Development of a Method of Analysis by Gas Chromatography-Mass Spectrometry using Selected Ion Monitoring for the Measurement of Fish Sex Steroids in Water

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Abstract

A method for the analysis of testosterone, androstenedione, 5β-dihydrotestosterone, 11-ketotestosterone, estrone, 17β-estradiol, 17α-hydroxyprogesterone, 17α,20β-dihydroxy-4-pregnen-3-one, 5β-pregnan-3α,17α,20α-triol, 5β-androstan-3α,17β-diol, 5β-androstan-3β,17β-diol, 5β-pregnan-3α,17α,20β-triol, 5β-androstan-3α,11β-diol-17-one, 5β-pregnan-3α,17α-diol-20-one, 17α,20β,21-trihydroxy-pregn-4-en-3-one and 11-deoxycortisol in water was developed using gas chromatography mass spectrometry with selected ion monitoring.

Fish sex steroids were extracted from holding water using C_{18} SPE cartridges, and eluted using methanol. Free and glucuronide conjugate levels of steroids were determined, glucuronide conjugates by hydrolysis using β-glucuronidase prior to derivatisation and free steroids by direct derivatisation.

The method thus developed was applied to twelve goldfish holding water samples. Androstenedione was found to be excreted in considerably higher quantities by female ovulatory goldfish than spermiating goldfish, in contrast to previous research in this area.

Acknowledgements

I would like firstly to acknowledge my supervisor Associate Professor Merilyn Manley Harris. Her support and guidance during this project has been unwavering despite the multiple ups and downs of this project. I could not have got this far without your continued help, and I appreciate greatly your ability to always be helpful despite how extremely busy you are.

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I would also like to thank all of the chemistry staff who have assisted me along the way, notably Professor Alistair Wilkins, Wendy Jackson, Annie Barker and Pat Gread.

Thank you to everyone at the chemistry department at The University of Waikato. Particular thanks must go to Jolene, Martyn, Jonathan, Megan, Bevan, Maria, Simon, Kelly and Ben. Whether you were just there for me to bounce ideas off, or to take my mind off things, all of it was greatly appreciated. I wish you all the best with your future endeavours.

Thank you to all my non-chemistry friends, who looked at me crazy when I was trying to explain my frustrations, and played along like they understood what I was talking about. Particularly my flatmates who have had to put up with my random working hours, mess and crazy ideas.

Finally I would like to thank my family who have always been there for me no matter what, even when the chips were down. To Mum and Dad for their continued love and support, and to my sister Michelle for her dedication to sending me motivational quotes every day when finishing seemed impossible.

“The only difference between the possible and the impossible lies in not giving up”- Tommy Lasorda.

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min	minutes
hr	hours
rpm	revolutions per minute
mL	millilitres
µL	microlitres
L	litres
°C	degrees Celsius
psi	pounds per square inch
V	volts
M	moles per litre
nm	nanometres
N_2	nitrogen gas
mg	milligrams
ng	nanograms
pg	picograms
amu	atomic mass units
17,20 β-P	17,20β-dihydroxy-4-pregnen-3-one
C_{18}	C-18 bonded silica stationary phase column
inHg	inches of mercury
pH	$-\log[H^+]$
UV	ultraviolet
r^2	coefficient of variation
Milli-Q	deionised and distilled water
EIA	enzyme immunoassay
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
SIM	Selected Ion Monitoring
HPLC	High Performance Liquid Chromatography

LCMS	Liquid Chromatography-Mass Spectrometry
RIA	radioimmunoassay
$gmol^{-1}$	grams per mole
M_r	molecular weight
MΩcm	mega ohm-centimetre
w/v	weight to volume ratio

1. Introduction

Introduced fish are becoming a large problem worldwide, with increasing numbers of species being considered to be invasive. Pest fish are overtaking important native species and detrimentally affecting the freshwater ecosystem in a number of countries including New Zealand.

A fish is defined as being invasive when it "significantly and adversely affects the long-term survival or genetic variation of native species, or the integrity or sustainability of natural communities"[4]. It is therefore possible that a native fish may be regarded as invasive however it is more common for introduced species to reach this classification.

1.1 Fish invasion in NZ

Prior to colonisation, freshwater fauna in New Zealand were very scarce, with only thirty nine species[5]. It is believed that there were few fish due to geographic isolation and unstable tectonic history[5]. After the arrival of European people, many exotic fish were introduced, both to provide another source of food, and to enable the Europeans to modify the landscape to make it similar to their homeland[5]. New Zealand is very fertile and productive, with a temperate climate which conditions, when combined with lack of fauna, enable introduced fish to thrive, and quickly achieve pest fish status[5].

In the past few decades there has been a dramatic surge in invasive fish that have become pests both in New Zealand and worldwide[6]. There have been twenty two fish species introduced to New Zealand, a rather high number considering there are only thirty nine native species[7]. It is becoming increasingly evident that certain introduced fish are also becoming a big problem here, and four fish have now been officially labelled as pests by the Department of Conservation- koi carp, rudd, catfish and gambusia[7]. These are by no means the only invasive fish that plague the fresh water ecosystem in New Zealand, perch and brown trout have

also been identified as the species posing the greatest threat to New Zealand biodiversity[8].

1.1.1 Negative impacts of invasive fish on the freshwater ecosystem

Negative impacts including increasing turbidity, consumption of eggs, spawn, juvenile fish, insects, plants, and otherwise competing with native species causing loss of habitat [9-12].

Most invasive fish are very prolific breeders, with fecundity increasing with age and size of the female this makes control or eradication very difficult[13].

1.1.2 Priorities in invasive fish research

Current priorities in invasive fish research include[4, 8]:

- the completion of biological studies of rudd, catfish and koi carp
- developing a better understanding of the dynamics of founder pest fish populations
- research in social behaviour
- development of socially and environmentally acceptable eradication tools
- refinement of surveillance monitoring techniques and development of predictive models
- development of containment barriers to safeguard pest free systems

1.2 Chemical Communication in Fish

Chemical communication in the aquatic environment is different from the terrestrial environment due to the difference in methods of transportation of chemical substances[14]. In the aquatic environment, molecules must be in solution while transported (rather than the gas phase), this can prove challenging, as water is slower than air at transporting chemicals (diffusion and current are both slower)[14]. Transportation of chemicals via diffusion is 10,000 slower in water than in air which greatly reduces the maximum size of space in which a receiver is able to detect a signal[15]. Therefore solubility is more vital than volatility in the choice of chemical signals[16]. Chemical communication in water can also be complicated by other dissolved compounds such as heavy metals that can disrupt neural

function, endocrine-disrupting signals that can mask natural signals, and humics that can bind odorants[15, 17, 18].

Stacey and Sorensen[15] theorise the evolution of chemical communication involves three separate phases: ancestral, spying and communication. During the ancestral phase, individuals emit a metabolic product which does not alter receivers, this phase may develop into the second phase of spying if conspecifics evolve to detect the released chemical which is termed a pheromonal cue[15]. Throughout the spying phase, producers of cues remain unspecialised in relation to the production and release of pheromonal cue(s), regardless of whether they benefit from the receivers response[15]. Spying may develop into the third phase, communication if producers also profit from cue production, and evolve in order to enhance efficiency of production[15].

Chemical stimuli are unlike sound and light, they are able to linger and produce non-directional gradients of concentration around a chemical source, which are easily disturbed by turbulence caused by animals, wind and currents[13]. Fish detect chemical stimuli through both gustation (taste) and olfaction (smell)[16, 19]. The gustatory system seems to be dedicated to instinctual identification of feeding cues, however, the olfactory system is reactive to a wider range of stimuli, including pheromones and geographic location[13]. Due to the high specificity and sensitivity with which they are detected by the vertebrate olfactory system, their ease of dispersal and detection in dark and noisy environments, odours are prone to act as social signals[20]. It can be difficult to ascertain whether chemical stimuli produced by fish act as pheromones-chemical signals developed to communicate among individuals- or incidental by-products of physiological processes[15]. Chemical cues are released by fish via the gills, urine, feces and gonadal fluid[15, 20] Little is known about the release of hormonal pheromones however it appears that free steroids are released quickly by gills, sulfated steroids are released slowly in urine, and glucuronidated steroids are released slowly in bile[15]. It is thought the release of free steroids across the gills represents a passive transfer of steroid as a result of differing concentration of steroids in plasma and water[21]. Concentrations of conjugated steroids are largely dependent on rates of defecation and urination of fish, and the time lag required for conjugation to occur[21]. Quantities of free steroids in water are therefore most likely to correlate to the concentration of

physiologically active steroid in the plasma close to the time the sample is taken[21]. The hormonal pheromone systems of a fish quickly transmit information about another individual's hormonal status by acting as a direct link between the individual's endocrine system and the nervous systems of conspecifics[22]. In goldfish, hormonal pheromones are released in amounts ranging from 10-100 ng/hr and detected by other goldfish at concentrations in the picomolar range[23].

Fish utilise olfactory cues for predator avoidance, feeding, and to recognise potential mates, conspecifics, offspring and individuals[13, 15]. When an individual releases water soluble sex hormones they are able to produce striking changes in the physiology and behaviour of the receiver[15]. In certain cases the release of steroids into the water can have an immediate physiological effect such as priming for reproduction[15].

1.3 Pheromones in Fish

Pheromones are defined as "an odour or mixture of odorous substances, released by an individual (the sender) and evoking in conspecifics (the receivers) adaptive, specific, and species-typical responses, the expression of which does not require prior experience or learning"[6].

Detection of pheromones by the receiver requires specialisation within the olfactory system, however specialisation of the donor is not required[6].

Pheromones may often be identified as single compounds, although they can also be mixtures of compounds[6]. A pheromone does not need to be a specialised compound[6].

1.3.1 Functions Pheromones serve in fish

Anti-predation and alarm cues

Fish reduce their predation risk by using various chemically mediated responses[6]. Some fish are able to exhibit evasive responses to the odour of damaged conspecifics or predators that have consumed conspecifics[6].

Non-reproductive aggregation

- *kin and individual recognition in dominance hierarchies and schooling*

in the social systems of some fish, there appears to have evolved chemosensory mechanisms that are used to recognise young, establish dominance relationship, schooling/shoaling and migration[6, 24]

- *Species recognition, aggregation and shoaling responses*

  in many fishes, including common carp and goldfish, the odour of conspecifics promotes aggregation and shoaling[6, 24]

- *Migratory attraction*

  a few species of migratory fish locate spawning and feeding habitats by tracking conspecific odour. Examples of both short and long range attractions are known[24]

Reproductive aggregants and stimulants

- *Gender recognition*

  Some fish species have evolved to distinguish the odour of mature female and male conspecifics well prior to spawning. The male goldfish increases swimming activity when exposed to vitellogenic females or estrogen-treated females, but not males[24]

- *Priming responses before spawning*

  Immediately before and during spawning, some teleost fish respond to the odour of conspecifics by causing hormonal changes that induce final gamete maturation[22]. These have so far been found to be prostaglandins, gonadal steroids or their precursors and metabolites[22, 24]

- *Releaser responses associated with spawning*

  Reproductive behavioural responses to conspecific odours that are commonly induced by released hormonal products[24]

A reproductive pheromone is defined as being a pheromone that induces any behavioural or physiological response associated with reproductive activity[15]. A hormonal pheromone is a term applied to "any reproductive pheromone that contains at least one compound derived from a chemical pathway that produces hormones"[15].

1.3.2 Types of compounds fish use as pheromones

The fish olfactory system has been shown to be very sensitive to amino acids, bile salts, prostaglandins and sex steroids[15, 22]. Electrophysiological studies have shown more than 100 fish species are highly sensitive to steroids and prostaglandins, with sea lamprey also being extremely sensitive to bile acids, which appear to function as pheromones that mediate sexual attraction and migration[15]. Goldfish are able to detect prostaglandins at levels between 10^{-12} and 10^{-10} M and steroid hormones at 10^{-13} to 10^{-12} M [19].

Released androgenic and estrogenic steroids are likely candidates for pheromonal function, as they convey information about the gender and reproductive status to conspecifics[15, 25]. They are also ideal candidates for pheromones as they are naturally produced and released, therefore production of these cues does not require specialisation[25]. The development of the ability to detect may only require a single step mutation where hormone receptors are expressed on external chemosensory receptor cells[25].

There is substantial proof that 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) functions as a pheromone in goldfish[26]. 17,20β-P is synthesised in the ovaries of female goldfish in response to a surge in gonadotropin levels 12 hours prior to ovulation and promotes final maturation of oocytes[26]. 17,20β-P is then released into the water where it functions as a priming pheromone for males, who, when exposed to low levels of 17,20β-P experience a rapid increase in blood gonadotropin combined with increased steroidogenesis, a small increase in sexual arousal and an increase in milt production within several hours of exposure in time for spawning[26].

C-21 steroids (steroids with 21 carbons) are released in the greatest quantity by ovulatory female goldfish, whilst C-19 steroids (steroids with 19 carbons) are released almost exclusively by male goldfish with the one exception being testosterone, which also released in considerable quantities by ovulatory females. Whilst considerable quantities of free steroids are released by goldfish, the majority of steroids released appear to be conjugated with sulfate or glucuronic acid.

1.3.3 How pheromones may be used to aid control of pest fish populations

Due to the scale of the problem of invasive fish, it is unlikely that one method of control would be able to singlehandedly eradicate established fishes in a reasonable period of time, across their entire range[6]. It seems most likely that successful control will be achieved through the use of an integrated pest management strategy that is specifically designed to target multiple weaknesses in the pest species[6]. These approaches are particularly suited to the use of pheromones, as they are typically environmentally safe and potent (therefore only a small amount is required) and have specific actions[6].

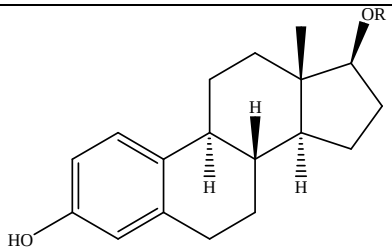
It is possible that 17,20β-P (17,20β-dihydroxy-4-pregnen-3-one) may be used as a synchronising agent to control population wide spawning of females and males, and as an attractant to capture adult males in an integrated pest management control program[6].

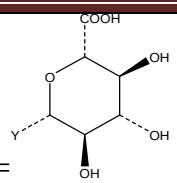
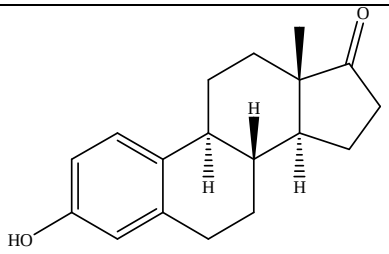
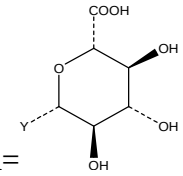
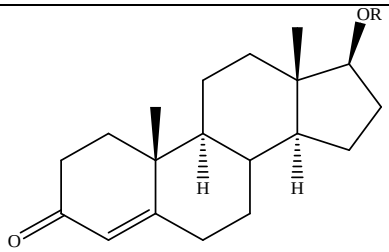
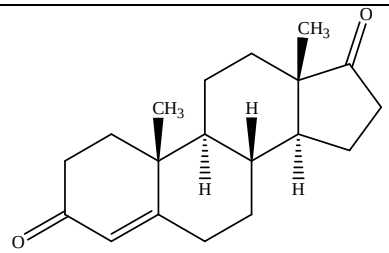
Pheromones can be used to facilitate trapping, disrupt and reduce reproductive success, disrupt movement and migration, promote success of sterilised fish, repel pest fish to prevent them spreading.

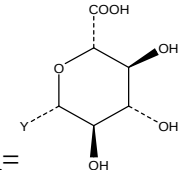
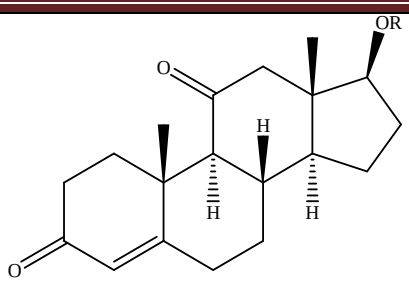
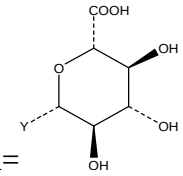
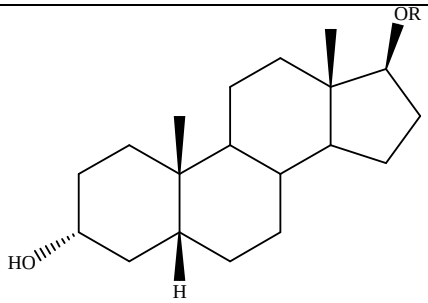
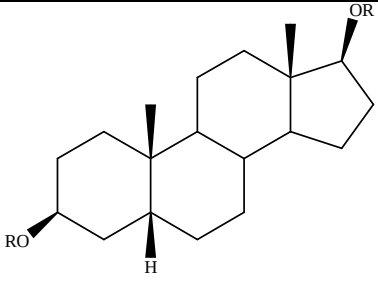
1.4 Free and conjugated steroids which may act as pheromones

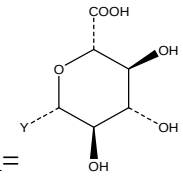
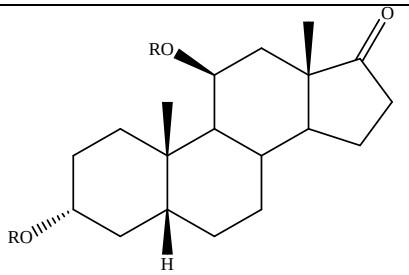
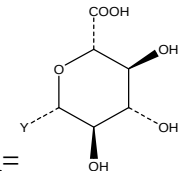
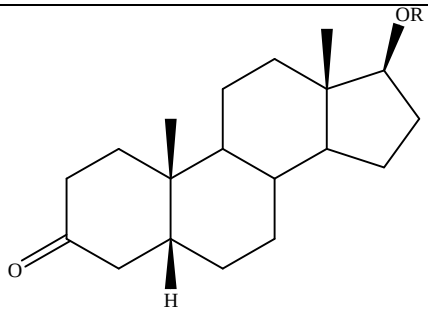
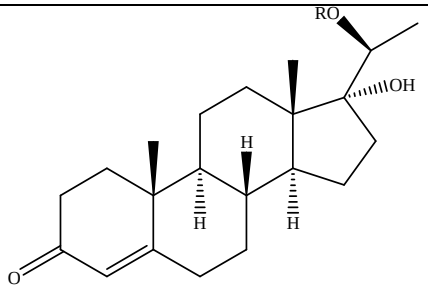
Steroids which may act as pheromones are shown in Table 1.1, only conjugation at C-17 and C-20 are shown (with the exception being 11-deoxycortisol) as this is what appears in the literature.

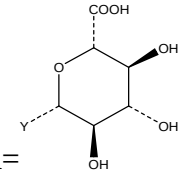
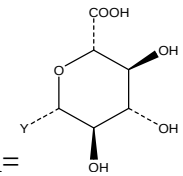
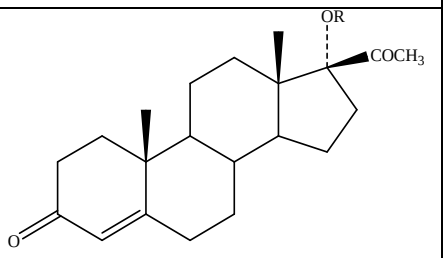
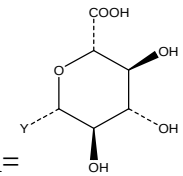
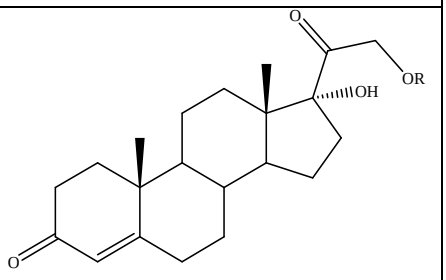
Table 1.1 Free and conjugated steroids which may act as pheromones

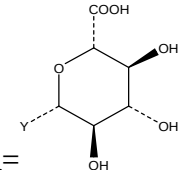
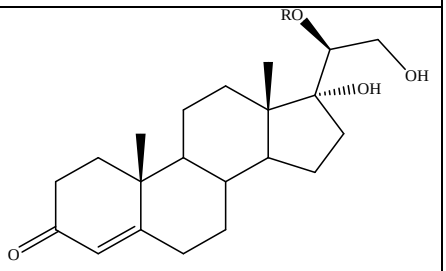
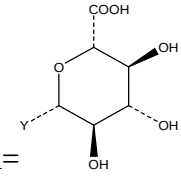
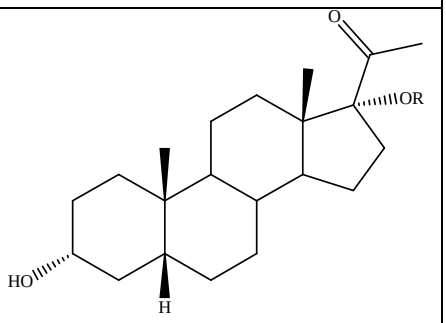
C-18 Steroids	
17β-estradiol (estra-1,3,5(10)-triene-3,17-diol) when R=H, M_r=272.42 $gmol^{-1}$ Estra-1,3,5(10)-triene-3-ol, 17-sulfate when R=SO_3H, M_r=352.49 $gmol^{-1}$ Estra-1,3,5(10)-triene-3-ol, 17-glucuronide when	

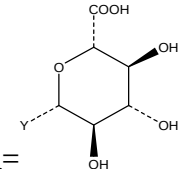
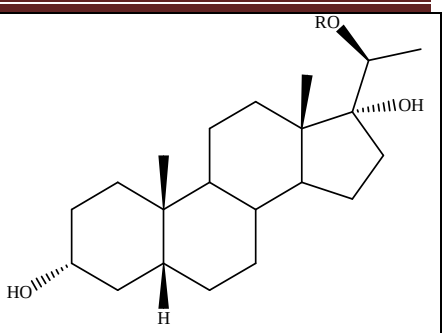
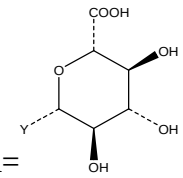
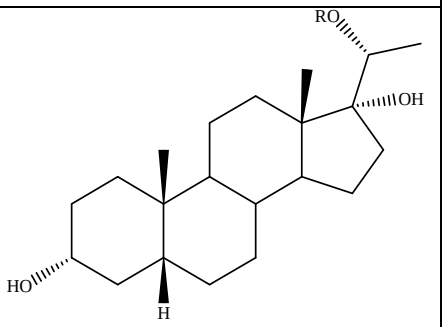
 R= where Y is point of conjugation. M_r= 448.56 $gmol^{-1}$	
Estrone M_r=270.37 $gmol^{-1}$	
C- 19 Steroids	
Testosterone (Androst-4-ene-3-one, 17-hydroxy) when R=H, M_r=288.209 $gmol^{-1}$ Testosterone sulfate when R= SO_3H, M_r=368.166 $gmol^{-1}$ Testosterone glucuronide when  R= where Y is the point of conjugation M_r=464.55 $gmol^{-1}$	
Androstenedione (Androst-4-ene-3,17-dione) M_r=286.193 $gmol^{-1}$	

11-Ketotestosterone (Androst-4-ene-3,11-dione, 17-hydroxy) when R=H, M_r=302.188 $gmol^{-1}$ 11-ketotestosterone sulfate when R=SO_3H, M_r=382.145 $gmol^{-1}$ 11-ketotestosterone glucuronide when  R= where Y is the point of conjugation. M_r=478.220 $gmol^{-1}$	
5β-androstan-3α,17β-diol when R=H, M_r= 292.51 $gmol^{-1}$ 5β-androstan-3α-ol, 17-sulfate when R=SO_3H, M_r=372.58 $gmol^{-1}$ 5β-androstan-3α-ol, 17-glucuronide when  R= where Y is the point of conjugation, M_r=468.65 $gmol^{-1}$	
5β-androstan-3β,17β-diol when R=H, M_r= 292.51 $gmol^{-1}$ 5β-androstan-3β-ol, 17-sulfate when R=SO_3H, M_r=372.58 $gmol^{-1}$	

5β-androstan-3β-ol, 17-glucuronide when  R= where Y is the point of conjugation, M_r=468.65 $gmol^{-1}$	
5β-androstan-3α,11β-diol-17-one M_r= 306.44 $gmol^{-1}$	
5β-dihydrotestosterone (5 β –androstan-17 β -ol-3-one) when R=H, M_r=290.49 $gmol^{-1}$ 5β-dihydrotestosterone 17-sulfate when R=SO_3H, M_r=370.56 $gmol^{-1}$ 5β-dihydrotestosterone 17-glucuronide when  R= where Y is point of conjugation, M_r=466.63 $gmol^{-1}$	
C-21 Steroids	
17,20β-dihydroxy-4-pregnen-3-one (Pregn-4-en-3-one, 17,20β-dihydroxy) when R=H, M_r=332.235 $gmol^{-1}$ 17-hydroxy-4-pregnen-3-one 20-sulfate when	

$R=SO_3H$, M_r=412.192 $gmol^{-1}$ 17-hydroxy-4-pregnen-3-one 20-glucuronide when  R= where Y is point of conjugation, M_r=508.67 $gmol^{-1}$	
17α-Hydroxyprogesterone (Pregn-4-ene-3,20-dione, 17-hydroxy) when R=H, M_r=330.219 $gmol^{-1}$ Progesterone-17-sulfate when $R=SO_3H$, M_r=410.50 $gmol^{-1}$ Progesterone- 17-glucuronide when  R= where Y is point of conjugation, M_r=506.65 $gmol^{-1}$	
11-deoxycortisol when R=H, M_r=346.51 $gmol^{-1}$ 11-deoxycortisol 21-sulfate when $R=SO_3H$, M_r=426.58 $gmol^{-1}$ 11-deoxycortisol 21-glucuronide when  R= where Y is point of	

conjugation, M_r=602.65 $gmol^{-1}$	
17,20β,21-trihydroxy-pregn-4-en-3-one when R=H, M_r=348.230 $gmol^{-1}$ 17,21-dihydroxy-pregn-4-en-3-one 20-sulfate when R=H, M_r=428 $gmol^{-1}$ 17α,21-dihydroxy-pregn-4-en-3-one 20-glucuronide when  R= where Y is point of conjugation, M_r=524 $gmol^{-1}$	
5β-pregnan-3α,17α-diol-20-one when R=H, M_r=334.49 $gmol^{-1}$ 5β-pregnan-3α-ol-20-one, 17-sulfate when R=SO_3H, M_r=414 $gmol^{-1}$ 5β-pregnan-3α-ol-20-one, 17-glucuronide when  R= where Y is point of conjugation, M_r=510 $gmol^{-1}$	

5β-pregnan-3α,17α,20β-triol when R=H, M_r=336.5 $gmol^{-1}$ 5β-pregnan-3α,17α-diol, 20-sulfate when R=SO_3H, M_r=416 $gmol^{-1}$ 5β-pregnan-3α,17α-diol, 20-glucuronide when R=  where Y is point of conjugation, M_r=512 $gmol^{-1}$	
5β-pregnan-3α,17α,20α-triol when R=H, M_r=336.5 $gmol^{-1}$ 5β-pregnan-3α,17α,-diol, 20-sulfate when R=SO_3H, M_r=416 $gmol^{-1}$ 5β-pregnan-3α,17α-diol, 20-glucuronide when R=  where Y is point of conjugation, M_r=512 $gmol^{-1}$	

1.5 Measurement of fish steroids in water

Advantages :

- Minimal intervention, no anaesthetic or bleeding is required therefore sampling stress is reduced[21, 27]
- No sacrifice of fish is required
- Ability to make repeat measurements on the same fish[21, 27]
- Ability to make concurrent behavioural observations
- Ability to measure fish that are too small to be bled[21, 27]

Potential problems include: release rate being affected by the affinity of the steroid for steroid binding proteins in the plasma, concentrations of steroids in plasma, rate of blood flow through gills, fish size/gill surface area, gill permeability, gill damage, salinity, water temperature concentration gradient, opercular beat rate[28].

Concentration of steroids in water is affected by all of the above, as well as fish biomass, water volume, steroid reuptake by fish (in static systems) steroid degradation, and adsorption of steroids to surfaces. Measurements can be affected by the stability of steroid during standing and extraction, and by analysis techniques.

Extraction from water is more costly and time consuming than plasma, and requires validation.

There is some evidence that fish are able to absorb steroids from the water[28]. A recent study on uptake of 17β-estradiol and testosterone in stickleback shows final concentrations of these steroids in plasma can reach 300 times the concentration in surrounding water[29].

1.5.1 Separation of fish sex steroids from other components in fish holding tanks

The concentration of fish steroids in the water from the holding tanks is too low to measure directly, therefore must be concentrated prior to measuring[30]. There are two methods of concentrating sex steroids currently known: the use of solid phase extraction (SPE) cartridges, and freeze drying.

1.5.1.1 Use of SPE cartridges in extracting fish steroids from water

SPE cartridges are very widely used in the extraction of fish steroids from water[25]. They have many drawbacks, including cost of the cartridges themselves, the cost of pre-filters, and the use of organic solvents which are not ideal for the environment and require special disposal[30].

The most common cartridges used for extraction of fish sex steroids from water are those with an octadecyl stationary phase[1, 21, 23, 25, 29, 31-37].

Cartridges are first primed with methanol, often followed by water, prior to use[1, 21, 23, 25, 31, 34]. Various volumes of holding water have been extracted, ranging from 450 mL[38] to 10 L[2, 39]. The most common volume appears to be either 500 mL[23, 25, 35, 36, 40, 41], or 1 L [21, 23, 34, 40, 42]. Flow rates of 5, 10, 16 and 25 $mLmin^{-1}$ are all

common[21, 23, 29, 38, 40-42]. After extraction, cartridges are frequently frozen (typically at -80°C) until a later date when elution is required[21, 23, 25]. Prior to elution, it is typical to wash the cartridge with water to remove water soluble contaminants, mainly proteins[31, 38].

1.5.1.2 Elution of fish steroids from SPE cartridges

Choice of elution solvent can be dependent on whether both free and conjugated steroids are required. The most common choice of solvent is methanol, which elutes both free and conjugated steroids[21, 23, 29, 37, 39, 41-46]. Diethyl ether or ethyl acetate have been used when elution of free steroids only was required[21, 27, 47, 48]. Ethanol and ethanol water mixes have also been used for elution[1, 35, 38, 49].

1.5.2 Alternative methods for extracting fish steroids from holding water

Van Weerd *et al*[3] used an alternative way of processing the fish holding water which involved freeze drying the whole fraction. The holding water (70 L) was first filtered and stored (-20°C, 10 L lots) in polyethylene bags, and the ice was later crushed and freeze dried (not exceeding 15°C)[3]. The residue was rinsed out of the freeze drier tray using distilled water, followed by a wash with 100% ethanol[3]. The ethanol fraction was reduced (~10 mL) and added to the water, to give a final volume of 2 L of concentrated holding water (stored at -20°C)[3].

1.5.3 Methods of separation of free and conjugated steroids

Separation of free and conjugated steroids is important as it is helpful to know how much of each steroid is present in the water, and the form in which it is present.

1.5.3.1 Separation of free and conjugated steroids

The most common way of separating free and conjugated steroids is by dissolving dried down eluent residue in water, and performing an extraction with dichloromethane[1, 3, 49] or diethyl ether[50]. Free steroids are quite non polar, and so dissolve in the organic phase, whilst conjugated steroids are more polar and remain in the water phase[1, 3, 49].

1.5.3.2 Separation of sulfate and glucuronide steroid conjugates

A mix of sulfate and glucuronide steroid conjugates remain in the water phase, and it is useful to distinguish how much of each is present.

Sulfate conjugates can undergo solvolysis to release them into the free form, where they can then be treated the same way as the initial free steroids, but will represent the amount of sulfated steroids were in the conjugate fraction[23]. Solvolysis is performed as following: the water phase is dried down and the residue dissolved in distilled water, solvolysing reagent (trifluoroacetic acid/ethyl acetate) is added, and heated overnight[21, 33, 37, 39, 42, 51-53]. Solvents are removed by evaporation under a stream of nitrogen, and the residue dissolved in sodium acetate buffer, sulfated steroids (now free) are extracted in diethyl ether[21, 42, 48].

Glucuronide conjugates are hydrolysed enzymatically using β-glucuronidase. This converts the conjugated steroid into a free steroid, which may then be processed in: the same way as the free steroid fraction, however it is representative of the glucuronide fraction.

If solvolysis is not performed, the residue containing conjugates is dissolved in sodium acetate buffer, treated with β-glucuronidase and left to incubate overnight[2, 3, 53]. The reaction is terminated with dichloromethane, and released steroids are extracted in dichloromethane[2, 3].

1.6 Preparation of steroids and conjugated steroids for analysis

Steroids tend to be thermally unstable and have a low volatility so are unsuitable for analysis using gas chromatography (GC) unless they are derivatised prior to undergoing gas chromatography[54].

1.6.1 Methods of derivatisation of steroids

There are three main ways of derivatising hydroxyl or acid groups prior to separation on the gas chromatograph:

- Methylation turns OH group to OCH_3
- Acetylation turns OH group to $OCOCH_3$
- Silylation turns OH group to $OSi(CH_3)_3$

All literature examples of derivatisation of fish steroids prior to GC analysis use various forms of silylation.

1.6.1.1 Preparation of oxime trimethylsilyl ethers

Formation of oxime

A fresh solution of hydroxylammonium chloride in pyridine is added to the residue and heated[1-3, 55, 56]. This causes the steroids possessing keto groups to be converted to oxime derivatives which are then silylated[56]. For certain steroids, this can result in two peaks in the chromatogram, one corresponding to the *cis* conformation, the other *trans* (refer to section 3.3.3.1 Development of derivatisation).

1.6.1.2 Preparation of trimethylsilyl ethers

Budzinski *et al*[31] tried the following methods, optimising conditions with testosterone, comparing peak areas of derivatised testosterone to establish which method was best. The molecular ion (288) was monitored to check efficiency of derivatisation[31].

The following derivatisation agents were tested:

1. *n*-methyl-trimethylsilyl trifluoroacetamide (MSTFA)
2. *n,o*-bis(trimethylsilyl) trifluoroacetamide (BSTFA)
3. Pentafluoropropionic acid anhydride. (PFPA)
4. Mix of BSTFA and TMCS (8:2 v/v)
5. Mix of MSTFA, β-mercaptoethanol and ammonium iodide (99.1%/0.5%/0.5%)

Derivatisation agents were tested on a testosterone solution at different temperatures, and time periods[31]. Response was similar for both temperatures, the exception being derivatisations with BSTFA/TMCS and MSTFA. MSTFA and MSTFA/BSTFA mixture gave a lower derivatisation yield at 60°C[31]. Low response and high variability was also seen with PFPA. Silylation efficiency was improved by the addition of a small amount of catalyser, i.e. NH_4I with MSTFA or TMCS with BSTFA[31]. The best sensitivity was achieved using the MSTFA/β-mercaptoethanol/NH_4I mix, with response being greater than one order of magnitude above the other agents[31].

The best method was found to be MSTFA, β-mercaptoethanol and ammonium iodide, heated (60°C, 30 min), cooled to room temperature and the volume adjusted with isooctane (to 100 µL)[31].

1.7 Methods of analysis of steroid hormones

1.7.1 High Performance Liquid Chromatography (HPLC)

1.7.1.1 Chromatographic Separation

A variety of eluents have been used on C_{18} columns to achieve separation of steroid hormones (Table 1.2)

Table 1.2 Various elution methods used to separate steroid hormones

Elution Method	Solvents used	Steroids Separated
Isocratic[57]	Methanol Tetrahydrofuran Water	11-deoxycortisol Androstenedione Testosterone 17α-hydroxyprogesterone 17β-estradiol Estrone 5α-dihydroxytestosterone
Isocratic[58]	Aqueous *N*-methylmorpholine Acetonitrile	Estrone 17β-estradiol
Isocratic[59]	Acetonitrile Aqueous NH_4 H_2PO_4	17β-estradiol Estrone
Isocratic[60]	Acetonitrile Methanol Aqueous Potassium Phosphate Buffer	17β-estradiol Estrone 17α-hydroxyprogesterone Testosterone Androstenedione 5β-dihydroxytestosterone
Gradient[61]	Acetonitrile Water	11-ketotestosterone 17β-estradiol 17,20β-P Testosterone Estrone Androstenedione 17α-hydroxyprogesterone
Gradient[62, 63]	Acetonitrile Water	17β-estradiol Estrone

		Progestogens
Gradient [46]	Acetonitrile Water	17β-estradiol Estrone 17α-hydroxyprogesterone Testosterone Androstenedione 5β-dihydroxytestosterone 11-deoxycortisol
Gradient[64]	Aqueous formic acid Methanol	Progesterone Dihydroxytestosterone 17α-hydroxyprogesterone 11-deoxycortisol 21-deoxycortisol Androstenedione Testosterone Cortisol
Gradient[65]	Acetonitrile Water	17β-estradiol Testosterone Estrone
Gradient[40]	Acetonitrile Water	17,20β-P 11-deoxycortisol 11-ketotestosterone
Gradient[66]	Methanol Aqueous NH_2PO_4	Testosterone 17-hydroxyprogesterone 5α-dihydroxytestosterone Progesterone Androstenedione 5α-androstan-3α,17β-diol 5α-androstan-3β,17β-diol

1.7.1.2 Detection

Ultraviolet

Detection of fish steroids has been achieved using the following wavelengths: 200, 225, 240, 245-247, 254, and 280 nm[18, 57, 60-63, 67].

Mass Spectrometry

Detection of estrogens

Electrospray ionisation was performed in the negative ion mode with the following optimum conditions nebuliser pressure 55 psi, drying gas temperature 300°C, drying gas flow 13 $mLmin^{-1}$, capillary voltage 3500 V, fragmentor voltage 110 V[62]. Analysis was carried out in selected ion monitoring (SIM) mode, 17β-estradiol was scanned at m/z=271 $[M-H]^+$ and estrone was scanned at m/z=269 $[M-H]^+$ [62].

It is also possible for estrogens with a catechol structure to be detected electrochemically, as ring A makes them electrochemically active, becoming oxidised as they pass through a polarization potential[68].

Detection of androgens and estrogens

Electrospray ionisation of 17β-estradiol, testosterone, and estrone was performed in the positive ion mode with the following conditions: nebuliser pressure 60 psi, drying gas (N_2) temperature 350°C, drying gas flow 13 $Lmin^{-1}$, capillary voltage 5000 V, fragmentor voltage 75 V[65]. Data acquisition was carried out in SIM mode[65].

1.7.2 Enzyme Immunoassay (EIA)

Performed for determination of 11-ketotestosterone; fractions were diluted in EIA buffer and assayed by EIA[33]. Cross reactivity of assay was 0.01% for 4-androsten-11β,17β-diol-3-one, <0.01% for 5α-androsten-3β,17β-diol, 5α-androstan-17β-ol-3-one, and testosterone[33]. The limit of detection for the assay of 11-ketotestosterone was 1.3 $pgmL^{-1}$ [33].

1.7.3 Radioimmunoassay (RIA)

The simplicity and low detection limits of RIA make it a good candidate for determining the level of fish steroids in water, however, RIA studies only deal

with free steroids, as they focus on specific steroids for which RIAs are readily available[31]. There have been concerns about the reliability of RIA for measurement of steroids in biological systems[31]. Measurements obtained from RIA should be regarded with caution due to the cross reactivity with other steroids and interference with other substances which can cause false positives[31, 46]. Disadvantages of RIA include limited availability of specific antisera, cross reactivities, safety, time intensive extraction and purification steps[60, 62, 63].

1.8 Gas Chromatography-Mass Spectrometry (GC/MS)

Analysis and quantitative interpretation of low levels of steroids using GC/MS is preferred over RIA methods, as it is possible to determine a total steroid profile compared to RIA whereby it is only possible to measure one steroid at a time[31, 54]. Although GC/MS is widely used in determining steroid levels in humans, they are little used for determining levels in aquatic organisms, mainly due to the low levels that must be reached[31].

Specificity of GC/MS is closely related to:

- Resolution capacity of the capillary column
- Resolution capacity of the mass spectrometer
- Optimisation of all stages of the analytical procedure (including extraction, hydrolysis, derivatisation[54]

1.8.1 Optimisation of GC/MS conditions

1.8.1.1 Temperature programmes

Injection port

The optimum temperature for the injection port is 250°C[1-3, 31, 49, 56, 69-71].

1.8.2 Type of columns used

Columns used to separate steroid hormones in gas chromatography are non polar, with various temperature programmes utilised.

1.8.3 Selection of scanning mode

Analysis of fish sex steroids in water is generally required to be performed in SIM mode, as the concentration of steroids in water is so small, the greatest sensitivity is required.

1.8.3.1 Selected ion monitoring

Electron multiplier operated at 2600-2800 V, however this may vary due to the cleanliness of the instrument[1-3, 49, 56, 71].

Ions selected for SIM mode

Ions previously selected for analysis in SIM are outlined in Table 1.3

Table 1.3: Steroids and ions used previously for selected ion monitoring[1-3]

Steroid	**Ions used for SIM analysis in GC/MS (m/z)**								
Testosterone di TMS	432	417	433	418					
Androstenedione-diTMS	430	432	431	415					
Dihydrotestosterone-diTMS	434	405							
11-ketotestosterone-triTMS	503	518							
Estrone-diTMS	414	399	309	231	415				
17β-estradiol-diTMS	416	285	417	401	231	232	286	418	
17α-hydroxyprogesterone-triTMS	546	316	445	370	301	147			
17α,20β-dihydroxy-4-pregnen-3-one-triTMS	431	548							
5α-androstan-3α,17β-diol-diTMS	256	346	436	241	215	421			
5β-androstan-3α,17β-diol-diTMS	240	256	331	346	347	436	435	241	215
5β-androstan-3α,17β-diol-11-one	306	307	345	360	361	450			
5β-pregnan-3α,17α,20β-triol-triTMS	255	256	345	435	436	437	504	386	
5β-pregnan-3α,17α,20α-triol-triTMS	255	256	345	435	436	437			

Previous results

SIM analysis of the holding water of catfish has shown the presence of , 5β-androstan-3α-ol-17-one, 5β-androstan-3α,17β-diol-11-one, 17β-estradiol, and estrone in the free steroid fraction, and 5β-pregnan-3α,17α,20α-triol, 5β-pregnan-3α,17α,20β-triol, 5β-androstan-3α,17β-diol, eticholanolone, 5β-androstan-3α,17β-diol-11-one, 17β-estradiol, and estrone in the glucuronide fraction[49]. These steroids were presumed to be present in concentrations greater than 10^{-11}-10^{13} M [3].

Quantification

Steroids were quantified in relation to the deuterated steroids (testosterone-*d3* and estradiol-*d4*) used as internal standards[31].

Limits of detection

Detection limits per injection (2 µL) were 100 pg-3.98 ng[49, 56]

Detection limits per litre of water were 0.1 ng for 17β-estradiol, testosterone and androstenedione, 0.2 ng for estrone[69].

For studies on levels in fish plasma, detection limits per gram of plasma were 33 pg for 17α-hydroxyprogesterone, 96 pg for androstenedione, 28 pg for testosterone, 54 pg for estrone, 100 pg for 17β-estradiol, 1100 pg for 17,20βP, 180 pg for 11-deoxycortisol[43].

1.9 Project Objectives

The purpose of this study was to develop an accurate analytical method which could determine the levels of sex steroids in fish holding water. This work could be extended to use for detection of steroids in different species of fish in order to obtain information about steroids released during various phases of the reproductive cycle. Steroids thus identified may then be investigated as potential pheromones. Pheromones can potentially be utilised in an integrated pest management system to aid the control of pest fish populations.

2. Experimental

2.1 Materials

2.1.1 Chemicals

Testosterone, 17β-estradiol minimum 98%, Androstenedione minimum 98%, 17α-hydroxyprogesterone minimum 95% and Stigmasterol minimum 95% were sourced from Sigma Aldrich Inc.

4-pregnen-17,20β-diol-3-one, 4-pregnen-17,20β,21-triol-3-one, 4-pregnen-17,21-diol-3,20-dione, 5β-pregnan-3α,17,20α-triol, 5β-pregnan-3α,17,20β-triol, 5β-pregnan-3α,17-diol-20-one, 5β-androstan-17β-ol-3-one, 5β-androstan-3α,11β-diol-17-one, 5β-androstan-3α,17β-diol, 5β-androstan-3β,17β-diol, 4-androstan-17β-ol,3,11-dione, and 4-androsten-17β-ol-3-oneglucosiduronidate were purchased from Steraloids Inc.

Silylating reagents *n*-trimethylsilylimidazole and Tri-Sil HTP Reagent HMDS/TMCS in pyridine (2:1:10) were sourced from Pierce Biotechnology (also known as Thermo Scientific).

Hydroxylammonium chloride (AR grade) was sourced from Ajax Chemicals Ltd.

Ammonium Carbonate used in HPLC was sourced from Ajax Finechem.

2.1.2 Enzymes

β-glucuronidase from *Escherischia coli* Type IX A was purchased from Sigma Aldrich Inc.

2.1.3 Solvents

Solvents used in this study were methanol, *n*-heptane, chloroform, acetonitrile, water, ethanol, pyridine and dichloromethane. Methanol used as a solvent during SPE in initial tests was of HPLC grade and sourced from either Scharlau or Ajax Finechem as available. Methanol used in the final method was distilled to remove an impurity (see section 3.3.3.5 Method limits of detection). *n*-heptane and chloroform were HPLC grade and sourced from Ajax Finechem. Acetonitrile was HPLC grade and sourced from Burdick & Jackson. Milli-Q water was used as a

solvent during SPE and HPLC and obtained from a Barnstead E-pure system (18.2 MΩcm). Ethanol (absolute) was analytical grade and sourced from Scharlau. Pyridine (minimum 99%, ACS grade) was sourced from Sigma Aldrich Inc. Concentrated nitric acid (27%) used for acid washing was obtained from Ajax Finechem.

2.2 Apparatus

Glassware

Glassware was washed with detergent, rinsed three times with water, and three times with distilled water and dried in an oven (100°C). Vials and inserts used in elution, derivatisation and GC analysis were washed with detergent, rinsed, soaked in concentrated nitric acid overnight and rinsed three times with tap water and three times with distilled water before being dried in an oven.

A Mettler Toledo AT201five figure balance was used to weigh standards for response factors and limits of detection

A Mettler Toledo AB204-S four figure balance was used to weigh larger amounts of reagents when making up solutions.

2.3 General Methods

2.3.1 Freeze Drying

A Labconco Bulk Tray Drier was used to freeze-dry when required. Liquid samples were frozen with liquid nitrogen prior to freeze-drying and left to dry overnight.

2.3.2 Evaporation

Large volumes of liquid were reduced using two rotary evaporators. One system consisted of an Eyela rotary evaporator equipped with an Eyela Water Bath SB-650 and a Büchi Vac® V-500 vacuum pump. The other system comprised a Büchi Rotavapor R-200, Büchi Heating Bath B-490 and a Büchi Vac® V-500 vacuum pump. The water bath was set to 30ºC on both systems.

Smaller volumes of liquid were evaporated using a Pierce Reacti-Therm Heating Module set to heat samples to 40ºC, and a Pierce Reacti-Vap Evaporating Unit which passes a stream of nitrogen gas over the samples.

2.4 Solid Phase Extraction

2.4.1 Sample collection conditions

Goldfish were injected with either: Domperidone- (10 $mgmL^{-1}$, 0.5 $mLkg^{-1}$ of fish) as the control, or Ovaprim- a spawning/ovulation inducer (0.5 $mLkg^{-1}$ of fish) as the treatment. Fish were held in tanks (12 L) for a period of twenty four hours in an approximate volume of two litres. Fish holding water (~2 L, 21-24°C) was pumped through a Waters Sep Pak® Classic C_{18} (360 mg, 55-105 µm) SPE cartridge (previously primed with methanol) using a peristaltic pump (~2 $mLmin^{-1}$). Cartridges were stored at -18°C until required for elution.

2.4.2 Extraction of standards from water

Standard solutions of varying concentrations of testosterone in water were passed through C_{18} SPE cartridges (primed with 5 mL methanol and washed with 5 mL Milli-Q water) using a peristaltic pump (~2 $mLmin^{-1}$, 1 L).

2.4.3 Elution from SPE cartridge

Elution using SPE cartridges was performed using a Varian Vac Elut 20 Extraction Manifold coupled to a Büchi V-700 Vacuum Pump.

Cartridges were washed with water (8 mL) and dried under vacuum (1 hr, 10 inHg) prior to elution. Free and conjugated steroids were eluted directly into a vial using methanol (8 mL).

2.5 Preparation of conjugated steroids for derivatisation

2.5.1 Separation of free and conjugated steroids

Methanol extracts were evaporated under a stream of nitrogen (40°C) and the residue dissolved in water. Free steroids were extracted into dichloromethane (3x2 mL) whilst conjugated steroids remained in the water phase. Both phases were evaporated, free steroids were now ready for derivatisation, conjugated steroids required hydrolysis prior to derivatisation.

2.5.2 Preparation of conjugated steroids for GC/MS analysis

Glucuronidated steroids

The dried residue was dissolved in potassium phosphate buffer (1 mL, pH 6.5) β-glucuronidase was added (100 µL) and left to incubate overnight (37°C).

Reaction was terminated using dichloromethane (6 mL) , and liberated steroids were extracted into dichloromethane (3x2 mL).

Sulfated steroids

For the purpose of this study sulfated steroids were ignored, as no sulfated steroid was obtained to test the validity and recovery of the solvolysis procedure.

2.6 Derivatisation of steroids

Derivatisation of steroid hormones was achieved using either Tri-Sil HTP Reagent or *n*-trimethylsilylimidazole. Residue was either reacted directly with Tri-Sil reagent or dissolved in a small volume of pyridine (200-500 µL), and *n*-trimethylsilylimidazole (100-250 µL) added. Samples were heated using an Accublock digital drybath D1200 (50°C, 1 hr) and evaporated under a stream of nitrogen (40°C).

Residue was dissolved in *n*-heptane (~1 mL), sonicated in an Astrason Ultrasonic Cleaner, (15 min), centrifuged in a Heraeus Sepatech Centrifuge (5000 rpm, 20 min) and supernatant transferred to another vial where it was evaporated to dryness under nitrogen (40°C). The residue was reconstituted in *n*-heptane (100 µL), sonicated and transferred to a GC vial.

2.7 Liquid Chromatography

A Waters 515 HPLC pump was used with a Waters inline degasser using either a Waters Resolve radial compression column (8x100 mm, 5 µm), or a low flow Zorbax C_{18} (2.1x150 mm, 5 µm) column, detection was with a Polymer Laboratories PL-ELS 100 Evaporative Light Scattering Detector.

2.8 GC/MS

The gas chromatograph used was a Hewlett Packard 6890 GC system which was coupled to a HP 5973 Mass Selective Detector and fitted with a 7683 Autosampler and Injector.

2.8.1 Method used

The autosampler was used to perform automated injections (2 µL). The gas chromatograph was operated in splitless mode, inlet temperature was set to 265°C, column flow was 1.1 $mLmin^{-1}$. Separation was achieved using a Zebron ZB5 5%

Polysiloxane 30.0 m x 250 µm x 0.25 µm column using the temperature programme outlined below (Table 2.1).

Table 2.1 Oven temperature programme used for GC of steroids

Time (min)	**Temperature (°C)**	**Temperature Increase (°Cmin^{-1})**	**Hold Time (min)**
0	160		0.5
6.5	250	15	1
27.5	260	0.5	0.5
43	290	2	10

2.8.1.1 Mass spectrometry of steroids

TIC (total ion chromatograph) mode was used to obtain spectra of individual steroids, optimise chromatography and determine ions to be used in SIM mode. Full method scans were of 50-800 atomic mass units (amu) with a solvent delay of 5 min.

Ions selected for SIM mode

SIM mode was used to obtain detection limits, response factors and to analyse fish holding water samples. The mass spectrometer scanned for ions outlined in Table 2.2. Ions were selected based on their intensity. The most abundant ions were chosen where possible, for example, in the 5β-androstan-3β,17β-diol-diTMS spectrum, the 346 ion is more abundant than the 215 ion, hence why it was chosen in preference to 215. Conversely, in the 5β-androstan-3α, 17β-diol-diTMS spectrum, the 215 ion was more abundant than the 346 ion, and was chosen as a confirmation ion rather than 346 for this reason (see section 3.3.3.2).

Table 2.2 Ions selected for SIM analysis

Standard	**Retention Time (min)**	**Ion 1 (m/z)**	**Ion 2 (m/z)**	**Ion 3 (m/z)**
5β-androstan-3β,17β-diol-diTMS	12.00	256	241	346
5β-androstan-3α,17β-diol-diTMS	12.17	256	241	215
5β-dihydrotestosterone-diTMS	12.65	246	362	347
Estrone-TMS	13.74	342	257	218
Androstenedione	14.11	286	244	201
17β-estradiol-diTMS	14.64	416	285	232
Testosterone-TMS	14.86	360	270	226
5β-androstan-3α,11β-diol-17-one-diTMS	15.38	270	360	394
5β-pregnan-3α,17α-diol-20-one	17.06	230	231	215
11-ketotestosterone-TMS	17.59	359	374	284
17α-hydroxyprogesterone-TMS	20.56	359	269	227
5β-pregnan-3α,17α,20β-triol	19.96	255	273	256
5β-pregnan-3α,17α,20α-triol	21.2	255	273	256
17,20β-dihydroxy-4-pregnen-3-one	25.7	287	371	269
11-deoxycortisol	31.41	359	269	227
17α,20β,21-trihydroxy-pregn-4-en-3-one	36.58	359	360	371
Stigmasterol (internal standard)	35.8	255	384	484

3. Method Development

3.1 Comparison of extraction methods

The two methods described in the literature for extracting fish steroids from water were:

1. Freeze drying large volumes of water or ice
2. Extracting steroids from water using C_{18} SPE cartridges

3.1.1 Freeze dry method

1 L solutions of testosterone were reduced to a small enough volume to freeze dry (~10 mL) using a rotary evaporator. Solutions were frozen with liquid nitrogen in the flasks used, and dried overnight. The flask was rinsed 5 times with methanol to dissolve the residue and to be sure to obtain all the material. The solution was transferred to a vial and evaporated to dryness under a stream of nitrogen prior to derivatisation, internal standard was also added in solution form (1 mL, ~200 ng) prior to silylation. These were derivatised and analysed using GC/MS/SIM.

3.1.2 SPE method

1 L solutions of testosterone were passed through C_{18} SPE cartridges using a peristaltic pump (~2 $Lmin^{-1}$). Cartridges were washed with Milli-Q water (5 mL), dried under vacuum for one hour and testosterone eluted from the cartridge using methanol (7 mL). Solution was evaporated to dryness under nitrogen, where the internal standard was also added. Samples were then derivatised, made up to a final volume of 100 µL and analysed using GC/MS/SIM.

SPE was chosen as it is a more practical method of extraction rather than evaporating large volumes of water and freeze drying. It has also been shown that the amount of steroids in solution is reduced upon storage of solutions[38].

3.2 Optimisation of elution for SPE extraction

Originally a 5 mL wash with Milli-Q water followed by a 5mL methanol wash was trialled, however poor results were obtained. Better results were obtained by using a 7 mL wash with Milli-Q water, followed by drying the cartridge under vacuum (1 hr, 10 inHg), and elution with methanol (7 mL)[31]. Recovery was still

poor, and it was determined this was due to the speed of throughput of the testosterone solution when attempting to extract testosterone (1 Lhr^{-1}). When extracting solutions for determination of method detection limits, the flow was reduced to a rate similar to rate used in sample collection (2 $mLmin^{-1}$).

3.3 Analysis Method

An HPLC method was initially developed to analyse for steroid hormones, as there is less sample preparation required because derivatisation is unnecessary.

3.3.1 HPLC method

Separation of 17α-hydroxyprogesterone, testosterone, 17β-estradiol, androstenedione, 17,20β-P, and 11-ketotestosterone was achieved on a low flow Zorbax C_{18} (2.1x150 mm, 5 µm) column using gradient elution (Table 3.1). Steroids were detected using an evaporative light scattering detector.

Table 3.1 Gradient system used for elution of steroids in HPLC

Time (min)	**Flow ($mLmin^{-1}$)**	**% Acetonitrile**	**% Aqueous Ammonium Carbonate (0.3%)**
	0.15	27	73
20.00	0.15	32	68
30.00	0.15	50	50
50.00	0.15	50	50
60.00	0.15	90	10
63.00	0.15	27	73
70.00	0.15	27	73

Initially a Waters Radial Pak column was used, however androstenedione and 17α-hydroxyprogesterone co-eluted, and by changing to a low flow analytical column greater resolution was obtained (Figure 3.1) and peaks eluted separately using the gradient shown above (Table 3.1). HPLC was not considered a useful technique for the analysis of fish steroids in water, as detection limits were not low enough (the instrumental limit of detection of testosterone was 0.3 ng per 20 µL injection).

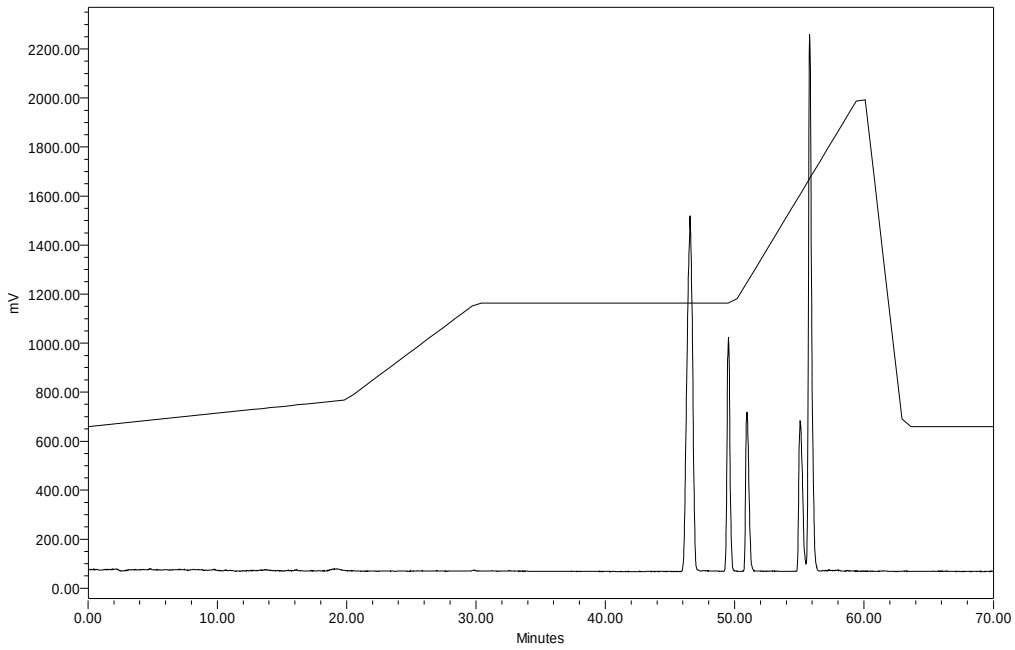


Figure 3.1: Mixed standard chromatogram with gradient overlayed showing separation achieved using HPLC low flow column

3.3.2 Liquid Chromatography-Mass Spectrometry (LCMS) method

Steroid hormones were analysed on a Thermo Finnigan LCQ advantage using atmospheric pressure chemical ionisation.

Original eluents used were acetonitrile and 0.3% aqueous ammonium carbonate. These eluents gave 6 or more peaks for some compounds $[MH]^+$, $[MNH_4]^+$, $[M+CH_3CN+H]^+$, $[M+CH_3CN+NH_4]^+$, $[M+NH_4]^+$, $[2M+H]^+$, and $[2M+NH_4]^+$. This situation was not ideal and it is preferable to see one main ion (ideally $[M+H]^+$). Eluents were changed to acetonitrile and 0.1% aqueous acetic acid which removed all ions which contained an ammonium ion, hence greatly reducing the variety of ions. Source induced dissociation was used at 30% to reduce the formation of $[2M+H]^+$ and $[M-OAc]^-$ ions.

Optimum source temperature was 350°C, this gave good response for most of the steroid hormones tested. Capillary temperature was kept at 200°C. Gradients were not fine tuned to gain optimum separation as method detection limits were not low enough and this technique was not pursued.

3.3.2.1 Limits of Detection for LCMS method

Limits of detection using the mass spectrometer as a detector were 20 ng per 10 µL injection for 17α-hydroxyprogesterone, androstenedione, testosterone,

11-ketotestosterone and 17,20β-P. The limits of detection using the photo diode array detector was an order of magnitude lower. LCMS was deemed an unsuitable technique for our requirements as detection of much lower levels was required.

Because, limits of detection were not low enough for HPLC or LCMS to be useful, gas chromatography mass spectrometry was used as the sensitivity is far greater, the only disadvantage being the requirement for derivatisation prior to analysis.

3.3.3 Development of GC method

3.3.3.1 Development of derivatisation

Oxime-trimethylsilyl ethers or trimethylsilyl ethers

Testosterone was used to observe the results of derivatisation to oxime-trimethylsilyl ether and compare to direct derivatisation to a trimethylsilyl ether.

Derivatisation to form oximes was carried out following the method used by Schoonen and Lambert[1]. A solution of hydroxylammonium chloride in pyridine (2 mL, 2% w/v) was prepared and added to testosterone (1 mg). The mix was heated (100°C, 1 hr), to convert the keto group to an oxime derivative, and evaporated to dryness under a stream of nitrogen (40°C). The residue was further derivatised by converting the oxime and hydroxyl groups into trimethylsilyl ethers.

The oxime was found to give two peaks in the chromatogram, one corresponding to *cis* configuration, and the other *trans*. This was considered to be undesirable as it provided unnecessary complications to the chromatogram, and might also affect the detection level. Therefore the chosen derivatisation method was direct silylation without prior formation of oximes.

3.3.3.2 Selection of ions for monitoring

Standards were silylated separately and analysed on the GC/MS using a method shown to separate most steroids (see section 3.3.3.3 Temperature Programmes) in order to establish the retention times for each steroid, and the ions produced for each one. The total ion spectra were used to decide which ions to monitor when using SIM mode. The most abundant ions were chosen where possible, however ions such as 73$[(CH_3)_3Si]^+$, 75$[(CH_3)_2Si{=}OH]^+$ and 129$[CH_2CHCHOSi]^+$ were

commonly the most abundant in many spectra, but were not a viable choice as they occur repeatedly in all silylated compounds.

The purity of standards was not always 100%, and some impurities are able to ionise better than the target steroid, making impurities look disproportionately larger than they are. Figures 3.2-3.35 give total ion chromatograms and mass spectra of the steroid standards and stigmasterol (internal standard). Ions chosen for SIM are listed underneath each mass spectrum. Accurate retention times with ratios of ion intensities of selected ions are given in Table 3.2.

5β-androstan-3β,17β-diol

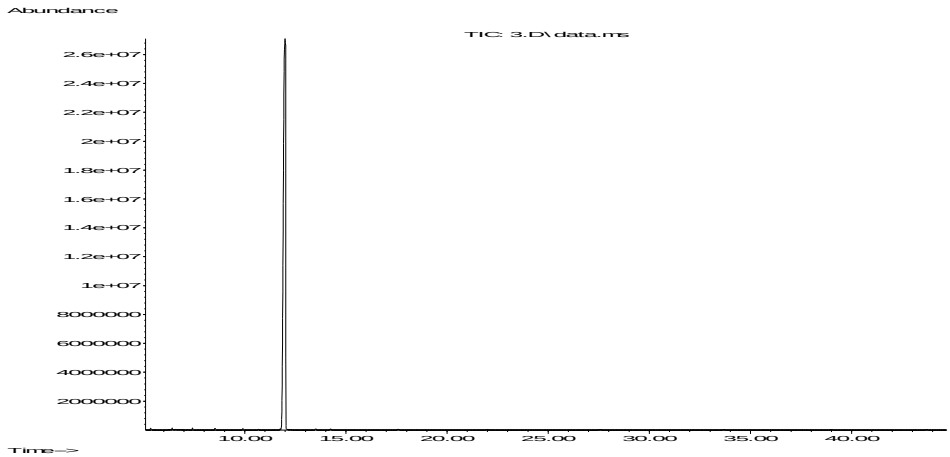


Figure 3.2 TIC trace of 5β-androstan-3β,17β-diol-diTMS

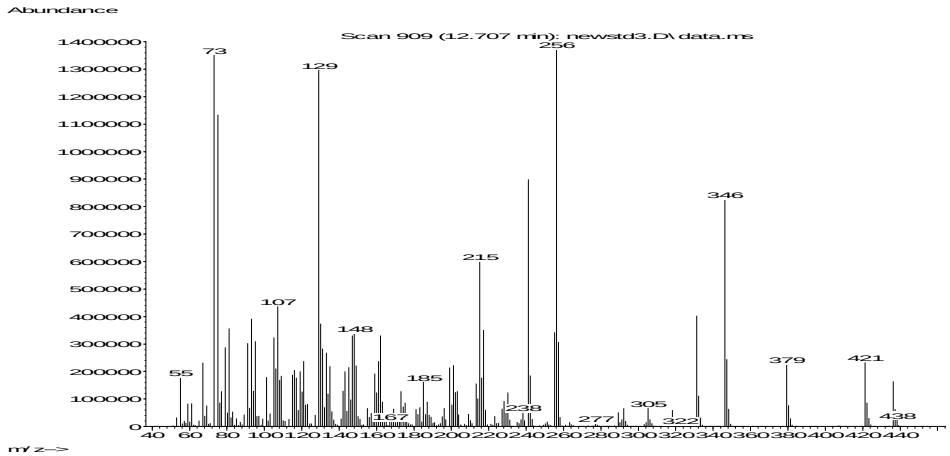


Figure 3.3 Mass spectrum of 5β-androstan-3β,17β-diol-diTMS

Ions selected for SIM analysis were 256[M-2OTMS]$^+$, 241[M-195]$^+$ and 346[M-OTMS]$^+$.

5β-androstan-3α,17β-diol

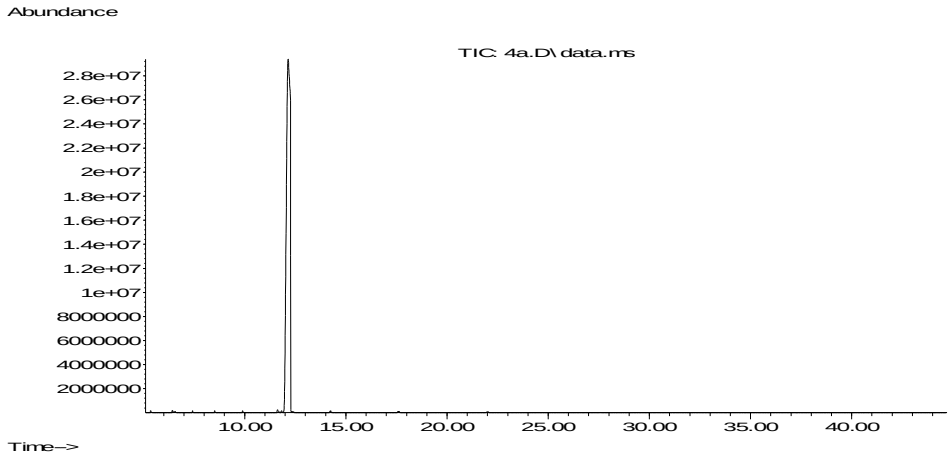


Figure 3.4 TIC trace of 5β-androstan-3α,17β-diol-diTMS

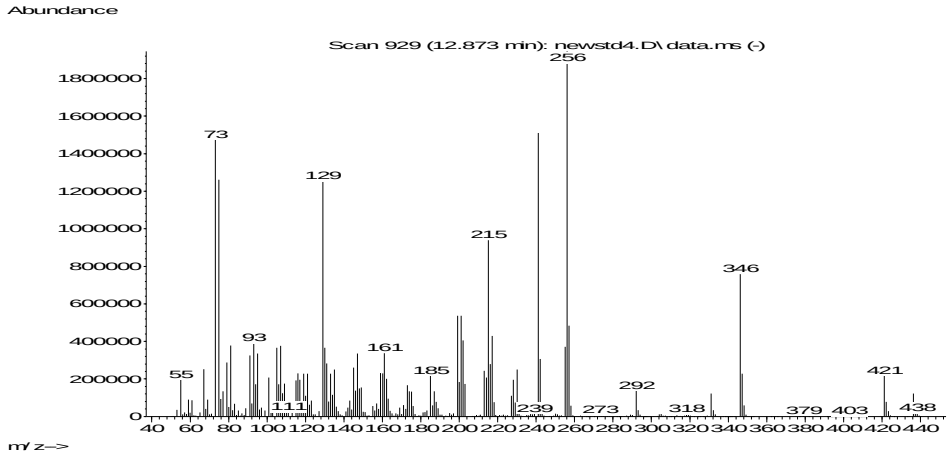


Figure 3.5 Mass spectrum of 5β-androstan-3α,17β-diol-diTMS

Ions selected for SIM analysis were 256[M-2OTMS]$^+$, 241[M-195]$^+$ and 215[M-221]$^+$.

5β-dihydrotestosterone

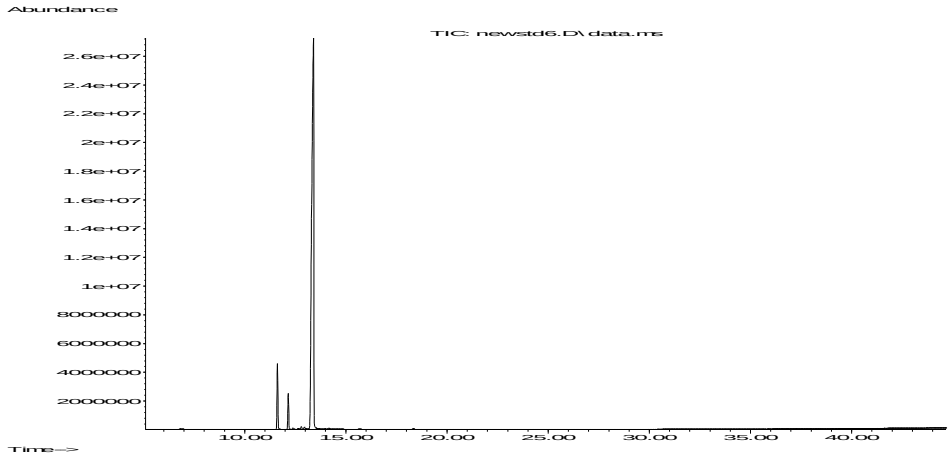


Figure 3.6 TIC trace of 5β-dihydrotestosterone-TMS

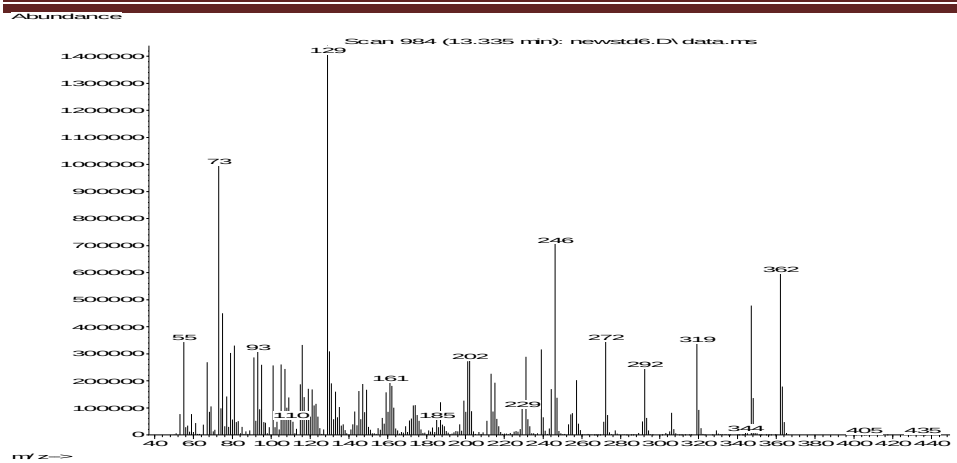


Figure 3.7 Mass spectrum of 5β-dihydrotestosterone-TMS

Ions selected for SIM analysis were 246[M-116]$^+$, 362[M]$^+$ and 347[M-CH_3]$^+$.

Estrone

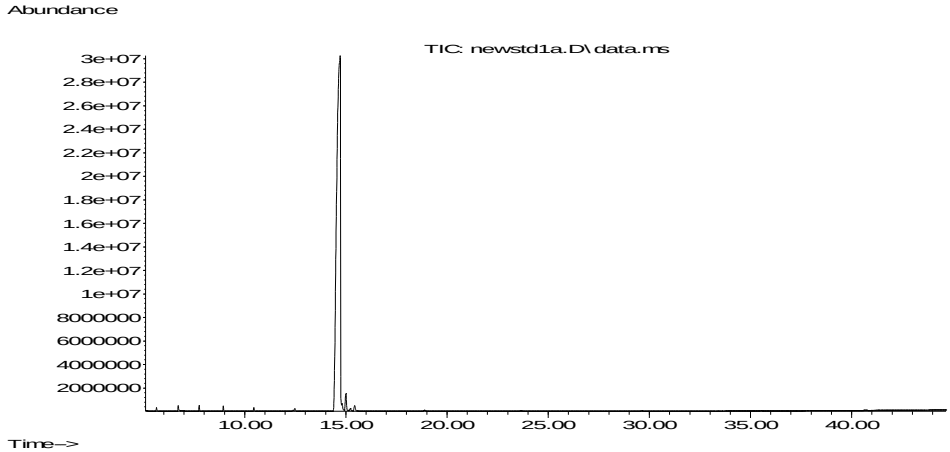


Figure 3.8 TIC trace of estrone-TMS

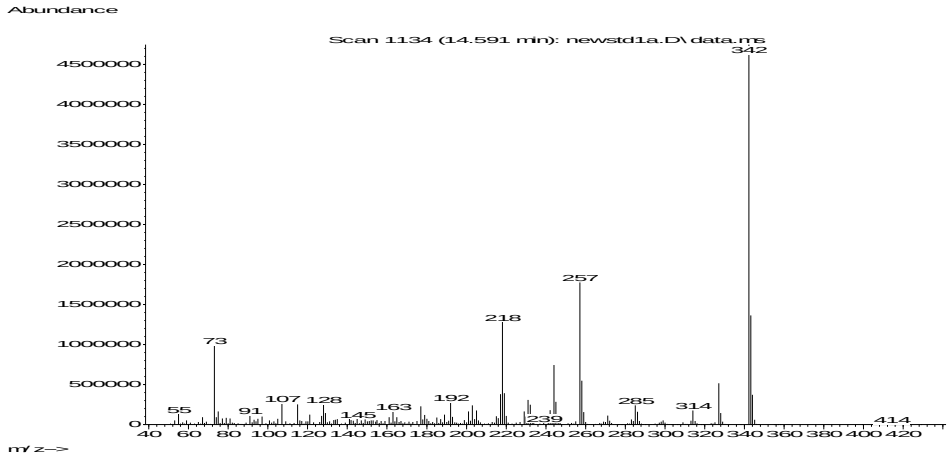


Figure 3. 9 Mass spectrum of estrone-TMS

Ions selected for SIM analysis were 342[M]$^+$, 257[M-85]$^+$ and 218[M-124]$^+$.

Androstenedione

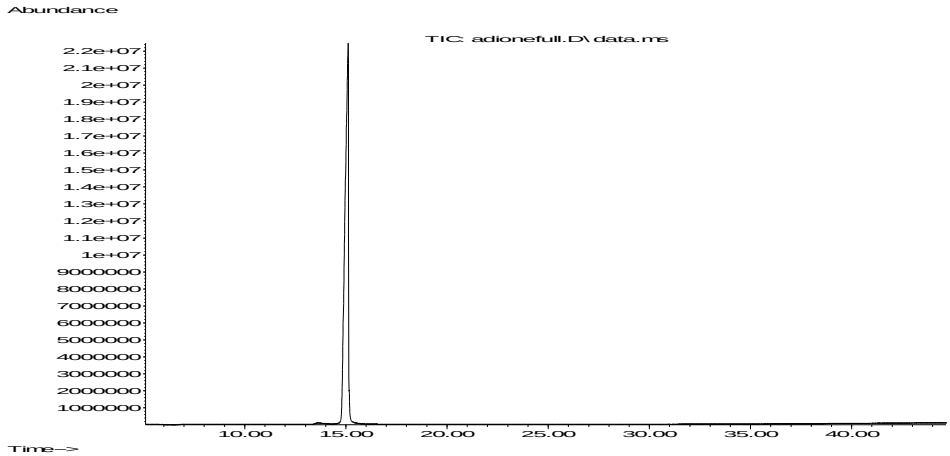


Figure 3.10 TIC trace of androstenedione

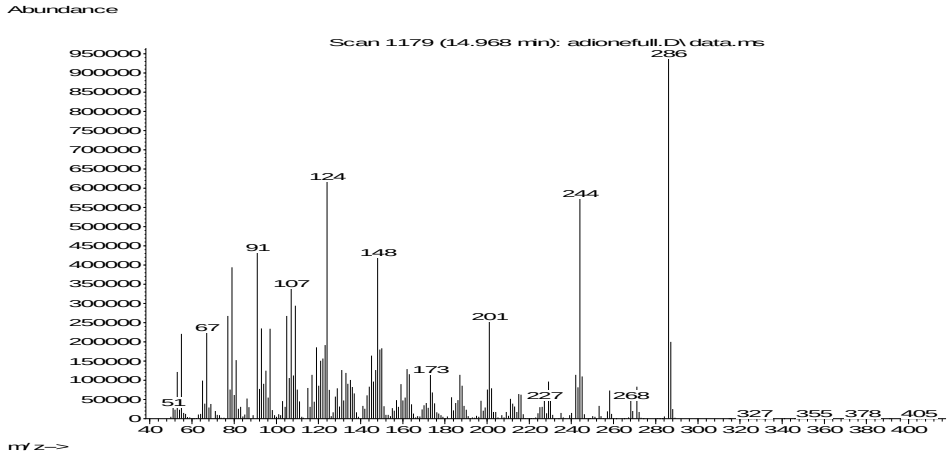


Figure 3.11 Mass spectrum of androstenedione

Ions selected for SIM analysis were 286$[M]^+$, 244$[M\text{-}C(O)CH_2]^+$, and 201$[M\text{-}85]^+$.

17β-estradiol

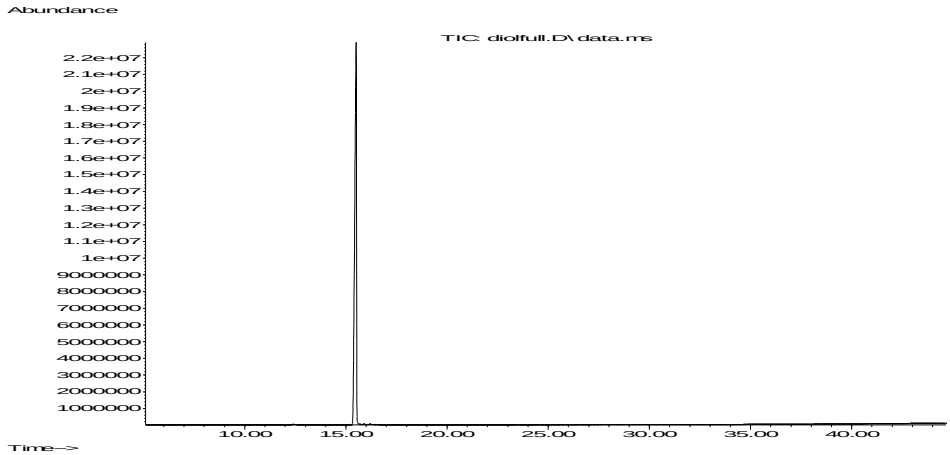


Figure 3.12 TIC trace of 17β-estradiol-diTMS

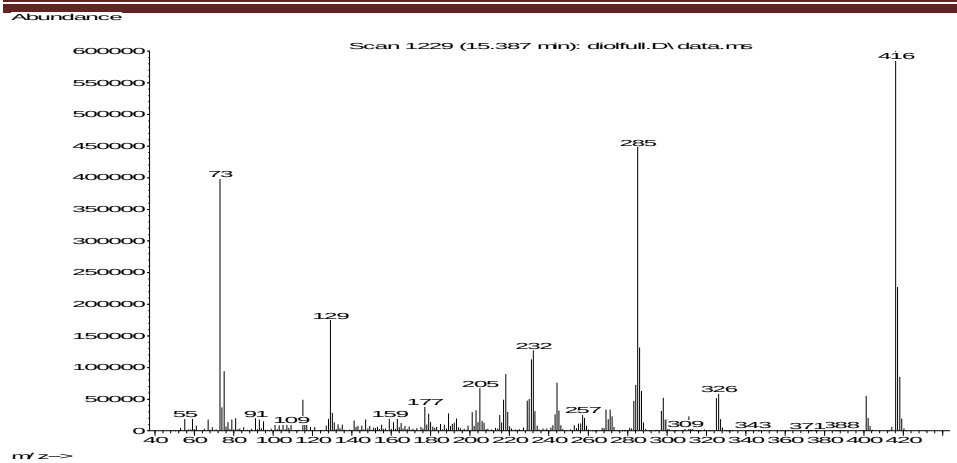


Figure 3.13 Mass spectrum of 17β-estradiol-diTMS

Ions selected for SIM analysis were 416[M]$^+$, 285[M-131]$^+$ and 232[M-184]$^+$.

It is important to note the relatively large intensity of the molecular ion in this spectrum. This may also be observed in the spectrum for estrone and androstenedione, but does not occur for any other steroids used. Estrone and 17β-estradiol both contain an aromatic ring at ring A. This assists in stabilising the molecular ion (416) and ion 326[M-TMSOH]$^+$ in the 17β-estradiol spectrum, which corresponds to loss of TMSOH at position C-17, as there is no such peak in the estrone spectrum, where C-17 had a ketone substituent. This stability reduces fragmentation of the molecular ion which is observed in the mass spectra of the other steroids.

Testosterone

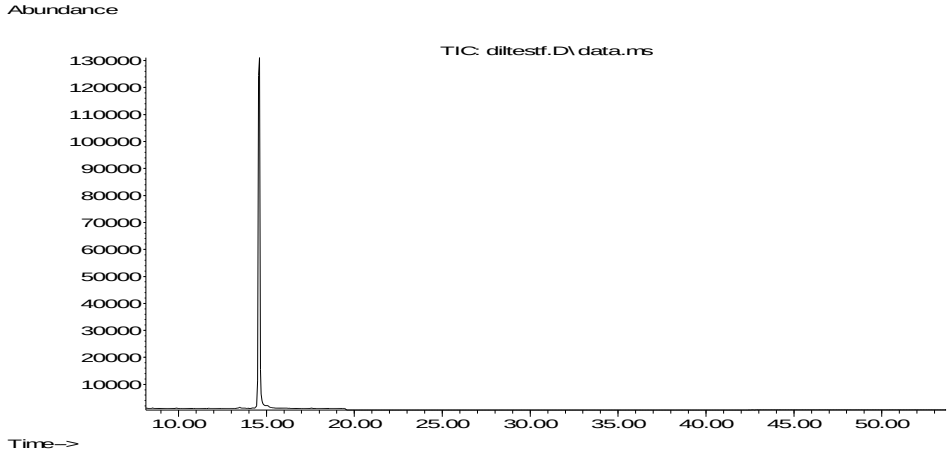


Figure 3.14 TIC trace of testosterone-diTMS

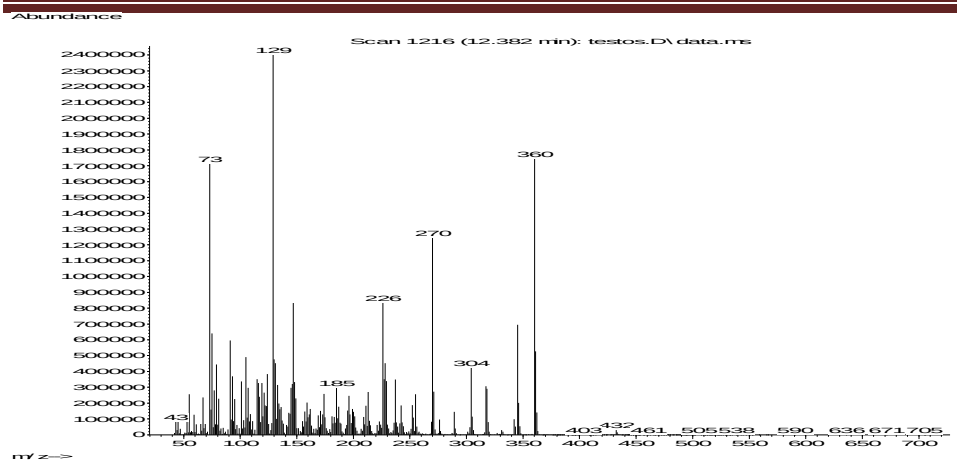


Figure 3.15 Mass spectrum of testosterone-diTMS

Ions selected for SIM analysis were 360$[M]^+$, 270$[M\text{-}OTMS]^+$ and 226$[M\text{-}134]^+$.

5β-androstan-3α,11β-diol-17-one

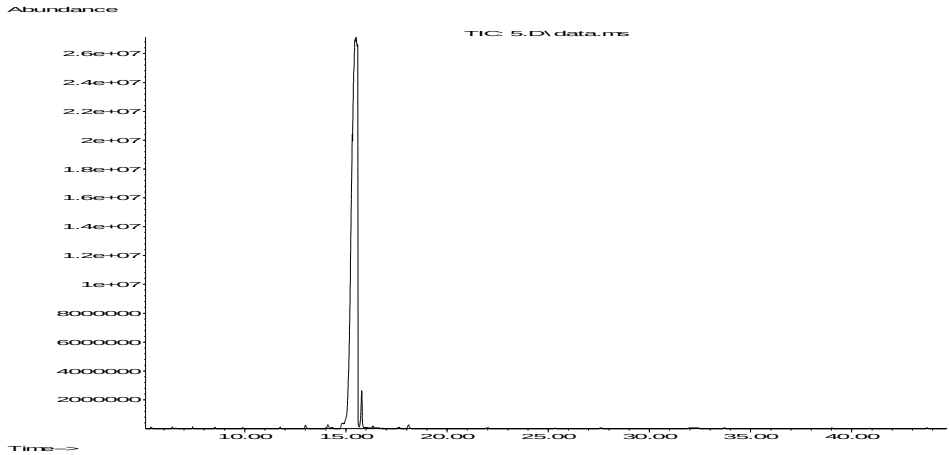


Figure 3.16 TIC trace of 5β-androstan-3α,11β-diol-17-one-diTMS

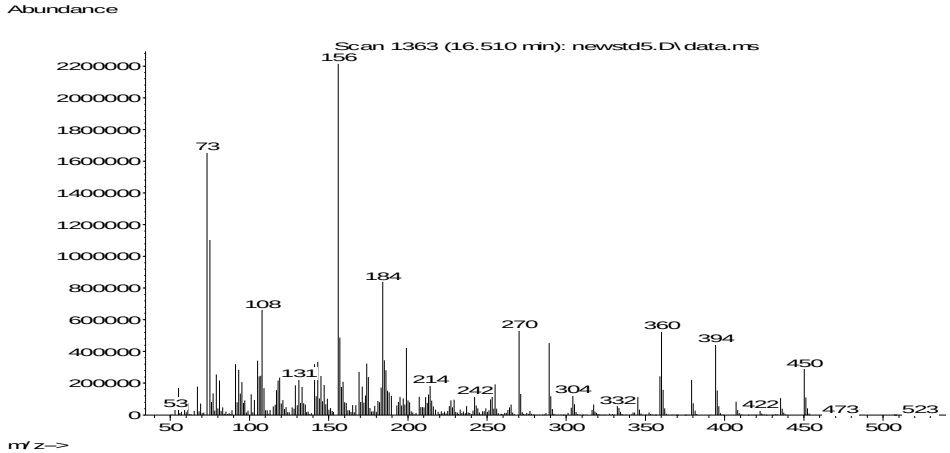


Figure 3.17 Mass spectrum of 5β-androstan-3α,11β-diol-17-one-diTMS

Ions selected for SIM analysis were 270$[M\text{-}OTMS]^+$, 360$[M\text{-}2OTMS]^+$ and 394$[M\text{-}66]^+$.

5β-pregnan-3α,17α-diol-20-one

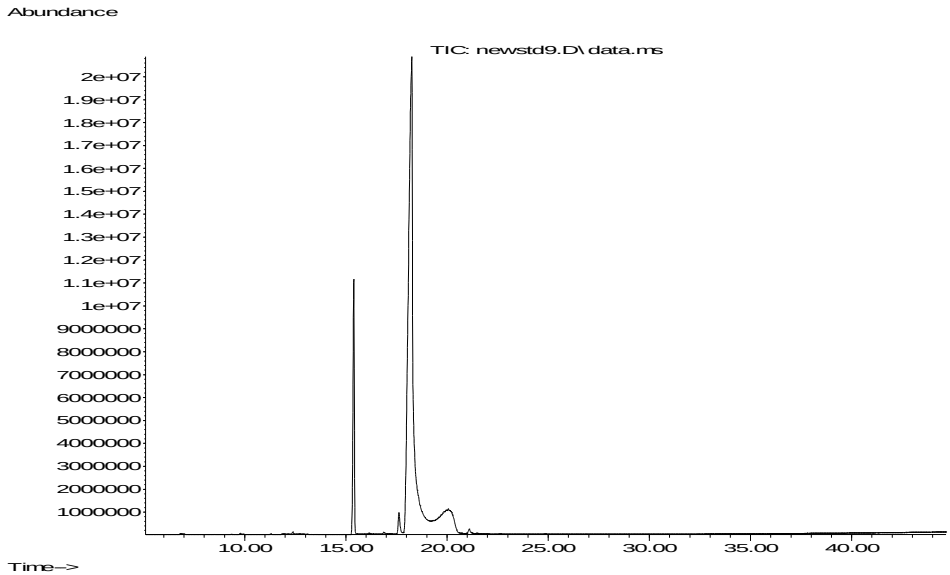


Figure 3.18 TIC trace of 5β-pregnan-3α,17α-diol-20-one

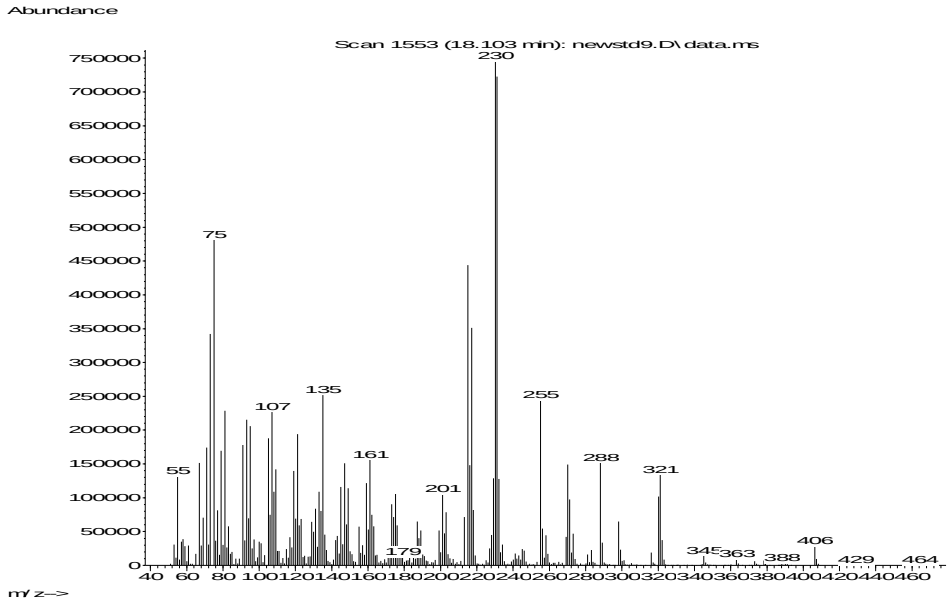


Figure 3.19 Mass spectrum of 5β-pregnan-3α,17α-diol-20-one

Ions selected for SIM analysis were 230$[M-176]^+$, 231$[M-175]^+$, and 215$[M-191]^+$.

11-ketotestosterone

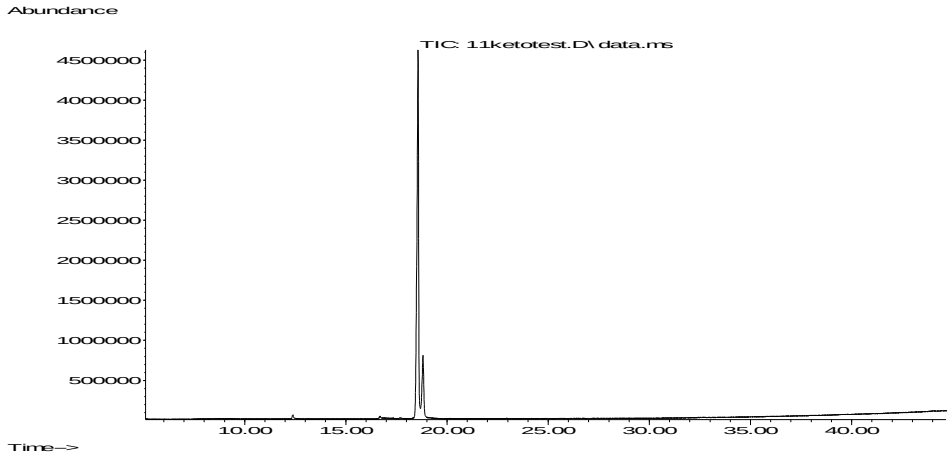


Figure 3.20 TIC trace of 11-ketotestosterone-TMS

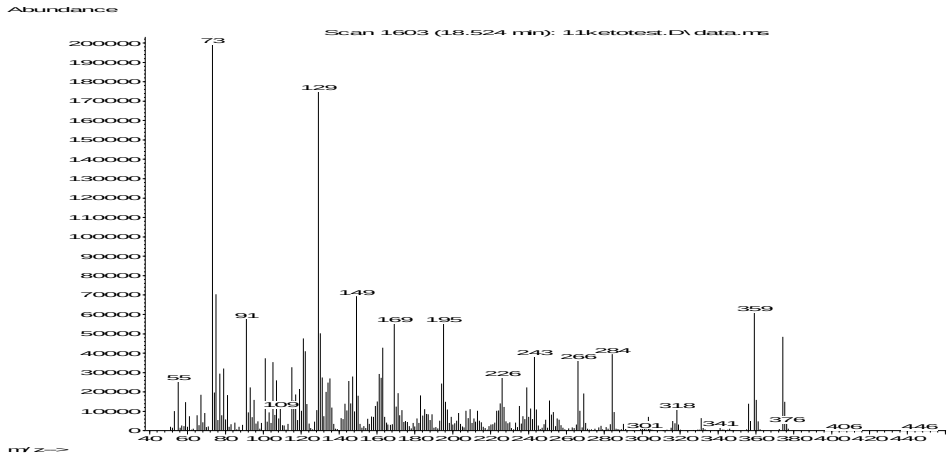


Figure 3.21 Mass spectrum of 11-ketotestosterone

Ions selected for SIM analysis were 359$[M\text{-}CH_3]^+$, 374$[M]^+$ and 284$[M\text{-}OTMS]^+$

17α-hydroxyprogesterone

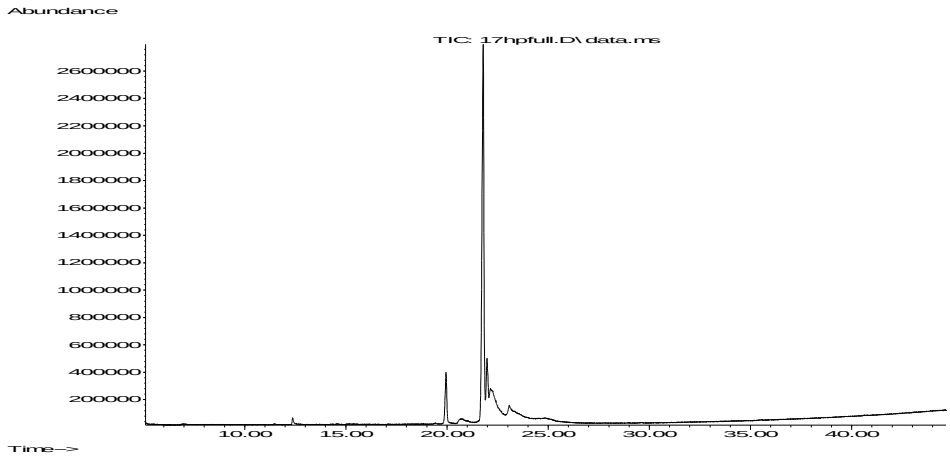


Figure 3.22 TIC trace of 17-hydroxyprogesterone-TMS

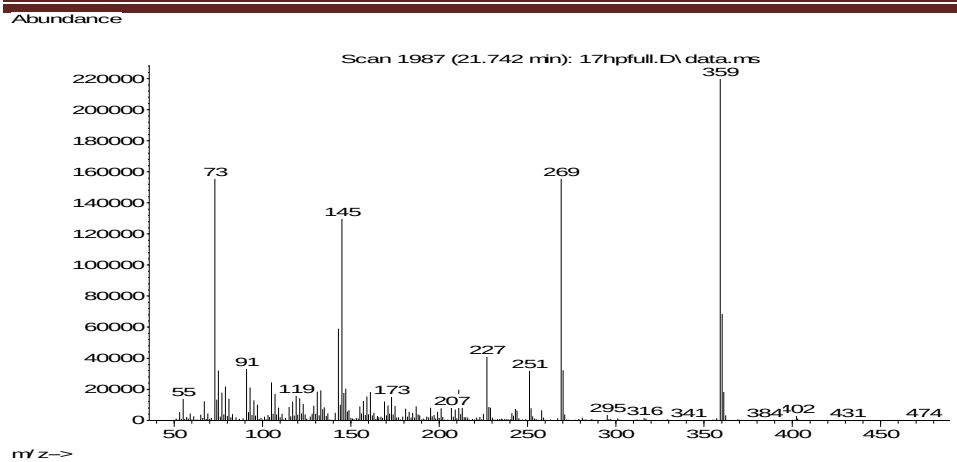


Figure 3. 23 Mass spectrum of 17α-hydroxyprogesterone-TMS

Ions selected for SIM analysis were 359[M-C(O)CH_3]$^+$, 269[M-133]$^+$ and 227[M-175]$^+$.

5β-pregnan-3α,17α,20β-triol

Elutes earlier (~1.5 min) than 5β-pregnan-3α,17α,20α-triol.

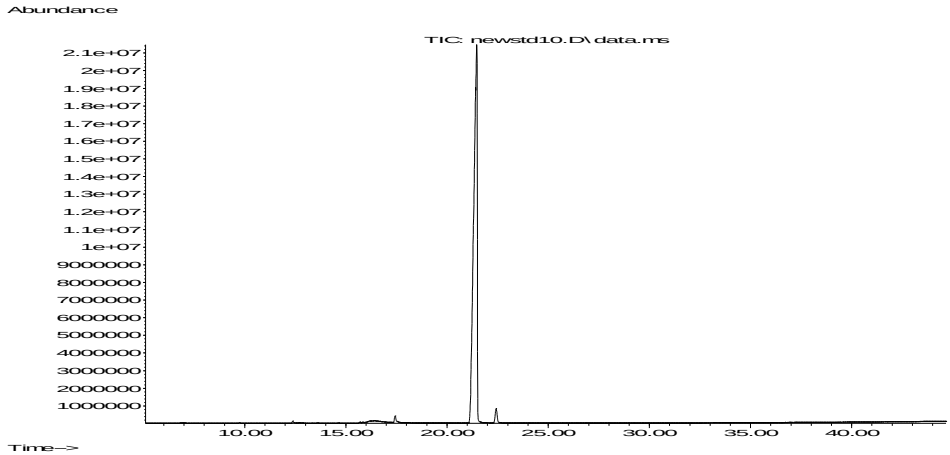


Figure 3.24 TIC trace of 5β-pregnan-3α,17α,20β-triol

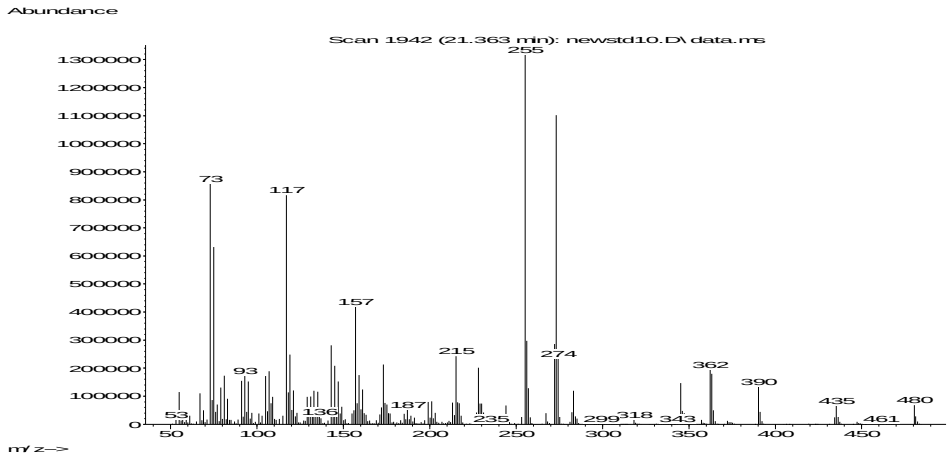


Figure 3.25 Mass spectrum of 5β-pregnan-3α,17α,20β-triol

Ions selected for SIM analysis were 255[M-225]$^+$, 273[M-207]$^+$ and 256[M-224]$^+$

5β-pregnan-3α,17α,20α-triol

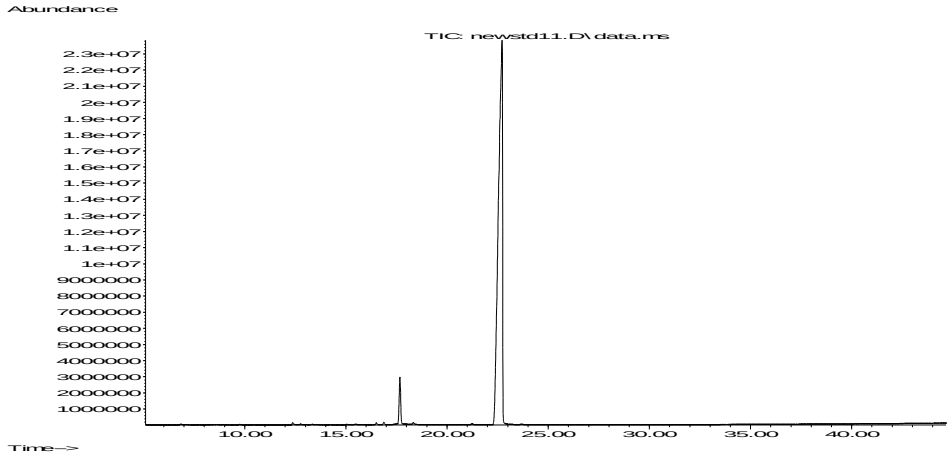


Figure 3.26 TIC trace of 5β-pregnan-3α,17α,20α-triol

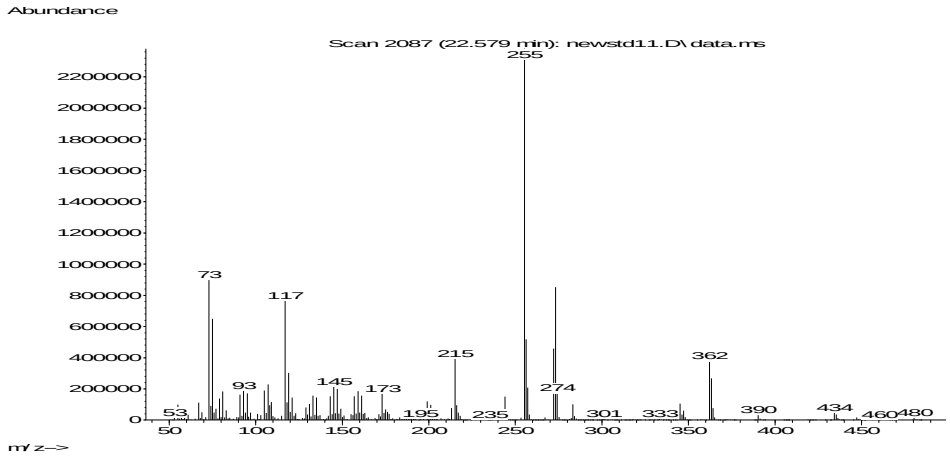


Figure 3.27 Mass spectrum 5β-pregnan-3α,17α,20α-triol

Ions selected for SIM analysis were 255[M-225]$^+$, 273[M-207]$^+$ and 256[M-224]$^+$.

11-deoxycortisol

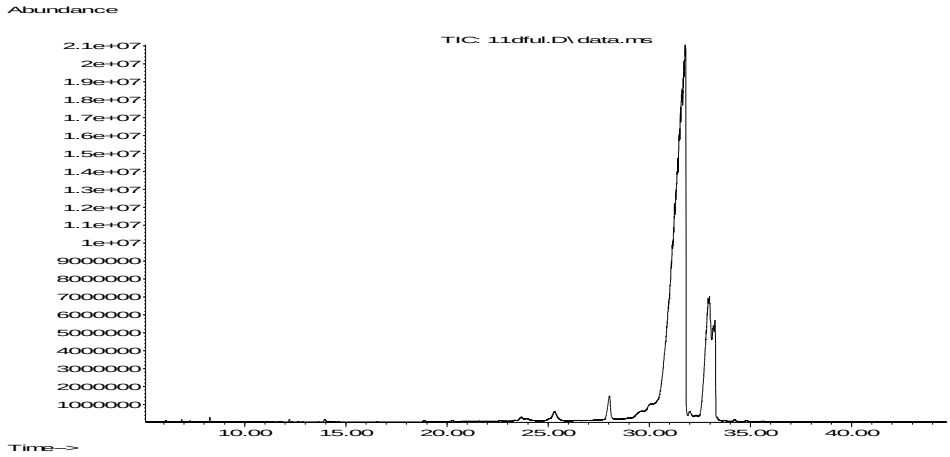


Figure 3.28 TIC trace of 11-deoxycortisol

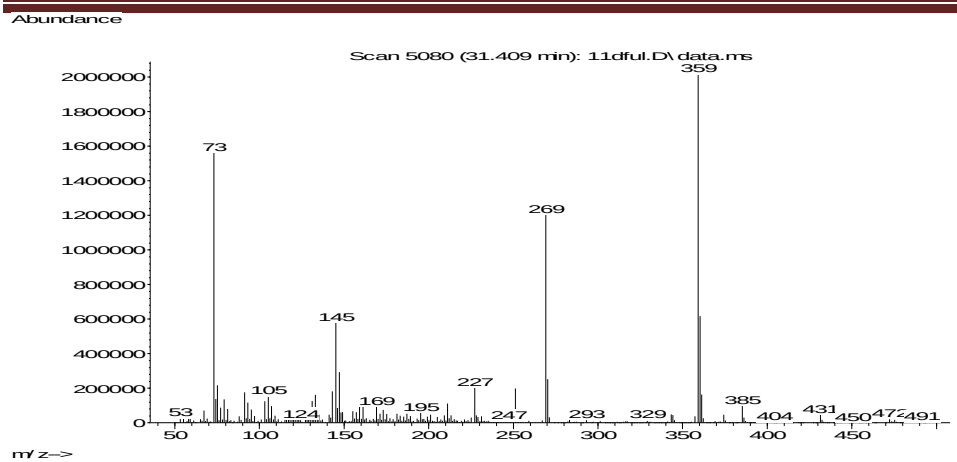


Figure 3.29 Mass spectrum of 11-deoxycortisol

Ions chosen for SIM analysis were 359[M-C(O)-$CH_2OSi(CH_3)_3$]$^+$, 269[M-221]$^+$, and 360[M-C(O)=$CHOSi(CH_3)_3$]$^+$.

17α,20β-dihydroxy-pregn-4-en-3-one

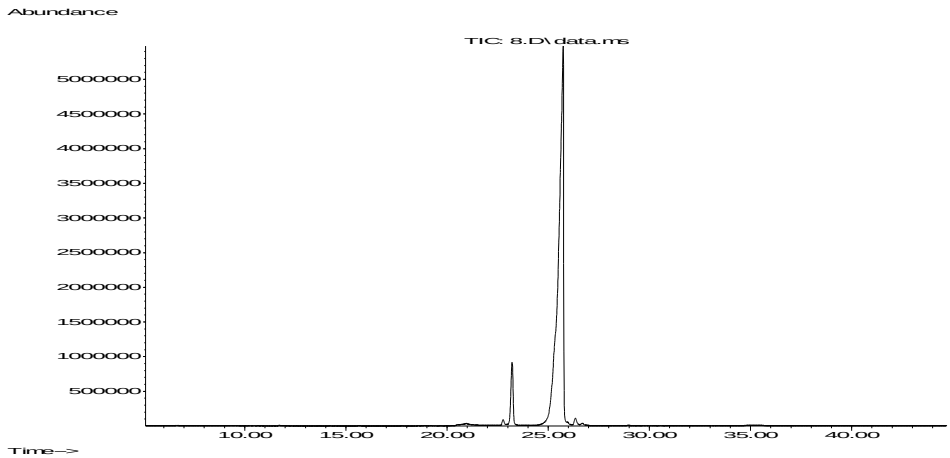


Figure 3.30 TIC trace of 17,20β-dihydroxy-4-pregnen-3-one

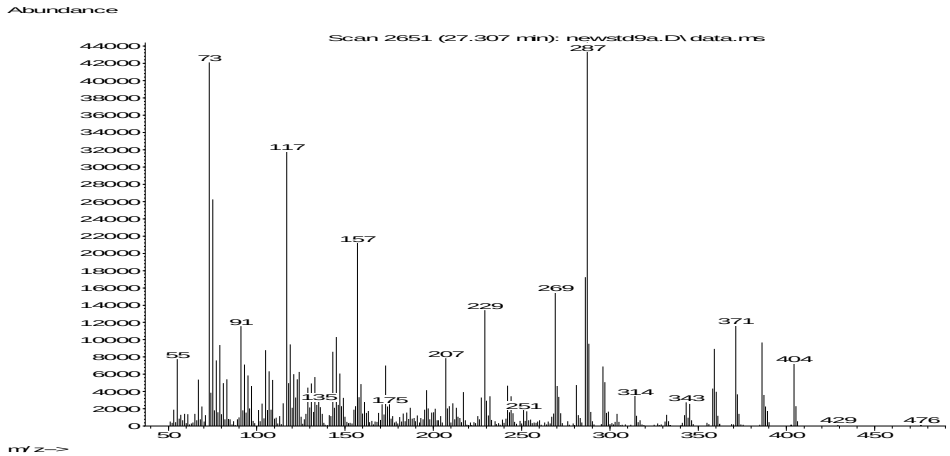


Figure 3.31 Mass spectrum of 17,20β-dihydroxy-4-pregnen-3-one

Ions selected for SIM analysis were 287[M-189]$^+$, 371[M-105]$^+$, and 269[M-207]$^+$.

17α,20β,21-trihydroxy-pregn-4-en-3-one

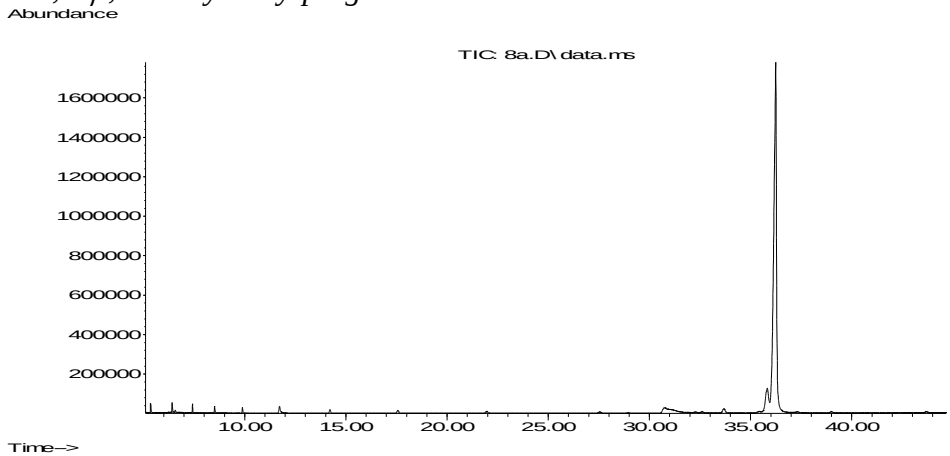


Figure 3.32 TIC trace of 17α,20β,21-trihydroxy-pregn-4-en-3-one

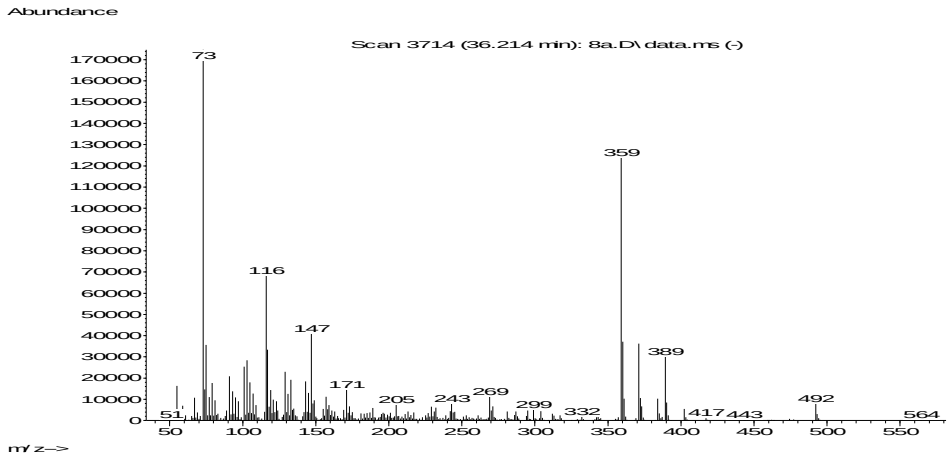


Figure 3.33 Mass spectrum of 17α,20β,21-trihydroxy-pregn-4-en-3-one

Ions selected for SIM analysis were 359[M-OTMS-CH-CH_2OTMS]$^+$, 371[M-193]$^+$ and 360[M-204]$^+$.

Stigmasterol (internal standard)

Figure 3.34 TIC trace of stigmasterol

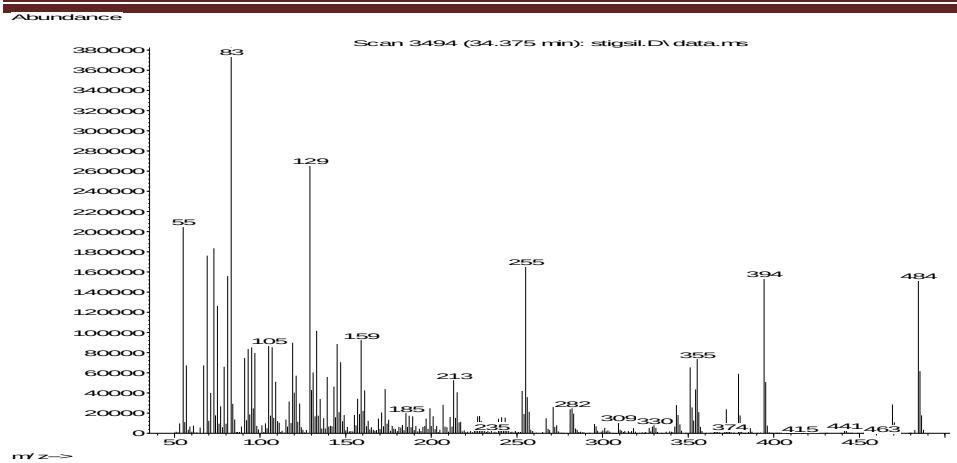


Figure 3.35 Mass spectrum of stigmasterol

Ions selected for analysis were 255[M-229]$^+$, 394[M-OTMS]$^+$, and 484[M]$^+$ as these were the most abundant higher mass ions.

Retention times and ion intensity ratios

Intensity ratios of ions in both individual steroids and steroids in a mixed steroid solution are compared to show there are no interferences in the mixed solution (Table 3.2).

Table 3.2 Retention times and ions selected for steroid analysis using GC/MS

Standard	**Retention Time (min)**	**Ion 1 (m/z)**	**Ion 2 (m/z)**	**Ion 3 (m/z)**
5β-androstan-3β,107β-diol	12.00	256	241	346
Intensity ratio in mixed standard[a]		0.94	0.63	0.56
Intensity ratio in individual		1	0.65	0.57
5β-androstan-3α,17β-diol	12.17	256	241	215
Intensity ratio in mixed standard		1	0.95	0.6
Intensity ratio in individual		1	0.77	0.37
5β-dihydrotestosterone	12.65	246	362	347
Intensity ratio in mixed standard		0.45	0.4	0.31
Intensity ratio in individual		0.48	0.4	0.33
Estrone	13.74	342	257	218
Intensity ratio in mixed standard		1	0.36	0.27
Intensity ratio in individual		1	0.36	0.27
Androstenedione	14.11	286	244	201
Intensity ratio in mixed standard		1	0.59	0.26
Intensity ratio in individual		1	0.60	0.25
17β-Estradiol	14.64	416	285	232
Intensity ratio in mixed standard		1	0.7	0.2
Intensity ratio in individual		1	0.61	0.16
Testosterone	14.86	360	270	226
Intensity ratio in mixed standard		0.64	0.43	0.3
Intensity ratio in individual		0.77	0.54	0.36
5β-androstan-3α,11β-diol-17-one	15.38	270	360	394
Intensity ratio in mixed standard		0.23	0.25	0.21
Intensity ratio in individual		0.24	0.23	0.19
5β-pregnan-3α,17α-diol-20-one	17.06	230	231	215
Intensity ratio in mixed standard		1	0.95	0.59
Intensity ratio in individual		1	0.95	0.39
11-ketotestosterone	17.59	359	374	284
Intensity ratio in mixed standard[a]		0.36	0.31	0.25
Intensity ratio in individual		0.30	0.24	0.20
17α-hydroxyprogesterone	20.56	359	269	227
Intensity ratio in mixed standard		1	0.61	0.16

a. Relative intensity ratio where the ion with the largest intensity is assigned a value of 1

Standard	**Retention Time (min)**	**Ion 1 (m/z)**	**Ion 2 (m/z)**	**Ion 3 (m/z)**
Intensity ratio in individual		1	0.64	0.18
5β-pregnan-3α,17α,20β-triol	19.96	255	273	362
Intensity ratio in mixed standard		1	0.76	0.1
Intensity ratio in individual		1	0.85	0.15
5β-pregnan-3α,17α,20α-triol	21.2	255	273	362
Intensity ratio in mixed standard		1	0.33	0.13
Intensity ratio in individual		1	0.35	0.15
17α,20β-dihydroxy-pregn-4-en-3-one	25.7	287	371	269
Intensity ratio in mixed standard		0.92	0.31	0.31
Intensity ratio in individual		1	0.30	0.35
11-deoxycortisol	31.45	359	269	227
Intensity ratio in mixed standard		1	0.42	0.07
Intensity ratio in individual		1	0.6	0.1
17α,20β,21-trihydroxy-pregn-4-en-3-one	36.58	359	360	371
Intensity ratio in mixed standard		1	0.29	0.27
Intensity ratio in individual		1	0.29	0.33
Stigmasterol (internal standard)	35.8	255	384	484
Intensity ratio in mixed standard		0.46	0.42	0.40
Intensity ratio in individual		0.45	0.41	0.39

Ion 1 generally agreed within ± 1%
Ion 2 generally agreed within ±2%
Ion 3 generally agreed within ±5%

Ion 1 was used as the quantification ion, and ion 2 and 3 were used as confirmation ions. This information was combined with retention times to determine the presence of steroids.

There is an excellent agreement for androstenedione which was detected most regularly in samples analysed, which means there is unlikely to be interference.

a. Relative intensity ratio where the ion with the largest intensity is assigned a value of 1

The selection of different ions for androstenedione and 17β-estradiol was necessary as they often co-elute, the temperature programme appears to have prevented this as 17β-estradiol elutes with testosterone in the oven temperature programme used (see Figures 3.38 and 3.39). This was more desirable as the spectrum of 17β-estradiol contains 286 ions, which would record a false positive result for androstenedione. However the ions selected for testosterone and 17β-estradiol were not found on the mass spectrum of the co-eluting compound.

3.3.3.3 Temperature programme:

Various oven temperature programmes were trialled in order to achieve the best separation of steroid hormones.

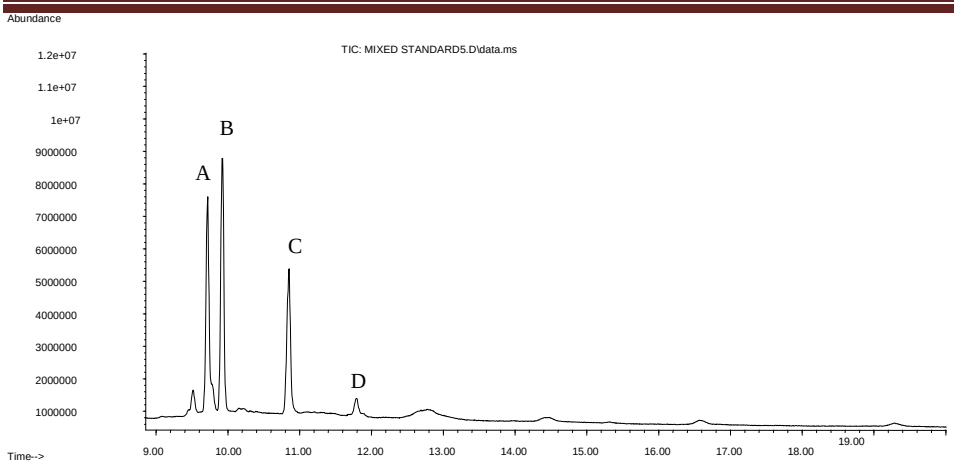
Method 1

The first method used was trialled with six steroid hormones: androstenedione, testosterone, 11-ketotestosterone, 17,20β-P, 17β-estradiol, and 17α-hydroxyprogesterone. The following oven temperature programme (Table 3.3) was used in conjunction with an inlet temperature of 250°C.

Table 3.3 Oven temperature programme of initial method

Temperature increase (°Cmin^{-1})	**Oven temperature (°C)**	**Hold time (min)**
	150	0.10
35	260	0.00
8	295	20.00

This method achieved separation of: 17α-hydroxyprogesterone, testosterone and 11-ketotestosterone and 17,20β-P (Figure 3.36). Androstenedione and 17β-estradiol were found to co-elute.



Where peak A= androstenedione and 17β-estradiol, B= testosterone, C= 11-ketotestosterone, D= 17α-hydroxyprogesterone

Figure 3.36 GC trace showing separation achieved using method 1

Method 2

This method also used the same six steroid hormones. The following oven temperature programme (Table 3.4) was used in conjunction with an inlet temperature of 265°C.

Table 3.4 Oven temperature programme of method 2

Temperature increase (°Cmin^{-1})	**Oven temperature (°C)**	**Hold time (min)**	**Run time (min)**
	150	0.5	0.5
30	270	2	6.5
5	300	18	30.5

This method achieved separation of testosterone, 17,20β-P, 17α-hydroxyprogesterone and 11-ketotestosterone. However, androstenedione and 17β-estradiol co-eluted.

Three methods with different temperature programmes were trialled to achieve separation of androstenedione and 17β-estradiol (Tables 3.5, 3.6 and 3.7).

Method 3a

Table 3.5 Oven temperature programme of method 3a

Temperature increase (°Cmin^{-1})	**Oven temperature (°C)**	**Hold time (min)**
	150	0.5
20	270	2.0
1	300	18

This method achieved separation of testosterone, 17,20β-P, 11-ketotestosterone, and 17α-hydroxyprogesterone. 17β-estradiol and androstenedione were partially separated.

Method 3b

Table 3.6 Oven temperature programme for method 3b

Temperature increase (°Cmin^{-1})	**Oven temperature (°C)**	**Hold time (min)**
	150	0.5
30	250	0
10	270	2
1	300	20

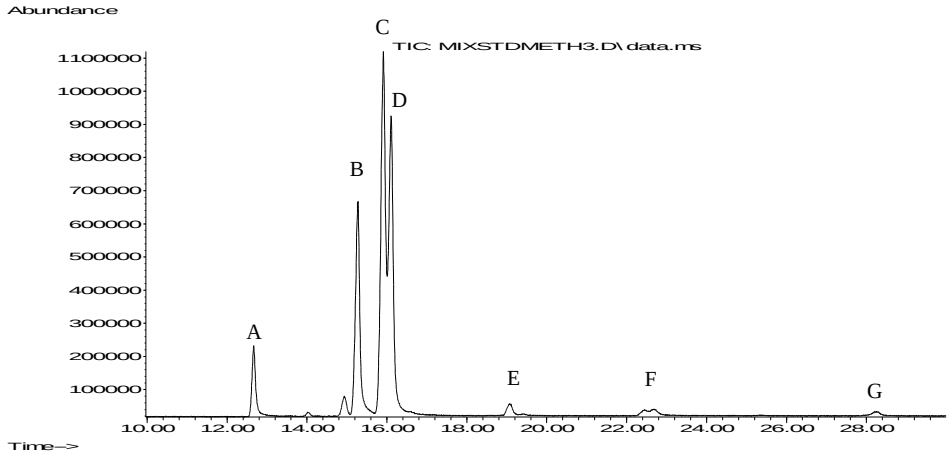
This method achieved separation of testosterone, 17,20β-P, 11-ketotestosterone, and 17α-hydroxyprogesterone. 17β-estradiol and androstenedione were partially separated.

Method 3c

Table 3.7 Oven temperature programme for method 3c

Temperature increase (°Cmin^{-1})	**Oven temperature (°C)**	**Hold time (min)**
	150	0.5
15	250	1
0.5	280	20

This method achieved separation of 17,20β-P, androstenedione, 17α-hydroxyprogesterone, and 11-ketotestosterone (Figure 3.37). 17β-estradiol and testosterone were partially separated, but separation of these two was less important as there were not as many clashing ions. The molecular ion of androstenedione was 286, and the 286 ion was also present in 17β-estradiol. In order to use this ion as a quantitative estimate of androstenedione, separation of androstenedione and 17β-estradiol was imperative.



Where A= Diisooctyl phthalate, B= androstenedione, C= 17β-estradiol, D= testosterone, E= 11-ketotestosterone, F= 17α-hydroxyprogesterone, G= 17,20β-dihydroxy-4-pregnen-3-one

Figure 3.37 GC trace showing separation achieved using method 3c

Method 4

Method 3b was the best of the methods outlined above, and it was used again when more steroid hormones were obtained. The temperature required further adjustment to achieve the separation of: androstenedione, testosterone, 11-ketotestosterone, 17,20β-P, 17β-estradiol, and 17α-hydroxyprogesterone, 11-deoxycortisol, estrone, 5β-androstan-3α,11β-diol-17-one, 5β-dihydrotestosterone, 17,20β,21-trihydroxy-pregn-4-en-3-one, 5β-pregnan-3α,17-diol-20-one, 5β-pregnan-3α,17α,20β-triol, and 5β-pregnan-3α,17α,20α-triol. The partial separation of 5β-androstan-3α,17β-diol and 5β-androstan-3β,17β-diol was also achieved. The TIC trace of the mixed standard solution is shown in Figure 3.38. Each section of the chromatogram is then expanded and labelled, to show separation of steroids (Figures 3.39-3.42).

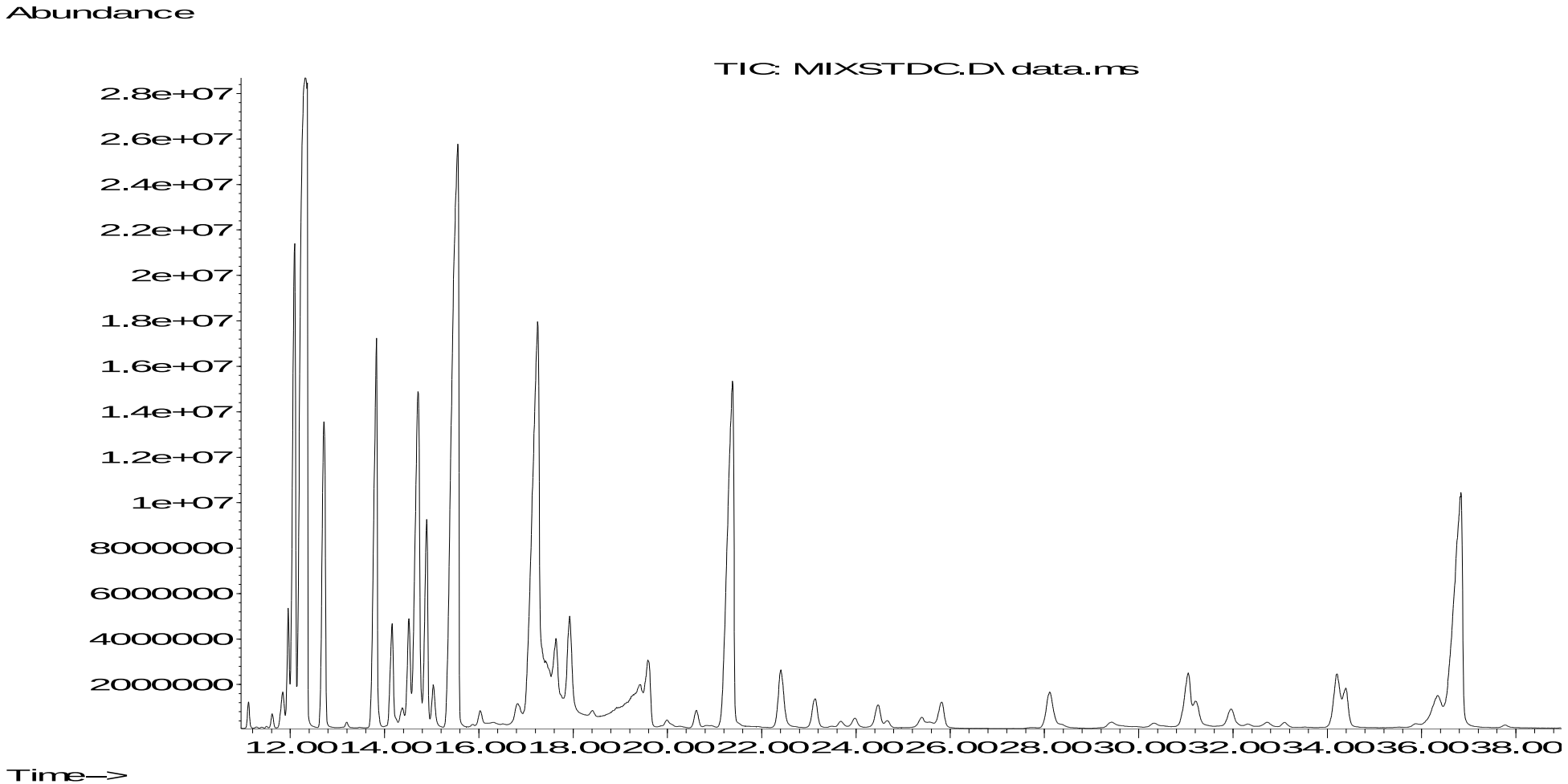
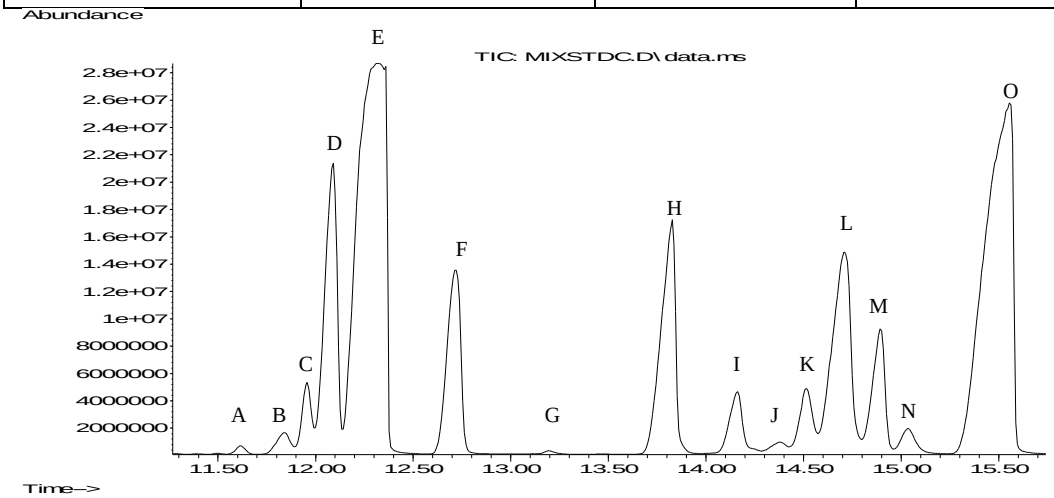


Figure 3.38 TIC trace of all 16 steroids separated using method 4

This oven temperature programme was the final method used (Table 3.8)

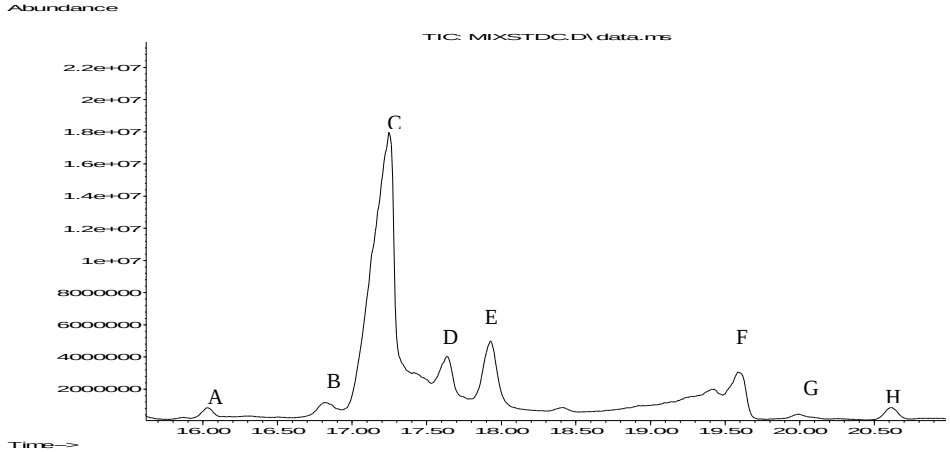
Table 3.8 Oven temperature programme for Method 4

Temperature increase (°Cmin^{-1})	**Oven temperature (°C)**	**Hold time (min)**	**Run time (min)**
	150	0.5	0.5
15	250	1	8.17
0.5	260	0.5	28.67
2	290	10	53.67



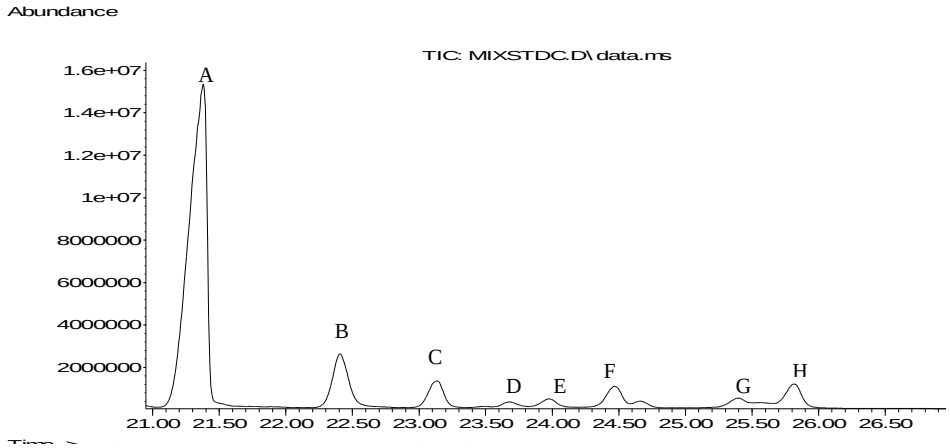
Where A,G,J &N= impurities found in standards, B= Diisooctyl phthalate, C, K= artefact of silylation, D= 5β-androstan-3β,17β-diol, E= 5β-androstan-3α,17β-diol, F= 5α-dihydrotesterone, H= estrone, I= androstenedione, L= 17β-estradiol-diTMS, M= testosterone-TMS, O= 5β-androstan-3α,11β-diol-17-one

Figure 3.39 TIC of mixed standard showing steroids with retention times from 11 to 15.7 min



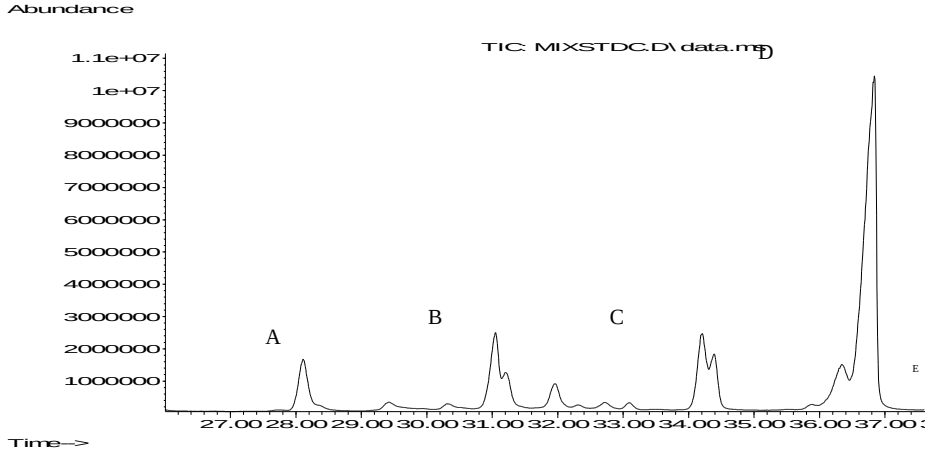
Where A, B & F are steroid impurities and E= artefact of silylation C= 5β-pregnan-3α,17α-diol-20-one, D= 11-ketotestosterone, G= 5β-pregnan-3α,17α,20β-triol, H= 17α-hydroxyprogesterone

Figure 3.40 TIC of mixed standard showing steroids with retention times from 15.7 to 21 min



Where A= 5β-pregnan-3α,17α,20α-triol, B= artefact of silylation, D,E, F & G= steroid impurities
H= 17,20β-dihydroxy-4-pregnen-3-one

Figure 3.41 TIC of mixed standard showing steroids with retention times from 21 to 27 min



Where A= artefact of silylation, B= 11-deoxycortisol, C= Stigmasterol, D= 17,20β,21-trihydroxy-pregn-4-en-3-one

Figure 3.42 TIC of mixed standard showing steroids with retention times from 26 to 37.5 min

3.3.3.4 Response factors

Response factors of testosterone, 17β-estradiol, 17α-hydroxyprogesterone and androstenedione were determined in relation to stigmasterol. These steroids were chosen as relatively large quantities were available.

Steroids were weighed out in ratios of approximately 1:2, 1:1 and 2:1 (steroid: stigmasterol), and derivatised using either Tri-Sil reagent (where available) or pyridine and *n*-trimethylsilylimidazole and heated (1 hr, 50°C). Solvent was removed by evaporation under a stream of nitrogen, and dissolved in either chloroform or *n*-heptane, sonicated (15 min) centrifuged (20 min, 5000 rpm) and the supernatant transferred to a volumetric flask (20 mL) and made up to volume.

1 mL of this solution was transferred to another volumetric flask (50 mL), and made up to volume. Aliquots (1 mL) of the final solution were transferred to a GC vial and analysed using GC-MS/SIM. SIM mode was used as this would be how ions would be detected in samples. Dilutions were necessary to acquire response factors for the lower levels the method would be used for, as relatively large initial weights were used in order to reduce error. Each main ion was extracted from the chromatogram, and integrated manually to gain the peak area. The ions used were testosterone (270), 17β-estradiol (416), androstenedione (286), 17α-hydroxyprogesterone (359) and stigmasterol (255).

Table 3.9 Response factors of testosterone, 17β-estradiol, androstenedione and 17α-hydroxyprogesterone

Steroid	**Response Factor**
Testosterone	1.8 ± 0.3
17β-estradiol	11.3 ± 0.3
17α-hydroxyprogesterone	1.1 ± 0.2
Androstenedione	1.9 ± 0.3

Testosterone

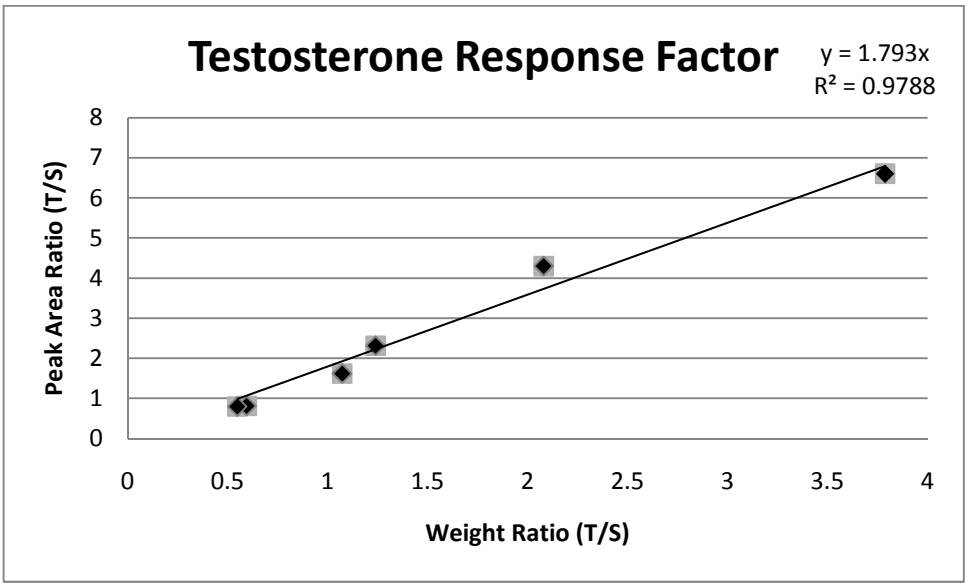


Figure 3.43 Response factor of Testosterone

Given the higher instrumental detection limit of testosterone, the response factor is surprisingly higher. This response factor would be used for compounds with similar structures such as 11-ketotestosterone.

17β-estradiol

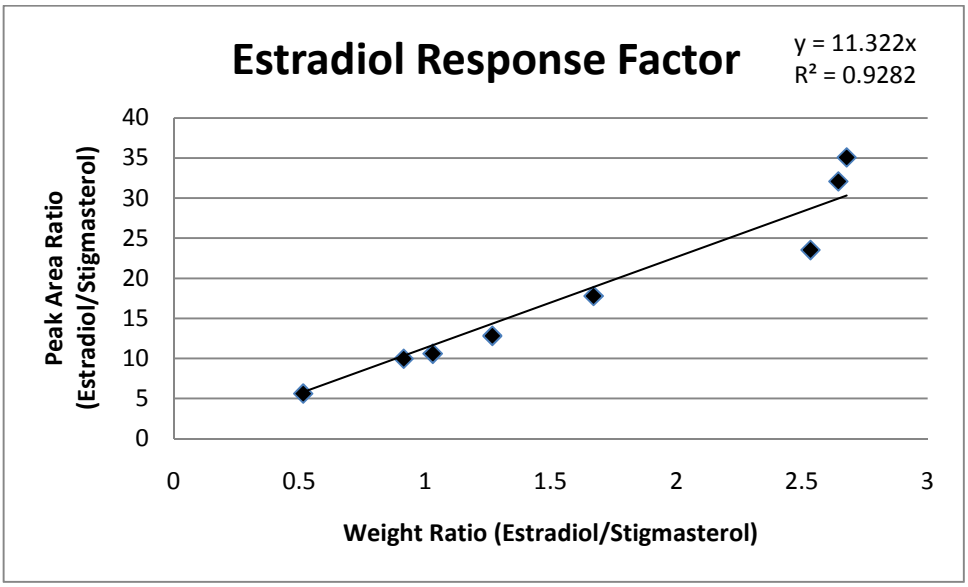


Figure 3.44 Response factor of 17β-estradiol

The response factor of 17β-estradiol is significantly higher than the other three response factors found for the other steroids. This is due to the slightly different structure (ring A is a benzene group) and this ring stabilises $[M]^+$ and $[M\text{-}TMSOH]^+$ so they do not fragment further.

This response factor would be used in practice for estrone, as it also has a similar structure to 17β-estradiol; with the only difference being estrone has a keto group at C-17, whilst 17β-estradiol has an alcohol group.

Androstenedione

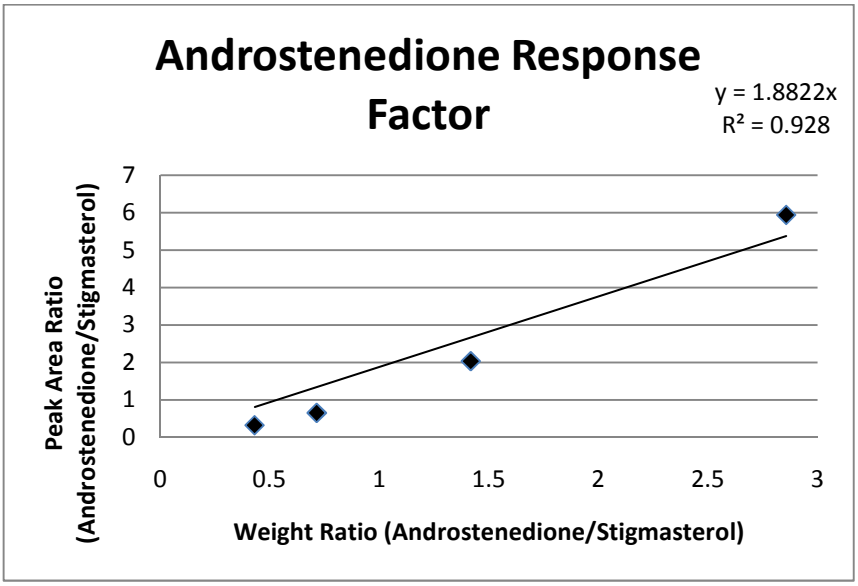


Figure 3.45 Response factor graph of androstenedione

This response factor would only be used for androstenedione in practice.

17α-hydroxyprogesterone

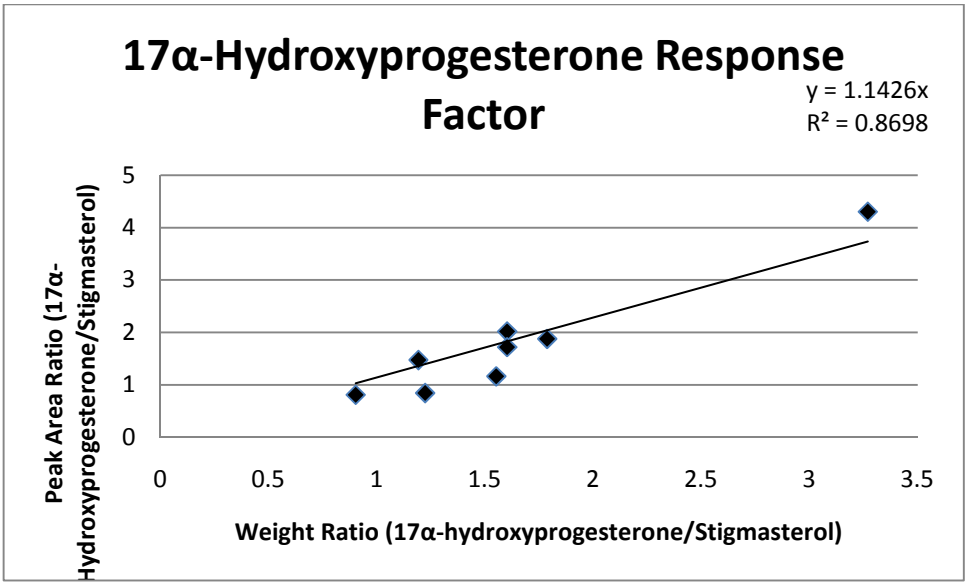


Figure 3.46 Response factor graph of 17α-hydroxyprogesterone

This response factor would be applied to compounds with similar structures, such as the C-21 compounds.

3.3.3.5 Limits of detection

When developing a method of analysis, which will be required to analyse low levels, it is important to know the detection limits. As it cannot be stated with certainty that a steroid is not present, only that it is not present at levels high enough to detect.

Detection limits of both instruments and methods must be determined. Detection limits are determined by obtaining the amount of compound that gives a signal to noise ratio of 2:1 or 3:1. It is likely that the limits of detection of the method itself, may be significantly higher than the instrumental detection limits.

Instrumental detection limits

Detection limits were established for testosterone, 17β-estradiol, 17α-hydroxyprogesterone, and androstenedione, as these were available in reasonable quantity.

Standards (~10 mg) were dissolved in methanol (100 mL). Dilutions were carried out, and precise volumes of varying concentrations were evaporated to dryness, derivatised and made up to the same volume then analysed. A larger mass was used to keep weighing errors as small as possible, along with keeping volume measurements to 1 mL or more. Instrumental response to injections of 2 pg quantities in 2 µL injections were as follows:

Testosterone (1.97 ± 0.03 pg/2 µL) signal to noise ratio of 13:1

17α-hydroxyprogesterone (1.93 ± 0.03 pg/2 µL) signal to noise ratio of 154:1

17β-estradiol (1.86 ± 0.03 pg/2 µL) signal to noise ratio of 417:1

Androstenedione (1.63 ± 0.02 pg/2 µL) signal to noise ratio of 11:1

These results showed it was clear that the limits of detection of the instrument were low and detection limits for the method were established to determine whether further investigation of instrumental detection limits was required.

Method Limits of Detection

To obtain method detection limits, solutions of various concentrations of testosterone were prepared and extracted with SPE cartridges. Testosterone was eluted from the cartridge and derivatised as would occur in the method. The aim was to establish a numerical concentration that gives a signal to noise ratio of 2:1 or 3:1.

After getting odd results, with false positives for testosterone solutions and blanks (360, 270, 226 ions at correct retention time), it was discovered that there was contamination occurring (Figures 3.45 and 3.46).

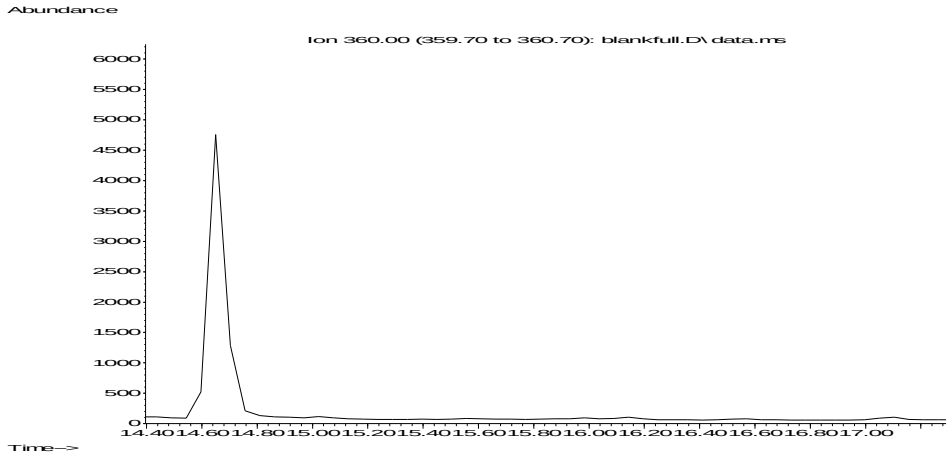


Figure 3.47 Extracted ion chromatogram of ion 360 showing interference ion at 14.5 min in blank SPE

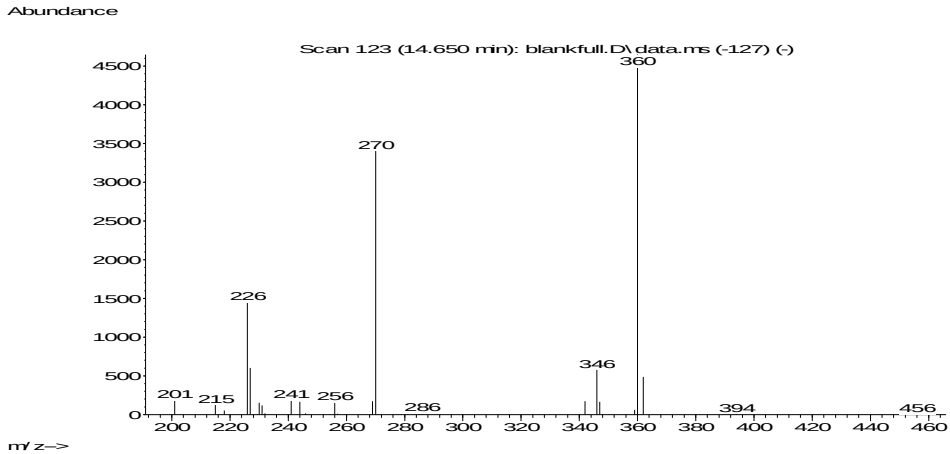


Figure 3.48 Selected ion mass spectrum of interference peak at 14.6 min in blank SPE

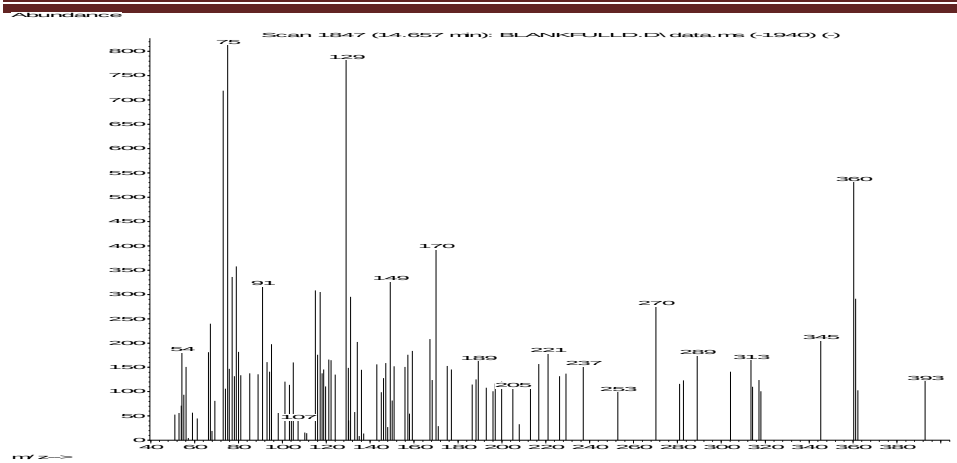


Figure 3.49 Full mass spectrum of interference as 14.6 min of blank SPE

It was thought that some testosterone could be adhering to the tubing used in the peristaltic pump setup, another blank run was completed after rinsing the tubing with methanol (30 mL). There were still false positives in both of these blanks.

In order to establish the source of contamination, a blank was carried out by reducing the volume of 1 L of Milli-Q water using a rotary evaporator, and freeze drying the small volume of liquid. This blank also contained the interference, indicating interference was not coming from the extraction procedure (Figures 3.44 and 3.48).

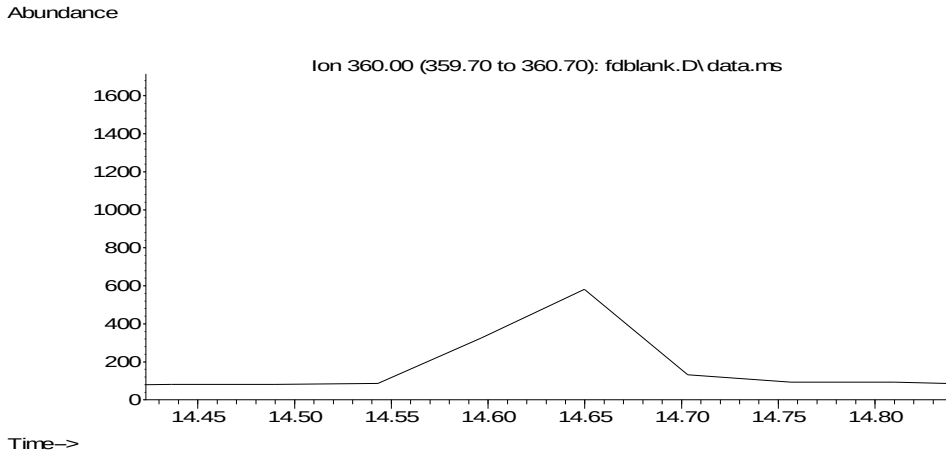


Figure 3.50 Extracted ion chromatograph of ion 360 showing interference ion at 14.6 min in freeze dried blank

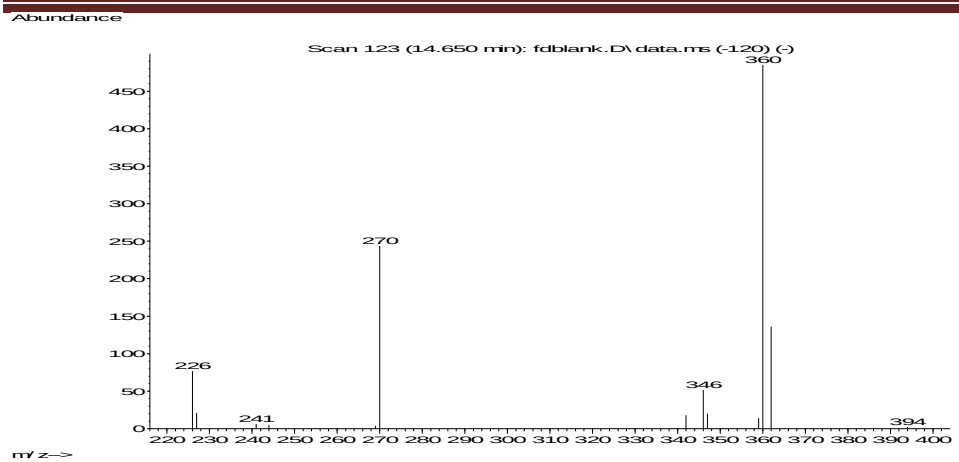


Figure 3.51 Selected ion mass spectrum of interference peak at 14.6 min in freeze dried blank

Closer analysis of the internal standard solution revealed the appropriate ions at the correct retention times, however after the discovery of an interference, internal standard was no longer being added prior to silylation until the source of contamination could be identified. The methanol was checked for the interference, as this was the solvent for the internal standard solution. The presence of all three ions selected for testosterone were confirmed in the spectrum at the correct retention time. A full spectrum was obtained and is shown below (Figure 3.51).

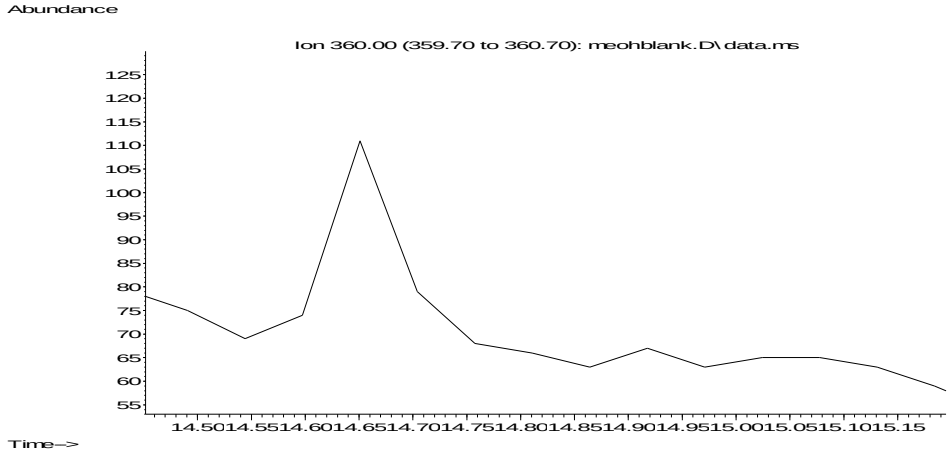


Figure 3.52 Extracted ion chromatogram of ion 360 showing interference ion at 14.6 min in methanol

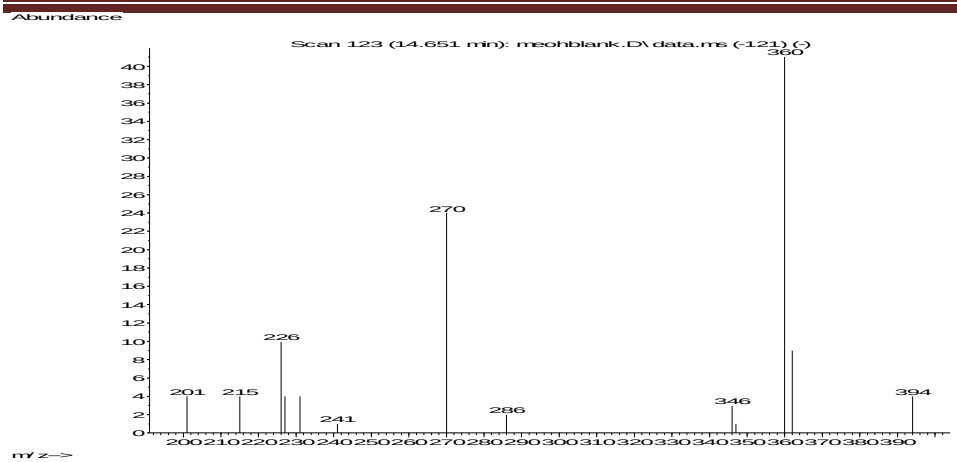


Figure 3.53 Selected ion mass spectrum of interference peak at 14.6 min in methanol

The interference in methanol was eliminated through distillation, a full spectrum of the impurity was unattainable due to the low levels at which it existed.

Interference was also sourced to the internal standard with a full scan shown below (Figure 3.52). There was no 270 ion present in this impurity and the quantitative ion was changed, and response factors recalculated using ion 270. This impurity may be attributed to another steroidal compound that is similar in structure to testosterone as the elution time is the same. The purity of the internal standard is only 95% therefore this is a very appropriate option.

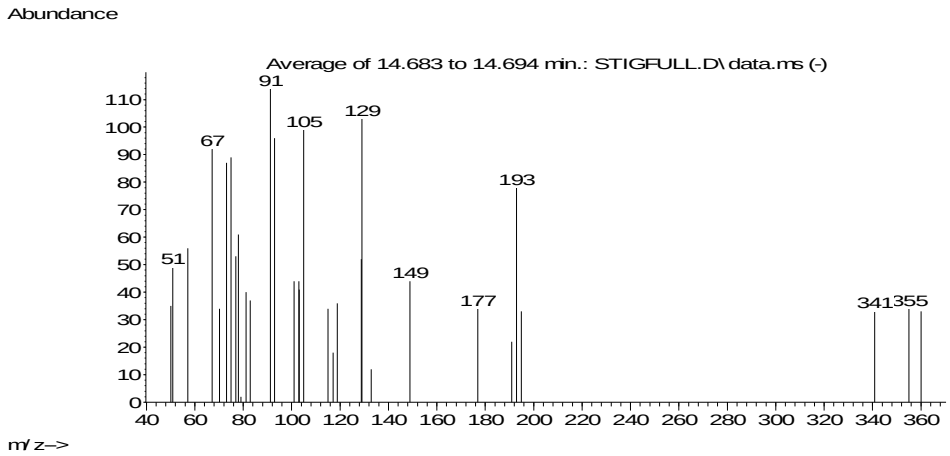


Figure 3.54 TIC trace of impurity at 14.6 min in Stigmasterol

The method detection limits for testosterone were found to be 3 ± 0.1 pg/2 µL injection, with a signal to noise ratio of 10:1 which is almost twice the instrumental detection limits.

Method detection limits were not determined for other steroids. However, for further work with this method, it is strongly suggested they are obtained, along with the recoveries of other steroids.

Recovery of testosterone

Solutions used in obtaining method detection limits were also used to calculate the recovery of testosterone from the cartridge. The reproducibility of the recovery of testosterone was extremely variable (see Table 3.10). More accurate determination of the recovery of testosterone and other steroids is required for further use of this method. A recovery value of 20% was used to adjust for testosterone levels measured in goldfish holding water (Tables 3.13 and 3.14), but this is only an approximation; the levels of other steroids were not adjusted as recovery was unknown. Poor recovery may be due to either incomplete extraction of steroids from the solution or incomplete elution of steroids from the cartridge. To establish where losses are occurring, it will be necessary to measure for the presence of any testosterone in the solution after it has passed through the extraction cartridge using the freeze drying method, and to perform elution at a much slower rate, allowing for equilibration of the stationary phase with the mobile phase. Elution was carried out at a greater rate than extraction, this was found to be an issue with the extraction, and is likely an issue with elution. It is very important that further work establishes and optimises recovery of as many steroids as possible. With improved recovery more steroids may be detected.

Table 3.10 Recovery obtained from testosterone method detection limit solutions

Concentration of solution (ng/L)	Weight Stig (ng)	Stig Peak Area 255	Test Peak Area 270	Weight Test (ng)	Recovery (%)
0.139	188	71905	2414	3.51	2510
0.139	188	77994	341	0.457	326
0.147	188	410269	14425	3.67	2450
0.147	188	168673	124	0.0768	51.2
13.9	188	27484	394	1.50	10.8
13.9	188	29733	127	0.446	3.21
14.7	188	464563	14700	3.30	22.5
14.7	188	507879	14215	2.92	19.9
1390	188	41074	17263	43.9	3.16
1390	188	52941	19647	38.8	2.79
1470	188	4521958	80960	1.87	0.13

Where test= testosterone and stig= stigmasterol

3.3.3.6 Hydrolysis of glucuronide conjugates

The efficiency and recovery of the hydrolysis of glucuronide conjugates using β-glucuronidase was determined using testosterone glucuronide as a substrate. Testosterone glucuronide (~2 mg) was hydrolysed following experimental procedures, and average recovery was 117%.

3.3.3.6 Selection of internal standard

Three internal standards were trialled before deciding on the use of stigmasterol. Glucitol was trialled first, but disregarded as it eluted too early (6.5 min) and not in the same region as other peaks. Sucrose was trialled next and looked promising until it was discovered to elute with 17β-estradiol. Stigmasterol and stigmasterol propionate were both trialled, however silylated stigmasterol was better resolved and eluted earlier, therefore was selected to be the internal standard (see Figures 3.55 and 3.56).

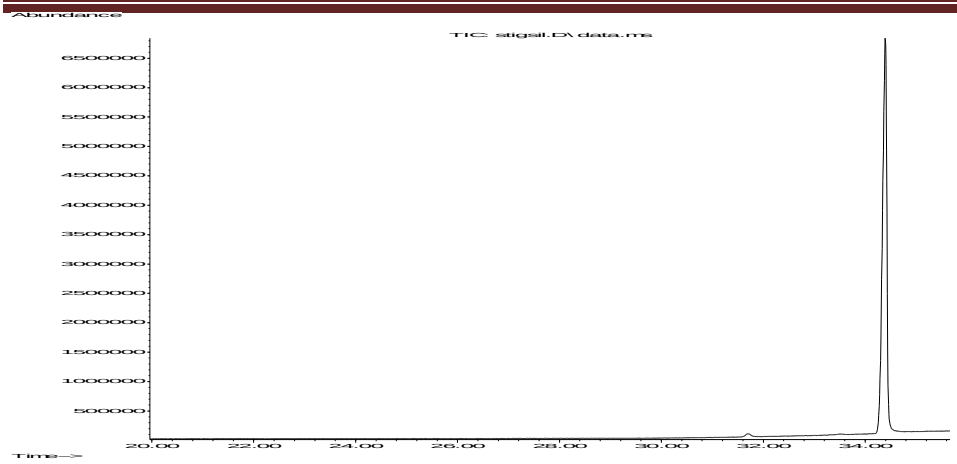


Figure 3.55 TIC trace of silylated stigmasterol

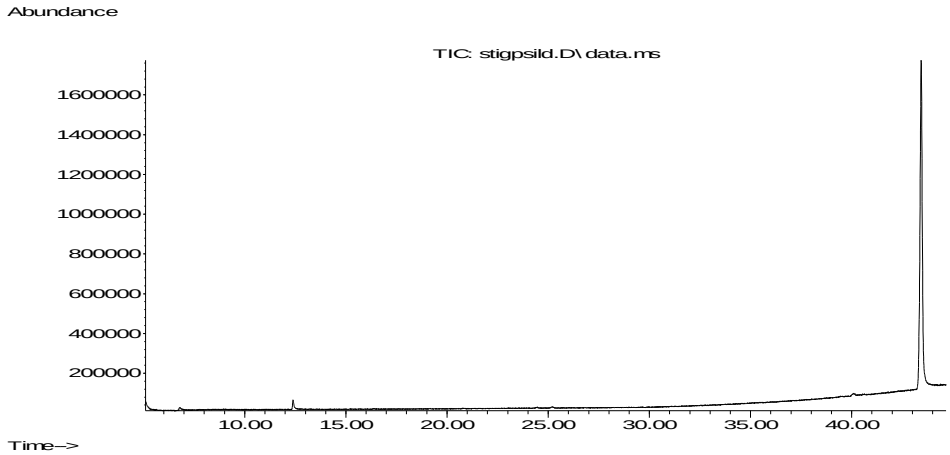


Figure 3.56 TIC trace of stigmasterol propionate

The internal standard was not the best for testosterone, deuterated testosterone-*d3* may have been a better choice however is currently priced at $1040 per 10mg.

3.4 Application of method

The method was applied to twelve samples, from the holding tanks of six goldfish injected with a Domperidone control, and the other half from goldfish treated with Ovaprim. Eight female goldfish were used, four were given the control and four were given the treatment. Four male goldfish were used, two were given the control, and two were given the treatment.

It was frequently observed that the correct ions for identification of a steroid were present at the correct retention time, however the ratio of ion intensity was often far away from original observations and the area of these was not recorded. A

mixed standard solution was run at the end of the samples to verify correct retention times.

The only steroids that were detected unambiguously were androstenedione (see Figure 3.53 for chromatogram), testosterone, 17α-hydroxyprogesterone (17OH-P) and 17,20β-21-trihydroxypregn-4-en-3-one (17,20,21-THP) see Tables 3.11 and 3.12. Quantities of steroids released in terms of fish bodyweight are outlined in Tables 3.13 and 3.14.

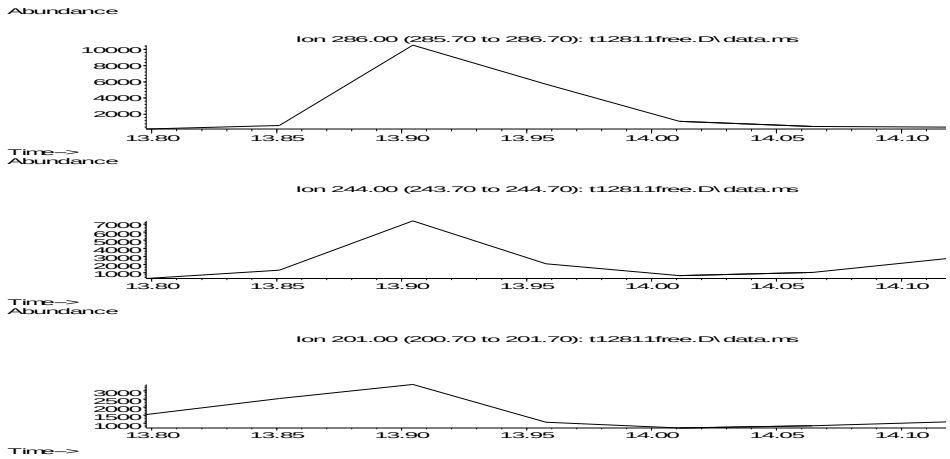


Figure 3.57 Extracted chromatograms of ions 286, 244 and 201 in a treated female goldfish sample at retention times 13.8-14.1 min

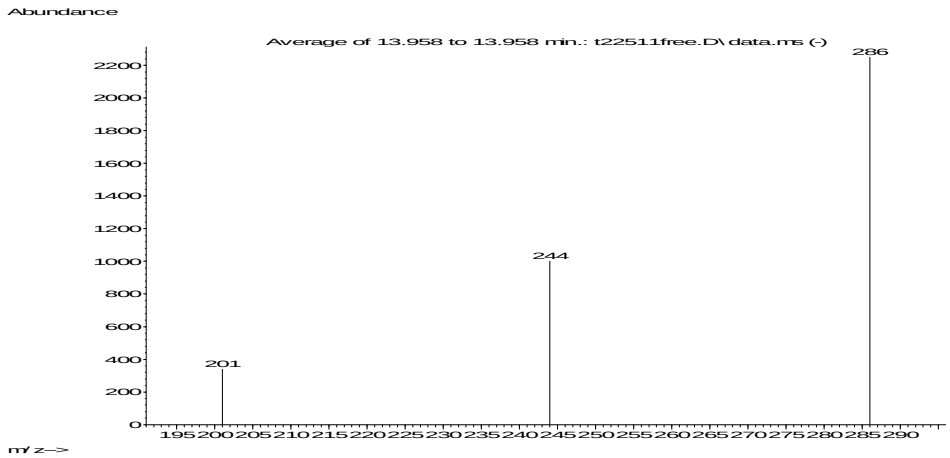


Figure 3.58 SIM Mass spectrum of peak with retention time 13.8-14.1 min in a treated female goldfish sample

Table 3.11 Table of results showing steroids detected in female goldfish holding water

	Dione (ng)	**Original Concentration of Dione (ng/L)**	**Test (ng)**	**Original Concentration of Test (ng/L)(not corrected)**	**17OH-P (ng)**	**Original Concentration of 17OH-P (ng/L)**	**17,20,21-THP (ng)**	**Original Concentration of 17,20,21-THP (ng/L)**	**Fish mass (kg)**
Control free	ND	-	10.58	6.47	ND	-	ND	-	0.196
Control gluc	ND	-	ND	-	ND	-	ND	-	0.196
Treatment free	0.37	0.20	2.53	1.78	0.18	0.10	0.18	0.10	0.164
Treatment gluc	ND	-	0.33	0.23	2.30	1.26	0.33	0.18	0.164
Control free	ND	-	4.77	3.06	0.47	0.24	0.59	0.30	0.232
Control gluc	ND	-	1.90	1.22	ND	-	ND	-	0.232
Treatment free	43.99	24.17	14.68	10.31	ND	-	6.26	3.44	0.219
Treatment gluc	ND	-	0.09	0.06	ND	-	ND	-	0.219
Control free	ND	-	ND	5.39	ND	-		-	0.186
Control gluc	ND	-	0.51	0.30	1.45	0.66	ND	-	0.186
Treatment free	107.9	49.97	8.59	5.08	ND		0.45	0.21	0.137
Treatment gluc	ND	-	5.64	3.34	8.38	3.88	ND	-	0.137
Control free	ND	-	4.95	2.97	ND	-	ND	-	0.140
Control gluc	ND	-	0.08	0.05	ND	-	ND	-	0.140
Treatment free	27.55	13.64	8.42	5.33	ND	-	ND	-	0.130
Treatment gluc	ND	-	0.6	0.38	ND	-	0.36	0.18	0.130

Where ND = not detected. Steroids which were not detected in any samples are not listed in order to simplify presentation of results.
Dione= Androstenedione 17,20,21-THP= 17**,**20β,21-trihydroxy-pregn-4-en-3-one 17OH-P= 17α-hydroxyprogesterone
Test= Testosterone Free= free steroid fraction Gluc= Glucuronide conjugate fraction

Table 3.12 Table of results showing steroids detected in male goldfish holding water

	Dione(ng)	**Original Concentration of Dione (ng/L)**	**Test(ng)**	**Original Concentration of Test (ng/L)(not corrected)**	**17OH-P(ng)**	**Original Concentration of 17OH-P (ng/L)**	**fish mass (kg)**
Control free	ND	-	1.36	0.67	ND	-	0.121
Control gluc	ND	-	1.07	0.53	1.98	0.98	0.121
Treatment free	4.96	2.41	4.00	1.94	ND	-	0.089
Treatment gluc	ND	-	1.03	0.50	ND	-	0.089
Control free	ND	-	ND	-	ND	-	0.156
Control gluc	ND	-	1.43	0.65	ND	-	0.156
Treatment free	1.77	0.83	5.54	2.61	ND	-	0.108
Treatment gluc	ND	-	1.86	0.88	2.92	1.38	0.108

Where ND = not detected. Steroids which were not detected in any samples are not listed in order to simplify presentation of results.
Dione= Androstenedione 17,20,21-THP= 17**,**20β,21-trihydroxy-pregn-4-en-3-one 17OH-P= 17α-hydroxyprogesterone
Test= Testosterone Free= free steroid fraction Gluc= Glucuronide conjugate fraction

Table 3.13 Total amount of steroids released by females per gram of goldfish

	Release of steroids (pg/g)			
	Dione	**Test (corrected)**	**17OH-P**	**17,20,21-THP**
Control free		2070		
Control gluc				
Treatment free	13.6	591	6.48	6.78
Treatment gluc		78.0	84	12.0
Control free		792	12.24	15.3
Control gluc		315		
Treatment free	120	2570		172
Treatment gluc		15.4		
Control free		1812		
Control gluc		117	46.8	
Treatment free	4730	2410		19.7
Treatment gluc		1580	367	
Control free		1360		
Control gluc		23.1		
Treatment free	1270	248		
Treatment gluc		176		

Table 3.14 Total amount of steroids released by males per gram of goldfish

	Release of steroids (pg/g)		
	Dione	**Test (corrected)**	**17OH-P**
Control free		1730	
Control gluc		1360	97.8
Treatment free	334	1730	
Treatment gluc		449	
Control free		121	
Control gluc		351	
Treatment free	97.8	1970	
Treatment gluc		660	162

Where dione= androstenedione, test= testosterone, 17OH-P= 17α-hydroxyprogesterone, free= free steroid fraction**,** gluc= glucuronide conjugate fraction
Testosterone values were adjusted by multiplying by 5, as recovery was found to be 20%
All values were multiplied by6 to get the total amount present (2 L of water was extracted from 12 L holding water)

The results for 17α-hydroxyprogesterone and 17,20β,21-trihydroxy-pregn-4-en-3-one are very close to detection limits, therefore the accuracy of quantitation may be reduced.

17,20β,21-THP was only detected in female goldfish holding water, which was expected.

It was disappointing that 17, 20β-P (primary component of preovulatory pheromone released by female goldfish)[6] was not detected, as it is produced solely by females as a luteinizing hormone, and excreted as a pheromone as a means of informing the male of spawning readiness[24]. Androstenedione and sulfated 17,20β-P are also components of this pheromone mixture. Androstenedione was detected in large amounts in the holding water of treated female goldfish. It is possible that 17,20β-P was also present, but not in high enough levels to detect. The variability in recoveries of testosterone indicate that there may also be problems in extracting other steroids, which need to be solved. Sulfated 17,20β-P may have been present in large quantities, however levels of sulfate conjugates were not determined, and future samples must be analysed for sulfate conjugates.

Significantly more androstenedione was produced by treated female fish than male fish. This is extremely unusual, despite it occurring in the preovulatory pheromone, and treated females have been shown to release significantly less amounts of androstenedione than even untreated males. All the literature shows that androstenedione is released into the water in greatest quantity by spermiating males, and that even non spermiating males release greater quantitities than ovulatory females[21, 25]. Results in our study do not agree with these published results, however only two treated male fish samples were analysed, and more male holding water samples should be measured to determine whether these results were usual or exceptions, as biological systems are prone to large amounts of variation. It has been suggested by Sorensen *et al*[23] that androstenedione may function as a male sex pheromone, as it is released in such great quantities by males, and strongly stimulates aggressive behaviour in male goldfish. Levels detected in the study were 3.3 $ngh r^{-1}$ in female goldfish, and 782 $ngh r^{-1}$ in male goldfish injected with human chorionic gonadotropin which increases milt

production[23]. These results were measured using radioimmunoassay, which has many limitations (Section 1.7.3).

Levels of testosterone were not any higher in treated fish than in control fish, or males rather than females as would be expected. However there were small amounts detected in the glucuronide fraction, indicating conjugation of some steroids is occurring prior to excretion.

It is important to note that these literature results were analysed by radioimmunoassay which whilst being very sensitive, is not without major disadvantages. Reliable results are dependent on specific antisera, with little cross reaction, and assays are only performed for compounds that are anticipated to be present.

Sulfate conjugate levels were not determined in this study, there may have been high levels of sulfate conjugates released, that were not measured.

More samples would need to be completed to establish the robustness of the method.

Conclusion

To enable this method to become a truly useful analytical method a few things must be addressed:

1. The recovery and method detection limits for substances other than testosterone should be obtained, the other steroids for which response factors were determined would be a good start
2. The recovery of steroids still requires considerable work to improve reproducibility and probably the best place to start is the rate of flow of the methanol during elution
3. Replacement of the internal standard with another compound that does not contain any interferences with ions of interest would be ideal, however this would require redetermination of response factors
4. Work on the hydrolysis of sulfate conjugates is recommended as it is essential to know the amount of sulfate conjugates that were released

When these issues are addressed, this method has the potential to be of greater use at measuring fish sex steroids compared to radioimmunoassay, as it is able to measure a range of fish sex steroids at once.

Appendix One- Chromatograms and Mass Spectra

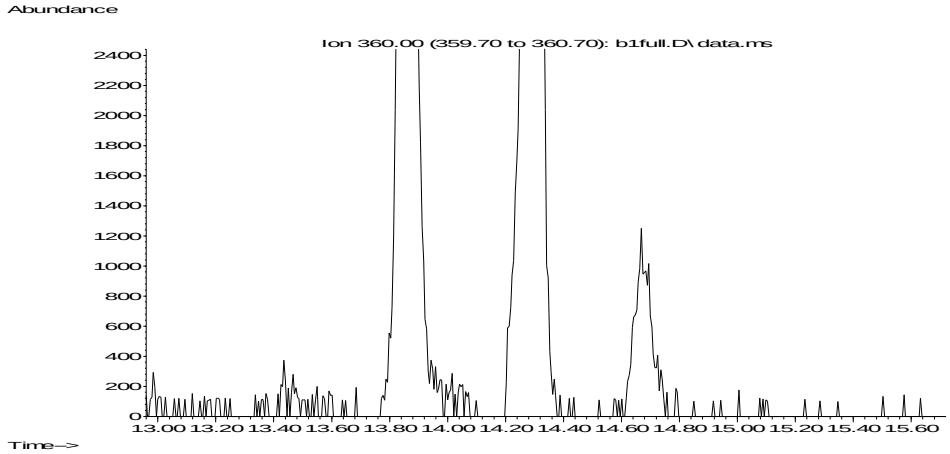


Figure 1 Extracted ion chromatogram (360) of blank SPE solution, showing impurity at 14.6 min

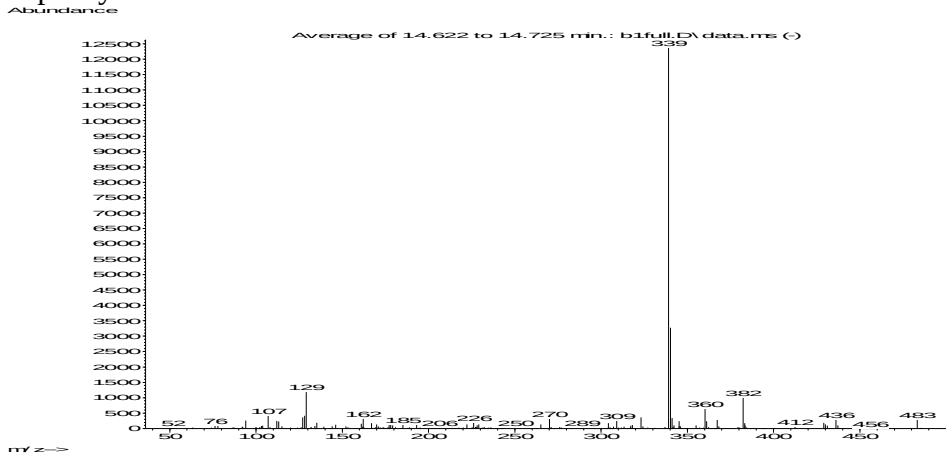


Figure 2 SIM spectrum of impurity at 14.6 min in blank SPE solution

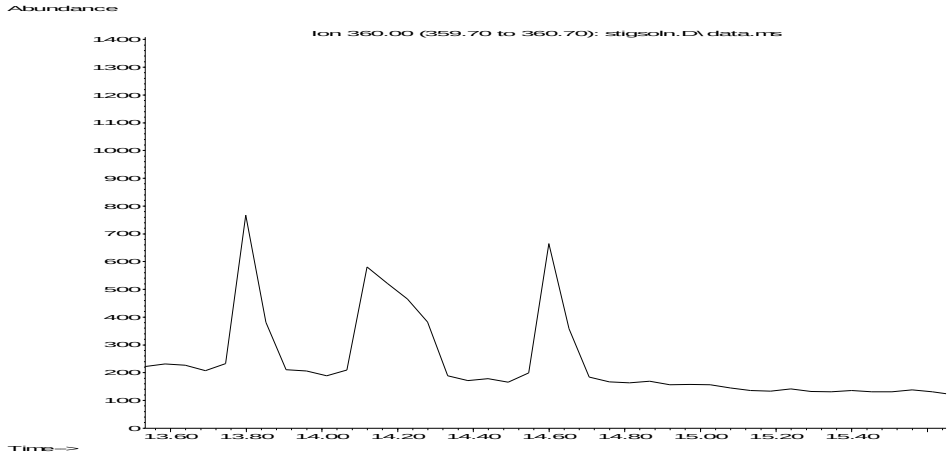


Figure 3 Extracted ion chromatogram (360) showing impurity at 14.6 min from solution of stilgmasterol in methanol

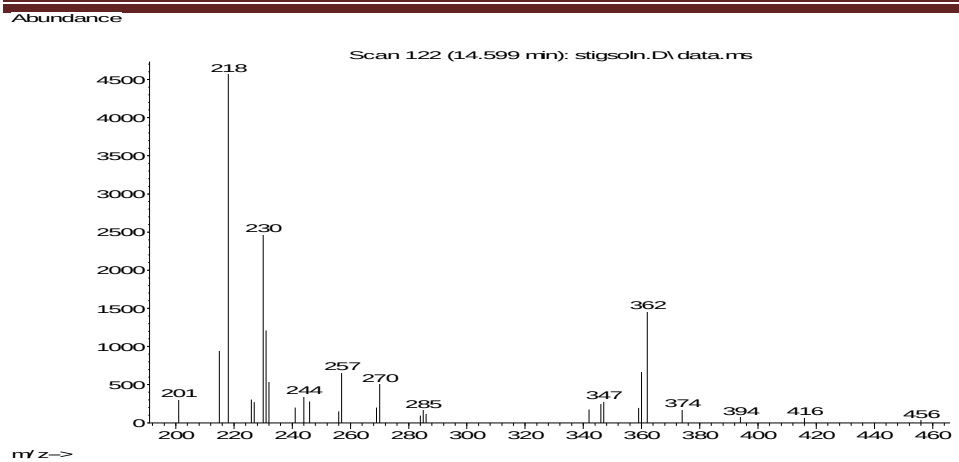


Figure 4 SIM spectrum of impurity at 14.6 min from solution of stigmasterol in methanol

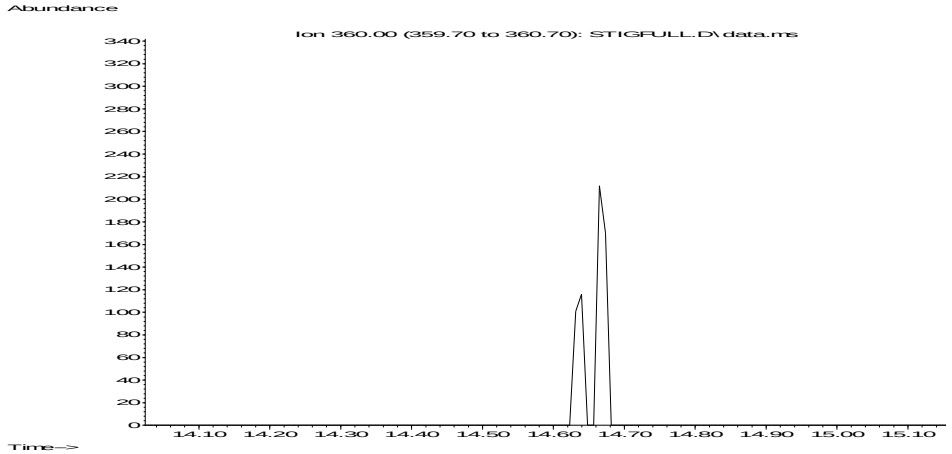


Figure 5 Extracted ion chromatogram (360) showing impurity at 14.6 min in stigmasterol

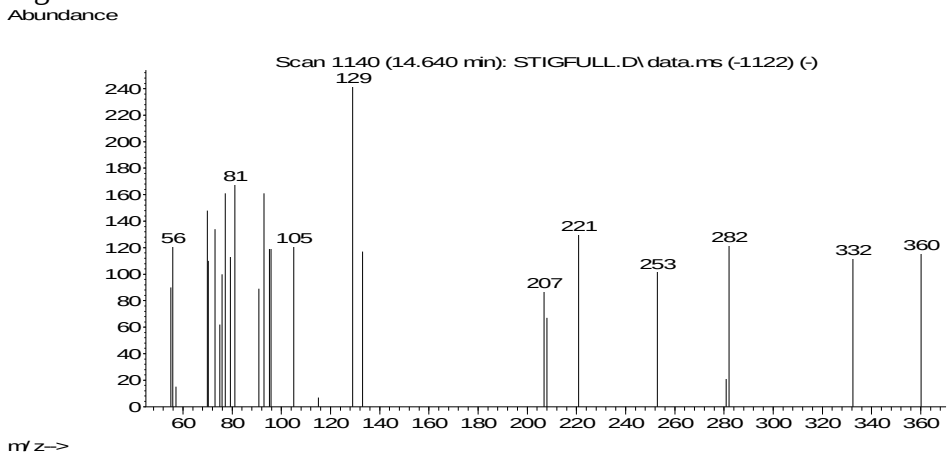


Figure 6 Full mass spectrum of impurity at 14.6 min in stigmasterol

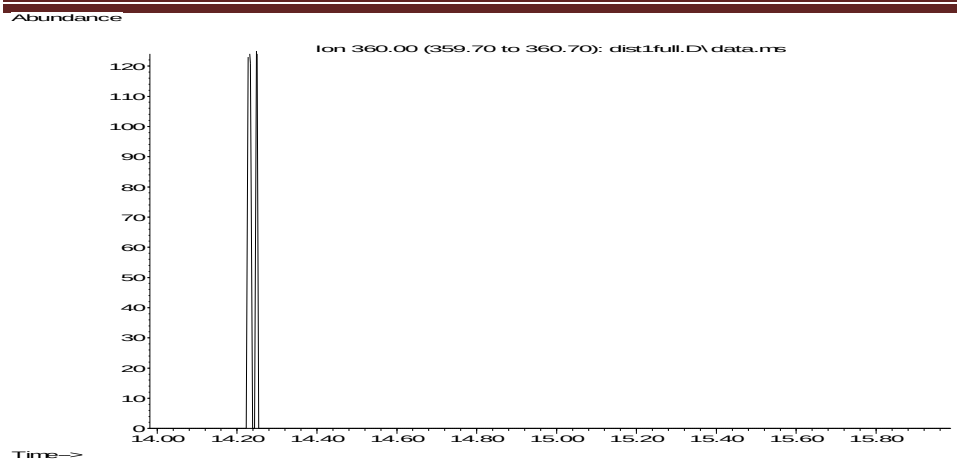


Figure 7 TIC of distilled methanol showing no interference peak at 14.6 min

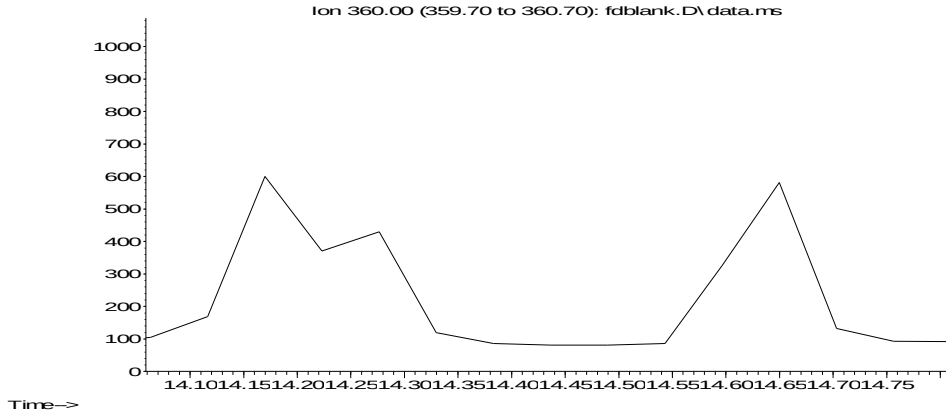


Figure 8 Extracted ion chromatogram (360) of blank solution that was freeze dried

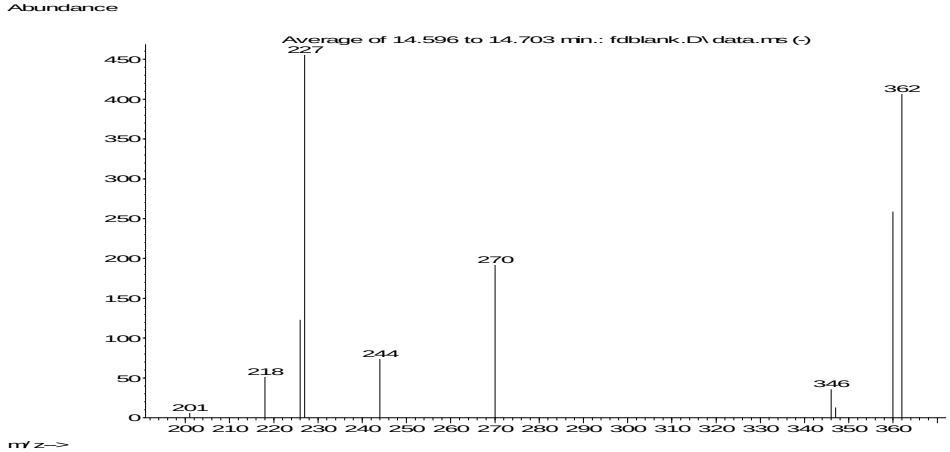


Figure 9 SIM spectrum of peak at 14.6 min in blank solution that was freeze dried

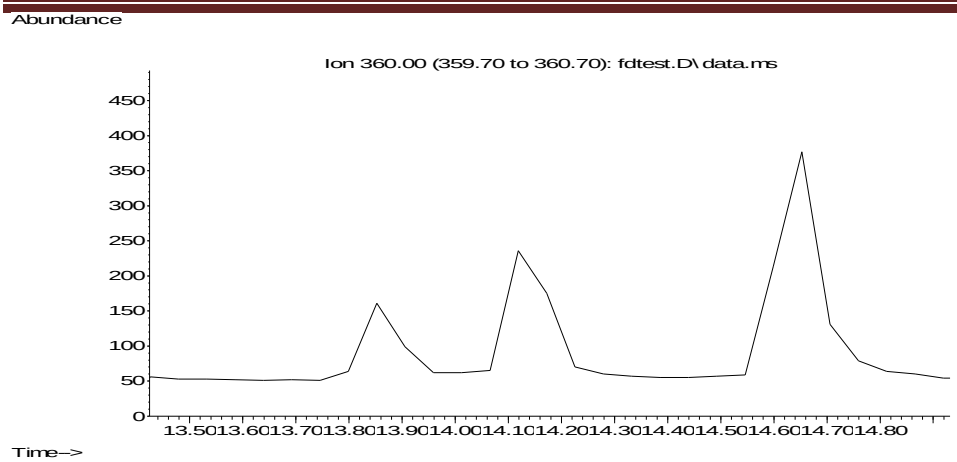


Figure 10 Extracted ion chromatogram (360) of freeze dried testosterone solution

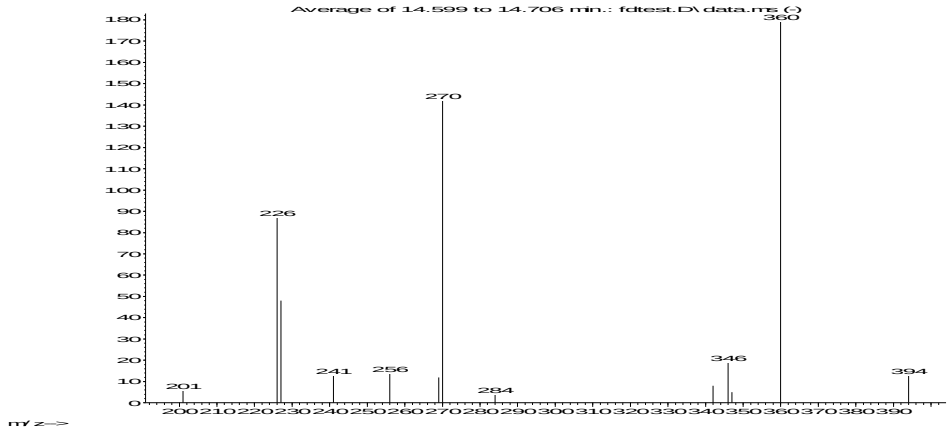


Figure 11 SIM spectrum of freeze dried testosterone solution at 14.6 min

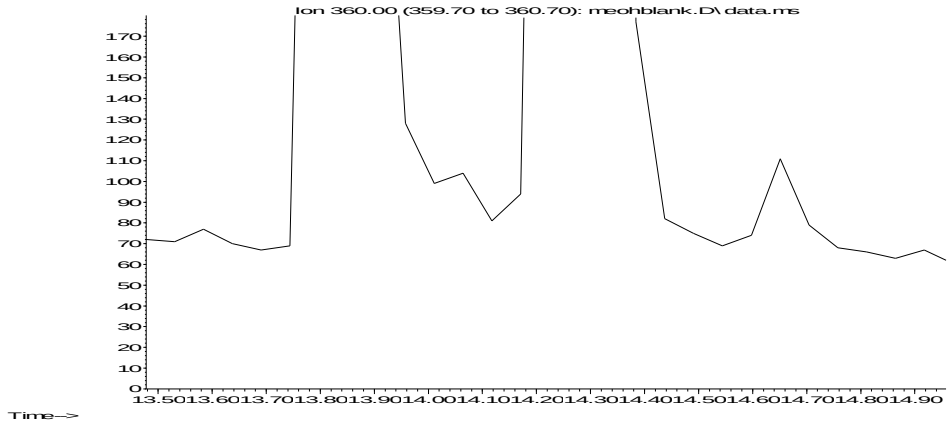


Figure 12 Extracted ion chromatogram (360) of methanol showing interference at 14.6 min

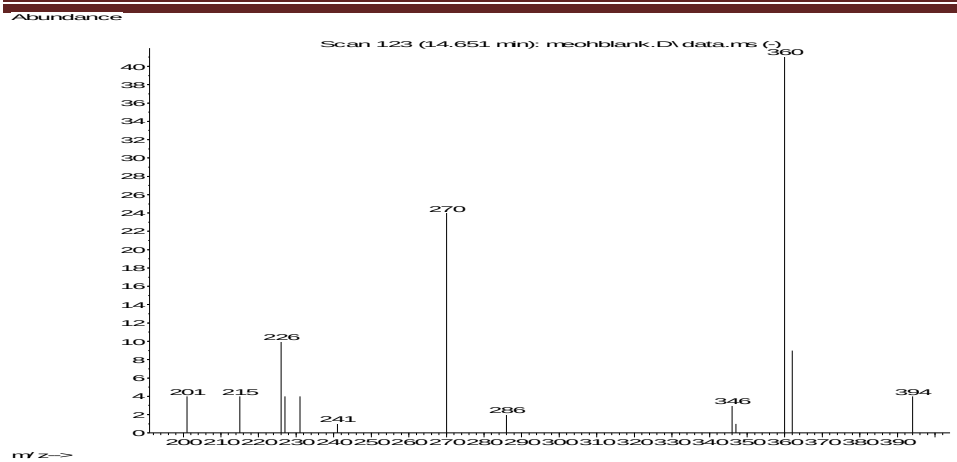


Figure 13 SIM spectrum of methanol at 14.6 min

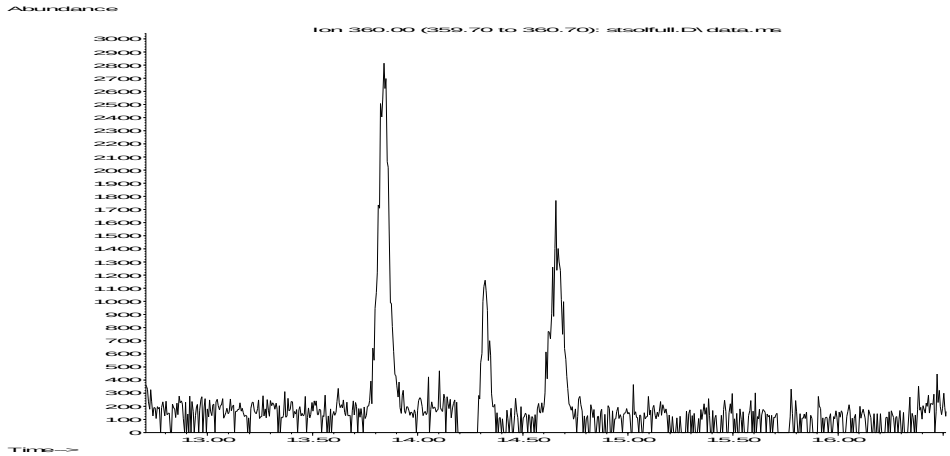


Figure 14 TIC of stigmasterol in methanol showing interference at 14.6 min

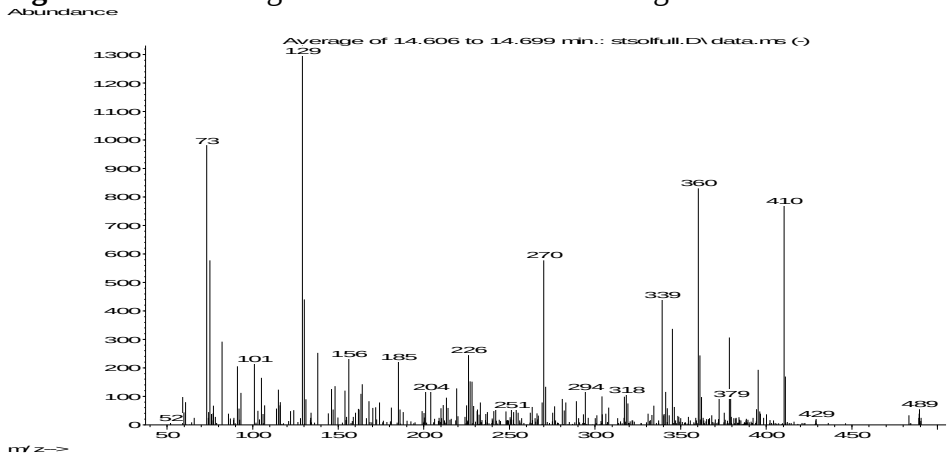


Figure 15 TIC of stigmasterol in methanol at 14.6 min

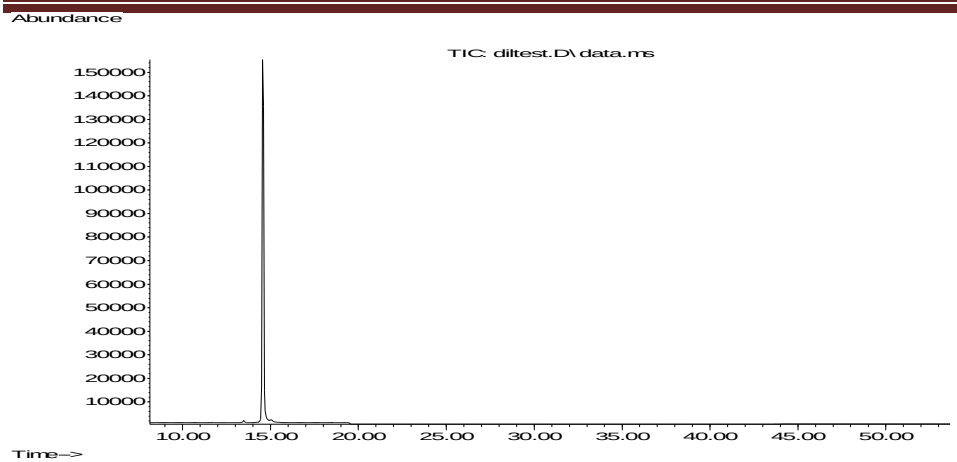


Figure 16 SIM chromatogram of testosterone

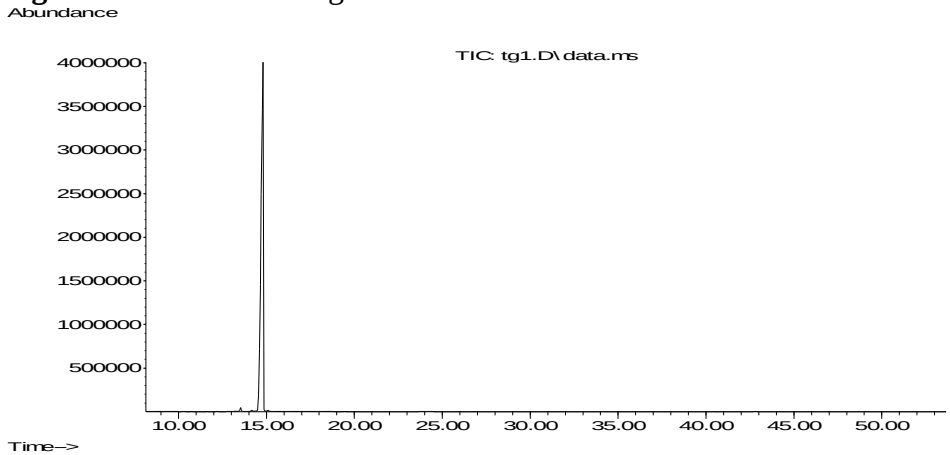


Figure 17 SIM chromatogram of testosterone glucuronide hydrolysis

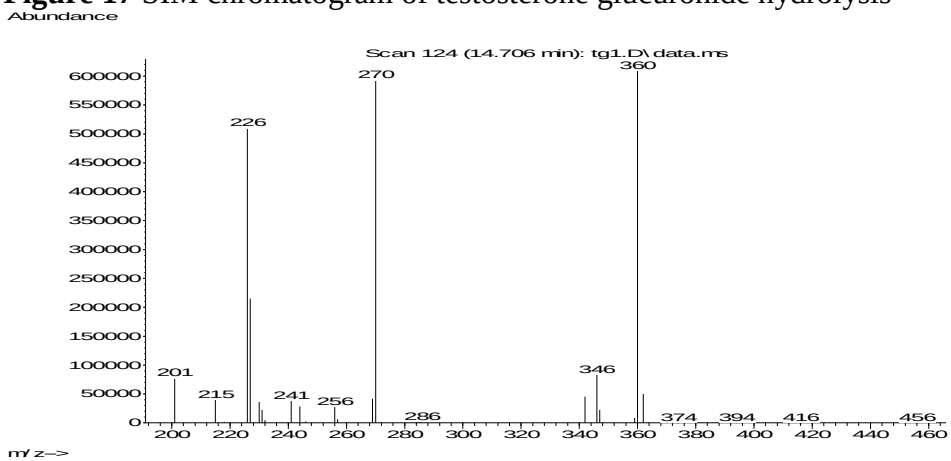


Figure 18 SIM mass spectrum of testosterone glucuronide hydrolysis

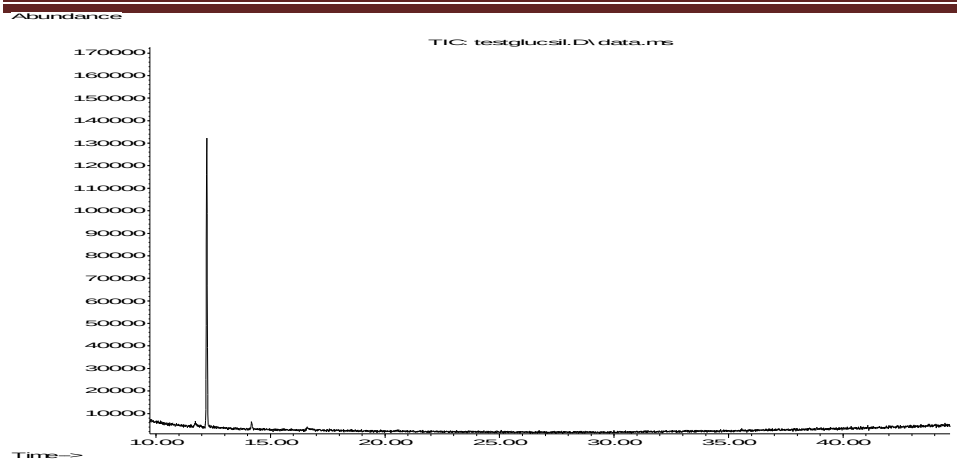


Figure 19 TIC of testosterone glucuronide

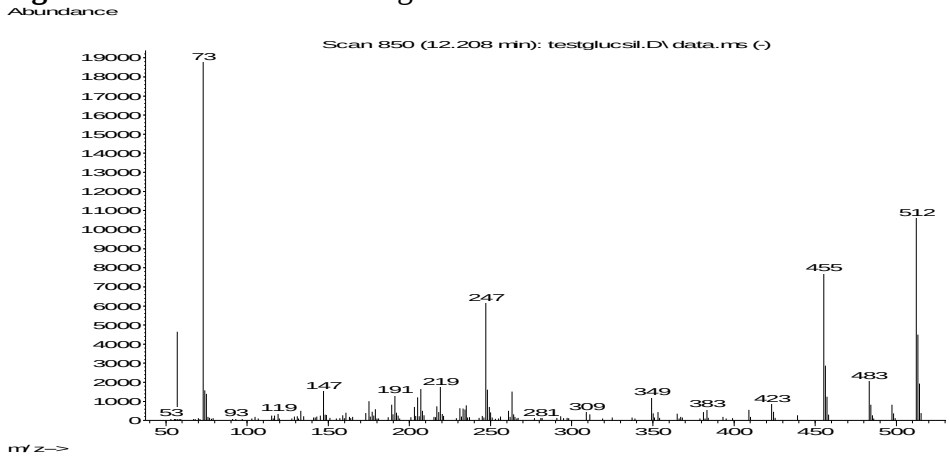


Figure 20 Mass spectrum of testosterone glucuronide

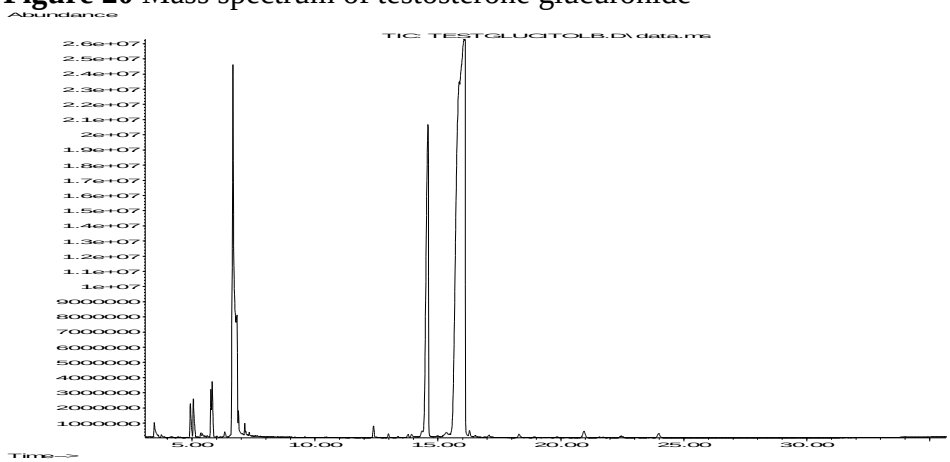


Figure 21 TIC trace of testosterone and glucitol

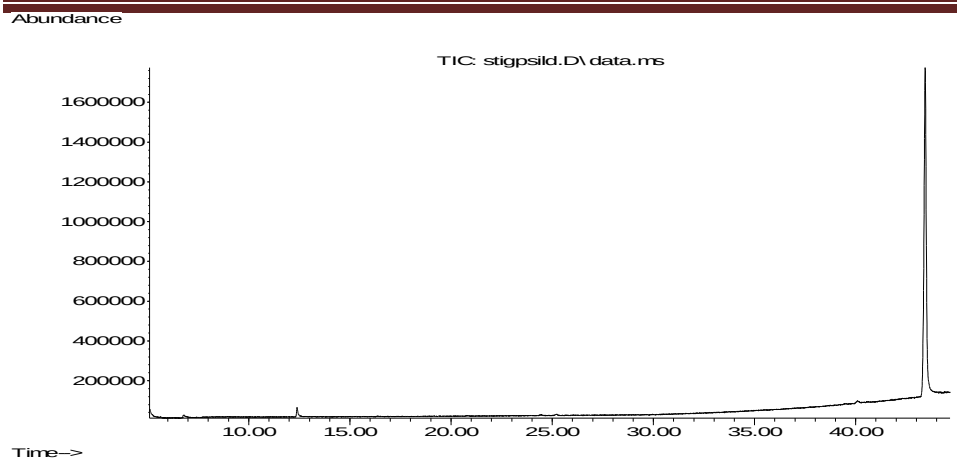


Figure 22 TIC of stigmasterol propionate

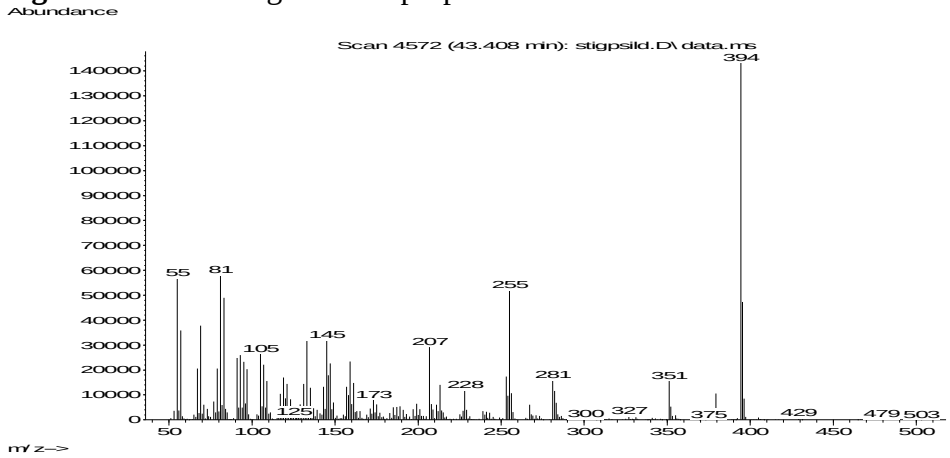


Figure 23 Mass spectrum of stigmasterol propionate

Figure 24 TIC trace of TMS ether of stigmasterol

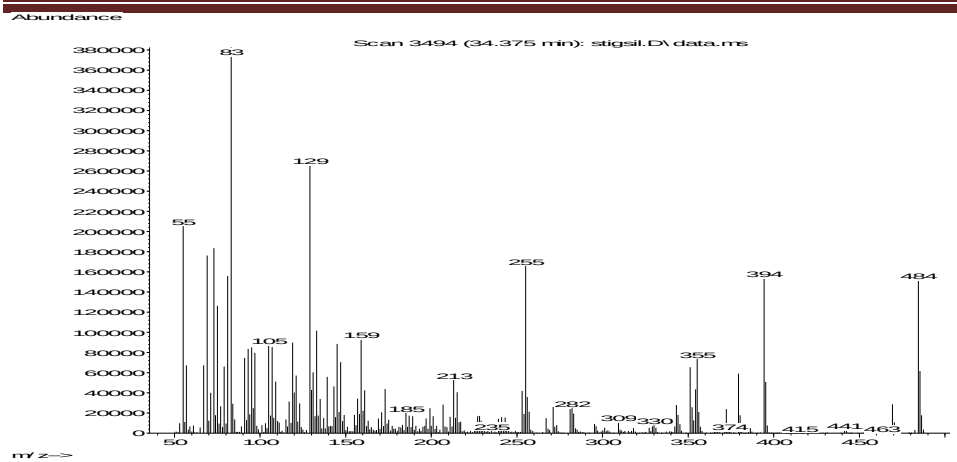


Figure 25 Mass spectrum of TMS ether of stigmasterol

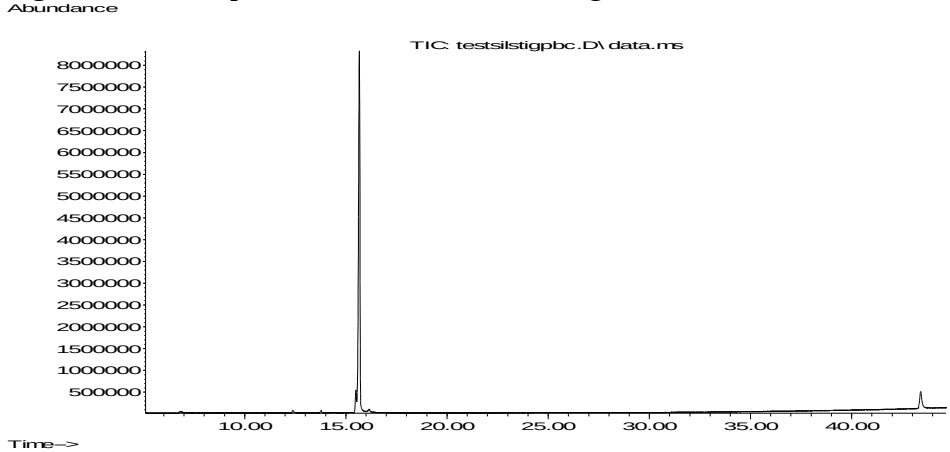


Figure 26 TIC trace of testosterone and stigmasterol

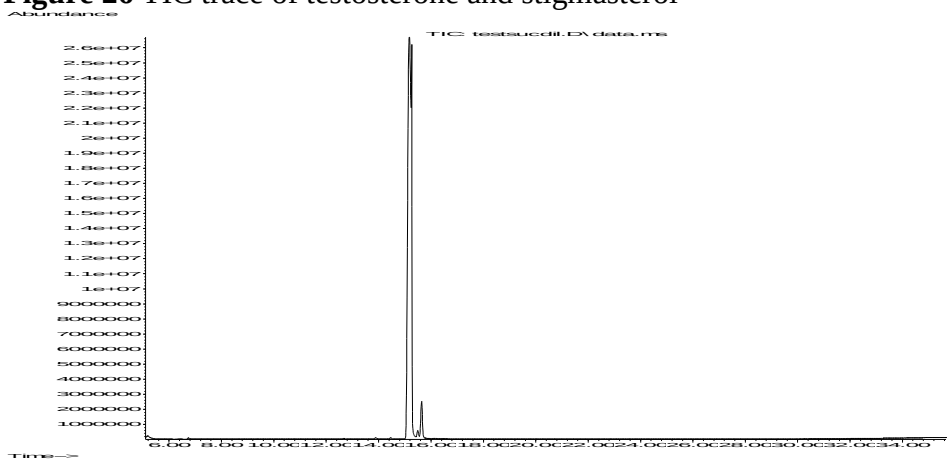


Figure 27 TIC trace of silylated sucrose and testosterone

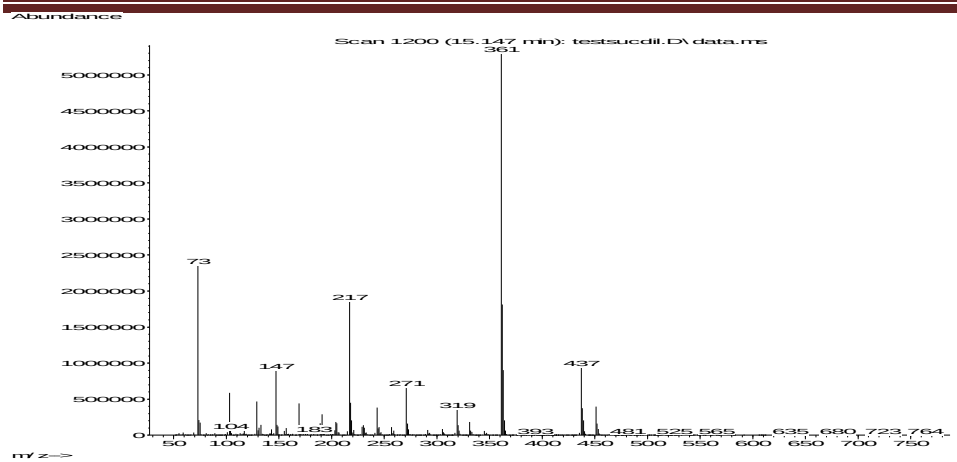


Figure 28 Mass spectrum of silylated sucrose

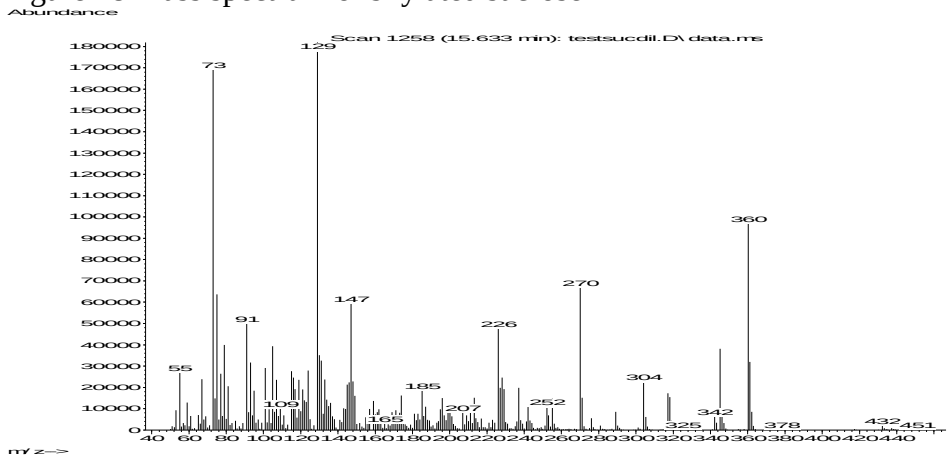


Figure 29 Mass spectrum of testosterone peak

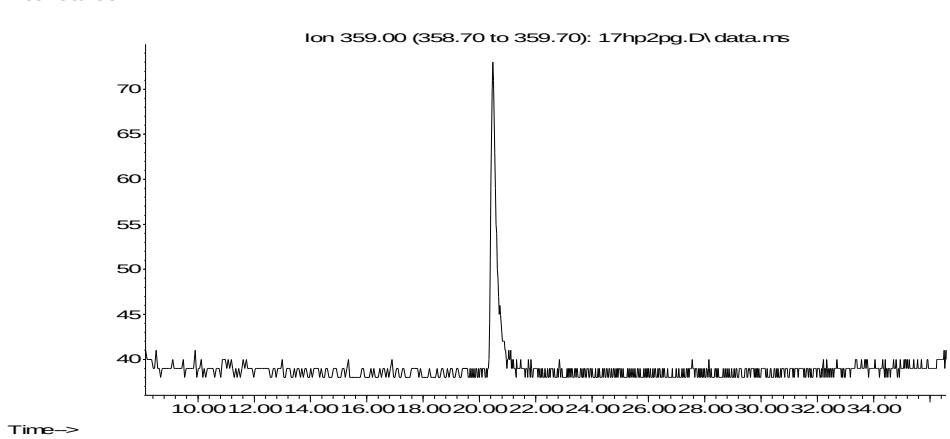


Figure 30 SIM trace of 2 pg injection of 17α-hydroxyprogesterone

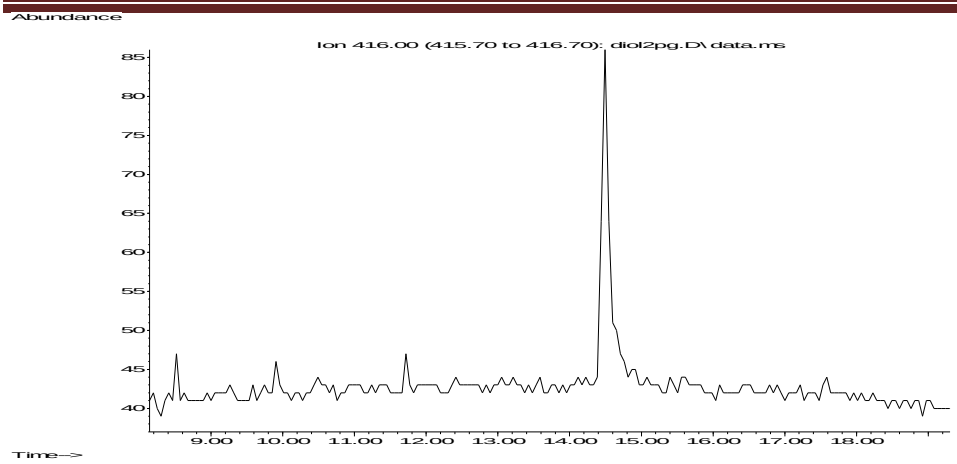


Figure 31 SIM trace of 2 pg injection of 17β- estradiol

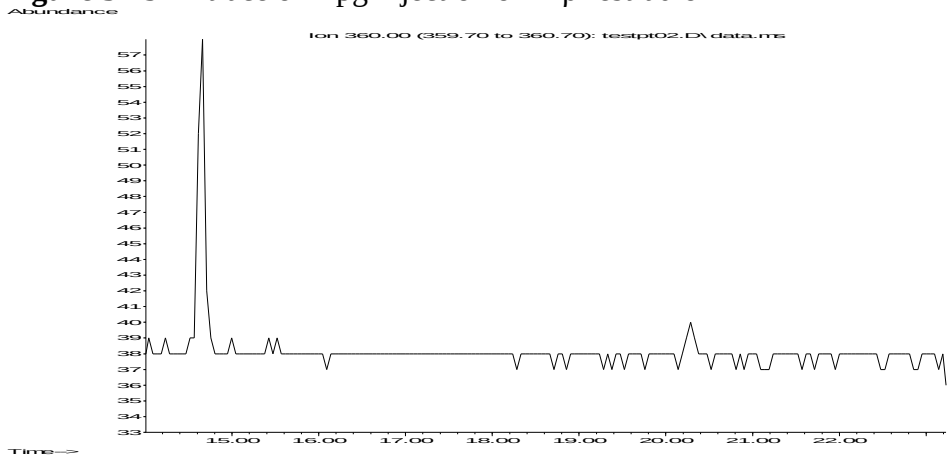


Figure 32 SIM trace of 2 pg injection of testosterone

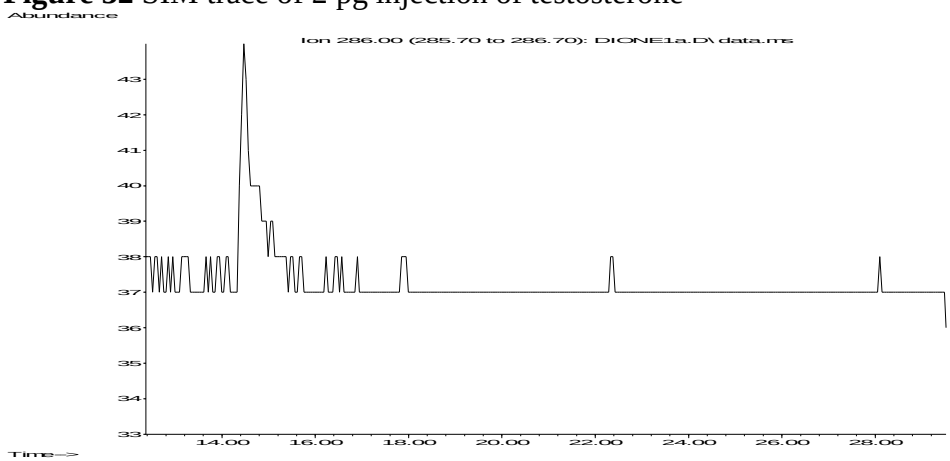


Figure 33 SIM trace of 2 pg injection of androstenedione

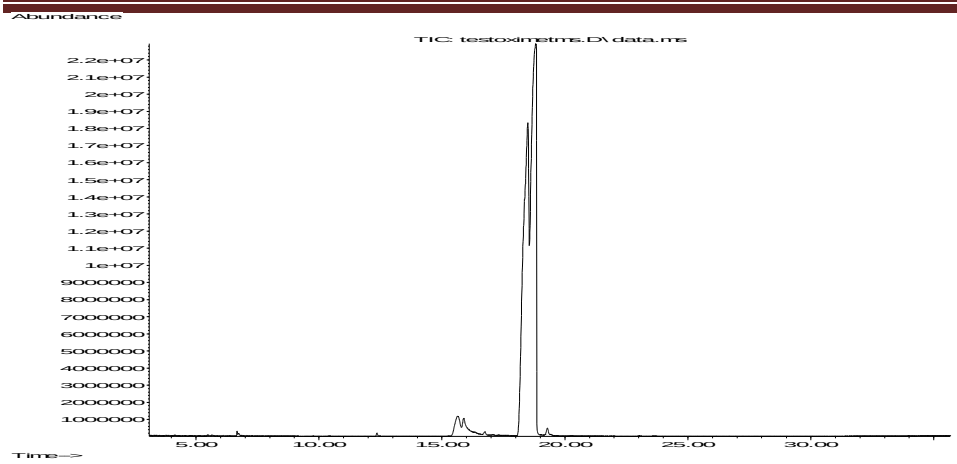


Figure 34 TIC trace of testosterone oxime di-TMS

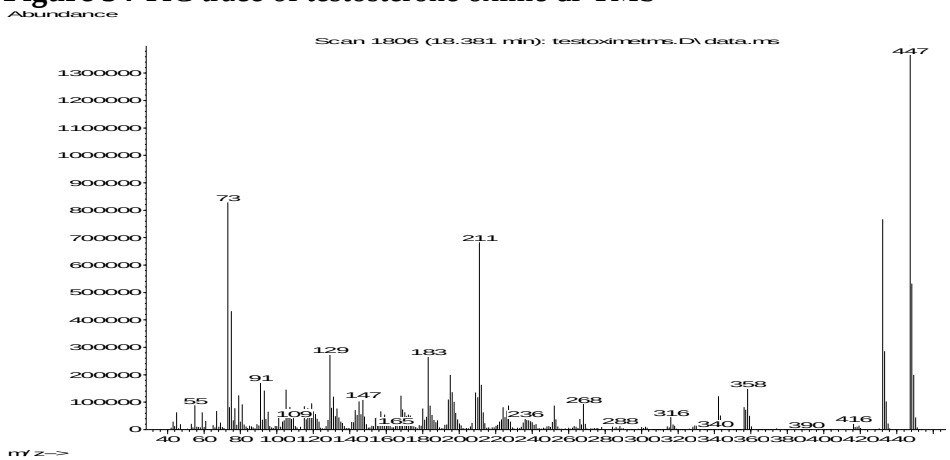


Figure 35 Mass spectrum of first peak

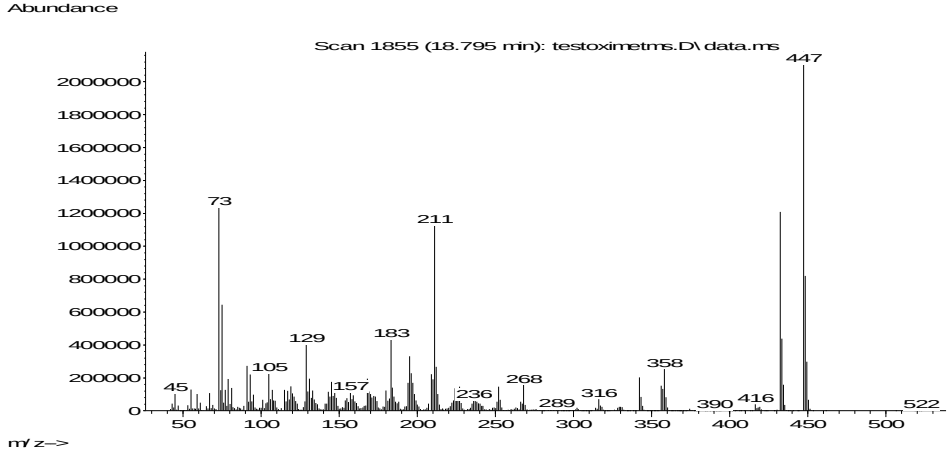


Figure 36 Mass spectrum of second peak

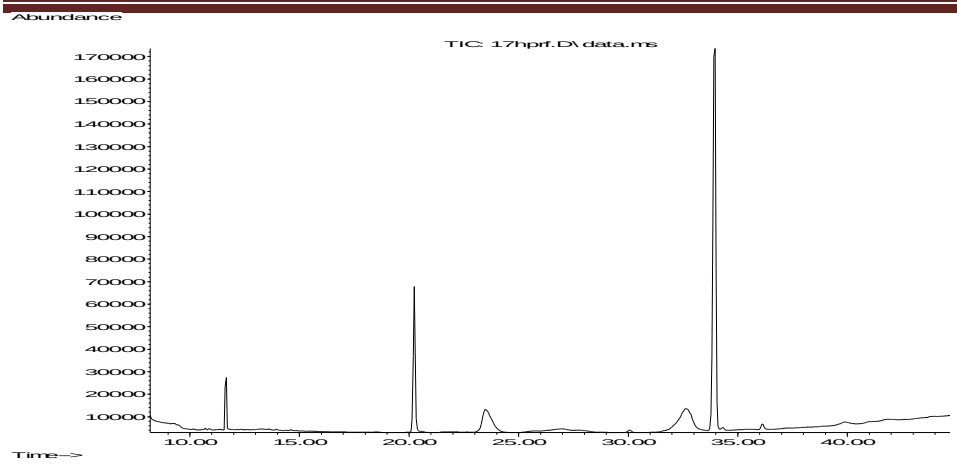


Figure 37 SIM trace of response factor solution for 17α-hydroxyprogesterone

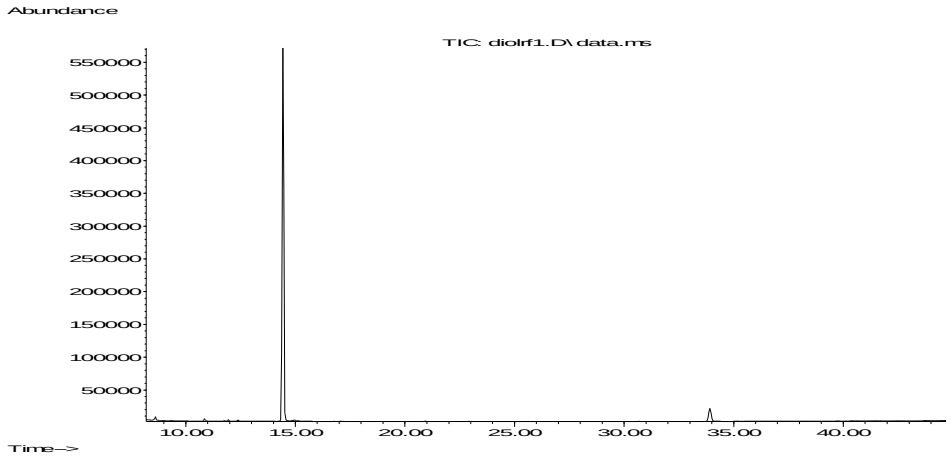


Figure 38 SIM trace of response factor solution for 17β-estradiol

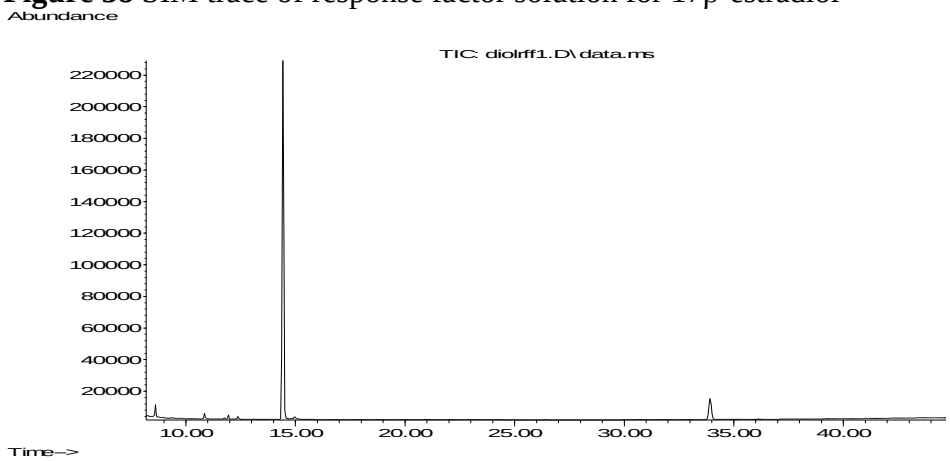


Figure 39 SIM trace of response factor solution for androstenedione

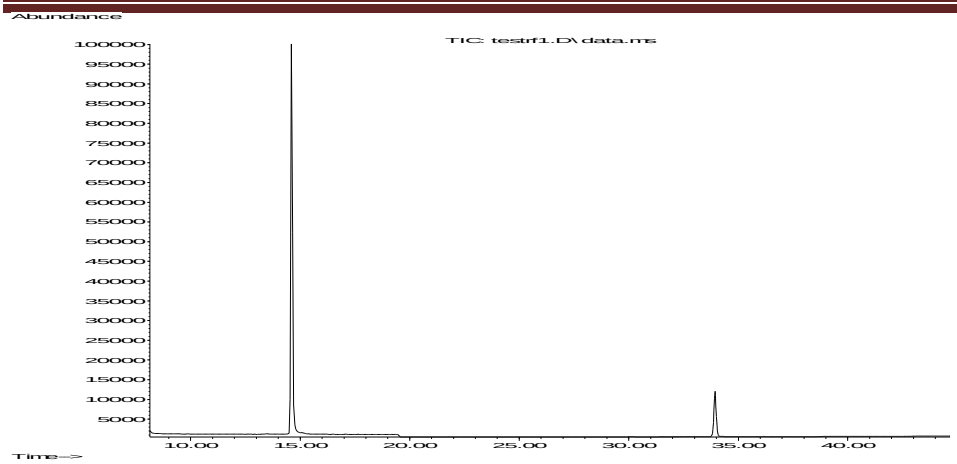


Figure 40 SIM trace of response factor solution for testosterone testosterone

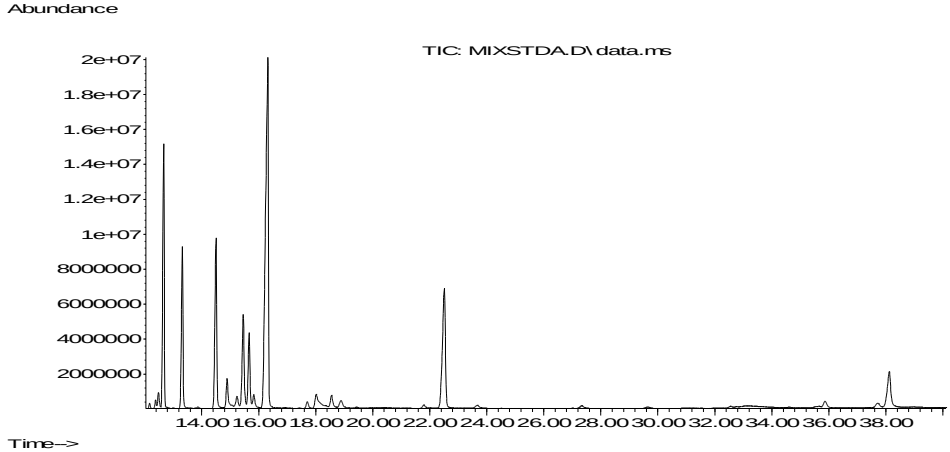


Figure 41 TIC of mixed standard solution using oven temperature method 3c

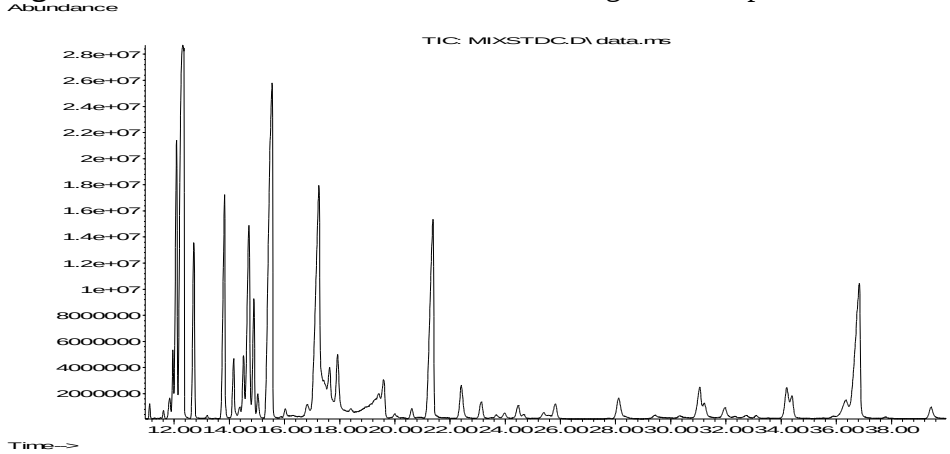


Figure 42 TIC of mixed standard solution using final oven temperature programme

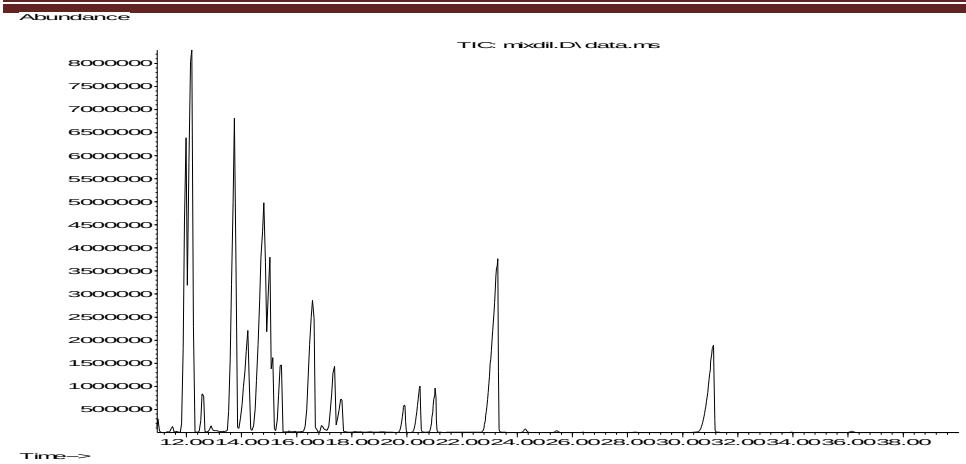


Figure 43 SIM chromatogram of mixed standard solution using final oven temperature programme

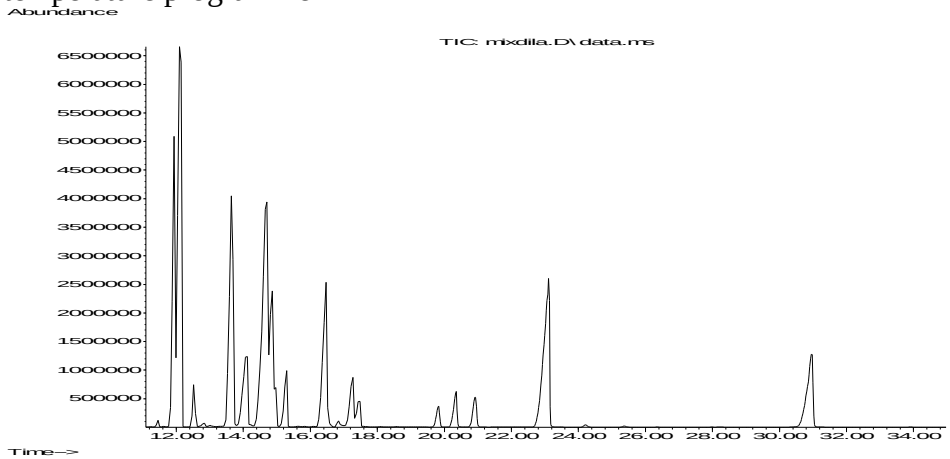


Figure 44 SIM chromatogram of mixed standard solution using final oven temperature programme

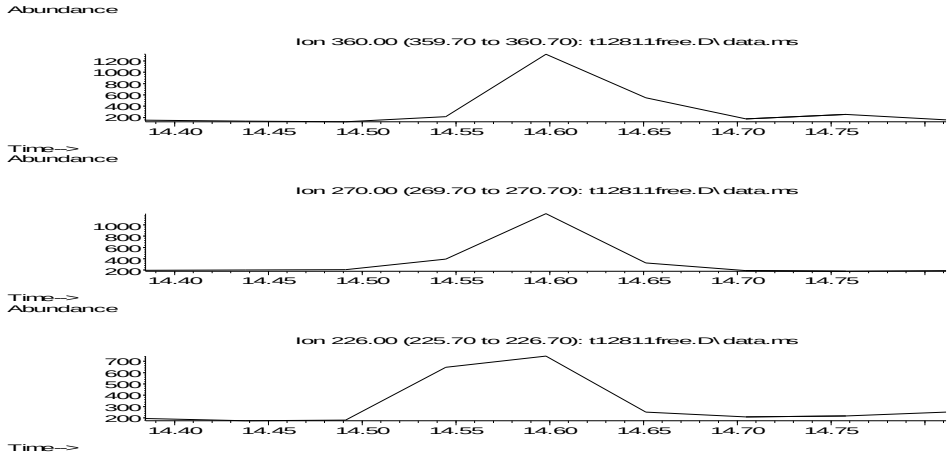


Figure 45 Extracted ion chromatogram (360, 270, 226) from treated female goldfish holding water, showing the presence of testosterone at correct retention time

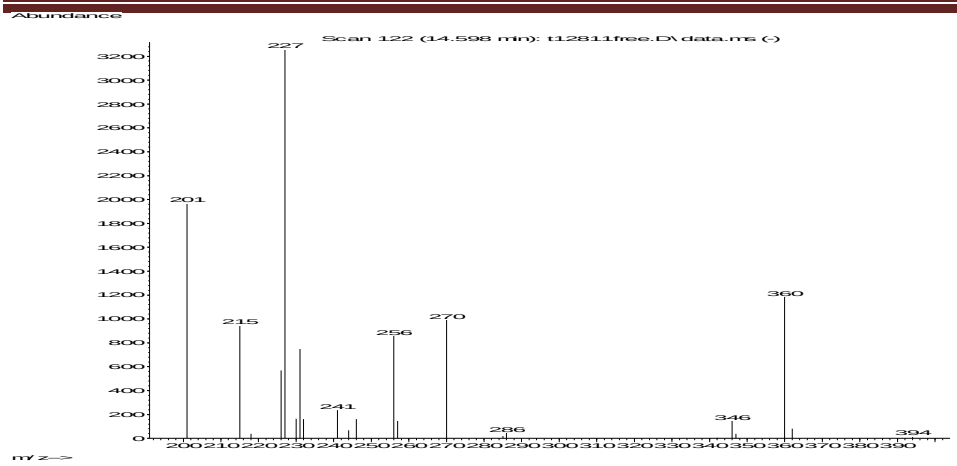


Figure 46 SIM mass spectrum of treated female goldfish holding water at 14.6 min

Appendix Two- Response Factor Data

Response Factors

Table 1 Response factor data for androstenedione

RF	Weight Dione (mg)	Weight Stig (mg)	Weight Ratio	Dione peak area 286	Stig peak area 255	Peak Area Ratio
DIONE A	3.43	7.96	0.4309045	89756	276202	0.3249651
DIONE B	4.98	6.97	0.7144907	143518	219937	0.6525414
Dione C	5.47	3.86	1.4170984	528492	259962	2.0329587
Dione D	13.08	4.58	2.8558952	2733755	460482	5.936725

Where dione= androstenedione, stig= stigmasterol

Table 2 Response factor data for testosterone

RF	Weight Test (mg)	Weight Stig (mg)	Weight Ratio	Test Peak Area 270	Stig Peak Area 255	Area Ratio
						0
TEST A	5.21	8.76	0.59475	427768	527975	0.81021
TEST B	5.62	5.24	1.07252	439359	271508	1.61822
TEST C	8.32	4	2.08	656856	152896	4.2961
TEST D	13.67	3.61	3.7867	2412662	365407	6.60267
TEST E	11.02	20.096	0.54837	1000658	1257176	0.79596
TEST F	7.97	6.43	1.2395	595270	257490	2.31182

Where test= testosterone, stig= stigmasteron

Table 3 Response factor data for 17β-estradiol

RF	Weight Diol (mg)	Weight Stig (mg)	Weight Ratio	Diol Peak Area 416	Stig Peak Area 255	Peak Area Ratio
DIOL A	4.94	9.59	0.515119	2557644	453989	5.63371
DIOL C	5.38	5.88	0.914965	3226426	323277	9.98037
DIOL Bb	7.25	5.72	1.267482	4170767	325037	12.8316
Diol D	7.98	3.15	2.533333	3390843	144140	23.5246
DIOL RF	11	4.16	2.644230	6139553	191386	32.0794
DIOL B	11.35	4.24	2.676886	7156108	204103	35.0612
DIOL E	7.16	6.95	1.030215	4197637	396027	10.5993
DIOL F	4.61	2.76	1.670289	2307080	129602	17.8012

Where diol= 17β-estradiol, stig= stigmasterol

Table 4 Response factor data for 17α-hydroxyprogesterone

RF	Weight 17-OHP (mg)	Weight Stig (mg)	Weight Ratio	17-OHP Peak Area 359	Stig peak area 255	Area Ratio
17-OHP A	6.12	8.5	0.72	57807	450699	0.12826
17-OHPRF	14.57	16.13	0.903285	895570	1101968	0.81270
17-OHPBb	7.5	4.83	1.552795	298357	256282	1.16417
17-OHPE	18.69	15.27	1.223968	916867	1084216	0.8456
17-OHPf	14	7.83	1.787994	881774	469333	1.87878
17-OHPg	20.37	6.23	3.269662	1479413	343734	4.30394
17-OHPh	9.15	7.67	1.192959	629688	426777	1.4754
17-OHPj	6.91	4.31	1.603248	488762	242079	2.01901

Where 17-OHP= 17α-hydroxyprogesterone, stig= stigmasterol

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