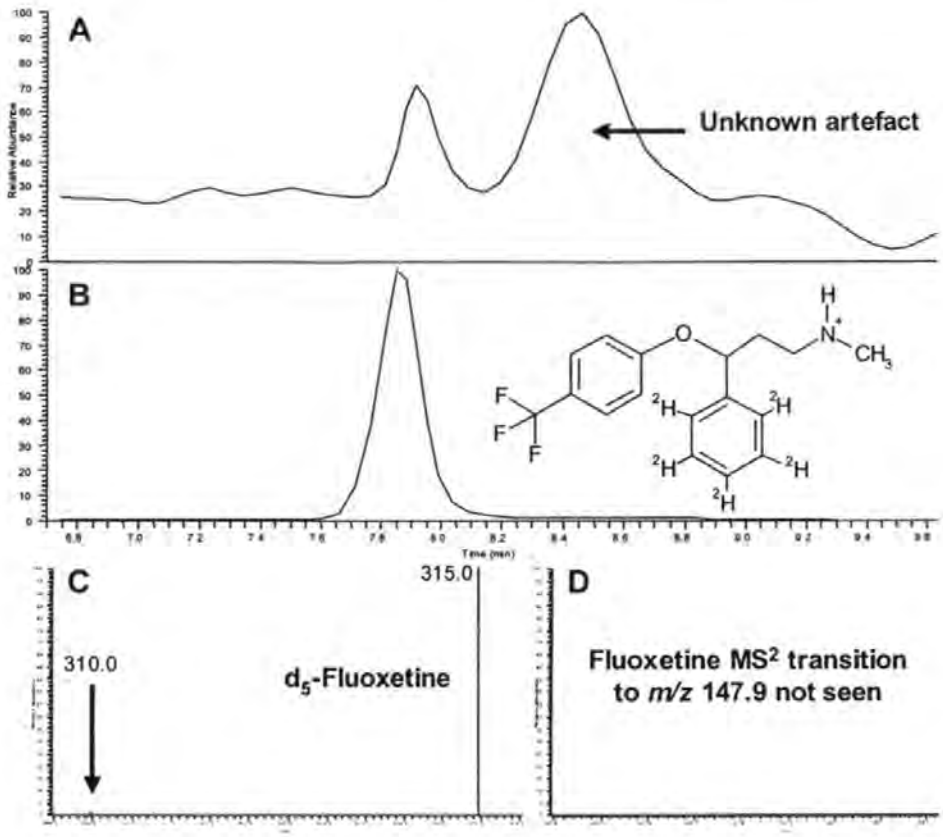


Figure 5.8. Example HPLC-ESI-MS^n extracted ion chromatograms and mass spectra for Fluoxetine-exposed cauliflower tissue culture (Sample 28, curd sub-sample)

A – SIM *m/z* 310; (Fluoxetine full MS $[M+H]^+$)
B – SIM *m/z* 315 (d_5-Fluoxetine full MS $[M+H]^+$)
C – Full MS spectra of IS and Fluoxetine; < 3% relative abundancey of *m/z* 310 (SIM analysis)
D – Fluoxetine MS^2 transition spectra (*m/z* transition 310 < 148; from SRM analysis)

Table 5.4 and Figure 5.9 summarise data for the uptake of Fluoxetine into Cauliflower T/Cs. Positive identification of Fluoxetine in all media and stem samples was achieved, and from 2 of the 4 leaf samples. As expected, the majority of Fluoxetine was found to remain in the medium (20%). The confirmed presence of the target compound Fluoxetine in all of the stems (mean 5 ± 2.4% of applied burden; n=5) and some of the leaves (mean 3 ± 3.5% of applied burden; n=4) indicate that xenobiotic transport did occur under these experimental conditions in some of the experimental plants. However it is clear that uptake to the leaves was not uniform in all experimental samples. No uptake to the curd was detected and neither was Fluoxetine detected in the roots.

	Positive identification	range (%*)	Mean Uptake (%*)	Standard deviation	Concentration (µg g^{-1} wet weight)	Concentration (µg mg^{-1} lipid)
Leaves	2 from 4	0 - 7	3	3.5	0.26	0.03
Curd	0 from 5					
Stem	5 from 5	3 - 8	5	2.4	0.49	0.29
Roots	0 from 4					
Media	5 from 5	5 - 50	23	20.3	n/a	n/a

Table 5.4. Summary of Fluoxetine Uptake: with regard to sub-sample type

* Percentage of total 9.8 µg Fluoxetine added to each growth pot
n/a =not applicable

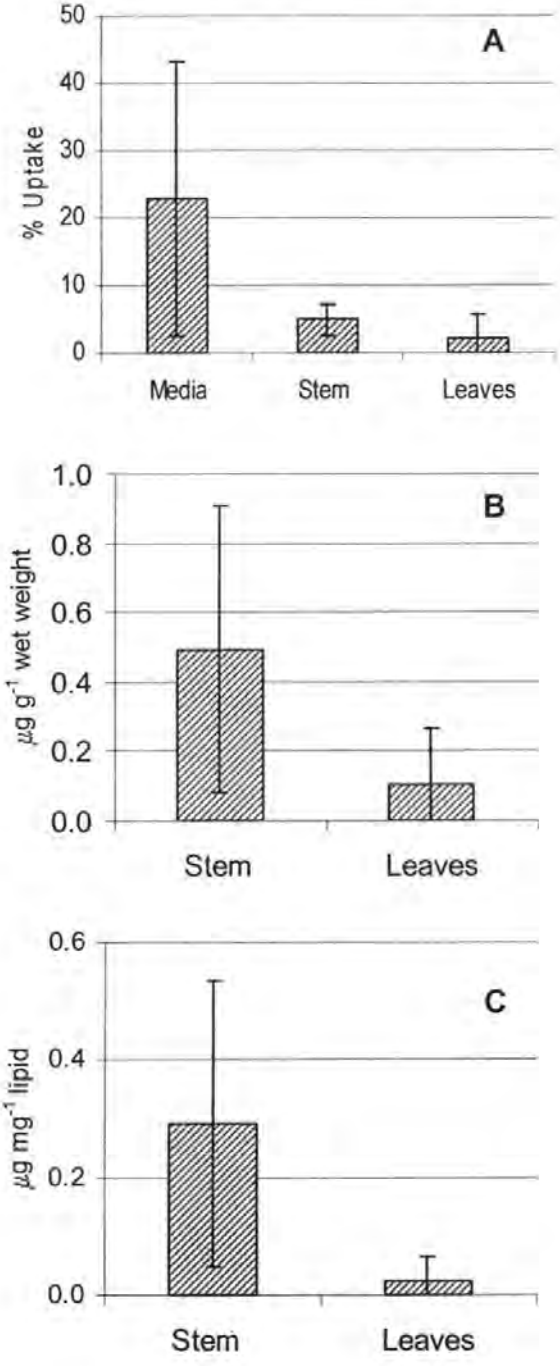


Figure 5.9. Summary of Fluoxetine Uptake: with regard to sub-sample type (Media, stem and leaves)

A. Percentage uptake of initial Fluoxetine added (9.8 µg growth pot^{-1})
B. µg Fluoxetine per gram of wet cauliflower tissue
C. µg Fluoxetine per milligram of lipid, for relevant tissue type
For media and stem samples n = 5 and n = 4 for leaf samples.

Expression of the data in terms of μg g^{-1} wet weight and μg mg^{-1} lipid removes some of the variability between data sets due to differences in tissue mass and lipid content between tissue types. The data show that mean Fluoxetine concentrations in the stems (0.49 μg g^{-1} wet weight; 0.29 μg mg^{-1} lipid) were considerably higher than those in the leaves (0.13 μg mg^{-1} wet weight; 0.03 μg g^{-1} lipid) although these differences were not statistically significant at the 95% confidence interval (t-test P-value = 0.08, 0.07, 0.17 for μg g^{-1}, μg mg^{-1} and % data respectively). Although the average Fluoxetine concentrations were higher in the stem than the leaf samples, the average lipid concentrations were significantly higher in the leaf samples (stem 1.69; leaf 4.15 mg g^{-1} wet weight; t-test P value = 0.02). Thus Fluoxetine concentration does not appear to be directly associated with lipid contents in the plants. It is also possible that more extensive uptake to the leaves did occur, but that the extraction method was unable to recover Fluoxetine that was tightly bound to these lipids.

These data, in combination with positive identification of Fluoxetine in only 2 of the 4 leaf samples, suggests perhaps that Fluoxetine was transported through the roots, to the stem and then onto leaves. This could be investigated further by performing the same experiment with a longer growth period and by sampling the plants at intermediate growth stages. Sampling at various growth stages would also provide more data regarding the connection between lipid content and xenobiotic storage to be investigated.

Fluoxetine was not detected in the roots of any of the plants, which is at first surprising if transport is *via* the roots to the stem. The most likely explanation for this is the root mass was so small and hence the LOD so high, that any

Fluoxetine present was below the LOD. It is also possible that Fluoxetine was 'stored' in the leaves and stems, whereas in the root system the passage of Fluoxetine may have been transient, resulting in concentrations below the LOD (Hellstrom, 2004; Collins *et al.*, 2006). Combining sub-samples of roots, or again extending the growth period so that more substantial root systems are developed might lead to the detection of Fluoxetine in the roots in future studies.

Consideration of the concentrations of Fluoxetine in individual tissue culture samples highlights considerable variability between the tissue culture clones (Figure 5.10). Total recovery of applied Fluoxetine, including Fluoxetine remaining in the medium, ranged from only ~ 15 to 57%, with an average of 30%. Thus an average loss of 70% of the originally spiked Fluoxetine had occurred.

It is highly likely that a proportion of these substantial losses were due to photodegradation. As discussed in Section 3.5.1, Fluoxetine is a photolabile substance with a measured half-life of 62 hours (West, 2007). The duration of the growth period in the present experiments (> 23 half-lives) would be more than sufficient to account for the observed losses. Plant metabolism and irreversible sorption to plant components may also have played a role (Zebrowski *et al.*, 2004). Consideration of only plant tissue sub-samples (i.e. excluding Fluoxetine remaining in the medium) showed uptake ranging from 2.7 to 10.5 % with an average of 8 %.

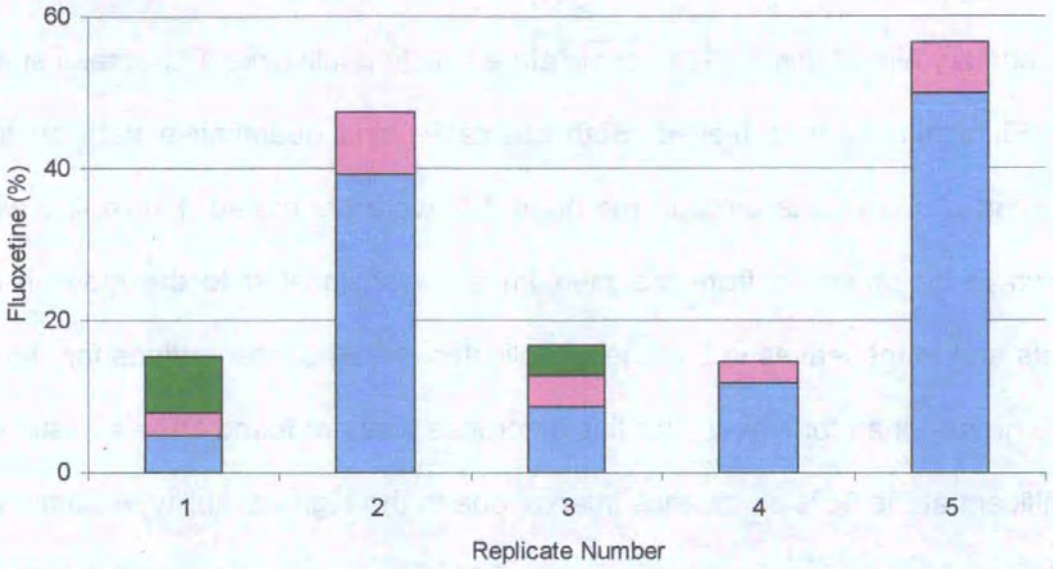


Figure 5.10. Summary of Fluoxetine uptake in tissue culture samples from individual plants

Percentage is expressed as % of total Fluoxetine added to each growth pot (9.8 µg)
(■ leaves; ■ stem; ■ medium)

The data also suggest that significantly higher quantities of Fluoxetine remained in the medium when uptake did not proceed as far as the leaves (Figure 5.10), with a larger proportion of Fluoxetine also remaining within the stems under these conditions. This provides support for transport from the medium, through the roots and into the stem and leaves. The data in Figure 5.10 also highlight the large losses of target compound when uptake to leaves did occur. These losses of Fluoxetine which appear to occur en route to the leaf may be due to plant metabolism (Zebrowski *et al.*, 2004).

5.9. Concluding Remarks

The primary aim of this work, to complete a simple cauliflower T/C uptake study with Fluoxetine, was achieved. Both qualitative and quantitative data on the transport of Fluoxetine through the plant T/C were generated. Fluoxetine was shown to be taken up from the medium and translocated to the stem in all plants and to the leaves in 2 of the 4 replicates. Mean concentrations for stems were greater than for leaves, but this difference was not found to be statistically significant at the 95% confidence interval due to the high variability. A summary of the uptake data is presented in Figure 5.11. These results suggest that there may potentially be a risk that Fluoxetine could be found in cauliflowers (and related *Brassica*; Chapter 3) grown on SS-amended soils due to plant uptake. However no evidence of uptake to the most commonly eaten part, the curd, was found and it should be emphasised that the plants were grown in artificial medium, not in soil. Uptake from soil may be the same or may differ significantly from the mechanism identified herein.

Growth data generated in this experiment indicated no significant phytotoxic impacts of Fluoxetine upon growth, as regards either total growth mass or the mass of different tissue types. Average lipid concentrations were found to be significantly higher in leaves (0.42 % wet weight) than stems (0.17 % wet weight), but there was no clear relationship between lipid concentrations and Fluoxetine concentrations. Leaf lipid data (0.42 % wet weight) were comparable with literature data for cauliflower florets stored at -85°C (0.4 % wet weight; (Baardseth, 1977)).

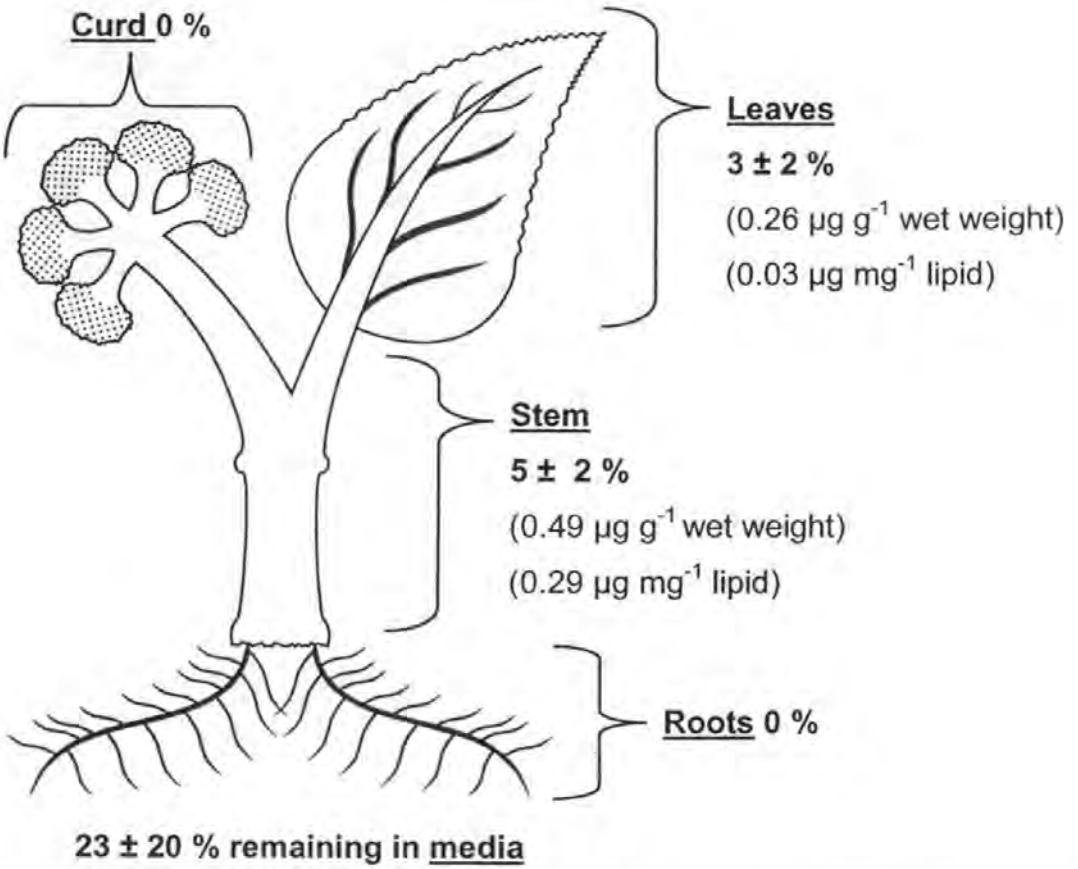


Figure 5.11. Summary of percentage Fluoxetine uptake into different tissues of cauliflower tissue culture samples

Data expressed is percentage of original amount of Fluoxetine HCl added to each pot (9.8 µg).

Conclusions drawn from this experimental work should be treated as preliminary due to the limited environmentally relevant conditions, notably the lack of soil. However these results do suggest that further research into the uptake of pharmaceuticals into crops will be of value, as discussed in Chapter 6.

CHAPTER SIX

CONCLUSIONS AND FUTURE WORK

6. Conclusions and Future Work

6.1. Findings and Conclusions of this project

This project, which involved an investigation into the transport and fate of selected generic pharmaceuticals in SS-amended soils, began with an extensive literature review to assess the then current state of knowledge and to aid in the selection of the target compounds. Selection criteria such as information on usage and ecotoxicty, were tested for a range of compounds, which resulted in the choice of Fluoxetine, Diazepam and their human metabolites as target analytes. Information gained from literature also allowed the calculation of PECss for Fluoxetine and Diazepam, which then ensured the use of environmentally relevant spiking / exposure concentrations for subsequent experimental work.

A method for the chromatographic separation of the target analytes, based on HPLC-UV, was developed to be used later for the analysis of samples from soil and water. An appropriate method for the separation of four target analytes (Fluoxetine, Diazepam, Temazepam and Oxazepam) was achieved using a Gemini C_{18}, 5µm, 15 x 2.1 mm i.d. column with formic acid (0.1%) modified eluents (Milli-Q & ACN) and gradient elution (20 to 100 % organic eluent over 10 mins).

All six target compounds were found to be compatible with analysis by infusion ESI(+)-MS, and CID mass spectral fragmentation pathways up to MS^4 were elucidated using optimised compound-specific parameters. Once the MS analysis conditions were optimised, modification of HPLC-UV chromatographic conditions for use with HPLC-ESI-MS^n was achieved (20 to 80% organic eluent

over 18 mins). The established mass spectral fragmentations, along with Rt data then allowed robust methods for the identification of the target analytes in later experiments to be established.

Due to the known limitations of ion trap mass spectrometers for the generation of quantitative data, calibration work involving ratio-calibration and matrix matching was performed. Ratio-calibration using deuterated internal standards was found to be suitable for the production of quantitative data for the target compounds when analysed using a Finnigan MAT LCQ™ quadrupole ion trap mass spectrometer. Matrix-matched calibration was used to investigate the phenomenon known as ion suppression particularly by the matrices resulting from extraction of plant material (specifically cauliflower tissues) on the response of Fluoxetine. Although the use of matrix-matched calibration standards revealed matrix interference to some extent in all samples tested, matrix matching was not found to produce a statistically significant advantage over the use of non-matched ratio calibration.

A simple extraction and clean-up method for four target compounds in water matrices was developed using Strata-X SPE cartridges, with recoveries ranging from ~ 70 to 100 % depending upon the compound in question. This extraction was then modified and optimised for use with soil matrices by the inclusion of liquid extraction (ACN with 1% formic acid) and filtration, prior to tandem SPE with Strata-X and Strata-SAX SPE cartridges (compound dependant recoveries ranged from 60 – 90%). The same extraction method was then examined for use with Fluoxetine-exposed cress and cauliflower T/C samples. Recoveries of ~ 28% (n = 3) for cress leaves were obtained, 71% (n = 3) for cress stems and

16 – 40% for cauliflower plant tissues (leaves > stem > curd > roots; n = 1) and 77% for T/C media. Unfortunately, due to project time constraints, it was not possible to further optimise the extraction conditions for plant tissues, but since deuterated internal standards were used and external calibration studies had been made, the recoveries were felt sufficient for a preliminary plant-uptake experiment; the first of its kind. The developed extraction methods were used for the extraction and clean-up of target analytes in all subsequent experiments.

Two types of biodegradation experiments were completed; 60 day simple shake flask experiments with Fluoxetine, Diazepam and their major human metabolites (Norfluoxetine, Temazepam and Oxazepam), and a 270 day simulated agricultural field soil study with Fluoxetine. Simple plating techniques and determination of the concentration and distributions of bacterial membrane marker chemicals (BHPs) for the test soil and a representative range of UK SS-amended soils, established that the microbial populations were stable but fungal dominated. Under the test conditions, no statistically significant losses of the SSRIs were seen in either experiment type. The most persistent of the 1,4-benzodiazepines tested, Diazepam, underwent neither biotic or abiotic losses. The same was probably the case for Temzepam but results were so variable that it was not possible to ascertain whether partial or no losses occurred (0 – 20%). Oxazepam was the only 1,4-benzodiazepine to undergo significant losses, due to both biotic (~ 40%) and abiotic (~ 40%) factors. HPLC-ESI(+)-MS experiments revealed one of the biotic metabolites and tautomerism experiments, conducted with Nordiazepam and Oxazepam, provided in conjunction with deuterium exchange experiments and HPLC-ESI(+)-MS^n produced evidence to support the theory that the metabolite was an enol form of

Nordiazepam (either 2-enol or 3-enol forms). Some evidence was provided that presence of a C3 hydroxyl group in 1,4-benzodiazepines (e.g. Temazepam and Oxazepam) produced an increased tendency to undergo sorption to the soil.

To the authors knowledge the cauliflower T/C work completed in this project is the first example of a study of the uptake of a pharmaceutical by a crop plant. The aim of this work was therefore to provide initial background knowledge into plant uptake as a potential transport route in the environment. A simple laboratory study, which delimited many environmental factors, was conducted. Fluoxetine was selected as the target analyte after consideration of the predicted K_{ow} (1 – 4.7 pH dependent), which placed the pharmaceutical within the risk bracket for moderate sorption to soil and possible uptake by plants. Cauliflower was chosen as the test plant as it is grown commercially as a crop in SW England which was the model area from which soils were obtained for the biodegradation studies. Results indicated that Fluoxetine underwent uptake from media and translocation to the stem (5 %) and leaves (3%). No evidence of uptake in to the edible portion, the curd, was found.

6.2. Environmental Implications

The lack of substantial degradation of all target analytes except Oxazepam under simulated but realistic SS-amended soil conditions is of environmental concern, and indicates their likely persistent nature. Although Oxazepam did undergo significant biotic (and abiotic) losses, the metabolite formed under biotic influences was hypothesised to be another bioactive 1,4-benzodiazepine, either 2-enol or 3-enol Nordiazepam which is likely to be resistant to further change. With persistent compounds there is a potential for accumulation within environments such as field soil to which SS is regularly added both as a disposal mechanism and as a fertiliser. When compounds undergo accumulation the risk of transport to other environmental components becomes more likely. From field soils these may include potential exposure to flora and fauna and possible bioaccumulation in terrestrial organisms and plants, including crops grown on the SS-treated soils. Although statutory monitoring of pathogens and heavy metals in SS-amended soil is carried out, there are no such requirements in the UK for organic chemicals such as PPCPs.

The subsequent finding herein that Fluoxetine underwent uptake into cauliflower stem and leaves is thus potentially a significant finding and extends significantly current knowledge of the transport and fate of pharmaceuticals in the environment. Further plant uptake work must be completed before any major conclusions can be drawn, but the results highlight the need for further research. Should plant uptake of pharmaceuticals prove to be a common phenomenon, further contamination risks arising from this should be assessed. For example, heavily contaminated crops could pose a risk to terrestrial organisms including livestock, any birds that may feed upon crop grains or

berries, and the human food chain, but the concentrations are likely to be very low. Disposal of pharmaceuticals may need to be reconsidered and further attempts to minimise environmental contamination be made. In the case of plant uptake, not only the plant species but also the soil type and environmental factors e.g. soil moisture, pH, temperature, may influence uptake of xenobiotics. Regulation of disposal of SS to land would therefore need to incorporate a range of risk based factors including soil and crop type, to minimise uptake. Another method for minimising contamination would be to ensure removal at the STW (cf Ternes, 2006). However this research field is still emergent and not enough is known about the transport of pharmaceuticals in the environment, especially the terrestrial environment, and therefore the cost of reducing the contamination risk cannot be fully assessed at present.

6.3. Recommendations for Future Work

Research into the fate of PPCPs in aquatic environments has been substantially more commonplace than studies of PPCPs in terrestrial environments. Monitoring and degradation studies, especially for STWs have resulted in the accumulation of enough knowledge to be able to model the fate of many pharmaceuticals in these systems, and evidence of accumulation in aquatic species is beginning to appear in the literature (Brooks *et al.*, 2005). Equivalent literature for terrestrial environments is still sparse. As over 3000 pharmaceuticals are licensed for use in the UK it is not feasible to test each of these extensively and it is in these circumstances that modelling becomes a useful tool. However for a model to be accurate a broad database of compound behaviour under environmentally relevant conditions must exist for the model to be developed. Not enough is currently known about the transport and fate of pharmaceuticals in the terrestrial environment for predictive behaviour or fate models to be developed.

This project attempted to use environmentally realistic conditions where possible. For example the inocula used in all biodegradation studies were isolated from lime treated SS amended field soil, and in the case of soil biodegradation of Fluoxetine, realistic environmental temperature regimes were employed. Future work into the biodegradation of pharmaceuticals needs to be performed under such specific conditions to build a catalogue of data required to develop predictive models.

Repetition of the agricultural field soil simulation type test with 1,4-benzodiazepines as the target analytes would be of interest. This would allow

differences in the biotic degradation of Oxazepam to be assessed and compared to results from the liquid culture biodegradation study. The advantages of using the simulated soil biodegradation study are that the environmental conditions are more relevant than in the liquid culture studies. In the long term, soil fungal degradation should be studied as should the bioavailability of target compounds to soil microbes. Results generated from the study of 1,4-benzodiazepines would also generate additional data regarding sorption, and links between structure and behaviour may be elucidated, especially if the range of 1,4-benzodiazepines studied was extended.

This research field in general not only requires more monitoring data to be collected, but also further research into the terrestrial transport and fate of pharmaceuticals. Future terrestrial biodegradation studies should account for field conditions and use groups of structurally related compounds from a wide range of pharmaceutical classes. These experiments should have long running durations, so that potential build up within an environmental compartment can be studied. Ideally all biodegradation studies should generate both quantitative and qualitative data on any metabolites formed, and consider their subsequent transport and fate. Generation of data for physical constants; such as K_{ow} or K_d for different compounds, soils, and SS types, alongside these experiments may also aid in interpretation of compound behaviours.

Future work is also required regarding the plant uptake of pharmaceuticals. This project provided the first example of pharmaceutical plant uptake but only results for a preliminary experiment were obtained. The first stage in further research should be further optimisation of extraction methodologies.

Unfortunately due to project time constraints method development for extraction and purification of the analyte from plant tissue had to be limited. Although detection of the target compound was good; with Gaussian peak shapes, high ion counts and peaks well above the required S:N ratio, IS recoveries were low, highlighting the need for further method development and optimisation. Average IS recoveries for the T/C experiment ranged from 17 to 35 % depending upon the sub-sample in question (roots > curd > stem > leaves > media; n = 9 or 10). These lower recoveries meant that lower LOD were required, hence the use of SIM and SRM. Plant metabolites of Fluoxetine formed during the T/C experiment were therefore unlikely to be detected using SIM. Improvement of extraction and clean up methodologies may also allow for use of full MS^n analysis methods, therefore potentially allowing identification of any metabolites formed.

Further method development for the extraction and clean-up of pharmaceuticals from plant tissue matrices should be completed. The use of ASE or LLE with MeOH and chloroform, might aid cell lysis and lipid extraction. This should improve compound recovery as there is evidence that organic xenobiotics tend to associate with lipid type materials. Further clean-up would lead to lower LODs, and may therefore allow the use of full MS for analysis and hence detection of any metabolites formed. Initially this work should be completed for cauliflower matrices but later extended to other plants considered at risk. For example results from this project showed no uptake to the edible curd, but uptake to leaves was demonstrated in cauliflowers and cress. Both of these plants come from the *Brassica* family, and it may be pertinent to focus future

research into Fluoxetine uptake on *Brassica* crops in which the leaves are eaten (e.g. cabbage).

Tissue cultures proved to be an easy and relatively quick way to screen for PPCP uptake in plants, and could be used in the future for routine screening, although extension of growth period to full maturity may be advisable. Plant uptake of xenobiotics are affected by a wide range of environment factors which should be taken into account. The most important of these influencing factors is soil, and the influence of soil on uptake must be considered for results to be of more environmental significance. The use of glasshouse pot trials or even larger scale field trials would perhaps be an appropriate choice. It is important that uptake studies provide information on where in the plant the compound is translocated as this has an impact on further transport and fate of the compound, and may provide a route for bioremediation.

To obtain true perspectives on the terrestrial fate of pharmaceuticals more research is required across the whole of this field. Monitoring data are beginning to emerge in the literature but there is still a lack of information on terrestrially relevant biotic and abiotic losses, including sorption. There are almost no terrestrial ecotoxicological data in the literature and no evidence of plant uptake studies. Considering the potential for accumulation of persistent compounds in soils future research needs to include more fate studies, especially ecotoxicological and plant-up take studies.

APPENDIX

US EPA Modelling Suite (EPI Suite V3.20) Results (Kowin v1.67; Henry v3.10; Biowin v4.01; PcKocwin v1.66; Hydrowin v1.67; BCF v2.17; Level III fugacity model; STP fugacity model)	
Diazepam	Log K_{ow} = 2.70 Henry's Law Constant (at 25°C) = 3.64 x 10^{-9} atm m^{-3} $mole^{-1}$ Linear model prediction = biodegrades fast; Non-linear model prediction = biodegrades fast; Ultimate biodegradation timeframe = weeks - months; primary biodegradation timeframe = days - weeks; MITI linear model prediction = does not biodegrade fast; MITI non-linear model prediction = does not biodegrade fast; anaerobic model prediction = does not biodegrade fast; ready biodegradability prediction = No. K_{oc} = 1.122 x 10^4 Hydrolysis rate extremely slow, $t_{1/2}$ > 1 year Log BCF = 1.471 Soil $t_{1/2}$ = 1800 hours Removal in STW: Total removal = 4.42 %; total biodegradation = 0.11 %; total sludge adsorption 4.31 %
Desmethyldiazepam	Log K_{ow} = 2.87 Henry's Law Constant (at 25°C) = 1.78 x 10^{-10} atm m^{-3} $mole^{-1}$ Linear model prediction = biodegrades fast; Non-linear model prediction = biodegrades fast; Ultimate biodegradation timeframe = weeks - months; primary biodegradation timeframe = days - weeks; MITI linear model prediction = does not biodegrade fast; MITI non-linear model prediction = does not biodegrade fast; anaerobic model prediction = does not biodegrade fast; ready biodegradability prediction = No. K_{oc} = 8847 Hydrolysis rate extremely slow, $t_{1/2}$ > 1 year Log BCF = 1.556 Soil $t_{1/2}$ = 1800 hours Removal in STW predictions: Total removal = 5.14 %; total biodegradation = 0.12 %; total sludge adsorption 5.02 %

Table A. 1. Selection criteria findings from literature for Fluoxetine HCl, Diazepam and their human metabolites: US EPI Modelling Suite Results

Kow: Octanol-water partition coefficient. Koc: Soil adsorption coefficient.
BCF: Bioconcentration factor. $t_{1/2}$: half-life. STW: sewage treatment works

Table A. 1 continued...

	US EPA Modelling Suite (EPI Suite V3.20) Results (Kowin v1.67; Henry v3.10; Biowin v4.01; PcKocwin v1.66; Hydrowin v1.67; BCF v2.17; Level III fugacity model; STP fugacity model)
Oxazepam	Log K_{ow} = 2.32
	Henry's Law Constant (at 25°C) = 5.53 x 10^{-10} atm m^{-3} $mole^{-1}$
	Linear model prediction = biodegrades fast; Non-linear model prediction = biodegrades fast; Ultimate biodegradation timeframe = weeks - months; primary biodegradation timeframe = days - weeks; MITI linear model prediction = does not biodegrade fast; MITI non-linear model prediction = does not biodegrade fast; anaerobic model prediction = does not biodegrade fast; ready biodegradability prediction = No.
	K_{oc} = 442.5
	Hydrolysis rate extremely slow, $t_{1/2}$ > 1 year
	Log BCF = 1.025
	Soil $t_{1/2}$ = 1800 hours
	Removal in STW: Total removal = 2.54 %; total biodeg = 0.1 %; total sludge adsorption = 2.44 %
Temazepam	Log K_{ow} = 2.15
	Henry's Law Constant (at 25°C) = 1.13 x 10^{-8} atm m^{-3} $mole^{-1}$
	Linear model prediction = biodegrades fast; Non-linear model prediction = biodegrades fast; Ultimate biodegradation timeframe = weeks - months; primary biodegradation timeframe = days - weeks; MITI linear model prediction = does not biodegrade fast; MITI non-linear model prediction = does not biodegrade fast; anaerobic model prediction = does not biodegrade fast; ready biodegradability prediction = No.
	K_{oc} = 561
	Hydrolysis rate extremely slow, $t_{1/2}$ > 1 year
	Log BCF = 0.986
	Soil $t_{1/2}$ = 1800 hours
	Removal in STW: Total removal = 2.46 %; total biodeg = 0.10 %; total sludge adsorption = 2.37 %

Table A.1. Selection criteria findings from literature for Fluoxetine HCl, Diazepam and their human metabolites: US EPI Modelling Suite Results

Kow: Octanol-water partition coefficient. Koc: Soil adsorption coefficient
BCF: Bioconcentration factor. $t_{1/2}$: half-life. STW: sewage treatment works

Table A. 1 continued...

	US EPA Modelling Suite (EPI Suite V3.20) Results (Kowin v1.67; Henry v3.10; Biowin v4.01; PcKocwin v1.66; Hydrowin v1.67; BCF v2.17; Level III fugacity model; STP fugacity model)
Fluoxetine HCl	Log K_{ow} = 4.65 Henry's Law Constant (at 25°C) = 8.90 x 10^{-8} atm m^{-3} $mole^{-1}$ Linear model prediction = does not biodegrade fast; Non-linear model prediction = does not biodegrade fast; Ultimate biodegradation timeframe = months; primary biodegradation timeframe = days - weeks; MITI linear model prediction = does not biodegrade fast; MITI non-linear model prediction = does not biodegrade fast; anaerobic model prediction = biodegrade fast; ready biodegradability prediction = No. K_{oc} = 2.07 x 10^5 Log BCF = 2.419 Soil $t_{1/2}$ = 2880 hours Removal in STW: Total removal = 32.40 %; total biodegradation = 0.34 %; total sludge adsorption = 32.06 %
Norfluoxetine HCl	Log K_{ow} = 4.18 Henry's Law Constant (at 25°C) = 4.05 x 10^{-8} atm m^{-3} $mole^{-1}$ Linear model prediction = biodegrades fast; Non-linear model prediction = does not biodegrade fast; Ultimate biodegradation timeframe = months; primary biodegradation timeframe = days - weeks; MITI linear model prediction = does not biodegrade fast; MITI non-linear model prediction = does not biodegrade fast; anaerobic model prediction = biodegrade fast; ready biodegradability prediction = No. K_{oc} = 1.497 x 10^5 Log BCF = 2.520 Soil $t_{1/2}$ = 2880 hours Removal in STW: Total removal = 38.89 %; total biodeg = 0.39 %; total sludge adsorption = 38.50 %

Table A.1. Selection criteria findings from literature for Fluoxetine HCl, Diazepam and their human metabolites: US EPI Modelling Suite Results

Kow: Octanol-water partition coefficient
Koc: Soil adsorption coefficient
BCF: Bioconcentration factor
$t_{1/2}$: half-life
STW: sewage treatment works

	Constants	Reference
Diazepam	Log K_{ow} = 2.82	(Ternes, 2004)
	Soil / water K_d = 4 to 20 (soil type dependant)	(Kreuzig *et al.*, 2003)
	Primary sludge K_d = 43.9 ± 26.1 L kg^{-1} SS, for secondary sludge Kd = 21.1 ± 7.6 L kg^{-1} SS	(Ternes, 2004)
	Soil / water K_d = 44 ± 26 L kg^{-1} for primary sludge; secondary sludge = 21 ± 8 L kg^{-1}. Log K_d = 1.6 for primary sludge; secondary sludge = 1.3. Log K_{oc} = 125 ± 75 L kg^{-1} for primary sludge, secondary sludge = 62 ± 23 L kg^{-1}	(Ternes *et al.*, 2004)
	Low solubility = 0.01 mg ml^{-1}	(Jjemba, 2006)
Desmethyldiazepam	No literature data available	
Oxazepam	No literature data available	
Temazepam	Low solubility = 0.1 mg ml^{-1}	(Jjemba, 2006)
Fluoxetine HCl	Log K_{ow} = 1 - 2.6 (pH 5 - 9)	El-lily website
	Log K_{ow} = 1.25 - 4.3 (pH 2 - 11)	(Brooks *et al.*, 2003a)
	Log K_{ow} = 4.05	(Kinney *et al.*, 2006)
	Log K_{oc} = 0.64 - 3.70 (pH 2 - 11)	(Brooks *et al.*, 2003a)
	BCF ~ 1; 2.00; 1071.52 (pH 2, 7, 11)	(Brooks *et al.*, 2003a)
	Moderately soluble = 33 mg ml^{-1}	(Jjemba, 2006)
	Solubility = 60.3 mg L^{-1}	(Kinney *et al.*, 2006)
Norfluoxetine HCl	Log K_{ow} = 0.97 - 4.06 (pH 2 - 11)	(Brooks *et al.*, 2003a)
	Log K_{oc} = 0.49 - 3.58 (pH 2 - 11)	(Brooks *et al.*, 2003a)
	BCF ~1; 6.97; 716.12 (pH 2, 7, 11)	(Brooks *et al.*, 2003a)

Table A.2. Selection criteria findings from literature for Fluoxetine HCl, Diazepam and their human metabolites: Constants

Kow: Octanol-water partition coefficient
Koc: (Kd ÷ % Organic content) x 100
Kd: Partition coefficient (matrix / matrices stated)
BCF: Bioconcentration factor

	Occurrence in the Environment	Reference
Diazepam	River water = 0.13 - 2.13 ng L^{-1}	(Calamari *et al.*, 2003)
	STW effluent = < 1μg L^{-1}; river ~ 10 ng L^{-1}; potable waters ~ 10 ng L^{-1}	(Halling-Sorensen *et al.*, 1998; Richardson and Bowron, 1985)
	STW effluent = 0.053 μg L^{-1}; river = 0.033 μg L^{-1}	(Ternes *et al.*, 2001)
	STW influent = 0.59 - 1.18 μg L^{-1}; effluent = 0.66 μg L^{-1}	(van der Ven *et al.*, 2004)
	Drinking water = 0.2 - 23.5 ng L^{-1}; river water = 0.5 - 1.2 ng L^{-1}	(Zuccato *et al.*, 2000)
	PEC = 0.055 μg L^{-1}; PNEC = 4.2 μg L^{-1}; PEC / PENC = 0.013	(Stuer-Lauridsen *et al.*, 2000)
	Waters of lake Mead, Nevada = 3 - 62 ng L^{-1}	(Snyder *et al.*, 2001)
	$PEC_{surface\ water}$ Sweden (worst case scenario) = 0.028 μg L^{-1}; refined $PEC_{sewage\ water}$ Sweden = 0.13 - 0.16 μg L^{-1}, refined $PEC_{surface\ water}$ Sweden = 0.006 μg L^{-1}	(Carlsson *et al.*, 2006)
Nordiazepam	No literature information available	
Oxazepam	No literature information available	
Temazepam	No literature information available	

Table A.3. Selection criteria findings from literature for Fluoxetine HCl, Diazepam and their human metabolites: Environmental Occurrence

PEC: Predicted environmental concentration (EU system). PNEC: Predicted no-effect concentration (EU system)
EIC: Environmental introduction concentration (US EPA system)
DWTP: drinking water treatment plant

Table A.3 continued...

	Occurrence in the Environment	Reference
Fluoxetine HCl	Stream = 0.012 μg L^{-1}	(Kolpin *et al.*, 2002)
	PEC / PNEC = 14.19	(Webb, 2000)
	STW effluent = 0.038 - 0.099 μg L^{-1}; Canadian surface waters = 0.013 - 0.046 μg L^{-1}	(Metcalfe *et al.*, 2003)
	EIC (assuming no metabolism & no dilution) = 0.439 μg L^{-1}; (assuming 90 % metabolism & no dilution) = 0.0439 μg L^{-1}	(Brooks *et al.*, 2003a)
	USA Reclaimed water facility effluent estimate = 1.23 - 5.40 ng L^{-1}	(Kinney *et al.*, 2006)
	Soil accumulation (soil cores irrigated with reclaimed water) = 366 to 14400 % of loading estimates; mean integrated mass equivalent from 3 sites = 109 ng, max = 376 ng	(Kinney *et al.*, 2006)
	EU PEC = 0.220 μg L^{-1}; US EIC 0.230 μg L^{-1}	(Johnson *et al.*, 2005)
	Refined (accounting for STW estimated removal & dilution factors) EU 99th centile PEC = 0.182 μg L^{-1}; US refined 99th centile EIC = 0.019 μg L^{-1}	(Johnson *et al.*, 2005)
	Fish (3 species, stream USA): Bioaccumulation seen, brain = 1.28 ng g^{-1}; liver = 1.34 ng g^{-1}; muscle tissue = 0.11 ng g^{-1}	(Brooks *et al.*, 2005)
	STW effluent = 40 μg L^{-1}; upstream river water < LOQ; detected downstream from STW (upto 8km)	(Furlong, 2007)
	STW effluent = < LOQ - 1.3 ng L^{-1}; STW influent = 0.4 - 2.4 ng L^{-1}; effluent average concentration = 73% of influent concentration	(Vasskog *et al.*, 2006)
	DWTP: detected in solid samples; average concentration in solids after clarification step = 49.5 μg kg^{-1}; average concentration in solids after filtration step = 58.6 μg kg^{-1}. These solids are returned to STW for disposal	(Stackelberg *et al.*, 2007)
Norfluoxetine HCl	Fish (3 species, stream USA): Bioaccumulation seen, brain = 8.86 ng g^{-1}; liver = 10.27 ng g^{-1}; muscle tissue = 1.07 ng g^{-1}	(Brooks *et al.*, 2005)
	STW effluent = ~ 1.2 ng L^{-1}; upstream river water < LOQ; detected downstream from STW (upto 8km)	(Furlong, 2007)

Table A.3. Selection criteria findings from literature for Fluoxetine HCl, Diazepam and their human metabolites: Environmental Occurrence

PEC: Predicted environmental concentration (EU system). PNEC: Predicted no-effect concentration (EU system)
EIC: Environmental introduction concentration (US EPA system). DWTP: drinking water treatment plant

	Human Metabolites	Reference
Diazepam	N-desmethyldiazepam & Temazepam N-methyloxazepam; then converted to Oxazepam; followed by glucuronide conjugation	(TOXNET, 2006a)
	1 % excreted as parent compound; metabolites = 22 - 43 % of applied dose	(Smith-Kielland *et al.*, 2005)
Fluoxetine HCl	Primary metabolite is S-Norfluoxetine. > 50 % of metabolic end products are unknown. Extensively metabolised in the liver to Norfluoxetine, and other unidentified metabolites. Norfluoxetine, the demethyl metabolite is also a SSRI	(TOXNET, 2006b)
	2.5 - 5 % of dose recovered in urine as parent compound, 10 % as Norfluoxetine, 5.2 % as fluoxetine glucuronide & 9.5 % as Norfluoxetine glucuronide. 16 % recovered in faeces	(Risley and Bopp, 1990)
	^{14}C-fluoxetine application: 65 % of radioactivity recovered in urine, of which 2.5 % is fluoxetine, 10 % is Norfluoxetine, 5.2 % as conjugated fluoxetine and 9.5 % as conjugated Norfluoxetine, 15 % is also recovered in faeces	(Lemberger *et al.*, 1985)

Table A.4. Selection criteria findings from literature for Fluoxetine HCl, Diazepam: Human Metabolites

	Degradation (Biotic & Abiotic)	Reference
Diazepam	Fungal degradation: N^1-demethylation & ring cleavage lead to formation of 3 metabolites (7-chloro-5-pheyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one, 2-acetamido-2-benzoyl-4-chloroacetanilide and 2-acetamido-2-benzoyl-4-chloro-N-methylacetanilide).	(Ambrus *et al.*, 1975)
	Under acidic aqueous abiotic conditions 7 degradation products were found	(Cabrera *et al.*, 2005)
	Marginal surface water degradation: Sediment / water system DT_{90} >> 365 days; DT_{50} = 311 ± 25 days; Surface water DT_{90} = 113 ± 17 days; DT50 = 34 ± 5 days. Classified as highly persistent	(Loffler *et al.*, 2005)
	Sediment sorption = 60 %	(Loffler *et al.*, 2005)
	Mineralisation < 2 %	(Loffler *et al.*, 2005)
	Hydrolysis (pH 1 - 11): formation of 2-methylamino-5-chlorobenzophenone	(Han *et al.*, 1977)
Nordiazepam	No literature information available	
Oxazepam	Classified as moderately persistent with limited sediment sorption tendencies: Sediment / water system DT_{90} = 179 ± 11 days; DT_{50} = 54 ± 3 days; Surface water DT_{90} = 63 ± 6 days; DT_{50} = 19 ± 2 days	(Loffler *et al.*, 2005)
	Sediment sorption = 19 - 29 %	(Loffler *et al.*, 2005)
	Hydrolysis (pH 1 to 11): formation of 2-amino-5-chlorobenzophenone	(Han *et al.*, 1977)
Temazepam	No literature information available	

Table A.5. Selection criteria findings from literature for Fluoxetine HCl, Diazepam and their human metabolites: Degradation

DTxx: Dwell time (xx days)

Table A.5 continued...

	Degradation (Biotic & Abiotic)	Reference
Fluoxetine HCl	Photodegradation: 3 products (o-dealkylation & photonucleophilic substitution)	(Lam *et al.*, 2005)
	Stability: Under acidic stress conditions α-[methylaminoethyl]benzene methanol and *p*-trifluoromethylphenol formed; otherwise stable under normal storage conditions	(Risley and Bopp, 1990)
	Biphasic dissipation nature in water column: mean 1st phase dissipation $t_{1/2}$ = 3.8 days; mean 2nd phase dissipation $t_{1/2}$ = 76.7 days (infinite 2nd phase at lower concentrations)	(Johnson *et al.*, 2005)
Norfluoxetine HCl	No literature information available	

Table A.5. Selection criteria findings from literature for Fluoxetine HCl, Diazepam and their human metabolites: Degradation

DTxx: Dwell time (xx days)

Compound Name	Therapeutic Use	Reference
Diazepam	Adjuvants, anaesthesia, anaesthetics, anti-anxiety agents, anticonvulsants, antiemetics, GABA modulators, muscle relaxants, sedatives	(TOXNET, 2006a)
Desmethyldiazepam	No therapeutic use	(British pharmacopoeia)
Oxazepam	Anxiolytic	(British pharmacopoeia)
Temazepam	Sedative and hypnotic	(British pharmacopoeia)
Fluoxetine HCl	Selective serotonin reuptake inhibitor (SSRI), Anti-depressant, Bulimia Nervosa treatment, Obsessive-compulsive-disorder (OCD) treatment	(British Pharmacopeia)
Norfluoxetine HCl	SSRI, Anti-depressant, Bulimia Nervosa treatment, OCD treatment. It has linear pharmacokinetics contributing to the long duration of action of fluoxetine	(British Pharmacopeia)

Table A.6. Selection criteria findings from literature for Fluoxetine HCl, Diazepam and their human metabolites: Therapeutic Usage

Compound Name	Ecotoxicity	Reference
	Hydra vulgaris(cnidarian): NOEC < 0.01 mg L^{-1}	(Crane *et al.*, 2006)
	Hydra vulgaris: inhibited regeneration of digestive regions (10 μg L^{-1})	(Pascoe *et al.*, 2003)
	Artemia parthenogenetica (crustacean): expressed oxidative-related stress response	(Nunes *et al.*, 2006b)
	Artemia parthenogenetica: alterations in cellular redox status & neurotransmission interference	(Nunes *et al.*, 2006a)
	Artemia parthenogenetica: LC_{50} = 12.16 mg L^{-1}	(Nunes *et al.*, 2005)
	Tetraselmis chuii (algae): IC_{50} = 16.46 mg L^{-1}	(Nunes *et al.*, 2005)
	Hyalella azteca & *Chironomus tentans* LC/EC_{50} = 15.2 mg kg^{-1}	(Brooks *et al.*, 2003b)
	Pseudokirchneriella subcapitata: cell density LC/EC_{50} = 39 μg L^{-1}; turbidity LC/EC_{50} = 24 μg L^{-1}	(Brooks *et al.*, 2003b)
	Ceriodaphnia dubia: LC/EC_{50} = 234 μg L^{-1}	(Brooks *et al.*, 2003b)
	Daphnia magna: LC/EC_{50} = 820 μg L^{-1}	(Brooks *et al.*, 2003b)
Diazepam	*Daphnia magna*: EC_{50} = 1.69 μmol L^{-1}	(Calleja *et al.*, 1993)
	Pimephales promelas: LC/EC_{50} = 705 μg L^{-1}	(Brooks *et al.*, 2003b)
	Streptocephalus proboscideus: EC_{50} = 2.55 μmol L^{-1}	(Calleja *et al.*, 1993)
	Rat: EC_{50} = 3.40 μmol kg^{-1}	(Calleja *et al.*, 1993)
	Mouse: EC_{50} = 2.89 μmol kg^{-1}	(Calleja *et al.*, 1993)
	Human oral lethal dose = 2.00 μmol kg^{-1}	(Calleja *et al.*, 1993)
	Photobacterium phosphoreum: EC_{50} (Microtox test) in > 35000 μmol L^{-1}	(Calleja *et al.*, 1994)
	Artemia salina: EC_{50} (Artoxkit M test) = 230 μmol L^{-1}	(Calleja *et al.*, 1994)
	Streptocephalus proboscideus: EC_{50} (Streptoxkit F test) = 362 μmol L^{-1}	(Calleja *et al.*, 1994)
	Daphnia magna: EC_{50} = 49.5 μmol L^{-1}	(Calleja *et al.*, 1994)
	Brachionus calyciflorus: EC_{50} (Rotoxkit F test) > 35100 μmol L^{-1}	(Calleja *et al.*, 1994)

Table A.7. Selection criteria findings from literature for Fluoxetine HCl, Diazepam and their human metabolites: Ecotoxicity

No literature regarding the other target 1,4-Benzodiazpines (Temazepam, Oxazepam and Nordiazepam) was available
LCxx: Lethal concentration (for xx % of population). ICxx: Growth inhibition concentration (for xx % of population)
ECxx: Effective concentration (for xx % of population). NOEC: No observed effect concentration

Table A.7 continued...

Compound Name	Ecotoxicity	Reference
	Wide range of known detrimental effects on organisms	(Fong, 2001)
	Green alga: $NOEC_{long\ term\ exposure} = 0.001\ mg\ L^{-1}$	(Crane *et al.*, 2006)
	Hyalella azteca (amphipod): $NOEC_{long\ term\ exposure} > 43\ mg\ kg^{-1}$	(Crane *et al.*, 2006)
	Ceriodaphnia dubia (waterflea): $NOEC_{long\ term\ exposure} = 0.056\ mg\ L^{-1}$	(Crane *et al.*, 2006)
	Lemna gibba (duckweed): $NOEC_{long\ term\ exposure} > 1.0\ mg\ L^{-1}$	(Crane *et al.*, 2006)
Fluoxetine HCl	*Gammarus pulex* (Crustacea, Amphipoda): $LOEC_{activity} = 100\ ng\ L^{-1}$	(De Lange *et al.*, 2006)
	Anolis carolinensis (Green anole lizard): reduction in aggressive behaviour	(Perreault *et al.*, 2003)
	Rodents: reduction in aggressive behaviour	(Perreault *et al.*, 2003)
	Thalassoma bifasciatum (bluehead wrasse): reduction in aggressive behaviour	(Perreault *et al.*, 2003)
	Territorial tree sparrows: reduction in aggressive behaviour	(Perreault *et al.*, 2003)
	Golden hamsters: reduction in aggressive behaviour	(Perreault *et al.*, 2003)
	Corynebacterium (group D2): $MIC_{90} = 32\ mg\ L^{-1}$	(Munoz-Bellido *et al.*, 2000)
	Two fish hepatocyte cell lines: oxidative stress induced, followed by cytotoxicicity (EC_{50} EROD = 77μM)	(Laville *et al.*, 2004)

Table A.7. Selection criteria findings from literature for Fluoxetine HCl, Diazepam and their human metabolites: Ecotoxicity

LCxx: Lethal concentration (for xx % of population).
ECxx: Effective concentration (for xx % of population)
NOEC: No observed effect concentration
LOEC: Lowest observed effect concentration
MICxx: Minimum inhibitory concentration (for xx % of population)

Table A.7 continued...

Compound Name	Ecotoxicity	Reference
	A. cygnea: laval release (10^{-6} M, 10^{-9} M some release); valve opening and foot extension (10^{-2} to 10^{-3} M); death of females after 5 - 6 hours	(Cunha and Machado, 2001)
	Daphnia magna: increased feundicity by ~ 3 times (36 μg L^{-1}); in combination with Clofibric acid (100 ug L^{-1}) 62.5 % mortality (over 6 days); with less Clofibric acid (10 μg L^{-1}) morphological abnormalities in ~ 19% occurred (including crinkled carapaces, malformed antennae, bent tail spines, which lead to mobility problems & death)	(Flaherty and Dodson, 2005)
Fluoxetine HCl	*Ceriodaphnia dubia*: 48 hr LC_{50} = 0.51 mg L^{-1}; $NOEC_{mean\ number\ of\ neonates}$ = 0.089 mg L^{-1}; reduced number of broods produced (7 days)	(Henry *et al.*, 2004)
	Lemma gibba (duckweed): LOECs for various endpoints (wet weight, frond number, Chlorophyll a & b, Carotenoids) all showed no significant difference	(Brain *et al.*, 2004)
	Japanese medaka (*Oryzias latipes*): 4 weeks exposure resulted in a low incidence of offspring developmental abnormalities, and significantly increased plasma estradiol levels in females	(Foran *et al.*, 2004)
	Fish (3 species, stream USA): Bioaccumulation seen, brain = 1.28 ng g^{-1}; liver = 1.34 ng g^{-1}; muscle tissue = 0.11 ng g^{-1}	(Brooks *et al.*, 2005)
	Lumbricus terrestris: reduced crawling rate	(Burns *et al.*, 1992)
Norfluoxetine HCl	Fish (3 species, stream USA): Bioaccumulation seen, brain = 8.86 ng g^{-1}; liver = 10.27 ng g^{-1}; muscle tissue = 1.07 ng g^{-1}	(Brooks *et al.*, 2005)

Table A.7. Selection criteria findings from literature for Fluoxetine HCl, Diazepam and their human metabolites: Ecotoxicity

LCxx: Lethal concentration (for xx % of population).
ECxx: Effective concentration (for xx % of population)
NOEC: No observed effect concentration
LOEC: Lowest observed effect concentration
MICxx: Minimum inhibitory concentration (for xx % of population)

	Usage / Demand	Reference
Diazepam	US: 12.475 prescriptions in 1995; featured on top 200 list from 1995 to current	(rxlist, 2006)
	Denmark: 0.207 tonnes in 1997; on top 25 list	(Ayscough *et al.*, 2000; Stuer-Lauridsen *et al.*, 2000)
	Sweden: 183 kg sold in 2002	(Carlsson *et al.*, 2006)
Nordiazepam	No literature information available	
Oxazepam	A pharmaceutical within it's own right	(British pharmacopeia)
	Sweden: 642 kg sold in 2002	(Carlsson *et al.*, 2006)
Temazepam	A pharmaceutical within it's own right	(British pharmacopeia)
Fluoxetine HCl	UK: number 83 in usage (2000); 2.83 tonnes	(Sebastine and Wakeman, 2003)
	US: on top 200 prescribed drugs since 1995 to current	(rxlist, 2006)
	Canada: 916 kg used / produced	(Johnson *et al.*, 2005)
	Norway: 320% increase in SSRI use in last 10 years	(Vasskog *et al.*, 2006)
Norfluoxetine HCl	No literature information available	

Table A.8. Selection criteria findings from literature for Fluoxetine HCl, Diazepam and their human metabolites: Demand

The EPI Suite from the Environmental Fate Database was used to generate data to predict the behaviour of Diazepam in a STW:

4.31% of Diazepam will enter the sewage sludge

95.58% of Diazepam will not be removed.

Assuming that all the Diazepam not removed enters the effluent;

Influent concentration x 0.9558 = effluent concentration

Influent concentration x 0.9558 = 1*

Influent concentration = 1 ÷ 0.9558

Influent concentration = 1.046 μg L^{-1}

4.31% of this then enters the sewage sludge;

Influent concentration x 0.0431 = Concentration in sewage sludge

1.046 x 0.0431 = 0.0451 μg Diazepam enters SS per L of effluent

Diazepam Concentration in SS =

Mass of Diazepam entering SS L^{-1} effluent ÷ †Mass of influent solids L^{-1}

0.0451 ÷ 720 = 0.00006261 μg mg^{-1} SS

Diazepam PEC_{SS} = 0.06262 μg g^{-1}SS

Figure A.1. Calculation of Diazepam PEC_{SS} from literature data

* This figure (1 μg L^{-1}) was selected as it was the highest effluent concentration of Diazepam found in the literature (Halling-Sorensen *et al.*, 1998) at the commencement of this project, and it represents a worst case scenario.

† Typical influent contains 720 mg L^{-1} of total solids (European Environment Agency, 2001).

Diazepam PEC_{SS} x 1 ton (10^6 g) = Diazepam concentration (μg ton^{-1}_{SS})

[‡]0.06261 x 10^6 = 62610 μg Diazepam in 1 ton of sewage sludge

= 0.06261 g ton^{-1}SS

0.06261 x [§]10^6 = 62610 g Diazepam in 1,000,000 tonnes of SS

= 62.61 kg Diazepam in 1,000,000 tonnes of SS

= 0.06261 tonnes Diazepam enter SS annually

By taking into account the mass of Diazepam expected to enter SS annually along with the predicted partitioning behaviour of Diazepam in STW (from EPI Modelling Suite) the total annual excretion of Diazepam can be calculated;

= (0.06261 ÷ 4.31) x 100

= 1.45 tonnes

Unfortunately Diazepam data for UK usage were not available in the literature, and therefore this calculated value cannot be compared and assessed against manufactured amounts, however 1.45 tonnes does appear realistic.

Figure A.2. Back-checking calculated Diazepam PEC_{SS}

[‡] The PEC_{SS} for Diazepam was previously calculated to be 0.06261 μg g^{-1}_{SS}.
[§] There are approximately 1,000,000 tonnes of sewage sludge produced per annum in the UK (Gendebien *et al.*, 1999).

The EPI Suite from the Environmental Fate Database was used to generate data to predict the behaviour of Fluoxetine in a STW:

32.06% of Fluoxetine will enter the sewage sludge

67.6% of Fluoxetine will not be removed.

Assuming that all the Fluoxetine not removed enters the effluent;

Influent concentration x 0.676 = effluent concentration

Influent concentration x 0.676 = 0.3708**

Influent concentration = 0.3708 ÷ 0.676

Influent concentration = 0.549 $\mu g\ L^{-1}$

32.06% of this then enters the sewage sludge;

Influent concentration x 0.3206 = Concentration in SS

0.549 x 0.3206 = 0.176 μg Fluoxetine enters SS per L of effluent

Fluoxetine concentration in SS =

Mass of Fluoxetine entering SS L^{-1} effluent ÷ ††Mass of influent solids L^{-1}

0.176 ÷ 720 = 2.44 x 10^{-4} $\mu g\ mg^{-1}$ SS

Fluoxetine PEC_{SS} = 0.244 ug g^{-1} SS

Figure A.3. Calculation of Fluoxetine PEC_{SS} from literature data

** At the commencement of this project there was no literature data available for Fluoxetine in STW effluents. However a predicted ambient exposure concentration of 1.2nM for effluents was found (Webb, 2001). The RMM of Fluoxetine = 309. This predicted concentration equates to:

$1.2x10^{-9}$ x 309 = $3.708x10^{-7}$ g L^{-1}

= 0.3708 $\mu g\ L^{-1}$

†† Typical influent contains 720 mg L^{-1} of total solids (European Environment Agency, 2001).

Fluoxetine PEC_{SS} x 1 ton (10^6 g) = Fluoxetine concentration (µg ton^{-1}_{SS})

‡‡0.244 x 10^6 = 244000 µg Fluoxetine in 1 ton of sewage sludge

= 0.244 g ton^{-1} SS

0.244 x §§10^6 = 244000 g Fluoxetine in 1,000,000 tonnes of SS

= 244 kg

= 0.244 tonnes Fluoxetine enter SS annually

By taking into account the mass of Fluoxetine expected to enter SS annually along with the predicted partitioning behaviour of Fluoxetine in STW (from EPI Modelling Suite) the total annual excretion of Fluoxetine can be calculated;

= (0.244 ÷ 32.06) x 100

= 0.76 tonnes

In the UK 2.83 tonnes of Fluoxetine HCl are prescribed per annum (Sebastine and Wakeman, 2003). Of this it is estimated that 65% is excreted in urine or faeces (this may include metabolites as this figure is obtained through radio tracer studies; (Risley and Bopp, 1990). Of that excreted it is predicted that 32.06% will enter the sewage sludge. Therefore;

(4.1 x 0.65) x 0.3206 = 0.59 tonnes of Fluoxetine HCl enter SS annually

This back checking of calculations suggested that the calculated PEC_{SS} for Fluoxetine may be a slight overestimate but is within the right magnitude.

Figure A.4. Back-checking calculated Fluoxetine PEC_{SS}

‡‡ The PEC_{SS} for Fluoxetine was previously calculated to be 0.244 µg g^{-1} SS

§§ There are approximately 1,000,000 tonnes of sewage sludge produced per annum in the UK (Gendebien *et al.*, 1999).

The application rate of sewage sludge for the field in which soil and sewage sludge samples where sourced from = 4400 g m^{-2}

As a m^2 = 10,000 cm^2 the application rate can be shown as;

4400 ÷ 10,000 = 0.44 g cm^{-2}

Figure A.5. Calculating sewage sludge application rates

No additional SS was to be added to SS amended-soil used for the biodegradation of Fluoxetine in soil experiment; however the application rate of sewage sludge is required to calculate spiking concentrations as the transport path of these drugs into soil is *via* sewage sludge application.

To calculate the sludge application rate assumptions first need to be made. As the sample mass of each chamber was 15 g, there was a choice of two assumptions;

A: That the 15g of soil is all surface area.

'B: That the 15g of soil is a core (as sewage sludge is ploughed into 20cm deep). The surface area of this core needs to be chosen.

Option A was chosen as this will give the greatest sewage sludge and hence drug application rate, therefore providing a 'worst case scenario'. It has been assumed that this 15g of soil is a 1cm deep layer.

Denbigh soil series (silty clay loam) is known to have a bulk density of 0.9 g cm^{-3} (data from Holtham (2006)). 15g of soil in a 1 cm deep layer with a bulk density of 0.9 g cm^{-3} equates to a volume of 13.5cm^3, i.e. surface area = 13.5 cm^2.

Therefore SS application rate for 15 g of soil

= 0.44 x 13.5

= 5.94g of SS

Figure A.6. Calculating sewage sludge application rates for biodegradation of Fluoxetine in SS amended soil experiment

Fluoxetine PEC_{SS}= 0.244 μg g^{-1} SS

SS application rate for 15 g soil = 5.94 g

Therefore spiking rate for Fluoxetine in soil biodegradation experiment;

5.94 x 0.244 = 1.45 μg of Fluoxetine to be added to the 15 g of soil

For easy and accuracy the actual amount of Fluoxetine added to the soil biodegradation experiments was 1.5μg (15μl of 0.1 mg/ml in MeOH).

Figure A.7. Calculating Fluoxetine HCl spiking rate for biodegradation in SS amended soil experiment

Liquid media was spiked with Fluoxetine HCl in MeOH (140µl of 1 mg mL^{-1} into 500ml of media) and stirred with a magnet flea. Then 35 mLs poured into plastic pots (6 cm diameter) with lids and allowed to set, in a laminar flow hood.

This concentration was selected as the pot surface area $= \pi r^2$

$= \pi \times 3^2$

$= 28.27\ cm^2$

In a field situation 0.44g of sewage sludge would be applied per cm^2 of soil, so in total = 28.27 x 0.44 = 12.44 g of sewage sludge would be applied.

Fluoxetine concentration = SS mass x Fluoxetine PEC_{SS} ***

= 12.44 x 0.244

= 3.04 µg of Fluoxetine HCl per pot

It was decided that a spiking rate of 10 µg Fluoxetine HCl per growth poit would be appropriate as it was within the same magnituded as the predicted concentration above. As the drug was spiked directly into 500ml of media before being pour the actual exposure concentration = 9.8µg per 35 ml media i.e. per pot.

Figure A.8. Calculating Fluoxetine HCl spiking rate for plant up-take of pharmaceutical experiment

*** Previously calculated Fluoxetine PEC_{SS} = 0.244 µg g^{-1} SS

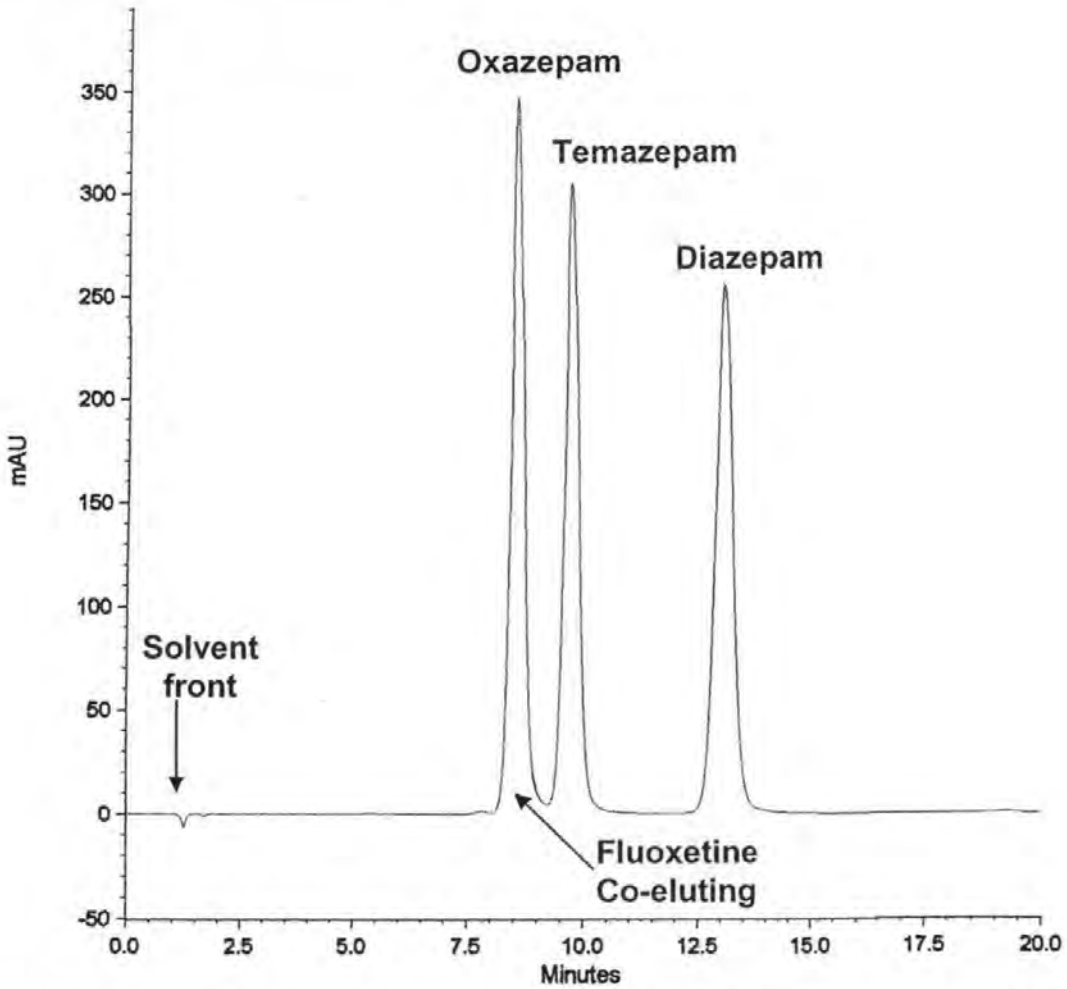


Figure A.9. Example 1: HPLC-UV method development chromatogram (at 254nm) of a mixture of four target compounds using Discovery HS C_{18}, 5µm, 10cm x 2.1mm i.d.

Aqueous phase (A): Milli-Q : MeOH : formic acid (95 : 5 : 0.1; v/v/v)
Organic phase (B): MeOH + 0.1% formic acid (v/v)
Gradient: 50 – 100% B over 20 minutes

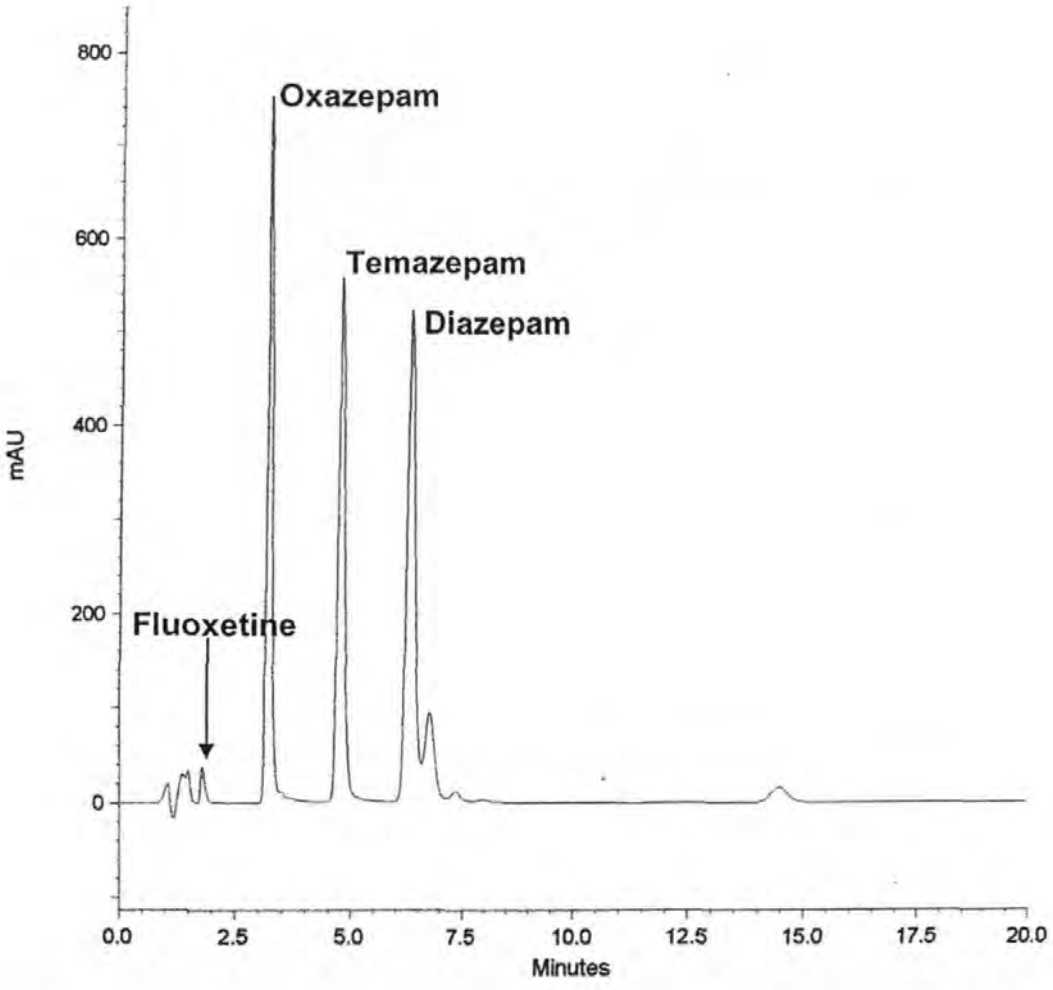


Figure A.10. Example 2: HPLC-UV method development chromatogram (at 254nm) of a mixture of four target compounds using Discovery HS C_{18}, 5µm, 10cm x 2.1mm i.d.

Aqueous phase (A): Milli-Q : MeOH : formic acid (95 : 5 : 0.1; v/v/v)
Organic phase (B): ACN + 0.1% formic acid (v/v)
Gradient: 50 – 100% B over 20 minutes

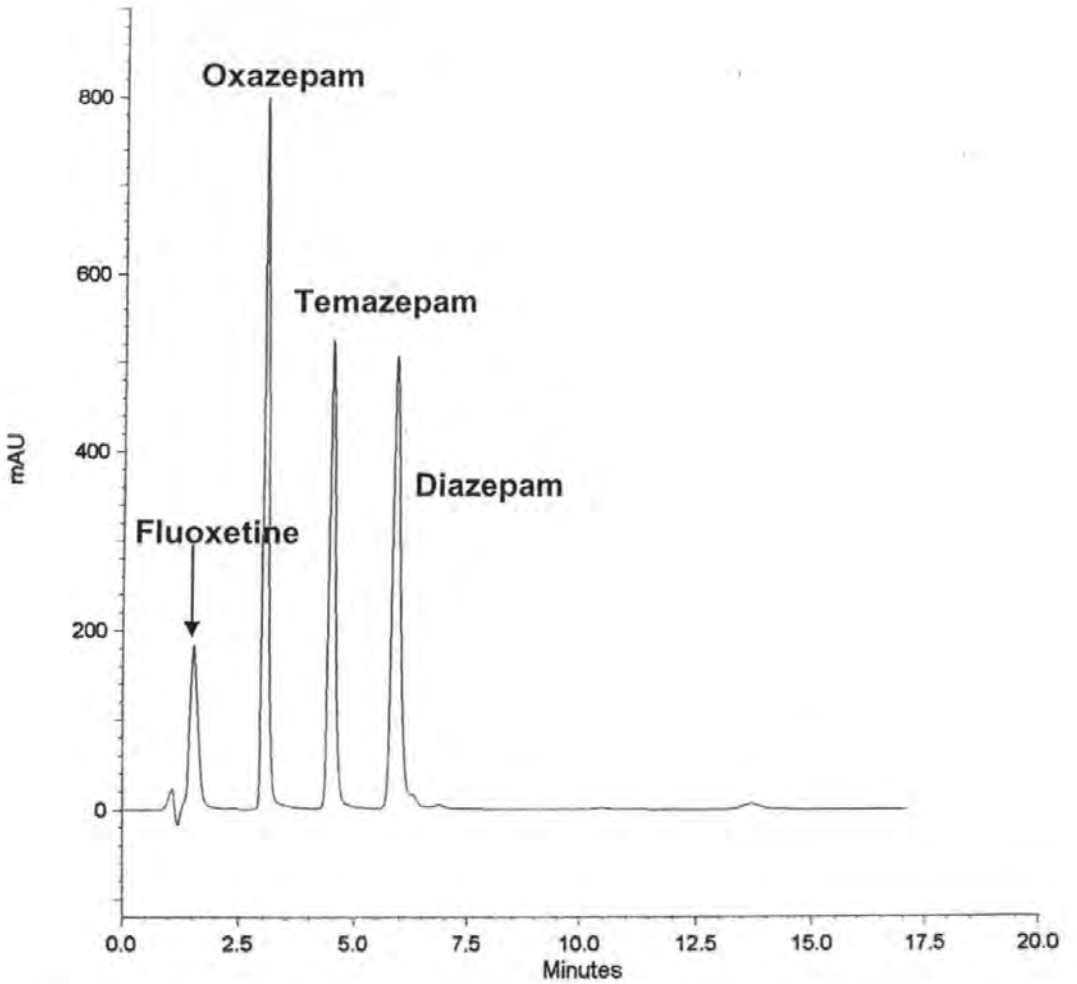


Figure A.11. Example 3: HPLC-UV method development chromatogram (at 254nm) of a mixture of four target compounds using Discovery HS C_{18}, 5µm, 10cm x 2.1mm i.d.

Aqueous phase (A): Milli-Q : MeOH : formic acid (95 : 5 : 0.1; v/v/v)
Organic phase (B): ACN + 0.1% formic acid (v/v)
Isocratic: 60 : 40; (A : B)

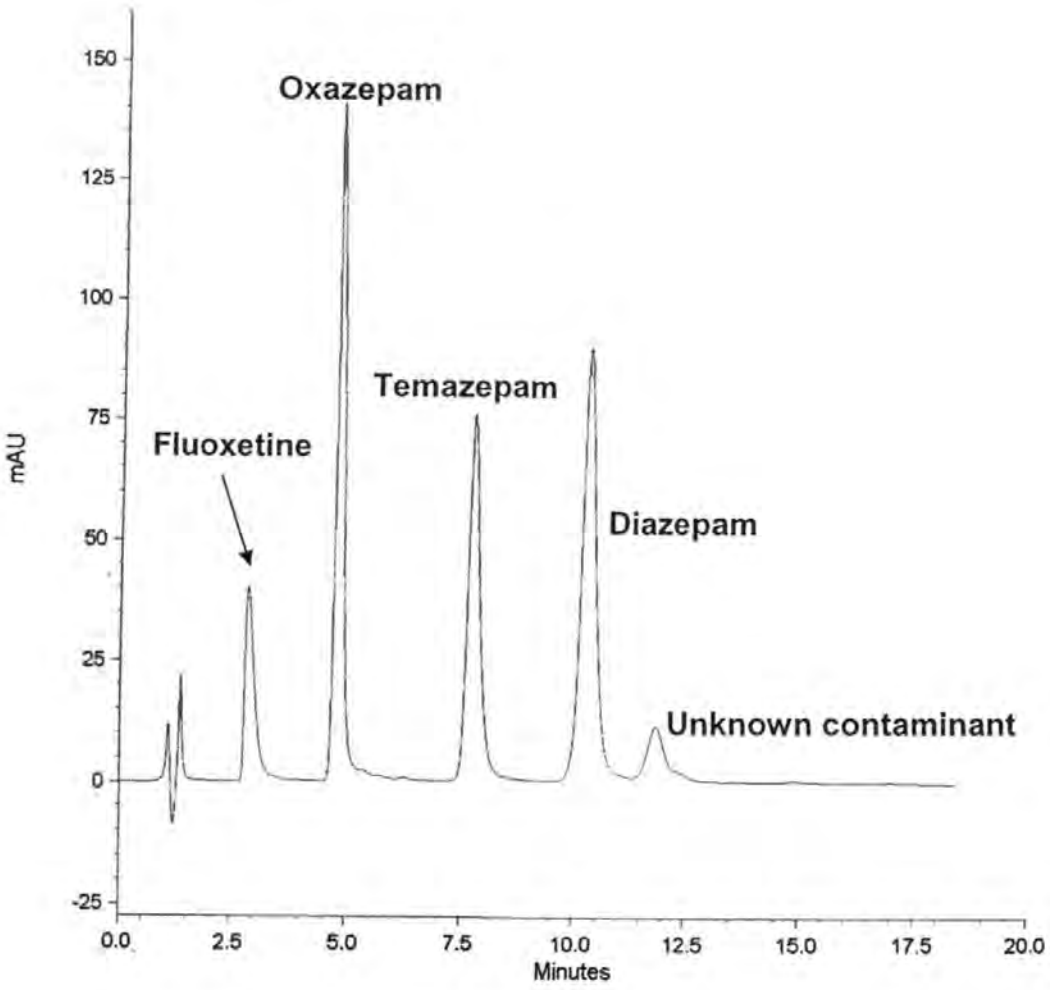


Figure A.12. Example 4: HPLC-UV method development chromatogram (at 254nm) of a mixture of four target compounds using Discovery HS C_{18}, 5µm, 10cm x 2.1mm i.d.

Aqueous phase (A): Milli-Q : MeOH : formic acid (95 : 5 : 0.1; v/v/v)
Organic phase (B): ACN + 0.1% formic acid (v/v)
Isocratic: 67 : 33; (A : B)

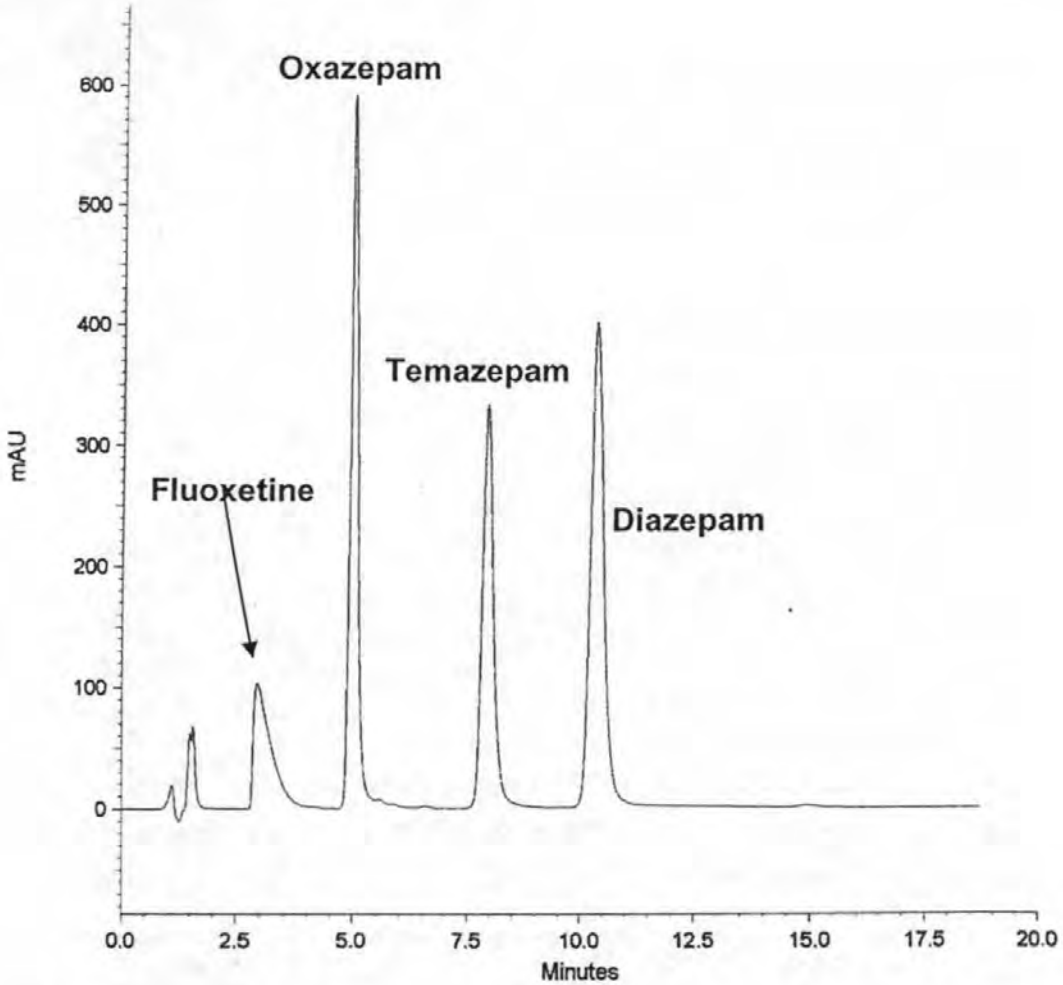


Figure A.13. Example 5: HPLC-UV method development chromatogram (at 254nm) of a mixture of four target compounds using Discovery HS C_{18}, 5µm, 10cm x 2.1mm i.d.

Aqueous phase (A): Milli-Q : MeOH : formic acid (95 : 5 : 0.1; v/v/v)
Organic phase (B): ACN + 0.1% formic acid (v/v)
Isocratic: 60 : 40; (A : B)
Freshly made standard solutions used

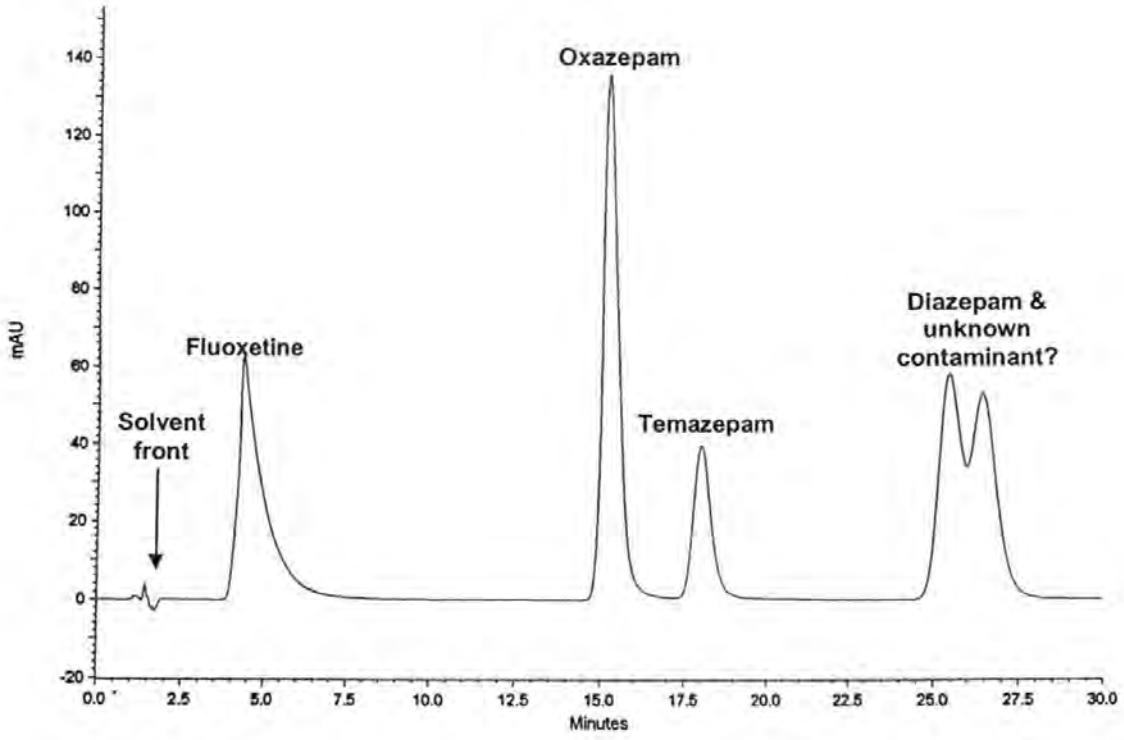


Figure A.14. Example 1: HPLC-UV method development chromatogram (at 254nm) of a mixture of four target compounds using Thermoquest, Hypercarb, 5µm, 10cm x 2.1mm i.d.

Aqueous phase (A): Milli-Q : MeOH : formic acid (95 : 5 : 0.1; v/v/v)
Organic phase (B): ACN + 0.1% formic acid (v/v)
Isocratic: 67 : 33; (A : B)

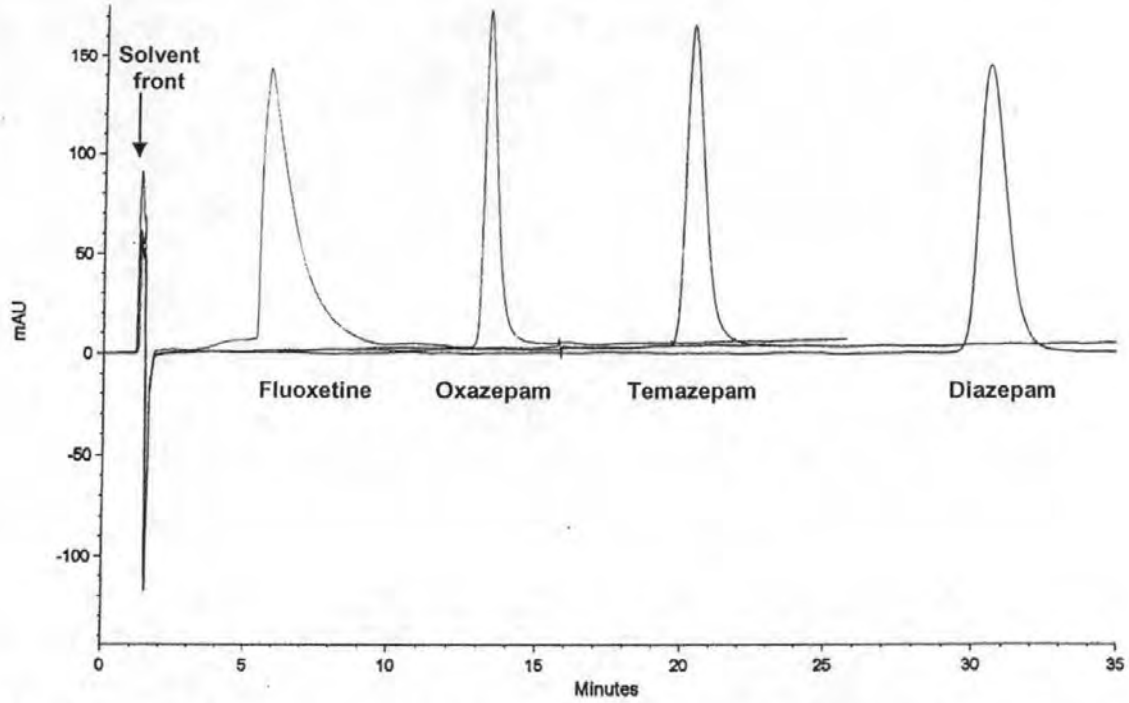


Figure A.15. Example 2: HPLC-UV method development chromatogram (at 254nm) of a mixture of four target compounds using Thermoquest, Hypercarb, 5µm, 10cm x 2.1mm i.d.

Aqueous phase (A): Milli-Q : MeOH : formic acid (95 : 5 : 0.1; v/v/v)
Organic phase (B): ACN + 0.1% formic acid (v/v)
Isocratic: 67 : 33; (A : B)
Note: This particular analysis was performed by C. West using individual standards 0.1 mg mL^{-1} (in 50% MeOH : 50% ACN : 0.1% formic acid v/v/v)

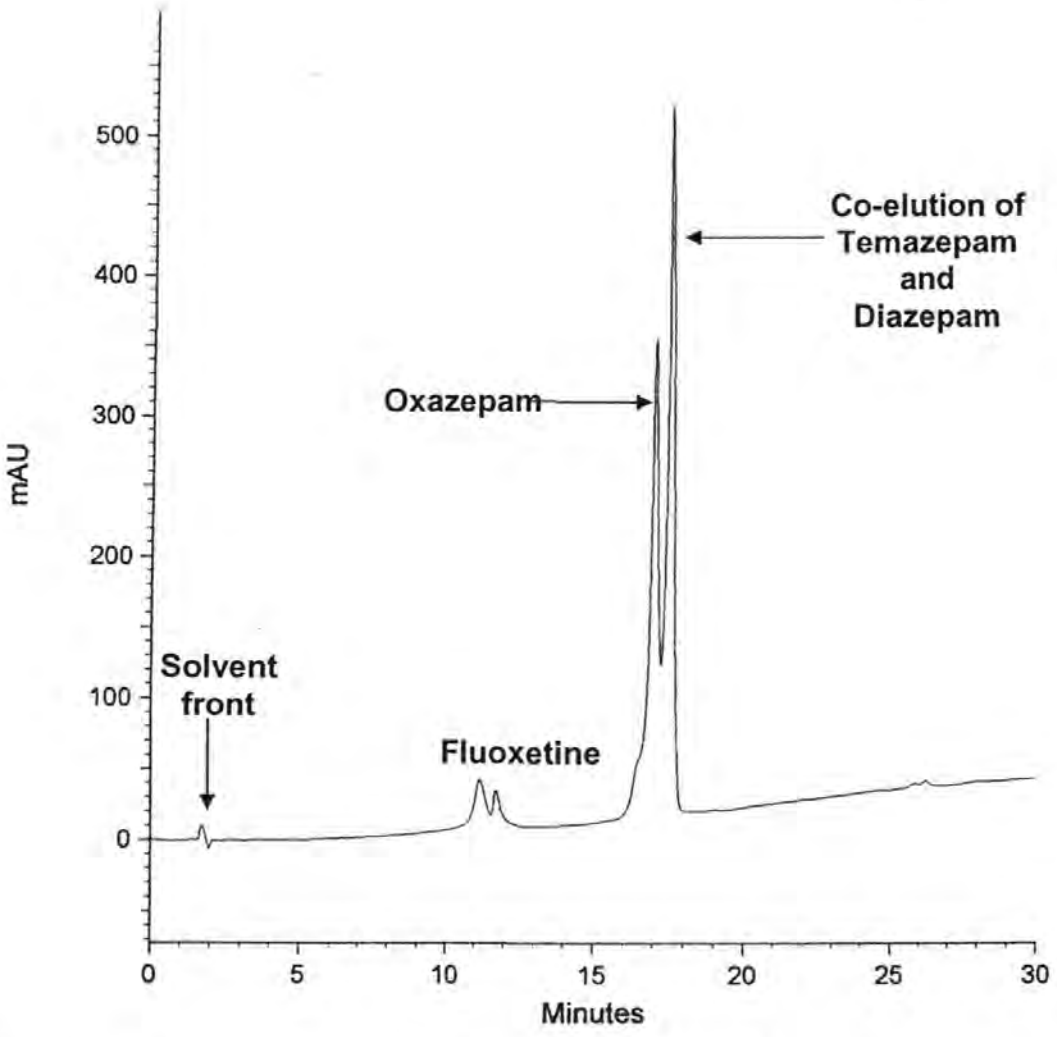


Figure A.16. Example 1: HPLC-UV method development chromatogram (at 254nm) of a mixture of four target compounds using Phenomenex, Gemini C_{18} hybrid, 5µm, 15cm x 2.1mm i.d.

Aqueous phase (A): Milli-Q + 0.1 % formic acid (v/v)
Organic phase (B): MeOH + 0.1% formic acid (v/v)
Gradient: 5 – 100% B over 30 minutes

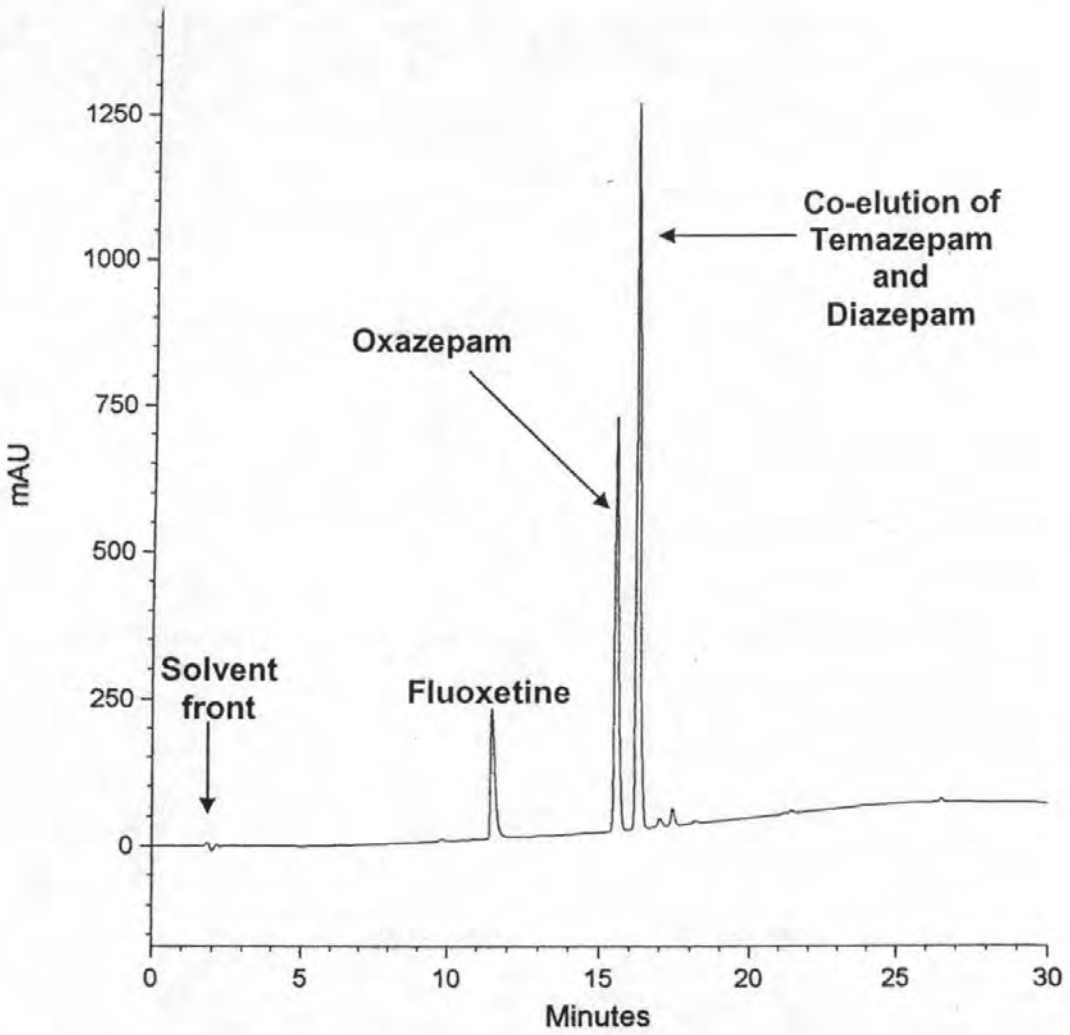


Figure A.17. Example 2: HPLC-UV method development chromatogram (at 254nm) of a mixture of four target compounds using Phenomenex, Gemini C_{18} hybrid, 5µm, 15cm x 2.1mm i.d.

Aqueous phase (A): Milli-Q + 0.1 % formic acid (v/v)
Organic phase (B): MeOH : ACN : formic acid (90 : 10 : 0.1; v/v/v)
Gradient: 5 – 100% B over 30 minutes

Minimal salts media (MSM) consisted of:

$MgSO_4.7H_2O$	0.2 g L^{-1}
$(NH_4)_2SO_4$	0.5 g L^{-1}
KH_2PO_4	0.5 g L^{-1}
K_2HPO_4	1.5 g L^{-1}
Na_2EDTA	0.12 g L^{-1}
NaOH	~ 0.02 g L^{-1} (to adjust pH to 7)

1ml of trace element solution per L of MM added. Trace element solution consists of:

$ZnSO_4$	0.4 g 100 mL^{-1}
$CaSO_4$	0.1 g 100 mL^{-1}
Na_2SO_4	0.01 g 100 mL^{-1}
Na_2MoO_4	0.1 g 100 mL^{-1}
$CoCl_2$	0.01 g 100 mL^{-1}
$MnSO_4$	0.04 g 100 μL^{-1}

Figure A.18. Constituents of minimal salts media used in liquid culture biodegradation studies for Fluoxetine, Norfluoxetine, Temazepam, Diazepam and Oxazepam

	Sample Types Compared		F-test		t-test	
			P-value	Significance	P-value	Significance
Fluoxetine	Sample Day 0	Sample Day 60	0.779	Variance not significantly different	0.507	Means not significantly different
	Control Day 0	Control Day 60	0.880	Variance not significantly different	0.170	Means not significantly different
	Sample Day 60	Control Day 60	0.735	Variance not significantly different	0.224	Means not significantly different
Norfluoxetine	Sample Day 0	Sample Day 60	0.036	Variance significantly different	0.304	Means not significantly different
	Control Day 0	Control Day 60	0.476	Variance not significantly different	0.604	Means not significantly different
	Sample Day 60	Control Day 60	0.458	Variance not significantly different	0.934	Means not significantly different
Diazepam	Sample Day 0	Sample Day 60	0.442	Variance not significantly different	0.180	Means not significantly different
	Control Day 0	Control Day 60	0.0004	Variance significantly different	0.8072	Means not significantly different
	Sample Day 60	Control Day 60	0.118	Variance not significantly different	0.2779	Means not significantly different
Temazepam	Sample Day 0	Sample Day 60	0.925	Variance not significantly different	0.009	Means significantly different.
	Control Day 0	Control Day 60	0.017	Variance significantly different	0.300	Means not significantly different
	Sample Day 0	Control Day 0	0.842	Variance not significantly different	1.000	Means not significantly different
	Sample Day 60	Control Day 60	0.015	Variance significantly different	0.497	Means not significantly different
Oxazepam	Sample Day 0	Sample Day 60	0.009	Variance significantly different	0.024	Means significantly different.
	Control Day 0	Control Day 60	0.254	Variance not significantly different	0.019	Means significantly different.
	Sample Day 60	Control Day 60	0.103	Variance not significantly different	0.0004	Means significantly different.

Table A.9. Results for the statistical analysis of normalised percentage recovery data generated in the liquid culture biodegradation of Fluoxetine, Norfluoxetine, Diazepam, Temazepam and Oxazepam

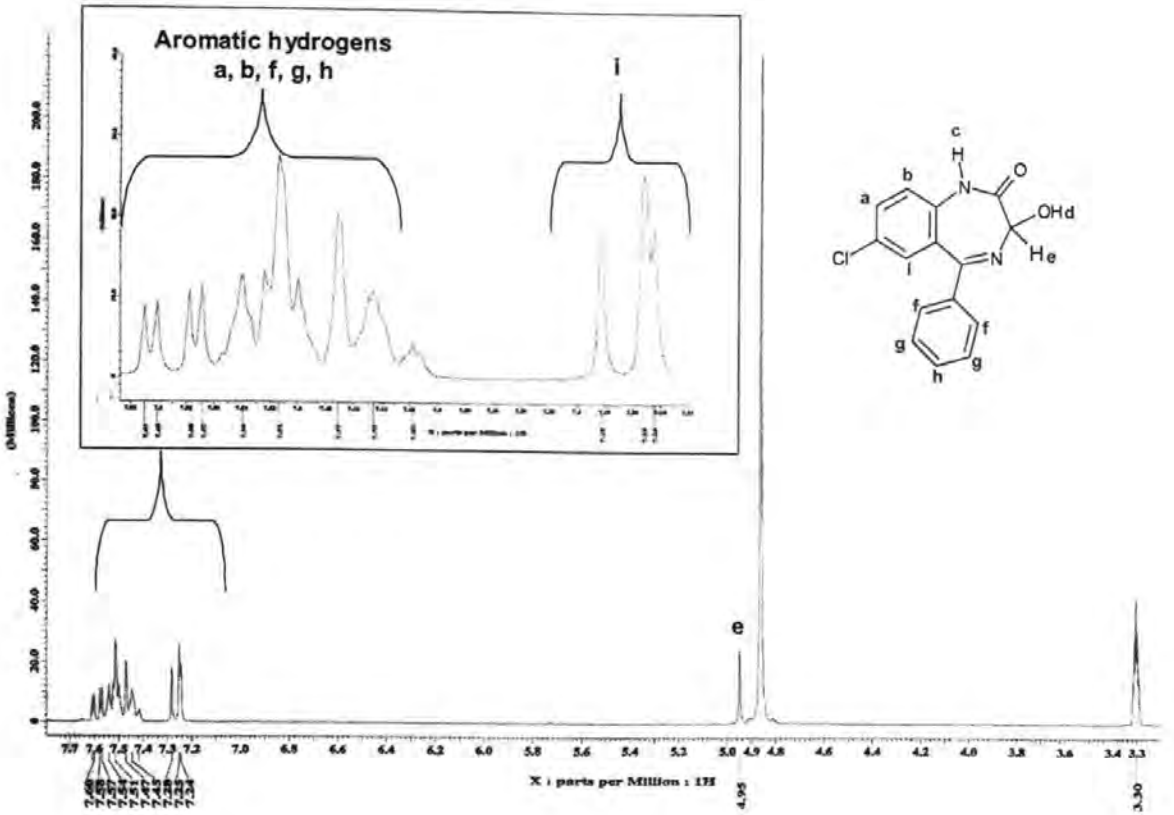


Figure A.19. ^{1}H NMR Spectrum of Oxazepam in CD_3OD (5 mg mL^{-1})

Jeol EX270 Hz high resolution FT-NMR spectrometer used.
16 scans
Proton i was found to have a coupling constant of 3 (usually 8), therefore indicating this H has no H neighbours. Protons c and d had undergone deuteration.
Evidence that keto tautomer is dominant isomer; as proton e was found to be present (as was quaternary carbon C = O).

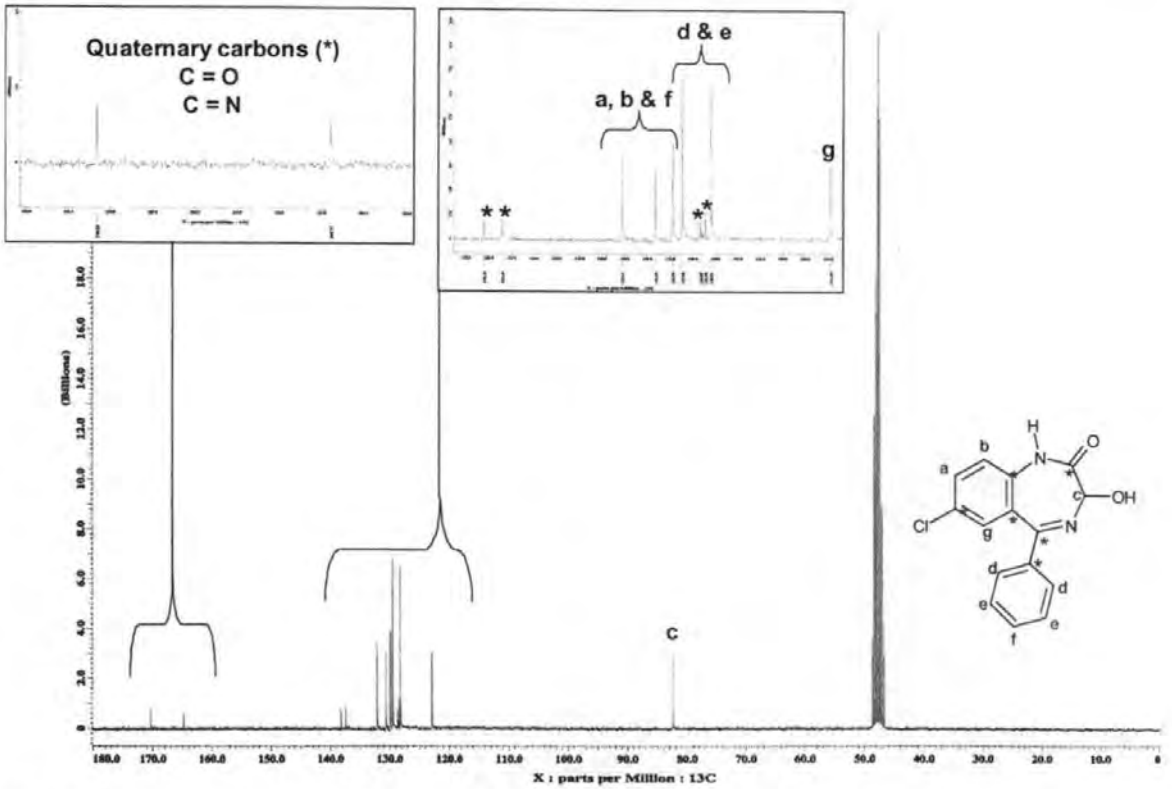


Figure A.20. ^{13}C NMR Spectrum of Oxazepam in CD_3OD (5 mg mL^{-1})

Jeol EX270 Hz high resolution FT-NMR spectrometer used.
16,000 scans
* indicates quaternary carbon
Evidence that keto tautomer is dominant isomer; as quaternary carbon C = O was found to be present (as was proton e).

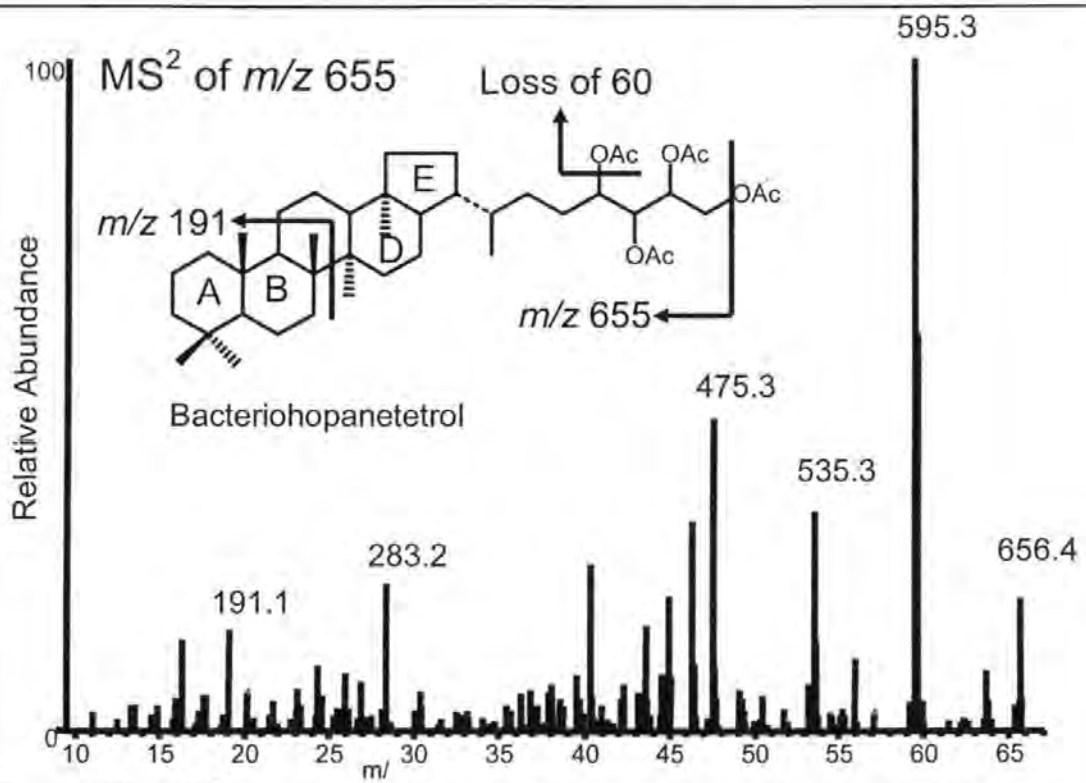
	Compound / Constituent	Concentration (g L^{-1})
Phosphate Buffered Saline	Sodium chloride (NaCl)	40
	Potassium chloride (KCl)	1
	Anhydrous potassium dihydrogen phosphate (KH_2PO_4)	1
	Anhydrous disodium hydrogen phophate (Na_2HPO_4)	4.6
Tryptone Soya Agar	Casein peptone (pancreatic)	15
	Soya peptone (papainic)	5
	Sodium chloride (NaCl)	5
	Agar	15
	Final pH 7.3 ± 0.2 at 37°C	
Potato Malt Agar	Potatoe infusion from solids	4
	Malt extract	20
	Peptic digest of animal tissue	1
	Sucrose	60
	Agar	20

Table A.10. Constituents of tryptone soya agar, potato malt agar and phosphate buffered saline used in viability testing during biodegradation of Fluoxetine HCl in SS amended-soil experiment

m/z	Known Bacterial Source
627	Originally in ammonia oxidising bacterium and subsequently found in Purple non-sulphur (Talbot et al 2007)
653	Acetic acid bacteria
655	Various
669	Cyanobacteria
712	Purple non-sulphur (one species only – Talbot et al 2007)
714	Various
728	Unknown species – possible in cyanobacteria as 2-methyl – (Talbot et al in review)
•	Purple non-sulphur, nitrogen fixing, ammonia oxidising
760	Nitrogen fixing bacteria (Talbot et al 2007)
761	Purple non-sulphur (Talbot et al 2007)
772	Methanotrophs (Types I and II)
775	Unknown species
•	Methanotrophs
830	Methanotrophs (Type I)
•	Methanotrophs
•	Cyanobacteria (Talbot et al – in review)
943	Cyanobacteria (Talbot et al – in review)
957	Cyanobacteria – especially if 2-Methyl (Talbot et al – in review)
1002	Methylotrophs, Cyanobacteria, Purple non-sulphur, acetic acid, *Burkholderia*
1016	Cyanobacteria as it is 2-Methyl
1060	Various
1074	Cyanobacteria as 2 methyl
1086	Facultative Methylotrophs
1118	Unknown species
1132	Unknown species – possibly cyanobacteria as 2-Methyl

Figure A.21. Bacteriohopanepolyols Analysis: Base Peak *m/z* and known bacterial sources

Compounds are referred to by their base peak *m/z* value for ease of identification and differentiation
Base peak m/z = $[M+H]^+$ or $[M+H\text{-}CH_3COOH]^+$
Please note this information is sourced from a report kindly prepared by (Cooke, 2007).



Prior to HPLC-MS^n the extracts were acetylated using pyridine and acetic anhydride to increase the amenability of the extracts to separation by HPLC-MS^n. This results in the acetylation of any hydroxyl groups and amines on the hopanoids.
These acetylated hydroxyls (OAc) have a characteristic mass of 59, but are lost as the neutral molecule (mass = 60) and as each is lost during the analysis the remaining fragment loses 60 from its mass give a spectra such as the one above (655-595-535-475) which equates to the loss of the 4 CH_3COOH groups from the molecule, in this case bacteriohopanetetrol.
In addition to the serial loss of hydroxyls BHPs have characteristic spectra based upon the fragmentation of the
ring system of the molecule. The remaining m/z 475 ion breaks on the C ring to give fragments of 191 and 283 depending where the charge is retained.
If the BHP was methylated at the C2 or C3 position then the 191 fragment would become 205.
If the A or B rings were unsaturated then the 191 would become 189.
For pentols the loss of all functional groups gives an m/z of 473 with corresponding 191 and 281 ring fragments
For hexols the loss of al functional groups gives m/z 471 and therefore 191 and 279 fragments.
BHPs with amine functional groups produce stable NH_3^+ ions that give different characteristic spectra (see m/z 714). The final method of identification is the relative retention time of the compounds. More polar compounds have a short retention time. Methylated compounds have a longer retention time than their non-methylated equivalents.

Figure A.22. Bacteriohopanepolyols Analysis: Identification of Bacteriohopanepolyols in samples based upon HPLC-MS^n fragmentation

Base peak m/z = $[M+H]^+$ or $[M+H-CH_3COOH]^+$
Please note this information is sourced from a report kindly prepared by (Cooke, 2007).

m/z	655	669	714	728	746	760	761	772	775
Bacterial source	Various	Cyanobacteria	Various	Unknown species (possible in cyanobacteria as 2-methyl)	Puirple non-sulphur, nitrogen fixing, ammonia oxidising	Nitrogen fixing bacteria	Purple non-sulphur	Methanotrophs (Types I & II)	Unknown species
Woburn	✔	✔	✔	✔	✔	✔	✔	✔	✔
Gleadthorpe	✔	✔	✔	✘	✔	✔	✔	✔	✔
Watlington	✔	✔	✔	✘	✔	✔	✔	✔	✔
Bridgets	✔	✔	✔	✔	✔	✔	✔	✔	✔
Rosemaund	✔	✔	✔	✔	✔	✔	✔	✔	✔
Pwllpeiran	✔	✔	✔	✘	✔	✔	✔	✔	✔
Shirlburn	✔	✔	✔	✘	✔	✔	✔	✔	✔
Launceston	✔	✔	✔	✔	✔	✔	✔	✔	✘

Table A.11. Bacteriohopanepolyols Analysis: Identification of Bacteriohopanepolyols in sewage sludge amended-soil samples from eight different sites across the UK

Base peak m/z = $[M+H]^+$ or $[M+H-CH_3COOH]^+$
Please note this data is sourced from a report kindly prepared by (Cooke, 2007).
Rothamsted Research kindly supplied SS amended soils from Woburn, Gleadthorpe, Watlington, Bridgets, Rosemaund, Pwllpeiran and Shirlburn. Soil from Launceston was used in extraction method development work and as an inocula source for all biodegradation studies.

Table A.11 continued...

m/z	830 aminopentol	1002 cyclitol ether	1016	1060	1074	1086	1118
Bacterial source	Methanotrophs (Type I)	Methylotrophs, Cyanobacteria, Purple non-sulphur, acetic acid, *Burkholderia*	Cyanobacteria	Various	Cyanobacteria	Facultative Methyltrophs	Unknown species
Woburn	✔	✔	✔	✔	✔	✔	✔
Gleadthorpe	✔	✔	✔	✔	✔	✔	✘
Watlington	✔	✔	✔	✔	✔	✔	✘
Bridgets	✔	✔	✔	✔	✔	✔	✔
Rosemaund	✔	✔	✔	✔	✘	✔	✘
Pwllpeiran	✔	✔	✔	✔	✔	✔	✔
Shirlburn	✘	✔	✘	✔	✔	✔	✘
Launceston	✘	✔	✔	✔	✔	✔	✘

Table A.11. Bacteriohopanepolyols Analysis: Identification of Bacteriohopanepolyols in sewage sludge amended-soil samples from eight different sites across the UK

Base peak m/z = $[M+H]^+$ or $[M+H\text{-}CH_3COOH]^+$
Please note this data is sourced from a report kindly prepared by (Cooke, 2007).
Rothamsted Research kindly supplied SS amended soils from Woburn, Gleadthorpe, Watlington, Bridgets, Rosemaund, Pwllpeiran and Shirlburn. Soil from Launceston was used in extraction method development work and as an inocula source for all biodegradation studies.

Table A.11 continued...

m/z	Total non-methylated tetra	Total methylated tetra	Total non-methylated penta	Total methylated penta	Total non-methylated hexa	TOTAL BHP
Woburn	8.8E-09	1.3E-09	2.9E-09	2.9E-09	5.8E-10	4.8
Gleadthorpe	1.2E-08	1.2E-09	2.6E-09	2.6E-09	5.9E-10	5.2
Watlington	1.8E-08	3.4E-09	3.6E-09	3.6E-09	1.2E-09	6.5
Bridgets	1.3E-08	1.4E-09	1.5E-09	1.5E-09	1.3E-09	6.5
Rosemaund	1.2E-08	1.7E-09	1.9E-09	1.9E-09	4.8E-10	5.4
Pwllpeiran	1.7E-08	1.4E-09	2.1E-09	2.1E-09	1.1E-09	9.8
Shirlburn	1.3E-08	2.6E-09	1.6E-09	1.6E-09	2.0E-09	4.1
Launceston	1.3E-08	2.3E-09	1.1E-09	1.1E-09	0.0E+00	3.7
					Mean	5.7

Table A.11. Bacteriohopanepolyols Analysis: Identification of Bacteriohopanepolyols in sewage sludge amended-soil samples from eight different sites across the UK

Base peak m/z = $[M+H]^+$ or $[M+H-CH_3COOH]^+$
Please note this data is sourced from a report kindly prepared by (Cooke, 2007).
Rothamsted Research kindly supplied SS amended soils from Woburn, Gleadthorpe, Watlington, Bridgets, Rosemaund, Pwllpeiran and Shirlburn. Soil from Launceston was used in extraction method development work and as an inocula source for all biodegradation studies.

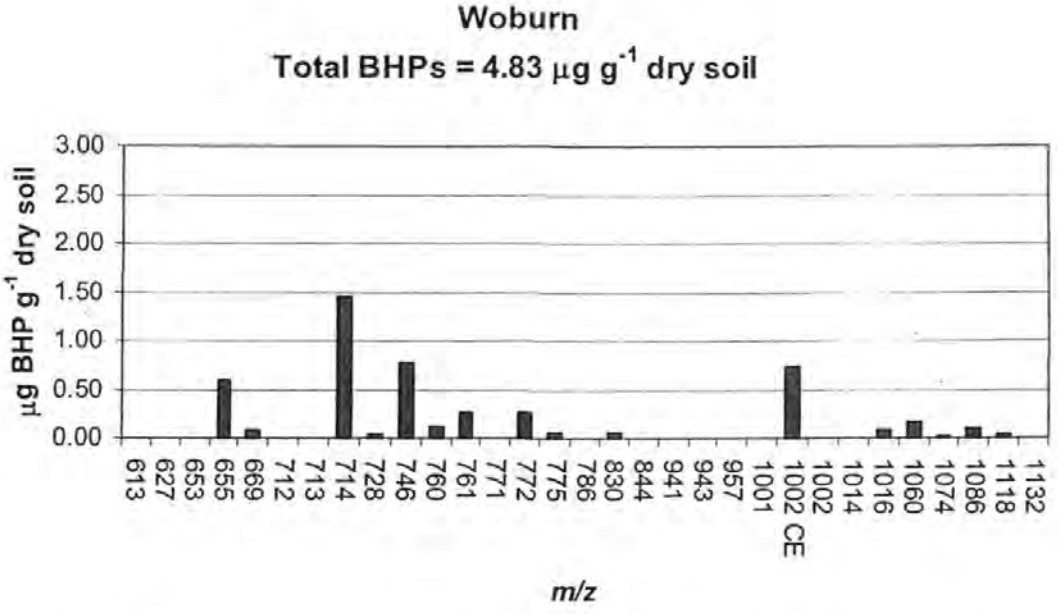


Figure A.23. Bacteriohopanepolyols Analysis: Identification of Bacteriohopanepolyols in sewage sludge amended-soil from Woburn

Base peak m/z = $[M+H]^+$ or $[M+H-CH_3COOH]^+$
Please note this data is sourced from a report kindly prepared by Cooke (2007). Rothamsted Research kindly supplied SS amended soils from Woburn.

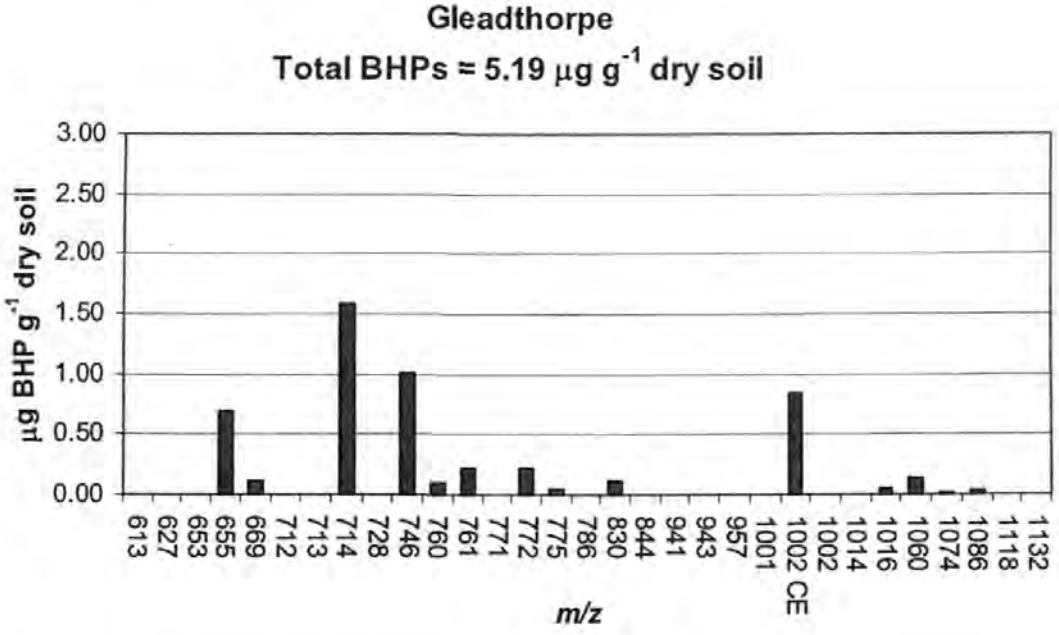


Figure A.24. Bacteriohopanepolyols Analysis: Identification of Bacteriohopanepolyols in sewage sludge amended-soil from Gleadthorpe

Base peak m/z = $[M+H]^+$ or $[M+H-CH_3COOH]^+$
Please note this data is sourced from a report kindly prepared by Cooke (2007). Rothamsted Research kindly supplied SS amended soils from Gleadthorpe.

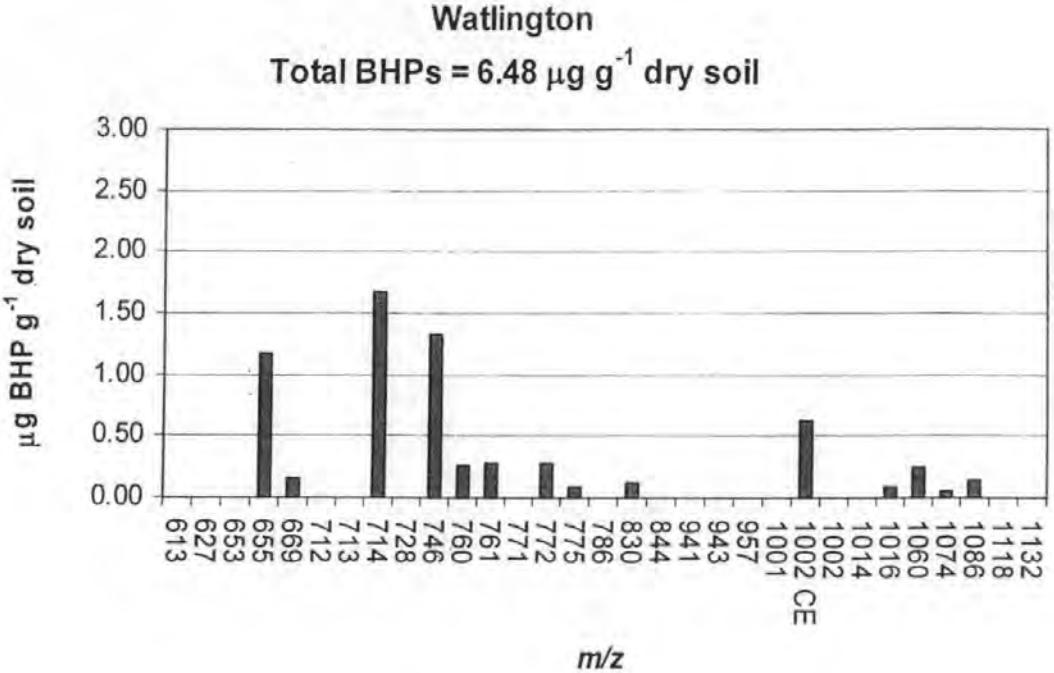


Figure A. 25. Bacteriohopanepolyols Analysis: Identification of Bacteriohopanepolyols in sewage sludge amended-soil from Watlington

Base peak m/z = $[M+H]^+$ or $[M+H\text{-}CH_3COOH]^+$
Please note this data is sourced from a report kindly prepared by Cooke (2007).
Rothamsted Research kindly supplied SS amended soils from Watlington.

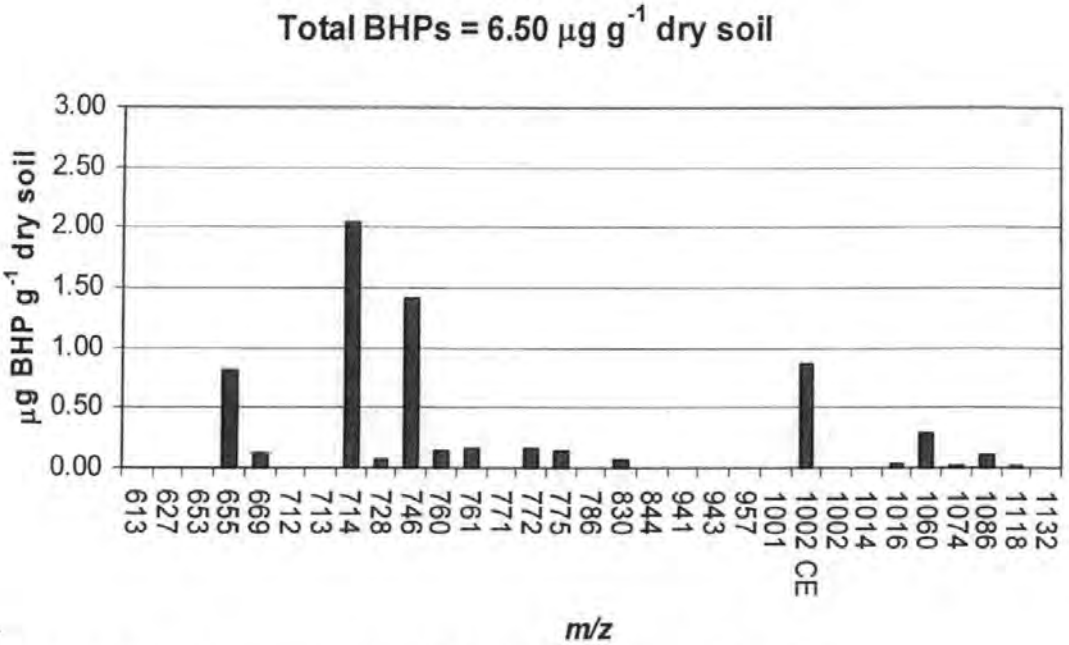


Figure A.26. Bacteriohopanepolyols Analysis: Identification of Bacteriohopanepolyols in sewage sludge amended-soil from Bridgets

Base peak m/z = $[M+H]^+$ or $[M+H\text{-}CH_3COOH]^+$
Please note this data is sourced from a report kindly prepared by Cooke (2007).
Rothamsted Research kindly supplied SS amended soils from Bridgets.

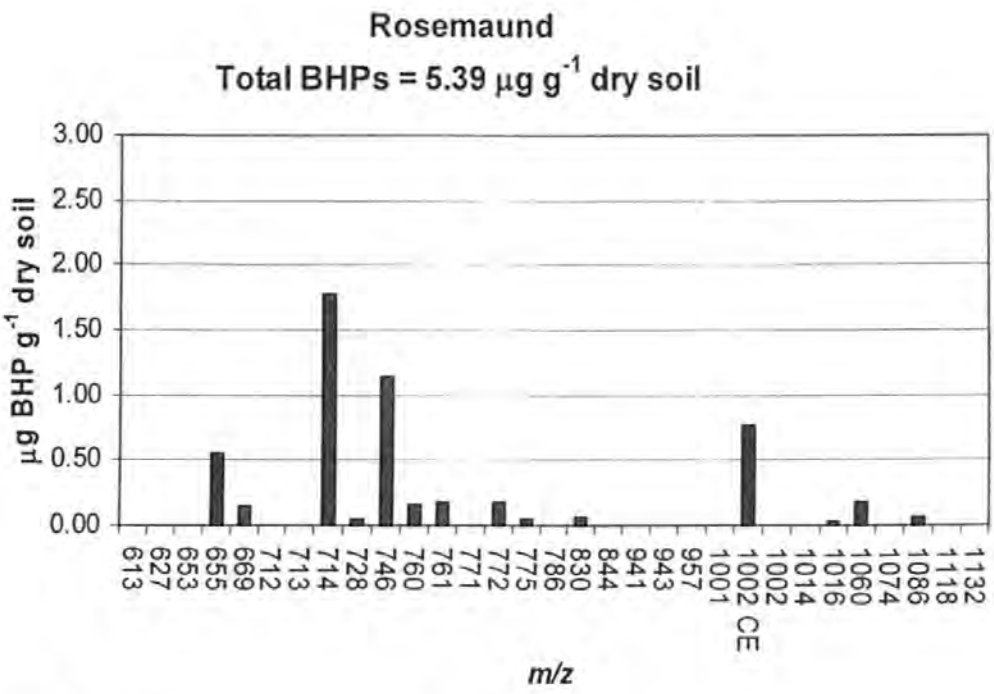


Figure A. 27. Bacteriohopanepolyols Analysis: Identification of Bacteriohopanepolyols in sewage sludge amended-soil from Rosemaund

Base peak m/z = $[M+H]^+$ or $[M+H-CH_3COOH]^+$
Please note this data is sourced from a report kindly prepared by Cooke (2007).
Rothamsted Research kindly supplied SS amended soils from Rosemaund.

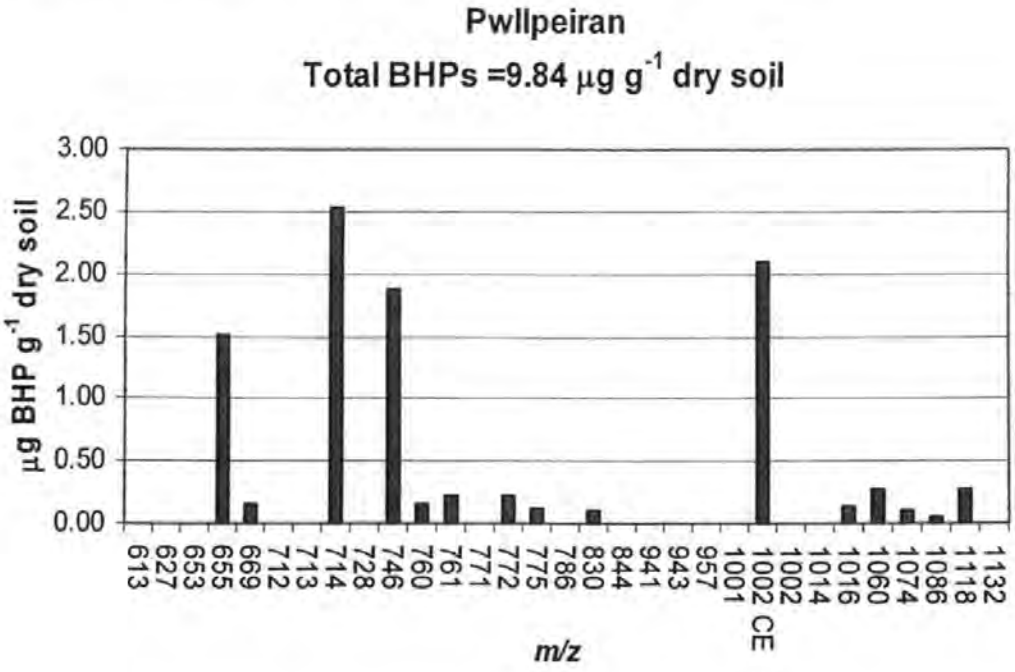


Figure A.28. Bacteriohopanepolyols Analysis: Identification of Bacteriohopanepolyols in sewage sludge amended-soil from Pwllpeiran

Base peak m/z = $[M+H]^+$ or $[M+H-CH_3COOH]^+$
Please note this data is sourced from a report kindly prepared by Cooke (2007).
Rothamsted Research kindly supplied SS amended soils from Pwllpeiran.

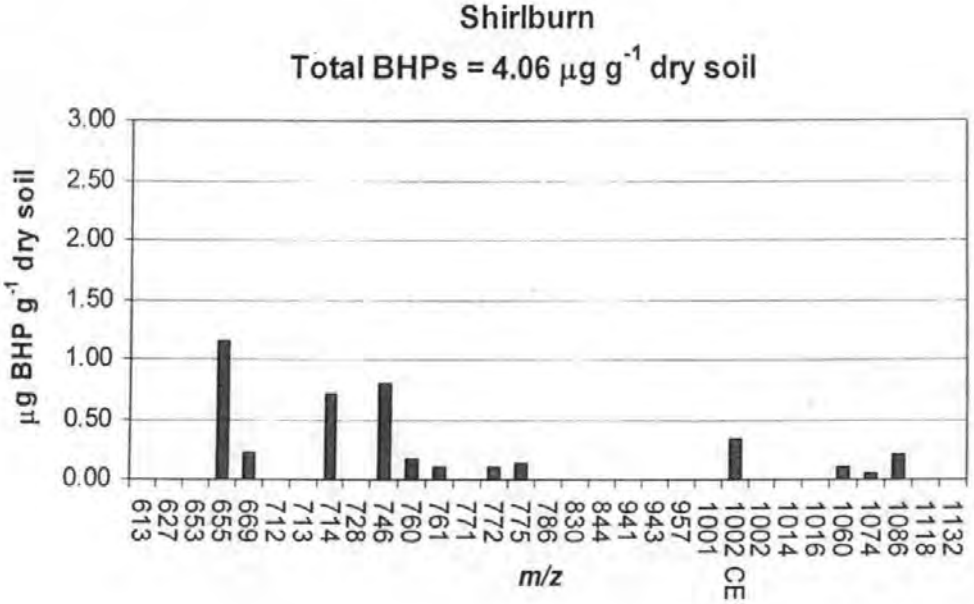


Figure A. 29. Bacteriohopanepolyols Analysis: Identification of Bacteriohopanepolyols in sewage sludge amended-soil from Shirlburn

Base peak m/z = $[M+H]^+$ or $[M+H-CH_3COOH]^+$
Please note this data is sourced from a report kindly prepared by Cooke (2007). Rothamsted Research kindly supplied SS amended soils from Shirlburn.

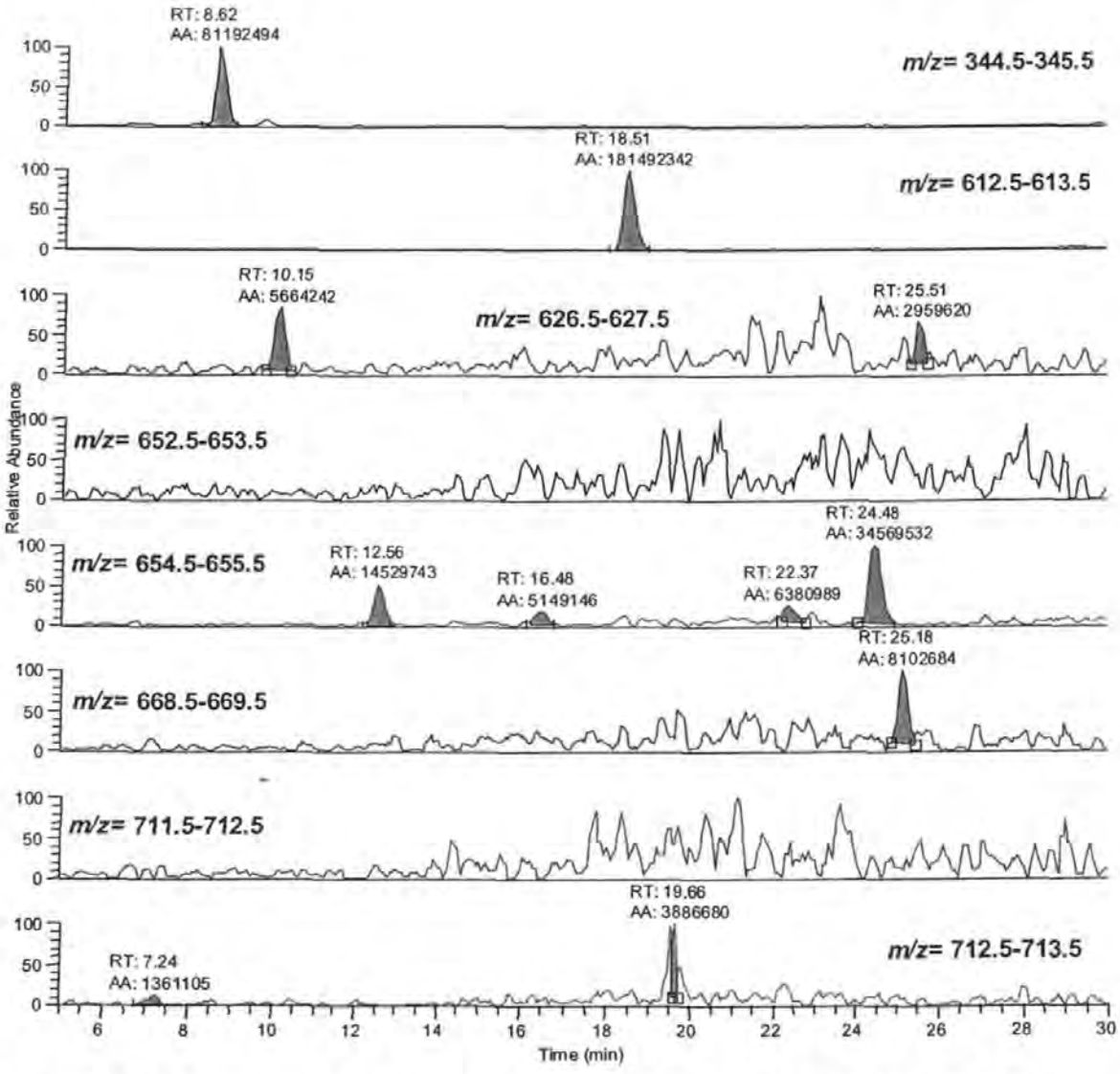


Figure A.30. Bacteriohopanepolyols Analysis: Example Full MS extracted ion chromatograms for the identification and quantification of Bacteriohopanepolyols in sewage sludge amended-soil from Cornwall, UK, low mass range data

Base peak m/z = $[M+H]^+$ or $[M+H-CH_3COOH]^+$
Please note this data is sourced from a report kindly prepared by Cooke (2007).
This soil was used in extraction method development and as inocula for all biodegradation experiments.

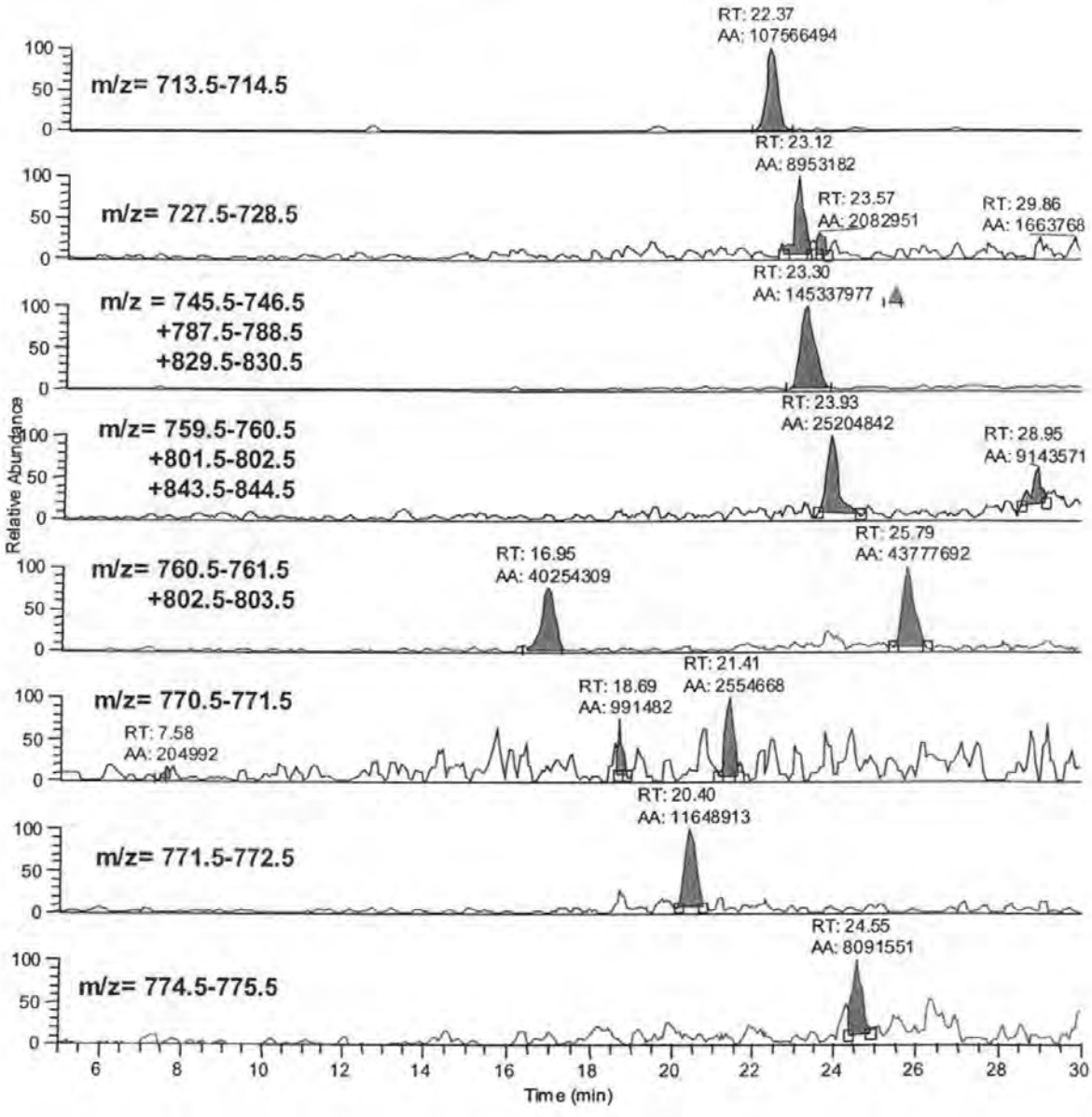


Figure A.31. Bacteriohopanepolyols Analysis: Example Full MS extracted ion chromatograms for the identification and quantification of Bacteriohopanepolyols in sewage sludge amended-soil from Cornwall, UK, low to mid-range mass data

Base peak m/z = $[M+H]^+$ or $[M+H\text{-}CH_3COOH]^+$
Please note this data is sourced from a report kindly prepared by Cooke (2007).
This soil was used in extraction method development and as inocula for all biodegradation experiments.

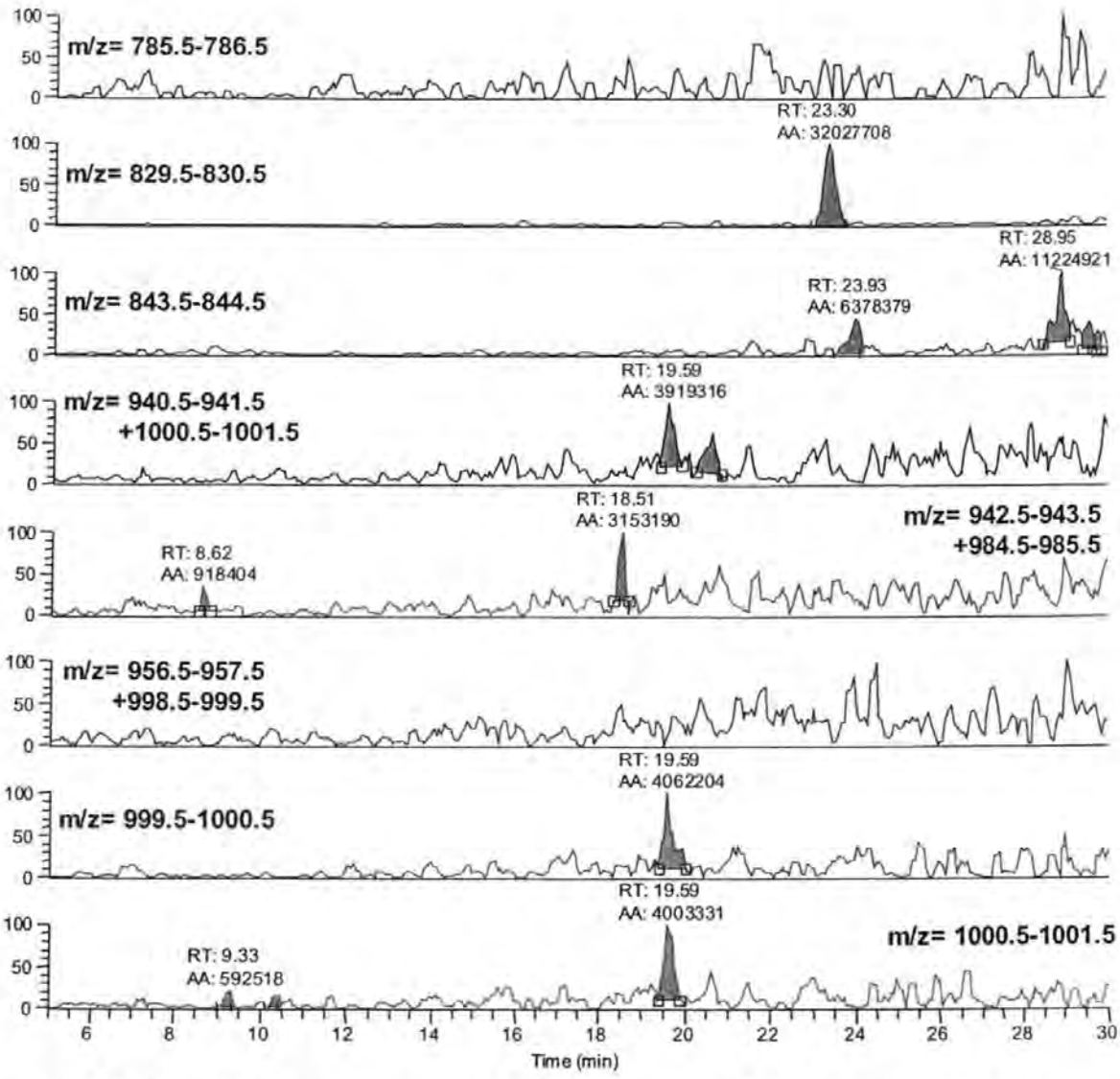


Figure A.32. Bacteriohopanepolyols Analysis: Example Full MS extracted ion chromatograms for the identification and quantification of Bacteriohopanepolyols in sewage sludge amended-soil from Cornwall, UK, mid-range mass data

Base peak m/z = $[M+H]^+$ or $[M+H-CH_3COOH]^+$
Please note this data is sourced from a report kindly prepared by Cooke (2007).
This soil was used in extraction method development and as inocula for all biodegradation experiments.

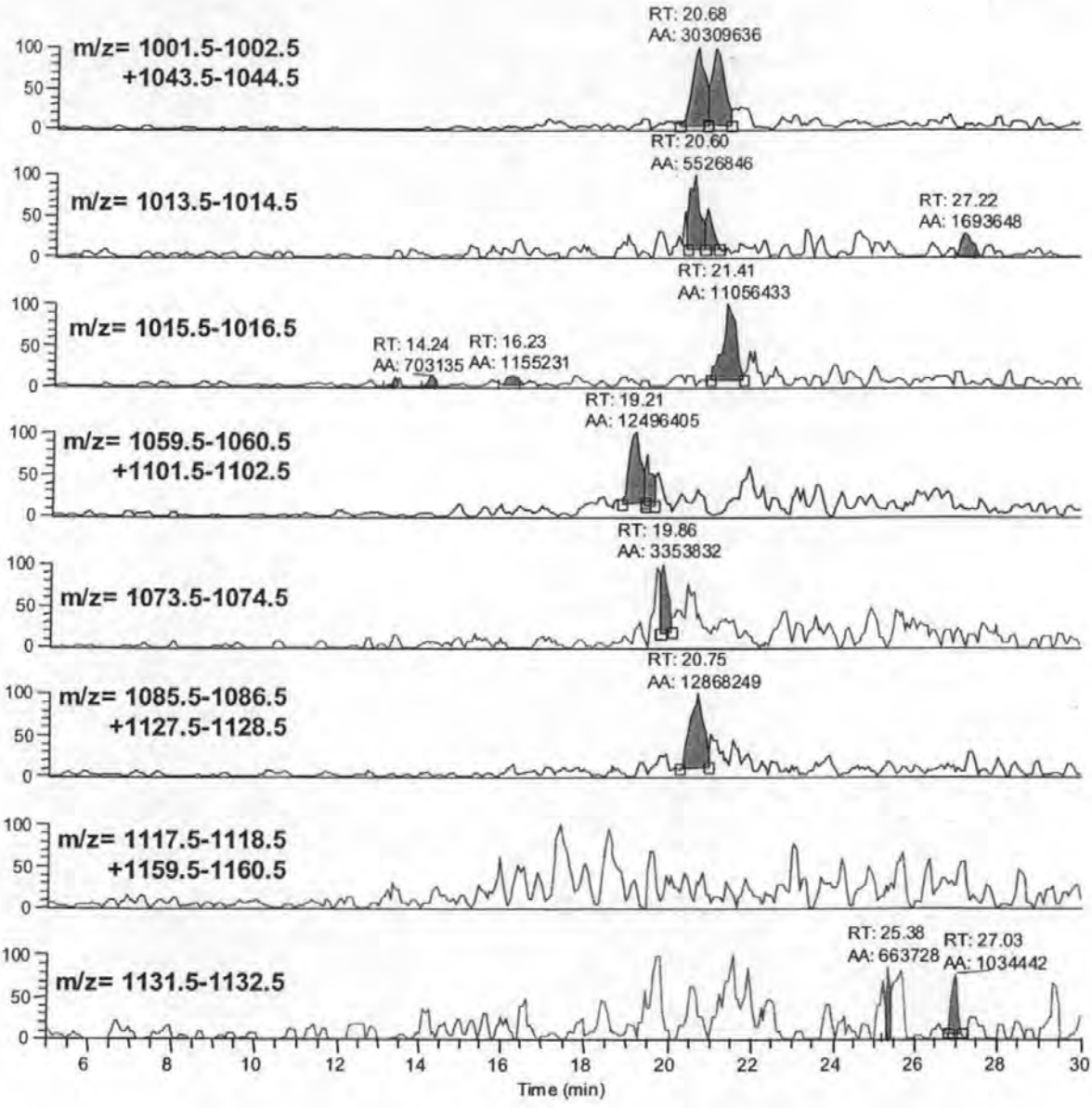


Figure A.33. Bacteriohopanepolyols Analysis: Example Full MS extracted ion chromatograms for the identification and quantification of Bacteriohopanepolyols in sewage sludge amended-soil from Cornwall, UK, high mass range data

Base peak m/z = $[M+H]^+$ or $[M+H-CH_3COOH]^+$
Please note this data is sourced from a report kindly prepared by Cooke (2007).
This soil was used in extraction method development and as inocula for all biodegradation experiments.

Macronutrients	Concentration (mg L^{-1})
Ammonium nitrate (NH_4NO_3)	1650
Boric acid (H_3BO_3)	6.2
Calcium chloride ($CaCl_2$)	332.2
Cobalt chloride ($CoCl_2$)	0.025
Magnesium sulfate ($MgSO_4$)	180.7
Cupric sulfate ($CuSO_4$)	0.025
Potassium phosphate (KH_2PO_4)	170
Ferrous sulfate ($FeSO_4$)	27.8
Potassium nitrate (KNO_3)	1900
Manganese sulfate ($MnSO_4$)	16.9
Potassium iodine (KI)	0.83
Zinc sulfate ($ZnSO_4$)	8.6
Disodium EDTA (Na_2EDTA)	37.2
Organic Additives	**Concentration (mg L^{-1})**
Indole-3-acetic acid (IAA)	0.1
Kinetine	3.8
Sucrose	20 g L^{-1}
Agar	8 g L^{-1}

Table A.12. Details of macronutrients and organic additives that constitute Murashige and Skoog medium used for cauliflower tissue culturing

LIST OF REFERENCES

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