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Development of a protocol to profile the gonadal steroid intermediates of vitellogenic ovaries from fathead minnows, pimephales promelas

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DEVELOPMENT OF A PROTOCOL TO PROFILE THE GONADAL STEROID INTERMEDIATES OF VITELLOGENIC OVARIES FROM FATHEAD MINNOWS, *Pimephales promelas*.

A Thesis

Presented to

The Department of Biology

of

Lakehead University

by

Christina M. Schumann ©

In partial fulfilment of the requirements

for the degree of

Masters of Science

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Abstract

This research developed a protocol to extract, isolate, detect, and measure the intermediates involved in ovarian steroid biosynthesis by *Pimephales promelas* to be used in bleached kraft mill effluent chronic toxicity studies. Steroid extraction from ovarian tissue was accomplished by sonication in methanol with 1:1 tissue:solvent. Four such extractions per sample successfully increased percent recovery from <58% to >74%, depending on the steroid. Isolation of steroids from the sample matrix was accomplished by C18 solid phase extraction columns. Adjustment of sample matrix pH to 6.5, and polarity of eluant to 7:3 hexane/t-butyl ether successfully removed free fatty acid interferences from the chromatographic analysis. The isolated steroids were derivatized to produce methoxime-trimethylsilyl derivatives that were detected and measured using selected ion recording - gas chromatography - mass spectroscopy. The finalized protocol had a percent recovery ranging from 74% to 101%, and a reproducibility of <11%, depending on the steroid. Detection limit standard curves were linear ($0.9740<r<0.9994$, depending on steroid). Dehydroepiandrosterone, 17β-estradiol, and pregnenolone had the lowest detection limits at 1 ppb.

The detection limits for the other steroids, androstenedione, 17-hydroxypregnenolone, progesterone, 17-hydroxyprogesterone, and testosterone were considerably higher (129ppb, 31ppb, 103ppb, 53ppb, and 24ppb, respectively). The developed protocol successfully measured dehydroepiandrosterone, 17β-estradiol, and pregnenolone in fathead minnow ovarian tissue. All other steroids were below the detection limit. Further sample concentration is required to detect the others. The presence of β-sitosterol in fathead minnow ovarian and intestinal tissue, and food was also confirmed in this thesis. With some method development, this protocol could be used to measure β-sitosterol levels in tissue.

Acknowledgements

I'd like to take this opportunity to express my thoughts on the people who have influenced me the most in the past three years.

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CHAPTER 1

GENERAL INTRODUCTION

Industrial effluent discharged into the Great Lakes has concerned both the public and government in recent years. Environment Canada and the Department of Fisheries and Oceans established regulations under the Fisheries Act to evaluate and establish the impact of industrial waste on the receiving waters. The pulp and paper industry is affected by this legislation. The legislation is of great importance to Lake Superior because of the high number of pulp and paper mills located on its north shore. The amended Pulp and Paper Effluent Regulations under the Fisheries Act now includes the Environmental Effects Monitoring Studies established to protect fish, fish habitat and the use of fisheries resources (Environmental Effects Monitoring Bulletin, 1992). The welfare of fish in Canadian surface waters is relevant to the public, to environmental control, and to sports and commercial fisheries (Hodson, *et al.*, 1991).

The establishment of biomarkers is necessary to evaluate the effects of industrial waste on fish living in polluted waters. A biomarker measures physiological or biochemical parameters that predict a biological effect in an organism (Martel and O'Connor, 1992). The two most widely accepted and established biomarkers for exposure of fish to pulp mill effluent are measurements of mixed function oxygenase activity, and of circulating levels of gonadal steroids.

Mixed function oxygenases (MFOs) belong to a large family of enzymes located in various tissues of both plants and animals. This makes MFOs a sensitive indicator of exposure to contaminants. MFOs play a critical role in controlling the levels of physiologically important compounds such as the reproductive steroid hormones (Payne, *et al.*, 1987). MFOs located in the liver also play an important role in the elimination of exogenous compounds such as drugs and pesticides. Since many lipophilic compounds entering the body have a low solubility in urine or bile they are poorly excreted. MFOs oxidize these molecules into more hydrophilic compounds which are much easier to excrete (Martel and O'Connor, 1992). Exposure to certain toxic contaminants causes the induction, or increased production, of these enzymes. The risk associated with these enzymes is they can produce toxic intermediates or varied levels of gonadal steroids which could result in a biological effect within the organism. Measurement of such MFO activity is therfore considered an important biomarker.

Circulating gonadal sex steroids such as estradiol (E) and testosterone (T) form a second popular biomarker. The circulating levels of these steroid hormones play an important role in reproduction of an organism. Changes in their levels caused by contaminant exposure have been associated with: alteration in gonadal size, fecundity, age to maturity and secondary sexual characteristics (McMaster *et al.*, 1993). Likely these alterations impact fish at the population level. This is because even small changes in reproductive success can lead to population declines (Cushing, 1979).

The initial concern for physiological responses of fish to bleached kraft mill effluent (BKME) exposure arose as a result of extensive Swedish studies in the Baltic Sea. They found that perch, *Perca fluviatilis*, living in polluted waters near the Norrsundet pulp mill suffered from: decreased gonad growth, liver enlargement, strong (5-20 times) induction of ethoxyresorufin-o-deethylase (EROD) cytochrome P-450, metabolic imbalance, and stimulated red blood cell production (Andersson *et al.*, 1988; Larsson *et al.*, 1988; Sodergren *et al.*, 1989).

Subsequently, a series of field studies were conducted on four fish species in Jackfish Bay, Lake Superior (Munkittrick *et al.*, 1991; Munkittrick *et al.*, 1992a; Munkittrick *et al.*, 1992b) . Jackfish Bay, an isolated bay, has a nearby pulp and paper mill as its only source of anthropogenic pollution (Munkittrick, *et al.*, 1991). Fish living in these polluted waters exhibited many reproductive dysfunctions (Munkittrick *et al.*, 1991; Munkittrick *et al.*, 1992a; Munkittrick *et al.*, 1992b). Both male and female white sucker, *Catostomus commersoni*, and lake whitefish, *Coregonus clupeaformis*, exposed to secondary BKME exhibited: decreased gonadal development, delayed age to maturity, decreased serum testosterone levels and increased mixed function oxidase activity. Females of both species had: decreased serum estradiol levels, lower fecundity with age and reduced egg size. These symptoms varied in magnitude depending on the age, sex, time of day, spawning status, size, and genetic strain of the fish.

Presently we do not know how steroidal control in fish is altered by exposure to pulp mill effluent (McMaster *et al.*, 1993). Reproduction in fish is complex involving the hypothalamus of the brain, the pituitary gland, gonads, liver and various other tissues. Various pituitary hormones and the gonadal steroid hormones interacting with these tissues regulate the growth and the maintenance of reproductive organs, production of gametes, and the control of sexual behaviour and secondary sexual characteristics (McMaster *et al.*, 1993). BKME could elicit the reproductive dysfunctions observed in field studies by interfering at multiple sites in the complex regulatory system by either binding with estrogen receptors, inducing or repressing the cytochrome P-450 system of the liver and/or gonads, or altering the availability of substrates such as cholesterol. Given the known relationship between MFO activity and effluent exposure, and the importance of MFO enzymes in steroid biosynthesis, such potential mechanisms are probably involved in physiological changes. I hypothesize that the observed peturbation of sexual development occurs in part due to repression or induction of various P450 enzymes.

One site acted upon by BKME involves reproductive alterations through steroid hormone biosynthesis in the gonads. Ovarian follicles of wild fish exposed to industrial effluents have a reduced capacity of producing the major functioning steroids (McMaster, *et al.*, 1993). Exposure to effluent decreased the activity of the various enzymes involved in the steroid biosynthetic pathway. Multiple sites in the biosynthetic pathway were affected by

effluent exposure but the sites seemed to differ with the reproductive state of the fish (McMaster, *et al.*, 1993).

One method to evaluate the impact that BKME has on steroid biosynthesis involves the measurement of the concentration of steroid intermediates relative to one another. This is referred to as steroid profiling. The term steroid profiling in the past has generally referred to profiling steroid metabolites in the urine of the organism. It serves as an indicator of certain endocrine dysfunctions such as hirsutism, Stein-Leventhal syndrome, and certain abnormalities in pregnancy (Leunissen, 1979, PhD thesis). It can profile steroids in human primary breast tumors (Millington, 1975b) and benign hyperplastic prostate tissue (Millington *et al.*, 1975a). More recently it has profiled steroids in plasma (Schoonen *et al.*, 1989a) and ovarian fluid (Schoonen *et al.*, 1989b & van Dam *et al.*, 1989) of African catfish, *Clarias gariepinus*. In this thesis, the term steroid profiling refers to the measurement of the various intermediates involved in the gonadal steroid biosynthetic pathway of extracted ovaries.

There are two commonly used methods for measuring the amount of steroids present in a biological sample; radioimmunoassay (RIA) and capillary gas chromatography. RIA is the method of choice for measuring individual steroids at very low concentrations (generally pg steroid/ml blood plasma) such as those found circulating in fish plasma. The procedure is simple involving little sample clean up and is relatively inexpensive. Although this

procedure is highly sensitive, it has many disadvantages for profiling multiple steroid intermediates:

- Lack of commercially available antisera and ^{3}H-labelled steroid intermediates
- The high cost of purchasing ^{3}H-labelled steroid intermediates for an entire intermediate profile
- Possible antiserum cross-reactivity between structurally similar intermediates providing misleading results
- Need for dealing with radioactive material

For these above reasons a more comprehensive, safe, and easily available method is needed for determining steroid profiles.

Capillary gas chromatography provides an alternate method of quantifying steroid intermediates in a biological sample. For example, it has been used to measure steroids in South African catfish ovarian fluid (Schoonen *et al.*, 1989a & 1989b) and human urine (Pfaffenberger and Horning, 1977). It is considered very convenient because all the intermediates can be measured in one sample analysis. It saves time, effort and money, however there are some difficulties associated with the application of capillary gas chromatography. They are:

- Detection of very low concentrations of steroid intermediates present in fish tissue is very difficult

- Extensive sample clean up procedures must be established in order to minimalize interference in the profile

These problems are rather difficult to overcome and have limited the use of capillary gas chromatography even though it is the preferable method for determining steroid profiles.

The objective of this thesis was to develop a protocol profiling the intermediates involved in gonadal steroid biosynthesis using capillary gas chromatography and mass spectroscopy - selected ion recording. My hope was to establish a protocol potentially acceptible as a biomarker for environmental pollution studies. In the future, this protocol could be used to determine and compare profiles of healthy and effluent exposed vitellogenic fathead minnows in order to evaluate the impact of exposure to pulp and paper mill effluent on steroid biosynthesis.

CHAPTER 2

THE GONADAL STEROID HORMONES OF FISH

2.1 GENERAL STRUCTURE

Steroids are a group of naturally occurring compounds. They include steroid hormones, sterols, bile acids, cardiac glycosides, steroid alkaloids and the vitamin D series.

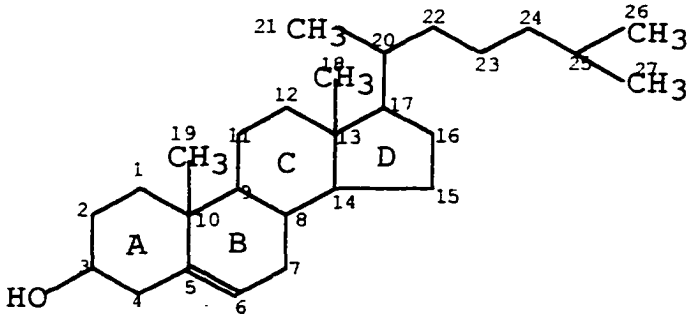


Figure 2.1: The precursor to all steroid hormones, cholesterol, and the IUPAC numbering scheme of the 27 carbon atoms and 4 cyclic rings.

The steroids considered in this thesis contain up to 27 carbon atoms, numbered as shown in Fig. 2.1 oriented in 3 cyclohexane and 1 pentahexane rings (Stryer, 1988, p565). Hydrogen atoms are attached to the carbons but are not written in the structure. Various functional groups can be at one or more sites. The gonadal steroids generally have a combination of either hydroxyl and/or ketone groups at the 3, 17 or 20 positions. IUPAC has definitive rules for the nomenclature of steroids but many are still referred to by their common names. A summary of the common and systematic names and abbreviations of the steroids discussed in this thesis are in Appendix I.

2.2 CLASSIFICATION AND BIOLOGICAL ACTION

Hormones are defined as organic compounds produced by endocrine glands (Berne and Levy, 1988, p819). The most important endocrine glands are the hypothalamus, pituitary (Berne and Levy, 1988, p895), thyroid (Berne and Levy, 1988, p932), adrenal gland (Berne and Levy, 1988, p950) or interrenal gland in fish, and gonads (Berne and Levy, 1988, p983).

There are three basic types of structure of hormones: a) hormones derived from amino acids, b) peptides or proteins and, c) steroid hormones (Berne and Levy, 1988, p819). This thesis will only deal with steroid hormones.

Steroid hormones are mainly secreted by the interrenal gland and gonads of fish. This thesis will deal with the gonadal steroid hormones which can be divided into three classes based on their molecular structure and physiological function. These three classes include androgens, estrogens, and progestins.

2.2.1 Androgens

Androgens consist of the four cyclic ring structure with methyl groups attached to the C-10 and C-13 sites. Androgens such as testosterone and 11-ketotestosterone play an important role in the differentiation of male secondary sex characteristics (Stryer, 1988, p564). Testosterone, androstenedione, and dehydroepiandrosterone (Fig. 2.2.1) are also

important in the female biosynthetic pathway because they are considered to be estrogen precursors in many fish (Nagahama, 1983). During vitellogenesis in rainbow trout, testosterone levels tend to be very low, which is likely related to its role as a precursor of estradiol (Zohar *et al.*, 1982). Plasma tesotsterone levels tend to be highest during the last part of vitellogenesis (Fig. 2.2) in some teleost species (Hoar *et al.*, 1983).

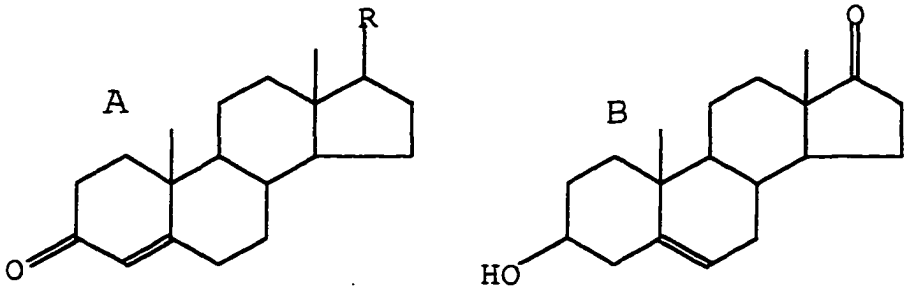


Figure 2.2.1: The androgens androstenedione, testosterone and dehydroepiandrosterone. Structure A is the basic structure for androstenedione (R = "=O") and testosterone (R = "-OH"). Structure B is dehydroepiandrosterone.

2.2.2 Estrogens

All natural estrogens lack a methyl group at the C-10 position and possess an aromatic A-ring (Pang and Screibman, 1991, p69). 17β-Estradiol (Fig. 2.2.2) and estrone have been found in most teleost species (Fostier *et al.*,, 1983). They are produced and excrected by the ovaries of most teleost fish (Fostier *et al.*, 1983). Estradiol is believed to be responsible for inducing vitellogenesis in *Onchorhynchus mykiss* (rainbow trout) (Lambert and Van Bohemen, 1979). In many teleost species the plasma levels of estradiol rise considerably during vitellogenesis (*Salmo trutta)* brown trout: Billard *et al.*, 1978; Breton *et al.*, 1983; rainbow trout: Fostier *et al.*, 1978; Scott and Sumpter, 1983;

Oncorhynchus kisutch coho salmon: Fitzpatrick *et al.*, 1986) but tend to decline towards oocyte maturation (Fig. 2.2).

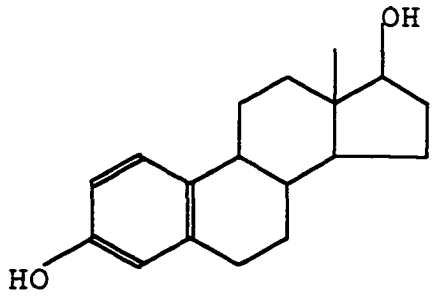


Figure 2.2.2: The estrogen 17β-estradiol.

2.2.3 Progestins

Progestins possess methyl groups at the C-10 and C-13 sites like androgens but they also have a characteristic short side chain at the C-17 position (Pang and Schreibman, 1991, p69). Pregnenolone, and progesterone and their hydroxylated analogs are all progestins (Fig. 2.2.3). Pregnenolone, 17-hydroxypregnenolone, progesterone and 17-hydroxyprogesterone are intermediates in the biosynthetic pathway synthesized *in vitro* in many isolated teleost fish ovaries (Fostier *et al.*, 1983). The progestin 17α-20β-dihydroxyprogesterone is considered a potent oocyte maturation hormone in many salmonid species (Nagahama, 1983; Fostier *et al.*, 1983). Progesterone has been demonstrated to induce oocyte maturation in many teleost fish but its stimulatory effect might be a result of conversion to the more active metabolite (Goetz, 1983). Progestins often reach the maximal concentrations during spawning (Hoar *et al.*, 1983). The level of 17α-20β-dihydroxyprogesterone in ovaries during vitellogenesis and postvitellogensis is

undetectible. Its production increases drastically during oocyte maturation (Schoonen *et al.*, 1989a) (Fig. 2.2).

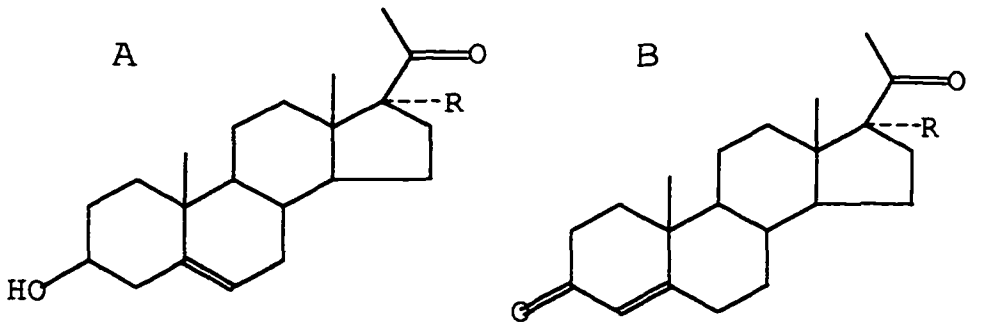


Figure 2.2.3: Examples of the class progestins. Compound A is the basic structure of pregnenolone (R = "-H") and 17-hydroxypregnenolone (R = "-OH"). Compound B is the basic structure of progesterone (R = "-H"), and 17-hydroxyprogesterone (R = "-OH").

2.3 STEROID BIOSYNTHESIS

The precursor to all steroids is cholesterol. It is produced in the liver and transported through the blood by phospholipids classified according to their density (Stryer, 1988, p560). All 27 carbon atoms of the cholesterol molecule are derived from acetyl CoA (Stryer, 1988, p554). Acetyl CoA (C_2) is converted to mevalonate (C_6) by the enzyme 3-hydroxy-3-methylglutaryl CoA reductase (or HMG CoA reducase). High dietary intake of cholesterol inhibits chlolesterol biosynthesis by inhibiting this enzyme. Mevalonate is converted to isopyrophosphate (C_5) and then to squalene (C_{30}) by a series of condensation reactions. Squalene undergoes a cyclization to lanosterol (C_{30}) which undergoes a demethylation resulting in the final product, cholesterol (Stryer, 1988, p558).

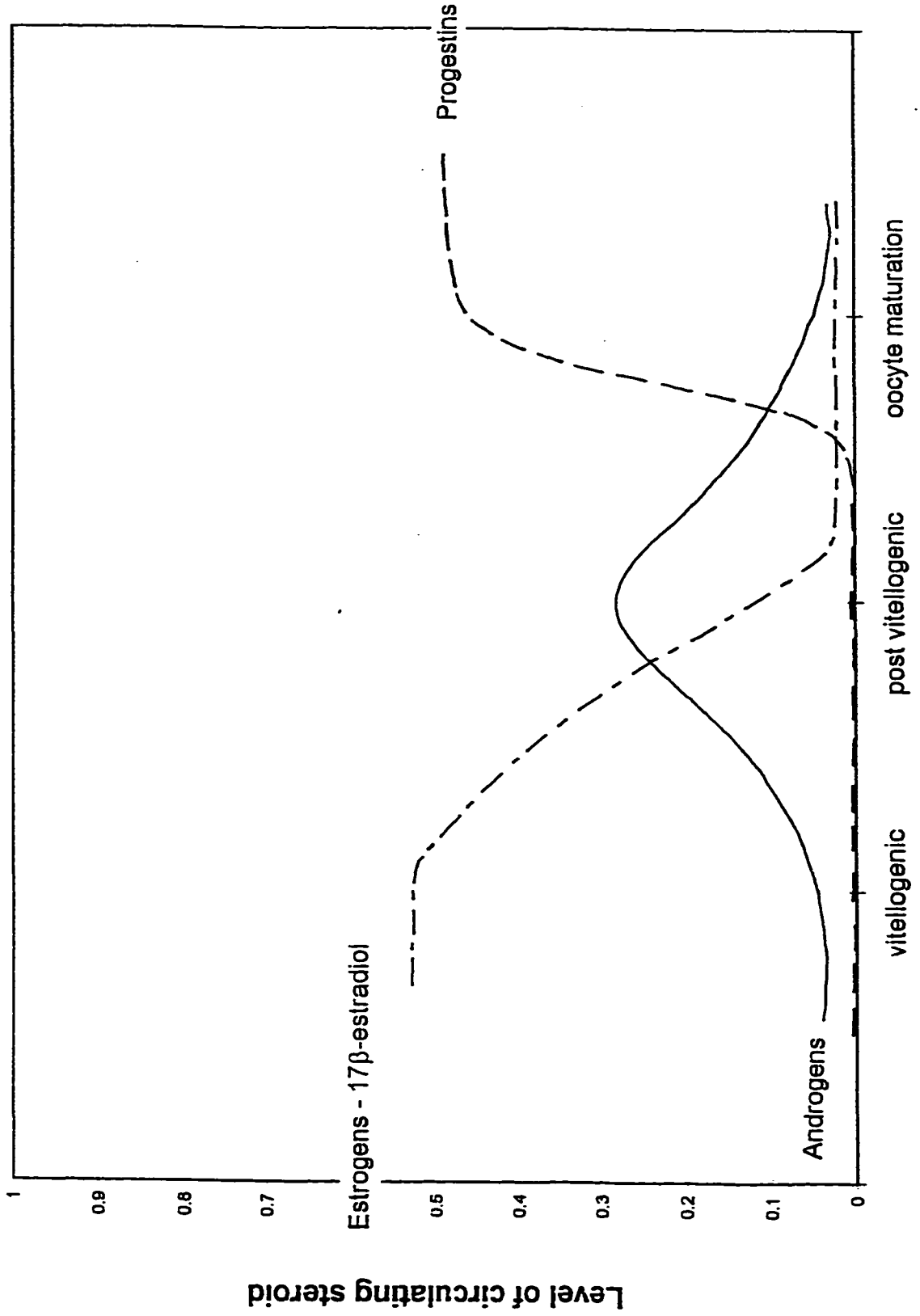


Figure 2.2: A general representation of the circulating levels of androgens, estrogens and progestins in fish based on available literature. It should be noted that this graph represents the relative level of these classes only and that species to species variations in this pattern are likely.

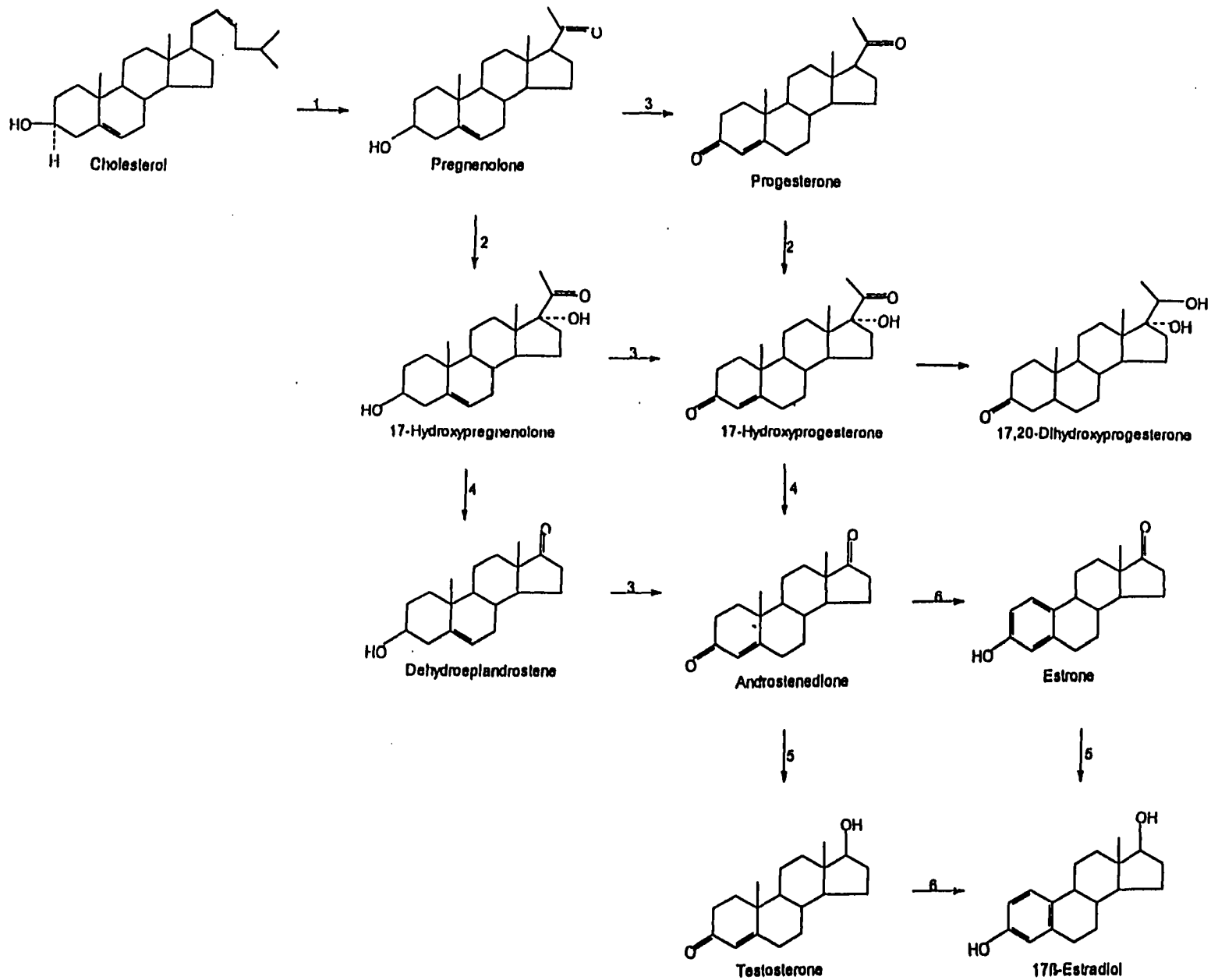


Figure 2.3.1: The gonadal steroid biosynthetic pathway in fish, including structures of steroid intermediates. The enzymes are numbered as follows: 1. cytochrome P-450$_{scc}$; 2. cytochrome P-450 17-hydrogenase; 3β-hydroxysteroid dehydrogenase; 4. C_{17}-C_{20} lyase; 5. 17-ketosteroid reductase/17-hydroxysteroid dehydrogenase; 6. aromatase. (After Pang and Schreibman, 1991, p70)

Once transported to the ovaries, the site of gonadal steroid production, cholesterol is converted into pregnenolone by the cytochrome P-450scc (side chain cleavage) enzyme which oxidizes the C-17 side chain at the C-20 position. From this point on, the pathway becomes more complex involving five main enzymes; 17-hydroxylase, 3β-hydroxysteroid dehydrogenase, C_{17}-C_{20} lyase, 17-ketosteroid reductase/17-hydroxysteroid dehydrogenase and aromatase. Some of these enzymes are considered part of the cytochrome P-450 family of enzymes. They are membrane bound enzymes which catalyze many oxidation and some reduction reactions. A summary of the biosynthetic pathway is in Fig. 2.3.1.

17-hydroxylase is a cytochrome P-450 enzyme. It adds a hydroxyl group to the C-17 position. It is responsible for converting pregnenolone into 17-hydroxypregnenolone and progesterone into 17-hydroxyprogesterone in the biosynthetic pathway.

3-β-hydroxysteroid dehydrogenase is a membrane bound enzyme which converts the hydroxyl group at the C-3 position into a ketone group, a dehydrogenation reaction. It is responsible for converting pregnenolone into progesterone and 17-hydroxypregnenolone into 17-hydroxyprogesterone. The other dehydrogenase reaction in the steroid biosynthetic pathway is catalyzed by 17-ketosteroid reductase/17-hydroxysteroid dehydrogenase. This enzyme is responsible for converting androstenedione into testosterone and estrone into 17β-estradiol.

C_{17}-C_{20} lyase is the enzyme responsible for removing the C-17 short side chain from the cyclopentane ring. It is responsible for converting 17-hydroxypregnenolone into dehydroepiandrosterone and 17-hydroxyprogesterone into androstenedione.

The final enzyme involved in steroid biosynthesis is aromatase. It is responsible for converting androstenedione into estrone and testosterone into estradiol by converting the A-cyclohexane ring to an aromatic ring.

The activities of the enzymes vary depending on the sexual stage of the fish and thereby affect the relative concentrations of the intermediates involved. Schoonen *et al.*, 1989, found that the activity of 3β-hydroxysteroid dehydrogenase and 17-hydroxylase decreased during ovulation in African catfish. They also found that in these same fish, the steroid biosynthetic pathway shifted towards the production of 5β-reduced pregnanes during oocyte maturation. For this reason it was very important to ensure that all fish used in an experiment were all at the same sexual stage of vitellogenesis.

Vitellogenesis is defined as the stage of production of vitellogin by the liver for the production of oocytes, oocyte growth, follicular layer development and yolk accumulation (Nagahama, 1983). During this stage, the ovaries are large with eggs and evidence of vitellogin production is observed. During this stage, increased estrogen levels act to induce the hepatic synthesis of vitellogenin, a phosphoprotein precursor of oocyte vitellus

(Hoar *et al.*, 1983). Just prior to ovulation in most telost fish, plasma estradiol levels drop.

Also observed in vitellogenic rainbow trout ovaries is a shift from Δ-4 (17-hydroxypregnenolone and dehydroepiandrosterone) to Δ-5 (progesterone and 17-hydroxyprogestrone) pathways (Lambert and van Bohemen, 1979). At the end of vitellogenesis, testosterone and dehydroepiandrosterone levels are the main end products in catfish ovary homogenates (Lambert and Van den Hurk, 1982) due to the relatively high activity of the enzymes C_{17}-C_{20} lyase and 17β-HSD.

2.4 DISRUPTIONS IN STEROID BIOSYNTHESIS

Fish exposed to bleached kraft mill effluent exhibit many reproductive dysfunctions including: decreased gonadal size, reduced fecundity with age, and delayed maturity. They also exhibit reduced levels of circulating estradiol and testosterone levels. Decreased steroid production in the ovarian follicles may account for the low levels of circulating steroids and may, at least in part, be responsible for the impaired reproductive performance observed in fish.

Steroid production and release from the ovaries is directly related to the rate of biosynthesis since the ovaries are incapable of storing steroids. Any interruptions in the biosynthetic pathway have the potential to effect steroid release and thereby alter circulating steroid levels. Ovaries of white suckers exposed to bleached kraft mill effluent

exhibited reduced steroid production which was associated with specific biochemical lesions within the steroid biosynthetic pathway (McMaster *et al.*, 1993). In preovulatory ovarian follicles, basal pregnenolone and 17-hydroxypregnenolone production was significantly lower in exposed fish. This resulted from reduced endogenous cholesterol stores rather than a decreased activity of P-450scc because this enzyme was capable of catalyzing exogenous cholesterol. Other lesions in preovulatory ovarian follicles were also found towards the end of the biosynthetic pathway. The production of both androstenedione and testosterone were significantly lower in exposed follicles but estradiol production was higher. This indicates the potential induction of the aromatase enzyme in the biosynthetic pathway. Another possibility is the repression of 3β-hydroxysteroid dehydrogenase and C_{17}-C_{20} lyase. However this is not as likely because it would result in decreased levels of estradiol production rather than the observed increase.

In ovarian follicles of vitellogenic whitefish, disruptions occurred near the top and throughout the pathway. Exposed follicles produced significantly lower levels of pregnenolone, androstenedione, testosterone and estradiol. Also there was a decrease in endogenous stores of cholesterol similar to that observed in preovulatory whitefish. However once excess cholesterol was provided, a build up of pregnenolone occurred indicating a reduced ability of BKME-exposed ovarian follicles to convert pregnenolone to further intermediates.

A laboratory study using fathead minnows was conducted in an attempt to determine whether laboratory toxicity tests are representive of the effects of pulp and paper mill effluent on receiving water wild-type fish (Robinson, 1994). Life cycle tests confirmed a correlation between decreased *in vitro* testosterone and 11-ketotestosterone production and reproductive performance (later spawning, fewer eggs produced, and fewer spawning events) in both males and females but only male fatheads demonstrated a dose-response relationship.

Robinson (1994) also found decreased *in vitro* basal and stimulation of testicular steroid production after 7 and 30 days, respectively, of BKME exposure. Sexually mature fish held under conditions conductive to spawning did not exhibit the detrimental effects on reproductive performance and *in vitro* steroid production in BKME exposed male fatheads.

Reseach into the effects of exposure to pulp and paper mill effluent on steroid biosynthesis in fish is still limited. The above studies indicate BKME exposure causes lesions in the steroid biosynthetic pathway of wildtype lake whitefish and and white suckers and the different lesions change and become more evident at different reproductive stages in fish, and that laboratory exposed male fathead minnows exhibit decreased *in vitro* steroid production which depends strongly upon the social environment in which the fish are exposed.

CHAPTER 3

ECOLOGICAL RISKS OF PULP AND PAPER MILL EFFLUENT

3.1 INTRODUCTION

Effluents from pulp and paper plants contain a complicated mix of wood extractives, and chlorinated degradation products of lignin. Only some 300 low-molecular-weight chlorinated organic compounds have been identified as of 1990, respresenting less than 10% of the total weight of those compounds in bleach pulp effluents (Bonsor *et al.*, 1990). There are various different production techniques associated with pulp and paper production and therefore various different products and by-products produced. The impact of chemicals released from pulp and paper production on the receiving waters is dependent on several factors including the wood species used, the degree of spill control and pulping liquor recovery, the bleaching process (if any) and the degree to which mill effluent is treated before being released (Solomon, *et al.*, 1994). Two factors which influence the toxicity of BKME and thereby its impact on receiving waters are (1) the implimentation of secondary treatment of effluent and (2) the use of chlorine dioxide to reduce the production of chlorinated organics during the bleaching process (Kinstrey, 1993; Earl and Reeve, 1990).

3.2 THE PULPING PROCESS

The production of paper from wood is a multi-step process including the use of many types of chemicals. The first stage is wood pulping. Its purpose is to remove lignin from

the wood by the separation of wood cellulose fibres. The most common type of wood pulping in North America is the kraft, or sulphate process, where wood chips are heated with a mixture of sodium hydroxide and sodium sulphide (Kringstad and Lindstrom, 1984). Other methods of wood pulping include using the sulphite process, or various types of thermal or mechanical pulping. Wood pulping is effective in solubilizing and removing approximately 90% of the lignin from the wood. Other compounds (Table 3.2.1) extracted from the wood during this process include hemicellulose, fats, waxes, terpenoids, resin acids and phenolics (Robinson, 1994).

The residual lignin remaining in the pulp after the wood pulping stage is removed and depolymerized using a multi-step bleaching process. Bleaching can be accomplished through the use of various bleaching agents including chlorine, chlorine dioxide, hypochlorite, oxygen, ozone, alkali or peroxide. During this stage chlorine is introduced to the organic mixture which will produce organochlorines and various chlorinated organic acids (Robinson, 1994).

Untreated pulp mill effluents from these plants have a high biochemical oxygen demand (BOD) production of suspended solids (SS), and are typically acutely lethal to fish. Secondary treatment, or biological treatment, of effluent was introduced to reduce BOD and SS levels and subsequently reduce acute toxicity levels to below Ontario Ministry of the Environment and Energy guidelines (Wilson *et al.*, 1992).

Table 3.2.1: Representative monomeric compounds of classes of organics from the various stages of bleached kraft mill effluent (BKME) production.

Stage	Group	Examples
Wood pulping		
•Polar extractives	• mono & disaccharides	• fructose • sucrose • glucose • lignans
• **Non polar extractives**	• fatty acids	• oleic • linoleic • 5,9,12-octadecatrienoic
	• resin acids	• pimaric • isopimaric • abietic • dehydroabietic • palustric
	• nonsaponifiables	• diterpenes • triterpenes • sterols • esterfied sterols
Bleaching	• phenols	• 2,4-dichlorophenol • 2,4,6-trichlorophenol • 2,3,4,6-tetrochlorophenol
	• guaiacols	• 4,6-dichloroguaiacol • 4,5-dichloroguaiacol • 3,4,5-trichloroguaiacol • 4,5,6-trichloroguaiacol • tetrachloroguaiacol
	• catechols	• 3,5-dichlorocatechol • 4,5-dichlorocatechol • 3,4,5-trichlorocatechol • tetracatechol

3.3 ORIGIN OF ENVIRONMENTAL CONCERN

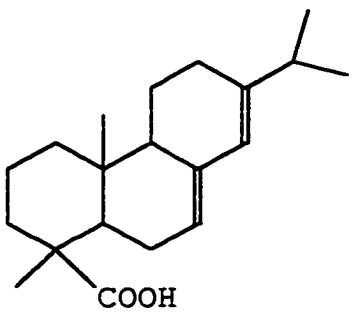
Public concern and environmental regulations have focused their attention on the presence of organochlorines in bleached kraft mill effluent (BKME). Various groups of low molecular weight organochlorines produced during the bleaching stage have raised concern. These include, chloro-phenols, -guaiacols, -catechols and -vanillins, chlorinated fatty and resin acids, and dioxins (Fig. 3.3.1). The relative toxicies of these monomeric components of BKME have been investigated.

Chlorophenols and high resin acid concentrations seem to cause most of the acute toxicity in BKME. However high toxicity levels are also found with low or zero concentrations of resin acids and chlorinated phenolics. This suggests the presence of unknown toxic compounds in the effluents (Priha and Talka, 1986). The introduction of secondary treatment to the processing system has greatly reduced the levels of acute toxicity in BKME. Major contributors to the acute toxicity of BKME, such as resin acids and other extractives present in cooking liquors, are generally amenable to detoxification by secondary treatment (Servizi *et al.*, 1986).

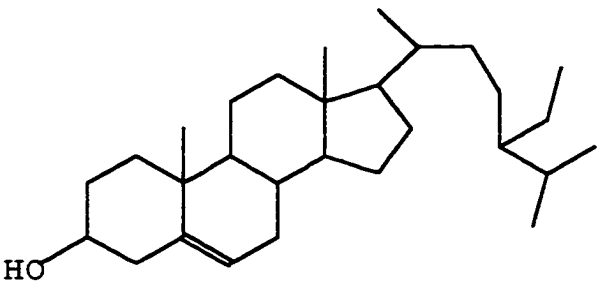
Another chlorinated organic 2,3,7,8-tetrachlorodibenzodioxin, a potent rat carcinogen, has been found in fish downstream of paper mills using elemental chlorine bleaching and subsequently in the mill effluent (Kinstrey, 1993). The use of chlorine dioxide reduces

levels of dioxin in BKME to close or below analytical detection limits of 1-10 pg/l. Mills on the north shore of Lake Superior now operate with dioxin levels at this range.

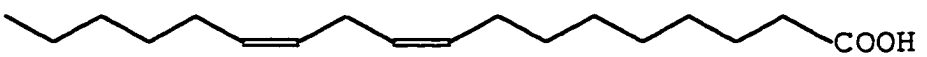
The organochlorines discussed above are frequently found on priority lists as chemicals of concern. Chlorinated organics tend to be very stable with increasing chlorine substitution. The environmental persistence of organochlorines in receiving waters and the tendency to bioaccumulate in lipophilic tissue of biological systems which live in these waters is the root of the environmental concern. Implimentation of secondary treatment systems and chlorine dioxide substitution in the bleaching stage are two measures which reduce the amount of acute toxicity, bioaccumulation and persistence of chemicals in BKME.



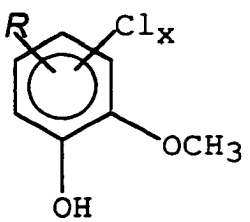
Resin acids (abietic)



Sterols (beta-sitosterol)



Fatty acids (linoleic)



Chlorinated guiacols and other lignin degradation products

Figure 3.3.1: Major classes of chlorinated and non-chlorinated monomeric organics found in bleached kraft mill effluent.

3.4 MEASUREMENT OF ORGANOCHLORINES

Pulp mill effluent is composed of numerous types of organochlorines as previously discussed. The quantity of chlorine in a sample which is retained on activated carbon is termed the adsorbable organic halogen, or AOX. It reflects the total quantity of organically bound chlorine (Solomon, *et al.*, 1994). The extractable organic halogen or

chlorine (EOX) is the non polar extraction of the total organically bound chlorine or AOX. EOX is regarded as giving a better indication of potential for bioaccumulation than AOX (Solomon, *et al.*, 1994). EOX is generally only a few percent of the AOX value indicating that most of the AOX is hydrophilic rather than lipophilic (Solomon, *et al.*, 1994). Only 0.2 % of the EOX portion of AOX has an octanol water coefficient or K_{ow} greater than 1000 resulting in potential bioaccumulation in lipophilic tissue.

3.5 ADVANTAGES OF CHLORINE DIOXIDE BLEACHING

A shift from bleaching with elemental chlorine to bleaching with chlorine dioxide has taken place in an attempt to reduce the impact on receiving waters. Use of chlorine dioxide is therefore believed to reduce the ecological risk associated with release of pulp mill effluent.

Elemental chlorine and chlorine dioxide react differently with organic material. When elemental chlorine reacts with lignin, 10% of the original Cl_2 applied remains as solubilized chlorinated organic material. The resulting chlorinated organics tend to have more chlorine substitution of 7-10 chlorine atoms per 100 carbon atoms, especially on aromatic substances, and 70-80% of the organics are high molecular mass (HMM) (>1000). The HMM tends to be part of the water soluble fraction due to the large number of carboxyl groups present.

Chlorine dioxide on the other hand has an oxidation potential of 2.5 times that of elemental chlorine. It is therefore on a weight basis more effective in oxidizing the lignin in pulp. The reaction can also be controlled by pH to reduce the total number of organochlorines produced. The resulting organochlorines tend to be less chlorinated (1 chlorine atom per 100 carbon atoms), less aromatic, and only 50% of the chlorinated organics are high molecular weight. The use of chlorine dioxide in bleaching also significantly reduces the AOX and EOX levels, while reducing the toxic dioxin and chlorophenol levels to close or below analytical detection limits.

3.6 CONCLUSIONS

The complicated nature and composition of BKME makes it difficult to assess its ecological impact on receiving water systems with less than 10% of the compounds present in BKME identified. Resin acids from the wood pulping process and chlorinated phenols from the bleaching process account for the majority of BKME acute toxicity. Secondary treatment of effluent reduces the levels of these compounds in effluent and subsequently effluent toxicity. Substitution of chlorine dioxide for elemental chlorine also reduces effluent toxicity and more importantly produces fewer organochlorines which tend to bioaccumulate in ecosystems and are environmentally persistant.

Such measures have not removed the chronic toxicity observed in fish associated with BKME-exposure. Fish suffer from reduced gonadal development, increased age to maturity, and low plasma estradiol and testosterone levels upon BKME-exposure. The chemical(s) in BKME, known as "compound(s) X", responsible for these symptoms remains unknown. Current research indicates that "compound(s) X" may be a wood extractive, not at all related to the use of chlorine for bleaching. This research is not yet published and therefore unavailable for review. However efforts to discover the identity of "compound X" are important from both an ecological and economic perspective.

CHAPTER 4

FATHEAD MINNOW AQUACULTURE

4.1 INTRODUCTION

Research into the effects of bleached kraft mill effluent exposure on steroid biosynthesis in fish have necessarily focused on wild-type fish to assess the true impact of BKME on fish populations. As with any field work, many variables are involved which may effect the stress level of the fish such as parasites, water temperature, food availability and others. Such factors effect the reproductive state of fish (Hoar, 1983). In order to evaluate the true impact of exposure on steroid biosynthesis in fish, fish must be exposed to BKME under controlled laboratory conditions.

While the field studies have focused their attention on lake whitefish, and white suckers living in the polluted waters, none have included fathead minnows (*Pimephales promelas*). One laboratory study conducted at the National Research Institute of Canada, Burlington, Ontario, Canada, evaluated the reproductive impact of BKME on laboratory reared fathead minnows, as discussed previously in chapter 2.

Fathead minnows were chosen as the test species for these laboratory controlled exposures because they are easy to culture by established EPA guidelines, they are inexpensive to rear, they are very fecund, have a short reproductive cycle and are one of the most widely distributed species of cyprinid in Canada (Scott and Crossman, 1970).

4.2 METHODS

The procedures are based on Environmental Protection Series Report EPS 1/RM/22. February 1992. Biological test method: test of larval growth and survival using fathead minnows.

The fathead minnow culture facility consisted of two rooms. One room contained the breeding stock and the second room contained the young culture minnows.

4.2.1 General Procedures:

All fish, including brood stock and culture were kept in glass aquaria, receiving dechlorinated water at a constant temperature of 25°C +/-2°C (temperature monitored daily) with a flow rate of 4 l every 10 minutes per 40 l tank. The photoperiod was 16 hours light/8 hours dark. The fish were fed once daily while on low maintenance and twice daily for reproductive priming and breeding. They received either frozen or newly hatched *nauplii* brine shrimp (San Francisco Bay Brand, Rolf Hagen, Montreal) depending on the age and sexual maturity of the fish. Newly hatched *nauplii* brine shrimp were hatched in aerated 4 litre jars filled with warm, salted, dechlorinated water. All tanks, jars and airstones were cleaned routinely using a bleach solution.

4.2.2 Brood Stock:

The initial brood stock was kindly donated by Mr. Al Smith of the Aquatic Toxicology Research Centre, Thunder Bay, Ontario, and is EPA stock. Four inch diameter PVC piping was cut longitudinally in half and then into lengths of 4 inches as outlined in the EPA report. Each 40 l breeding tank contained one female, one male and one piece of PVC piping. Brood stock was fed twice daily with frozen brine shrimp supplemented with live newly hatched brine shrimp whenever possible to increase their nutritional intake.

The tanks were checked daily for eggs. The PVC plates were removed and checked for any fungus infected eggs which were removed immediately. The plates with eggs were placed length-wise in a culture tank with a functioning airstone beside it. The plates were checked daily for fungus and any infected eggs were removed. Once hatching was complete, the PVC plate and the airstone were removed. The PVC plate was washed and disinfected before reuse. The newly hatched fish were fed *naulpii* brine shrimp twice daily until they reached an age of one month at which point they received frozen brine shrimp once daily until experimental use.

The tanks were cleaned daily using a siphon hose. Notes were taken on the health and behavior of the fish, dates of eggs laid, productivity of breeding pairs, and percentage lost due to fungus and infant mortality.

Water temperature was monitored daily and dissolved oxygen, conductivity and pH were monitored weekly.

4.2.3 Culture Tanks:

Culture tanks containing young minnows were kept at 26-27° C. The young fish were sorted in tanks by age (monthly) and numbers were recorded. They were fed frozen brine shrimp once daily until use. Once needed the newly matured minnows were fed freshly hatched *naulplii* shrimp and frozen shrimp twice daily.

4.3 RESULTS AND DISCUSSION

The aquaculture conditions were quite successful. From the initial five pairs of breeding stock, two pairs produced 2363 young minnows. Spawning of these two pairs began in March 1994 and continued through till May 1994. The egg masses ranged from 50 to 150 eggs per spawning. Repeat spawning took 4-6 days. Some eggs had to be removed due to fungus but the percentage infected never exceeded 10%. The airstone added to the incubating eggs successfully decreased the amount of infected eggs. The tanks were kept very clean to avoid the presence of green or brown algae which increase the occurrence of infection.

The young minnows increased in mass quite quickly reaching a size large enough to consume frozen food after only one month. The fish began reaching sexually maturity around six months. Once mature, some of the young male and female minnows were removed and isolated in the brood stock tanks for further breeding. One of these pairs began spawning to produce 131 young minnows in August 1994.

The mortality of young minnows was no greater than five per month with an average loss of 3 fish per month from all tanks combined. Overflow problems did arise and some fish were lost but again these numbers were very low. EPA guidelines state that tanks with a mortality rate greater than 10% should not be used for experimental purpose. None of the fathead minnow culture tanks exceeded this value and were thus considered adequate for experimental use.

The water quality of the tanks was monitored regularly. Water temperatures were taken daily and ranged from 24°C to 27°C. The EPA guidelines recommend a temperature of 25°C+/-1°C. This was not difficult to maintain under normal circumstances. However during summer, water temperatures tended to rise so it was necessary to vent the rooms in order to kept the water temperatures from rising. This method was effective at maintaining the water temperature except for one point during the summer of 1995. In June of this year, the water temperatures reached 30°C because of the extremely warm weather conditions. It was necessary to turn off one of the water heaters until the ambient water temperatures began to decline.

Other water quality parameters measured weekly included dissolved oxygen, conductivity and pH. These parameters remained constant and the average readings and ranges are found in Table 4.3.1. The most stressful parameter, dissolved oxygen, averaged 7.4 mg/l, well above the minimum of 4.0 mg/l. Dissolved oxygen levels below 4 mg/L cause oxygen

stress in fish characterized by fish swimming towards the surface of the water. This symptom was never observed supporting the fact that the fish were not under stress due to lack of oxygen.

Table 4.3.1: Average pH, conductivity and dissolved oxygen readings of the cultured fathead minnow tanks while raising the young minnows (December 1994 - May 1995). The range and standard deviation (S.D.) of these parameters is also given.

Parameter	Average	Range	S.D.
pH	7.38	7.19-7.64	0.11
Conductivity (usec)	108.6	104-115	3.2
Dissolved Oxygen (mg/l)	7.33	7.0-8.4	0.43

Fathead minnows are fairly easy species to culture. They require small tanks that are easy to maintain. Fecundity ranges from of 50-150 eggs per spawning. Their spawning cycle is short, ranging from 4-6 days during high maintenance diets and they reach maturity in as little as six months. These factors combine to make fathead minnows an excellent choice for use in laboratory controlled exposure experiments.

CHAPTER 5

PROTOCOL FOR DETERMINING STEROID PROFILES

5.1 INTRODUCTION

In many cases it is very difficult to determine steroid profiles directly from tissue extracts because the presence of large quantities of many non-steroidal components interfere with chromatographic analysis. For this reason, a multi-step protocol is required to remove steroids from a tissue extract and to isolate them for analysis. This process includes extraction of the steroids from the tissue; further purification or clean up of the sample to remove any non-steroidal compounds; formation of suitable derivatives; and removal of excess derivatization reagents.

Extraction is accomplished by using a steroid soluble solvent which depends on the chemical and physical properties of the steroids. Steroids are fairly large, lipophilic molecules which easily dissolve in a range of solvents from methanol to ether. Any of these are excellent extraction solvents to profile steroid intermediates because they will denature protein and cause it to precipitate therby releasing protein bound steroids into solution. These solvents, however also extract numerous other lipophilic components. These are mostly lipids present in the tissue, called co-extractives.

Sample clean up is required to remove any excess co-extracted non-steroidal compounds which interfere with analytical detection. The co-extractives can inflate or mask actual

steroid levels or raise the background and reduce sensitivity of the instrument. Liquid-liquid extraction was once the most popular clean up method. This tedious, time consuming and costly method had many disadvantages including requirement of several sample handling steps, large volumes of solvent, impure and wet extracts, and nonquantitative and irreproducible extractions (Zief and Kiser, 1994).

The second method of sample clean up, and the one used in this protocol, uses solid phase extraction (SPE) columns (Zief and Kiser, 1994) which require less solvent volume and are much more efficient. The method consists of disposable extraction columns filled with a variety of sorbents. It is based on the selective adsorption of the co-extractives on an active solid and the elution of the steroids with a given volume of solvent. The column is initially conditioned with a solvent to solvate the functional groups of the sorbent. The column is then further conditioned with the sample matrix solvent. Once the sample is passed through the column and the analytes are adsorbed to the sorbent, the column is washed with a solvent which selectively elutes the impurities from the column but retains the analyte on the column. The purified analyte is then eluted with a solvent strong enough to displace the analyte from the sorbent. Many factors affect the selectivity of the columns including: pH of the solution, flow rate of the solvent, and column capacity. In the case of steroids, SPE columns containing reverse phase (C18) and/or amino packing have been used to clean up urine steroid samples (Schmidt, *et al.*, 1985).

Even after sample clean up the isolated steroids cannot be analysed by gas chromatography because of their low volatility, their instability at high temperatures, and their tendency to adsorb on the glass surfaces of the instrument. To solve this problem, the steroids are derivatized by reaction with reagents which respond to reactive and polar moieties such as hydroxyl, carbonyl or ketone groups. This is commonly known as derivatization. Numerous methods for derivatizing steroids have been described but a commonly used method involves the formation of methoxime-trimethylsilyl (MO-TMS) derivatives. These steroidal derivatives are very volatile and thermally stable. They are commonly used in research and a good literature base exists outlining reaction procedures, removal of excess reagent procedures, mass spectra, and selected ion recording analysis.

Gas chromatography is a powerful analytical tool capable of separating complex mixtures of biological origin into their individual components. The compounds are carried down a long, thin capillary column by the carrier gas. They are then separated according to the relative affinity of the solute and the liquid phase. The time required for the steroids to reach the detector depends on: the column temperature, the type of column and the chemical and physical properties of the steroids.

Confirmation is achieved by a mass spectrometer connected to a gas chromatograph which act as a detector. Combined with the identifying ability of mass spectroscopy (MS), gas chromatography-mass spectroscopy (GC/MS) becomes a very powerful analytical

technique. As a steroid enters the MS, it is hit by an electron beam and fragmented. A magnet attracts the charged fragments and allows the measurement of their mass/charge ratio (m/z). The steroid is identified by the intensity of different fragment mass sizes and may be used to identify substances by comparison to reference spectra. Thousands of reference spectra are stored in software libraries facilitating the identification of an unknown chemical. Modifications of this technique can enhance resolution and detect very low concentrations (ng/ml to pg/ml) of a chemical. One modification, selected ion recording (or SIR) selects two characteristic ions from the chemical's spectral scan for detection, identification and quantification. A protocol to extract, isolate, identify and quantify profiles of steroid intermediates from vitellogenic ovaries of fathead minnows using gas chromatography/mass spectroscopy - selected ion recording was developed in this thesis.

5.2 METHODS

5.2.1 Materials

The steroid standards including 4-androstene-3,17-dione, 5-androsten-3β-ol-17-one, estratriene-3,17β-diol, 1,3,5[10]-estratriene-2,4-d_2,3,17β-diol, 4-pregnene-3,20-dione, 4-pregnen-17α-ol-3,20-dione, 5-pregnen-3β-ol-20-one, and 5-pregnene-3β-17α-diol-20-one were obtained from Sigma (Mississauga, Ontario, Canada). Testosterone was obtained from Mr. H.W Avdovich from the Bureau of Drug Research of the Pharmacology and Chemistry Division of Health and Welfare Canada, Ottawa, Ontario, Canada. Optima grade hexane, and

ethyl acetate, and HPLC grade methanol were obtained from Fisher Scientific (Toronto, Ontario, Canada). Non-UV grade, distilled in glass, acetonitrile and t-butyl ether were obtained from Caledon (Georgetown, Ontario, Canada). The C18 and amino solid phase extraction (SPE) columns were obtained from Supelco (Mississauga, Ontario, Canada). All derivatizing reagents were obtained from Pierce (Rockford, Illinois, USA). They included N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA), MOX® reagent (2% solution of methoxyamine-HCl in pyridine), heptafluorobutyric anhydride (HFBA), FLOROX™ reagent (2.5 mg/ml 0-(pentaflurobenzyl)hydroxylamine-HCl in pyridine, and Aqua-Sil™ (water soluble siliconizing fluid).

5.2.2 Dissection and Extraction (Fig. 5.2.2.)

Female fathead minnows were removed from culture tanks and immediately killed by a blow to the head and severing just posterior to the medulla oblongata (PAPRICAN, pers. comm.). The body weight of each fish was recorded. The ovaries were removed, weighed, and recorded. The optimum amount of tissue per sample was determined by evaluating the sample cleanup capacity. This was accomplished by spiking varying amounts of lake whitefish ovarian tissue (1.0g, 2.0g, 3.0g, 4.0g, and 5.0g) with a fixed concentration of each steroid. The amount of tissue just prior to the point where sample breakthrough occurred (the amount of interfering coextractives which competitively bind to the C18 packing and cause loss of the analyte of interest) was considered to be the optimum amount of tissue per sample.

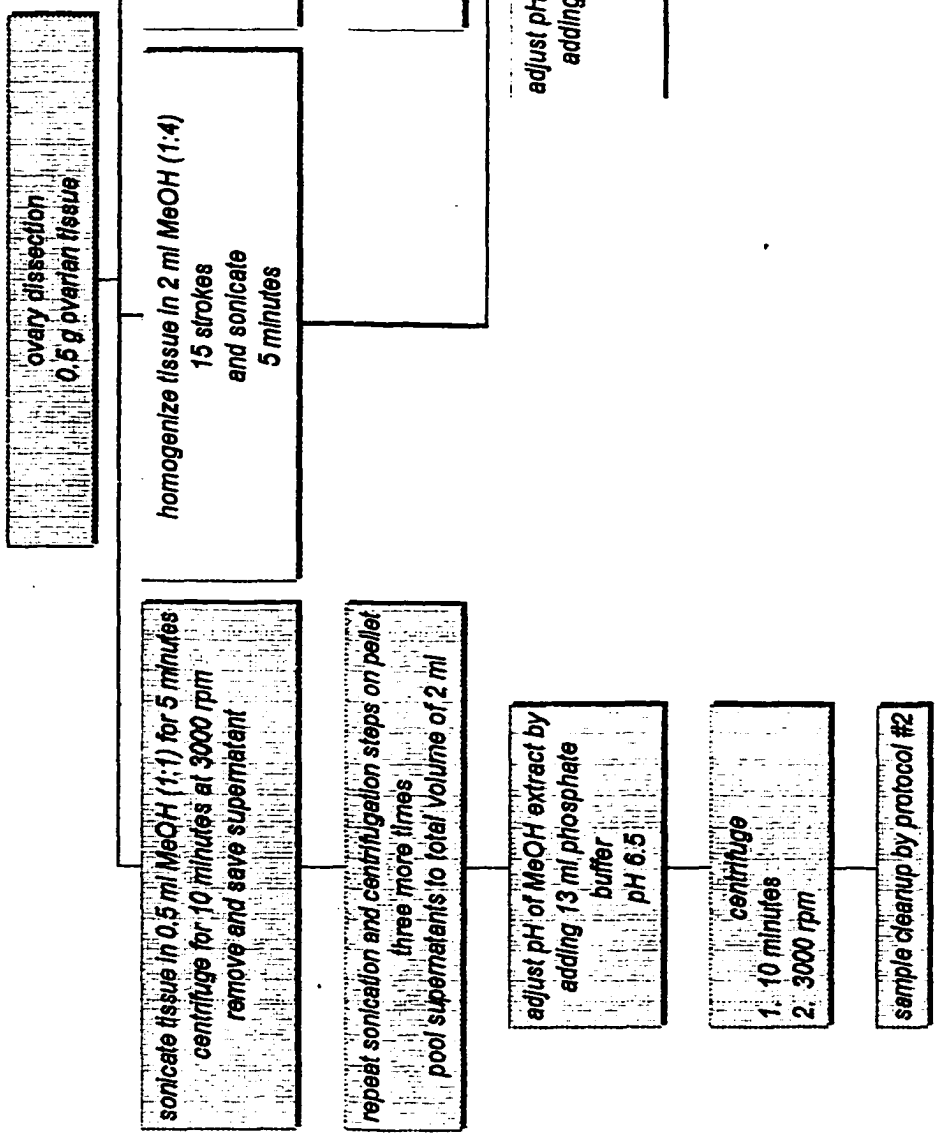


Figure 5.2.2: A schematic representation of the steroid extraction procedures used to isolate the steroid biosynthetic intermediates from fathead minnow ovarian tissue. The shaded path indicates the most efficient extraction procedure. Abbreviations are as follows: MeOH - methanol. The centrifuge radius was 25 cm. The ratios of 1:1, 1:4, and 1:10 indicate the ratio of tissue to solvent used to extract the steroids.

Methanol was used as the extraction solvent. Three extraction solvent volume to tissue ratios were evaluated (10:1, 4:1, and 1:1, respectively) in order to optimize steroid extraction from the fish tissue. Tissue was either homogenized with 5 strokes of a 10 ml telflon pestle with 0.125 cm clearance, or sonicated with a Sonifier cell disruptor for 5 minutes. The resultant mix was then centrifuged at 3000rpm for 10 minutes. The 1:1 solvent to tissue ratio was repeated 4 times and the resulting supernatants pooled in order to help maximize steroid extraction.

The pH of the supernatant was adjusted to either pH 4.6 using an acetate-boric acid buffer, pH 4.6 (Schmidt, *et al.*, 1985) or to pH 6.5 using a 0.1 M phosphate buffer pH 6.5. The ratio of buffer to supernatant required to adjust the sample pH to the desired value was determined using a freshly calibrated (two point calibration of pH 7.00 and pH 4.01) Orion model 920A pH probe. Once this was determined, the fixed amount of buffer was added to each sample without the use of the pH probe in order to minimalize sources of contamination and steroid loss.

A summary of the extraction procedure development and the measurements used can be found in Fig. 5.2.2.

5.2.3 Sample Clean Up (Figs. 5.2.3.1, 5.2.3.2, and 5.2.3.3)

The basic clean up procedure was originally based on the protocol outlined by Schmidt *et al.*, 1985, which isolated steroid metabolites and their conjugates from human urine. Their

general protocol, with slight modifications to accomodate available instrumentation, is outlined below.

The pH adjusted samples (pH 4.6) were added to preconditioned (2X5ml methanol followed by 2X5ml deionized water) 1 gram packing C18 SPE columns using capillary pipettes. The samples were slowly eluted through the columns using a stream of nitrogen at a rate of 4 ml/minute which required a pressure of 2 psi. After all the sample volumes were eluted through the columns, the columns were washed with 3X5ml deionized water. The steroids were eluted from the C18 SPE columns with 1X2ml and 2X1ml washes of Optima grade ethyl acetate into clean silanized test tubes containing 2 grams of anhydrous sodium sulphate to dry the samples. After confirming that the samples were completely dry of water, the ethyl acetate was removed using clean capillary pipettes and aliquoted to cleaned but unconditioned 500 mg packing amino SPE columns (cleaned with 1X3ml ethyl acetate). The samples were eluted through these amino columns and through a second series of cleaned, unconditioned 500 mg packing amino SPE columns. The eluants were collected in clean, silanized 4 ml reaction vials. The samples were dried down under a gentle stream of nitrogen and either derivatized or stored in a refrigerator for future derivatization.

This protocol (referred to as protocol #1 in Results) resulted in highly contaminated tissue samples which interfered with steroid analysis. The protocol was subsequently modified to

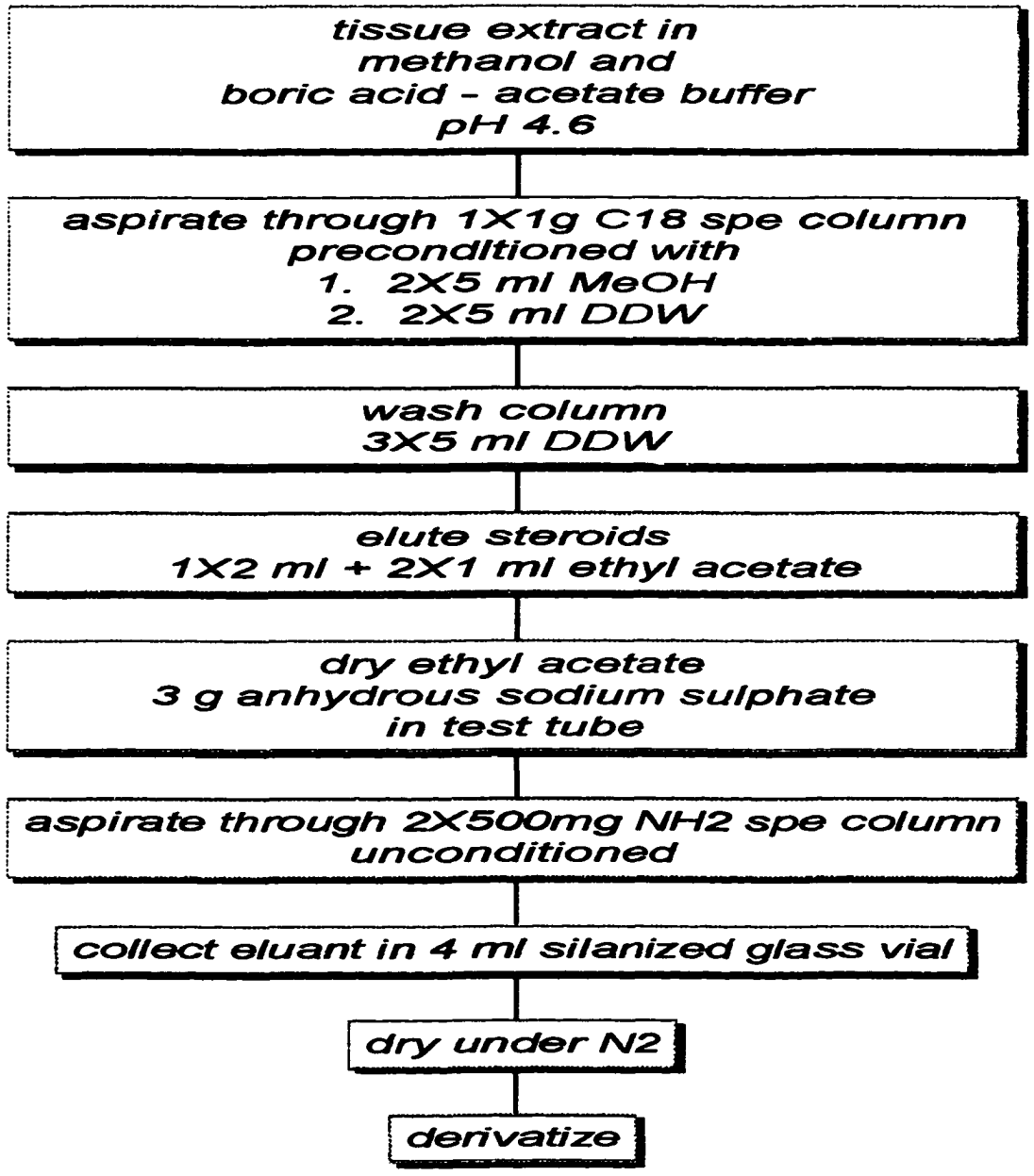


Figure 5.2.3.1: A schematic representation of protocol #1 clean up procedures used to isolate the steroid biosynthetic intermediates from fathead minnow ovarian tissue. Abbreviations are as follows: MeOH - methanol; DDW - distilled, deionized water; N_2 - nitrogen gas; spe - solid phase extraction; NH_2 - amino column.

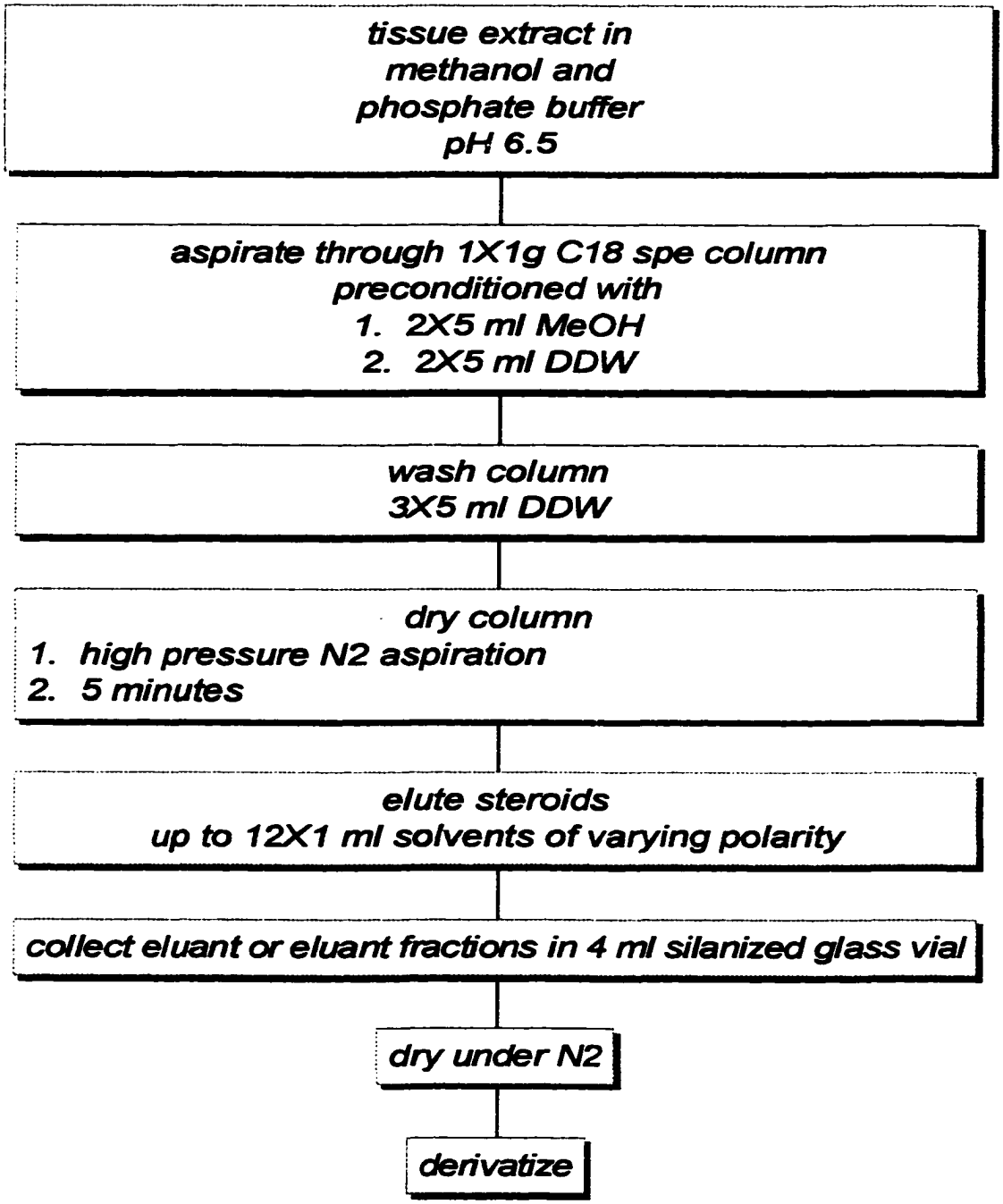


Figure 5.2.3.2: A schematic representation of protocol #2 clean up procedures used to isolate the steroid biosynthetic intermediates from fathead minnow ovarian tissue. Abbreviations are as follows: MeOH - methanol; DDW - distilled, deionized water; N_2 - nitrogen gas; spe - solid phase extraction.

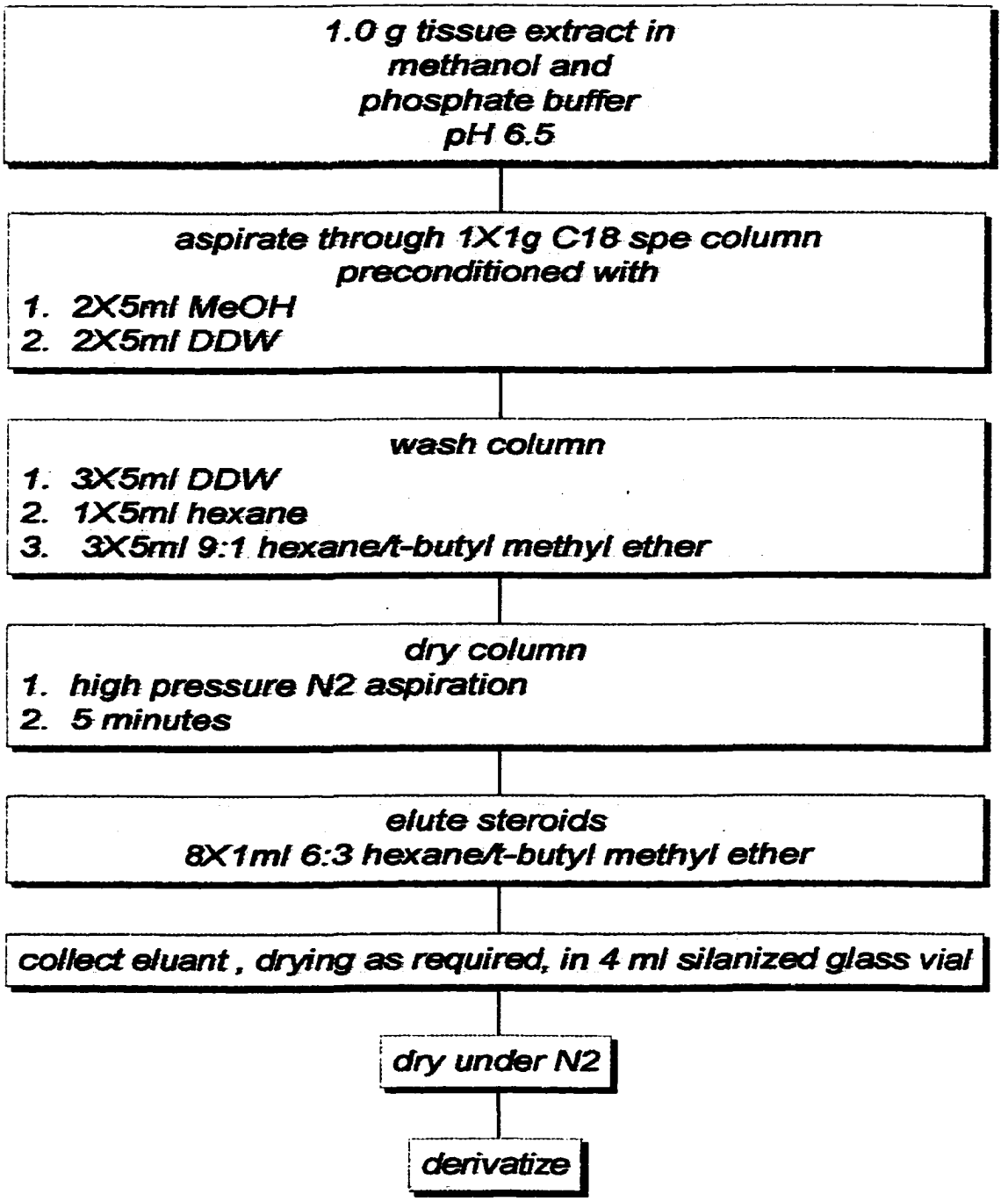


Figure 5.2.3.3: A schematic representation of finalized protocol #2 clean up procedure used to isolate the steroid biosynthetic intermediates from fathead minnow ovarian tissue. Abbreviations are as follows: MeOH - methanol; DDW - distilled, deionized water; N_2 - nitrogen gas; spe - solid phase extraction.

optimally clean up tissue samples. The modifications are outlined below and are represented in Fig. 5.2.3.2. This protocol was refered to as protocol #2 in Results.

The pH adjusted samples (pH 6.5) were added to preconditioned (2X5ml methanol followed by 2X5 ml deionized water) 1 gram packing C18 SPE columns using a stream of nitrogen at a rate of 4 ml/minute which required a pressure of 2 psi. After all the sample volumes were eluted through the columns, the columns were washed with 3X5 ml deionized water. The steroids were eluted from the C18 SPE columns with varying amount of 1 ml aliquots of various solvents and solvent mixes of increasing polarity (Tables 5.3.2.1 and 5.3.2.2 of the Results section) into clean silanized vials to determine the eluant, and eluant volume, which best separates the steroids from the contaminants. The amino SPE columns were eliminated from the protocol because they did not significantly reduce tissue contamination levels but did result in up to 10% (depending on the steroid) steroid loss. The samples were dried down under a gentle stream of nitrogen at 80°C and were immediately derivatized for GC/MS analysis.

A summary of the finalized sample clean up protocol #2, after all modifications, can be found in Fig. 5.2.3.3.

5.2.4 Derivatization (Fig. 5.2.4)

Two different derivatization protocols were investigated. The desired steroidal derivatives depended on the type of analytical unit used. For gas chromatrograph with electron

capture detection (GC/ECD), fluorinated derivatives were desired to enhance sensitivity. In order to accomplish this a 2.5 mg/ml O-(pentafluorobenyl) hydroxylamine-HCl in pyridine (FLOROX™) reagent was used to react with ketone groups to form pentafluorobenzyl (-PFB) derived steroids (Nambara *et al.*, 1975; Koshy *et al.*, 1975). 200 ul of the FLOROX™ reagent was added to the dried samples and incubated at 65°C for 1 hour. The samples were cooled and dried under nitrogen. 20 ul of heptaflurobutyric anhydride and 200 ul of 0.2 M triethylamine in benzene were added to derivatize the free hydroxyl groups to form heptofluorobutyric (-HFB)-derived steroids (Challis and Heap, 1970; Walle and Ehrsson 1970 & 1971). The samples were incubated at 50°C for 15 minutes. Once removed from the heat and cooled, 1.0 ml deionized water was added to the samples and shaken for 60 seconds. Next, 1.0 ml of 0.2 N ammonia solution was added to the samples and shaken for 5 minutes. The benzene layers were removed and placed in clean vials for GC/ECD analysis of the PFB-HFB derived steroids.

The second derivatization involved the preparation of derivatives which were extremely stable for gas chromatography combined with mass spectroscopy detection. The derivatization consisted of two individual reactions which resulted in the formation of methoxime-trimethylsilyl derivatives (MO-TMS). In the first reaction 200 ul of MOX® reagent was added to the dried samples to react with all ketone groups (Pierce catalog, 1987). The vials were capped and allowed to react for 3 hours in a preheated sand heating mantle at 80°C. After the 3 hours, the samples were removed and 100 ul of BSTFA was directly added to the vials for the second reaction. This reaction bound a trimethylsilyl

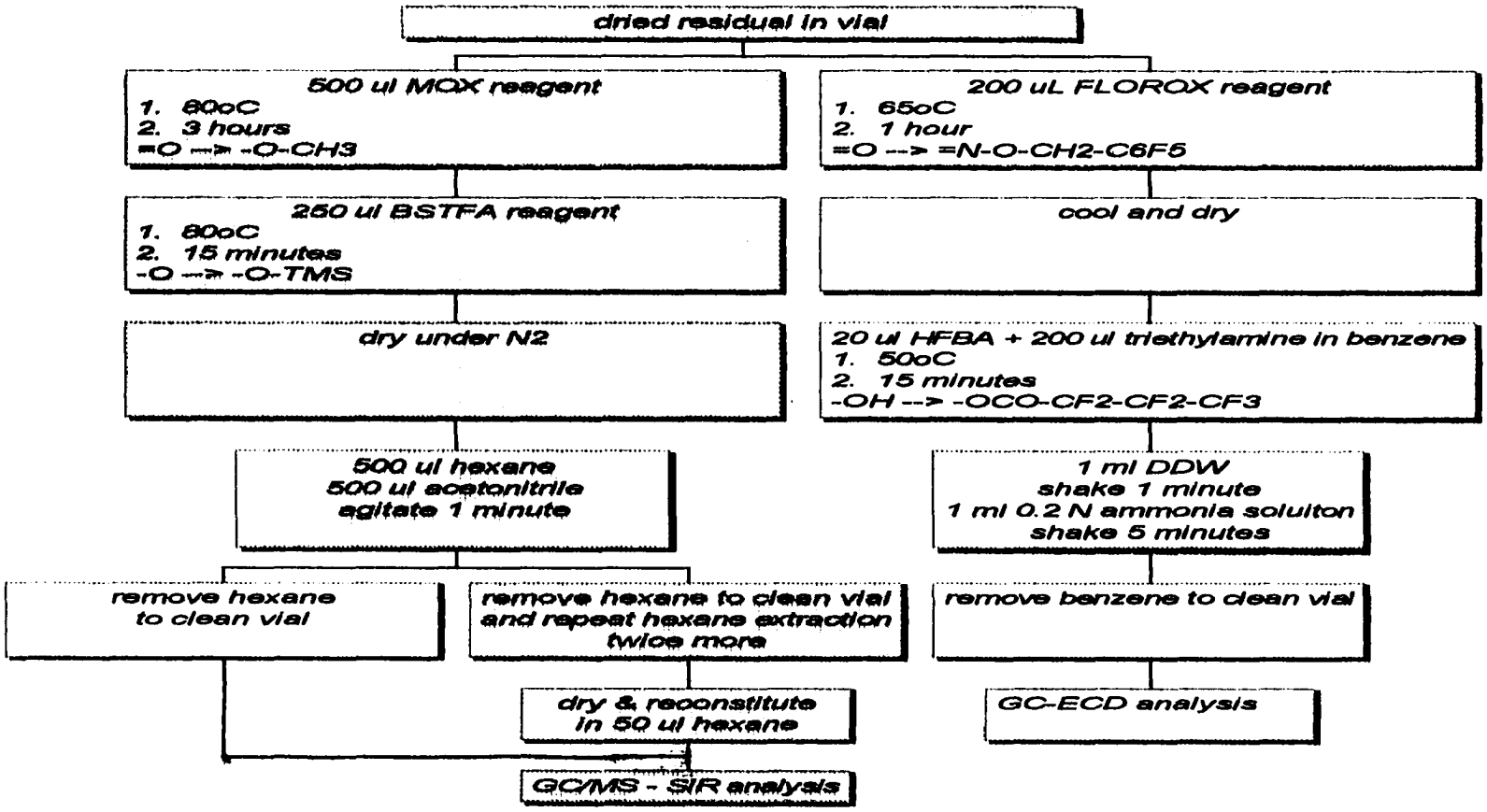


Figure 5.2.4: A schematic representation of the derivatization procedures used to derivatize the steroid biosynthetic intermediates from fathead minnow ovarian tissue. The shaded path indicates the finalized derivatization procedure. Abbreviations are as follows: MOX® - 2% solution of methoxyamine-HCl in pyridine; BSTFA - N,O-bis(trimethylsilyl)trifluoroacetamide; N_2 - nitrogen gas; FLOROX™ - 2.5 mg/ml O-(pentafluorobenzyl)hydroxylamine-HCl in pyridine; HFBA - heptafluorobutyric anhydride; DDW - distilled, deionized water; GC/MS-SIR - gas chromatography combined with mass spectroscopy using selected ion recording; GC-ECD - gas chromatography using electron capture detector.

(TMS) group to the free hydroxyl groups (Pierce catalog, 1987). The reaction vials were placed back on the heating mantle at 80°C for 15 minutes. Once the reactions were complete, the samples were removed from the heat and allowed to cool prior to drying under a stream of nitrogen. The dried residues were reconstituted in 500 ul of Optima grade hexane, shaken and then 500 ul of non-UV grade acetonitrile was added to wash the hexane solution of any polar derivatized compounds. The upper hexane layers were then removed using a clean capillary pipette and transferred to new clean vials for GC-MS analysis within the next 24 hours. The acetonitrile fractions were discarded.

A summary of the two derivatization procedures and the measurements used can be found in Fig. 5.2.4.

5.2.5 Glassware Cleaning:

All glassware was cleaned by soaking for at least 24 hours in chromic acid. The glassware was rinsed using tap water, distilled water (X3), deionized water and acetone. The glassware was then baked at a temperature greater than 120°C for 24 hours. After cooling, the glassware was silanized using a 1% Aqua-Sil™ solution in deionized water, rinsed with deionized water and acetone, and allowed to dry for 24 hours.

5.2.6 GC/ECD and GC/MS Analysis

The parameters used to detect the steroids on the GC/ECD and GC/MS are summarized in Table 5.2.6.

Table #5.2.6: GC/ECD and GC/MS settings used to measure steroids with a splitless injection. N/A is not applicable.

Parameter	GC/ECD Setting	GC/MS Setting
Column	15m DB-1 0.1 um film thickness id .25 um	30m DB-5 0.1 um film thickness id 0.25 um
Injection Temperature	250°C	250°C
Temperature Program	150°C isothermal for 5 minutes 150°C to 210°C at 10°/minute 210°C to 240°C at 1°/minute 240°C isothermal for 19 minutes	160°C isothermal for 1 minute 160°C to 190°C at 15°/minute 190°C isothermal for 1 minute 190°C to 235°C at 2°/minute 235°C isothermal for 25 minutes
Head Pressure	25 psi	20 psi
Carrier Gas	Nitrogen	Helium
Interface Temperature	N/A	230°C
Source Temperature	N/A	230°C
Resolution	N/A	3000
Lock Mass	N/A	Tuned on lock mass PFK 431

5.2.7 Quantification Calculations

The quantification of steroids was accomplished using the internal standard method. 500 ppb of 2,4-d_2-17β-estradiol was added to each sample either prior to sample clean up or prior to derivatization. Relative response factors (RRFs) were calculated for each steroid

using standard profiles containing known concentration of each steroid. RRFs were calculated using the following equation:

$RRF_s=(A_s*C_{is})/(A_{is}*C_s)$ Standard Methods for Examination of Water and Wastewater, p.6-23.

where: A_s = area of standard,

A_{is} = area of internal standard. A correction factor was made in the summation of M1 and M2 areas due to the presence of naturally occurring d_2-estradiol in a sample. As determined from previous mass spectrographs, naturally occurring estradiol contributes 17% of the total abundance of the M1 (287) d_2-estradiol peak and 10% to the total abundance of the M2 (418) d_2-estradiol peak. These percentages were subtracted from the M1 and M2 areas of d_2-estradiol before further calculation.

C_s = concentration of the standard,

and C_{is} = concentration of the internal standard.

Using these relative response factors, the concentration of steroid in a 1 ul injection (C_i) of an unknown sample was calculated as follows:

$C_i=(A_s*C_{is})/(A_{is}*RRF_S)$.

The concentration of steroid per gram of ovarian tissue (C_t) was calculated as follows:

$C_t=C_i*500ul*(g\ tissue/sample)^{-1}$

All steroid concentrations were reported as picogram steroid per gram of ovarian tissue.

5.3 RESULTS

5.3.1 Tissue Capacity of C18 SPE Column

Capacity is defined as the total quantity of compounds (analytes and interferences) which can be retained from a specific sample matrix solution by a given mass of sorbent (Zief, M. & Kiser, R., 1994). The capacity of the finalized clean up procedure was determined by spiking varying amounts of tissue with a fixed concentration of each steroid. The point at which the amount of tissue began to interfere with steroid recovery from the C18 SPE column was referred to as the point of breakthrough. As observed in Fig. 5.3.1.1, breakthrough for dehydroepiandrosterone, testosterone and estradiol began to occur between 1.0-2.0 mg tissue/sample. In contrast to this, the levels of androstenedione, 17-hydroxypregnenolone and 17-hydroxyprogesterone rose quite dramatically as the amount of tissue per sample increased (Fig. 5.3.1.2). This resulted either from the actual presence of these steroids in the tissue, or from interference compounds with identical retention times. Interferences in the chromatogram made it impossible to quantify pregnenolone in samples containing more than 2.0 mg tissue/sample. Scan analysis of these samples contained large amounts of interfence compounds which are most likely the cause of the elevated androstenedione, 17-hydroxypregnenolone and 17-hydroxyprogesterone levels. It is for these reasons that the maximal amount of fathead minnow ovarian tissue for the finalized clean up procedure, or tissue capacity was 1.0 mg tissue/sample.

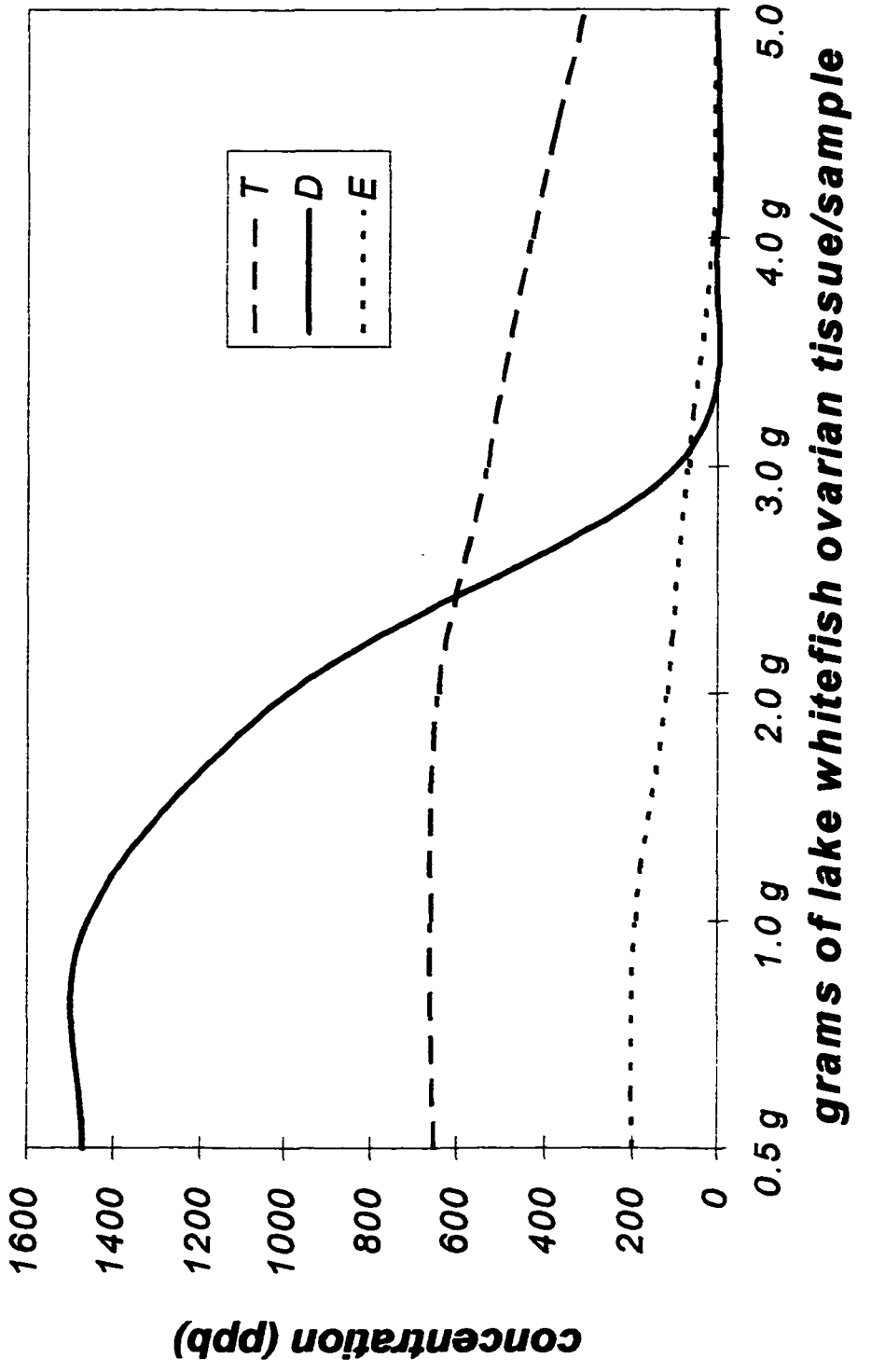


Figure 5.3.1.1: The effect of increasing ovarian tissue per sample on testosterone (T), dehydroepiandrosterone (D), and estradiol (E) C18 spe column retention.

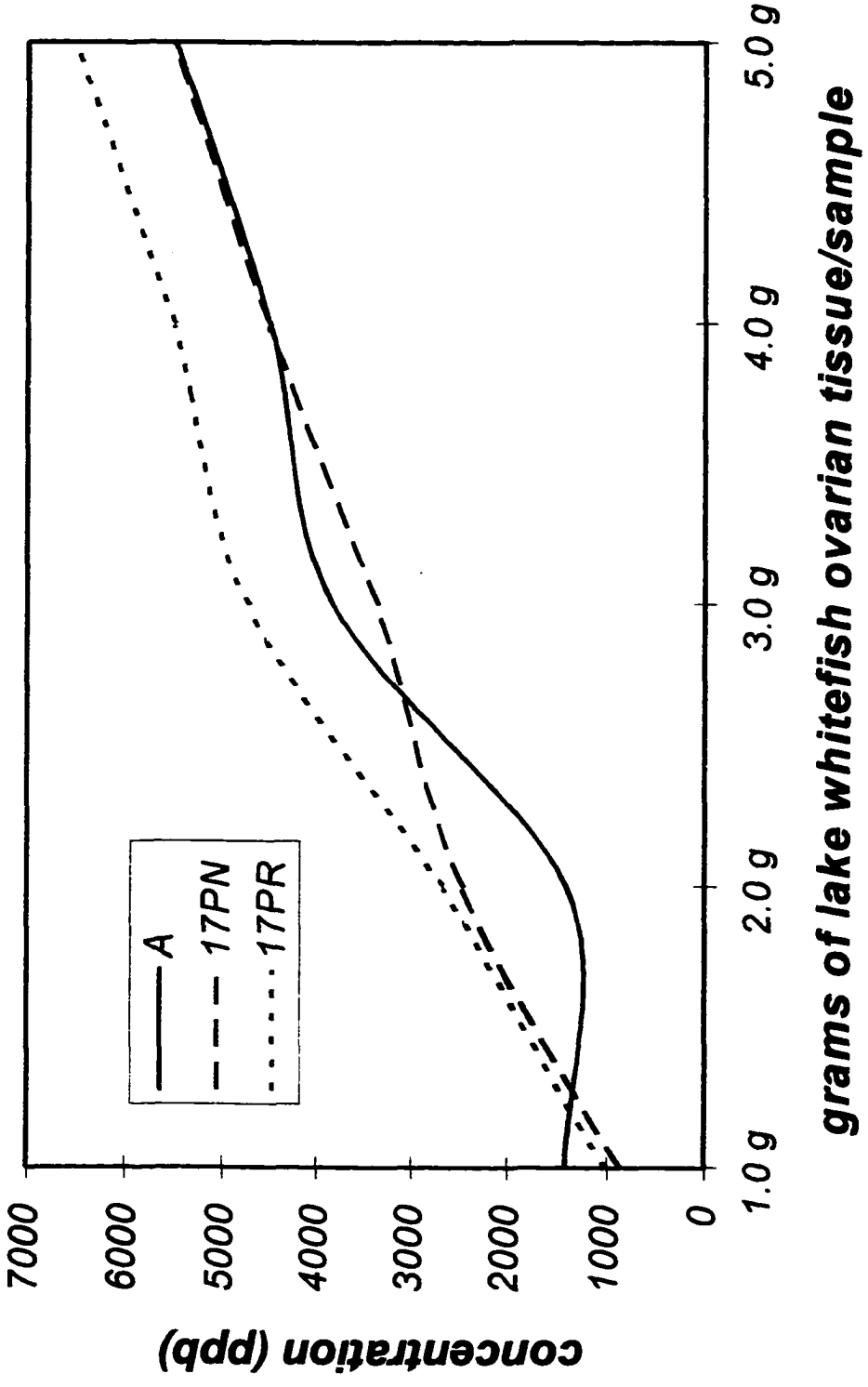


Figure 5.3.1.2: The effects of increasing ovarian tissue per sample on androstenedione (A), 17-hydroxypregnenolone (17PN), and 17-hydroxyprogesterone (17PR) retention on a C18 spe column.

5.3.2 C18 Fractionation of Steroids from Contaminants

In the original cleanup protocol (#1), ethyl acetate was used as the C18 SPE column eluant. This resulted however, in large amounts of contaminants, mostly coextracted lipids, which interfered with GC/MS analysis (see Fig. 5.3.2.1A&B for total ion current chromatogram). It was necessary to select a more appropriate eluant which selectively fractionated the steroids from the contaminants (in protocol #2). In order to do this, a series of experiments were performed using series of solvents of increasing polarity to elute the steroids and contaminants from a loaded C18 SPE column. From the first series of mobile phases it was found that t-butyl ether eluted the steroids along with some of the other contaminants (Table 5.3.2.1). The t-butyl ether total ion current (TIC) chromatogram containing the eluted steroids is in Fig. 5.3.2.1C&D.

Table 5.3.2.1: First series of mobile phases used in the C18 SPE column for the fractionation of steroids from other lipids.

Solvents, volume ratios	Compounds eluted
Hexane	• very few contaminants
Toluene	• very few contaminants
t-Butyl ether	• steroids • few contaminants
Methylene chloride	• contaminants
Methylene chloride/acetone (1:1)	• contaminants
Acetone	• contaminants

Although the contamination was reduced by using 100% t-butyl ether, it was still necessary to separate the steroids from the remaining lipids. For this reason a series of hexane:ether ratios were used as mobile phases in an attempt to fractionate the steroids

from the remaining lipids (Table 5.3.2.2). The TIC chromatograms in Figs. 5.3.2.2 - 5.3.2.4 demonstrates the elution of steroids and contaminants at the varying polarities.

Table 5.3.2.2: Second series of hexane:ether mobile phases used in the C18 SPE column for the fractionation of steroids and neutral lipids.

Solvent volume ratios	Compounds eluted
9:1 hexane/t-butyl ether	• contaminants
7:3 hexane/t-butyl ether	• 40% of steroids in 4X1 ml washes
5:5 hexane/t-buytl ether	• remaining steroids in 4X1ml washes • contaminants
3:7 hexane/t-butyl ether	• contaminants
9:1 hexane/t-buytl ether	• contaminants

Since the 7:3 hexane/t-butyl methyl ether solvent removed 40% of the steroids from the C18 SPE column with 4X1ml washes (Fig. 5.3.2.2 C&D), it was necessary to determine the optimal eluant volume to remove 100% of the steroids from the C18 SPE column. In order to do this, up to 12 mls of eluant (7:3 hexane/ether) were passed through a loaded C18 SPE column and fractions thereof were taken and analysed by GC/MS-SIR. The steroids testosterone, dehydroepiandrosterone and estradiol required up to 8 X 1ml washes of eluant in order to completely remove them from the column (Fig. 5.3.2.5) where as androstenedione, pregnenolone, progesterone, 17-hydroxypregnenolone and 17 hydroxyprogesterone required only 6 X 1 ml washes of eluant (Fig. 5.3.2.5 and Fig. 5.3.2.6).

Figure 5.3.2.1: Selected ion recording total ion current (SIR-TIC) chromatogram of spiked *Pimephales promelas* ovarian tissue using protocol # 1 clean up procedure (A&B) (pH 4.6, 1 NH_2 SPE column, and ethyl acetate as eluant). A is function #1 from 16.00 to 21.00 minutes containing dehydroepiandrosterone (D), native estradiol (E), deuterated estradiol (E_{is}), androstenedione (A) and testosterone (T). B is function #2 from 21.00 to 30.00 minutes, containing pregnenolone (PN), progesterone (PR), 17hydroxypregnenolone (17PN), and 17hydroxyprogesterone (17PR). For comparison, the SIR-TIC chromatogram of spiked *Pimephales promelas* intestinal tissue determining that t-butyl ether elutes the steroids from the C18 SPE column with less contaminants than ethyl acetate is shown in C&D. C is function #1 from 16.00 to 21.00, containing D, E, E_{is}, A, and T. D is function #2 from 21.00 to 30.00 minutes, containing PN, PR, 17PN, and 17PR.

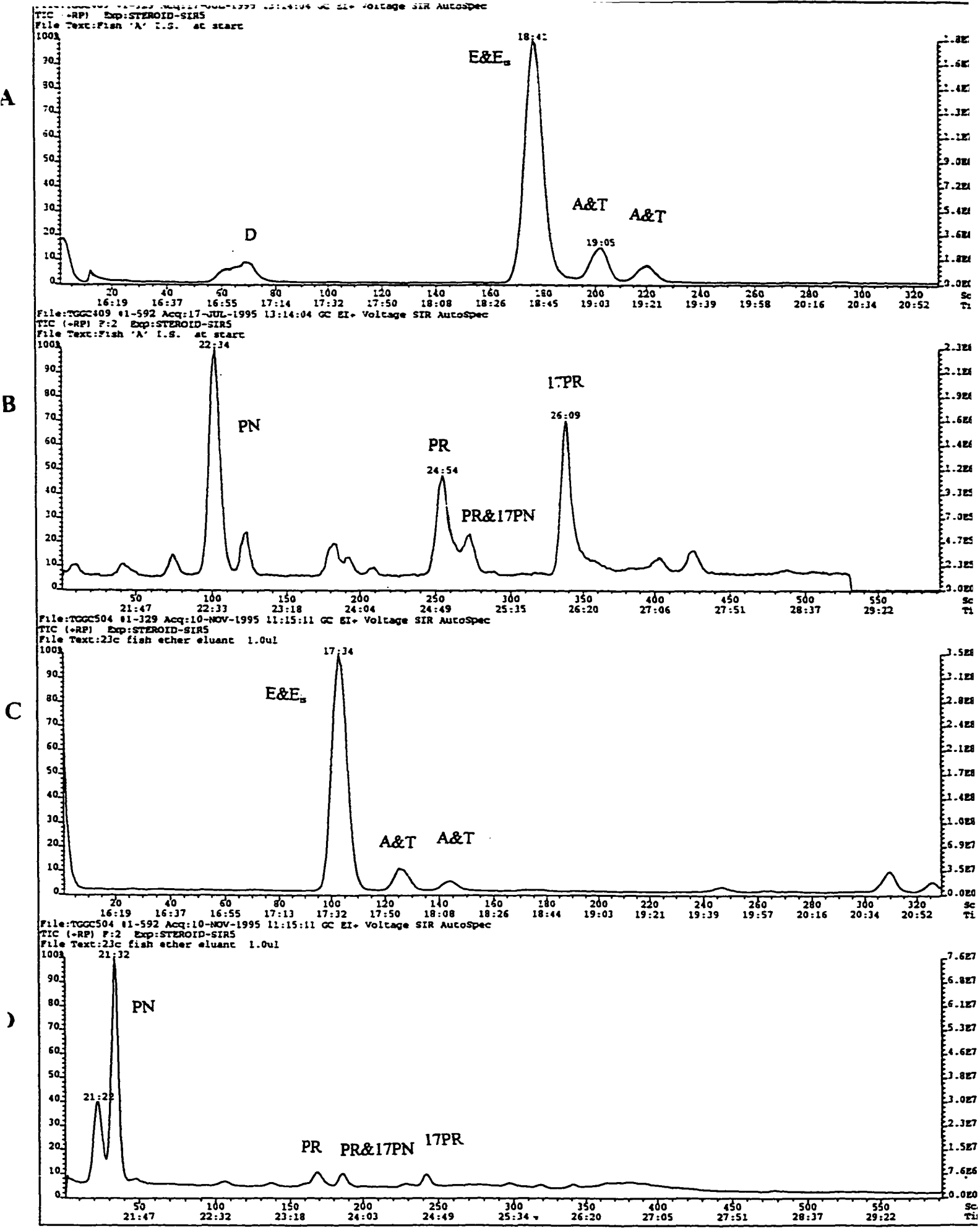


Figure 5.3.2.2: F1 and F2 SIR-TIC chromatograms of 9:1 (A&B) and 7:3 (C&D) hexane/t-butyl ether demonstrating the selective fractionation of steroids from a C18 SPE column using the 7:3 solvent. Abbreviations are as follows: D - dehydroepiandrosterone, E - native estradiol, E_{is} - deuterated estradiol as internal standard, A - androstenedione, T - testosterone, PN - pregnenolone, PR - progesterone, 17PN - 17-hydroxypregnenolone, and 17PR - 17-hydroxyprogesterone.

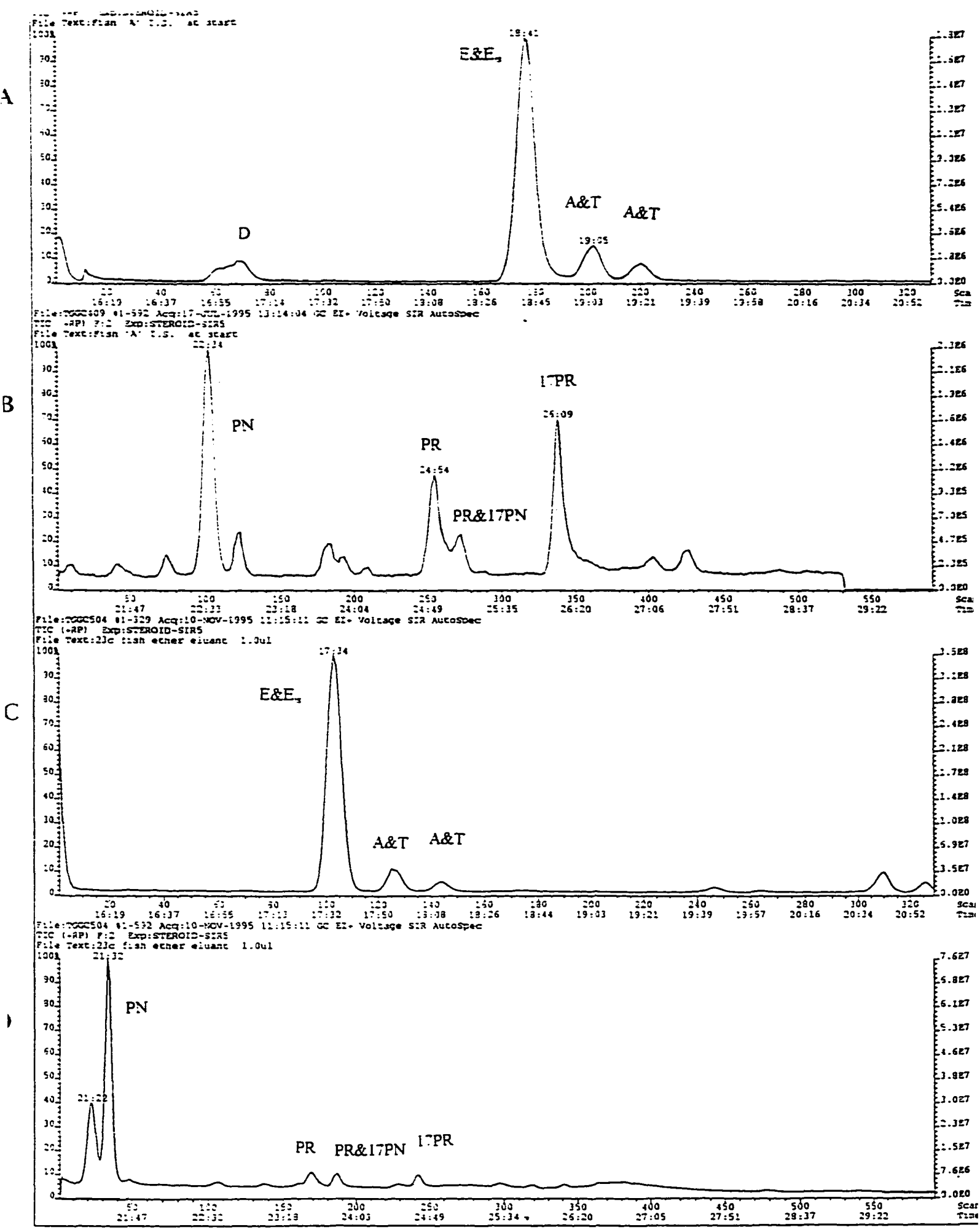
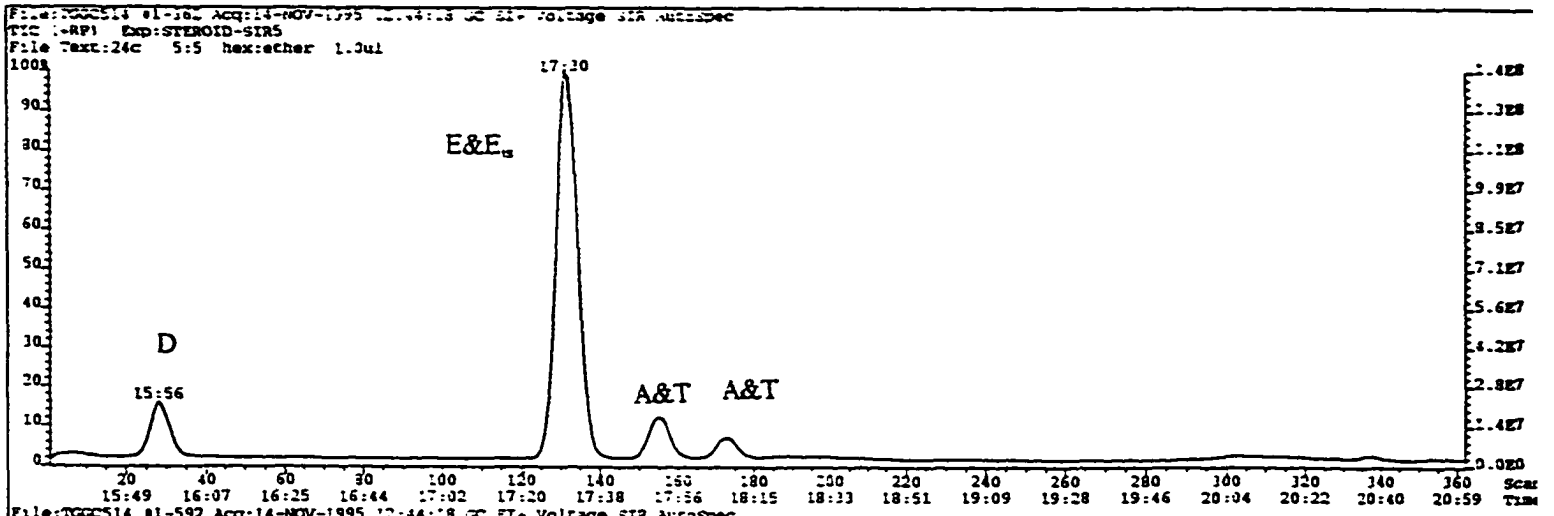
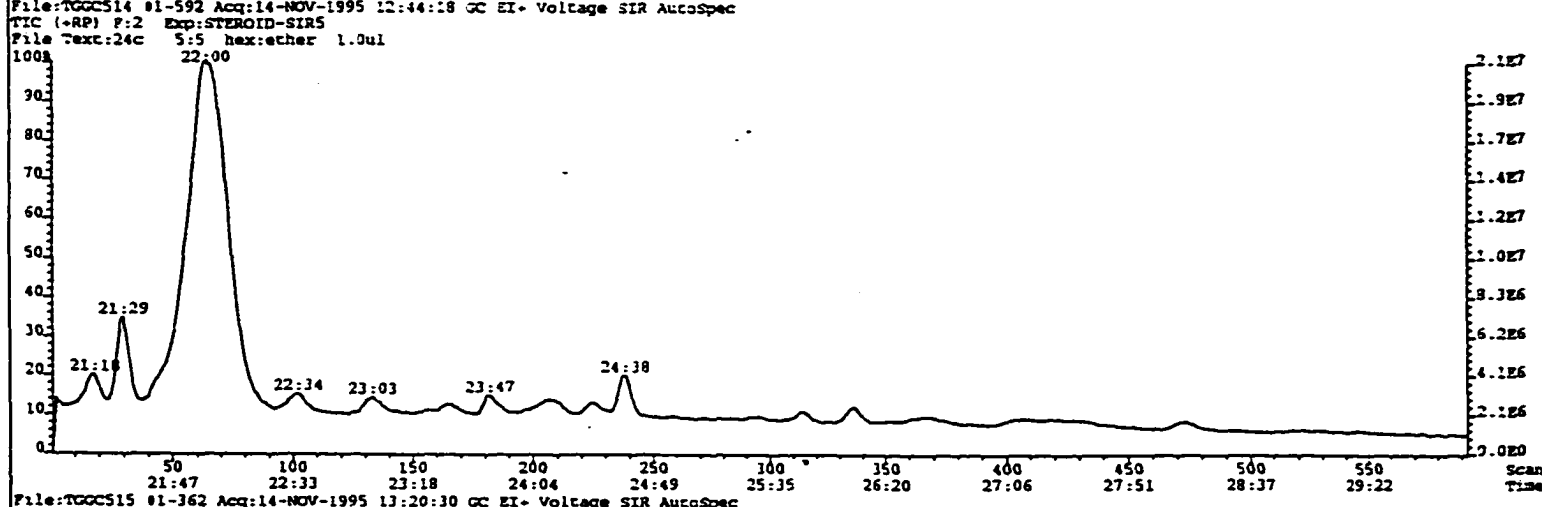
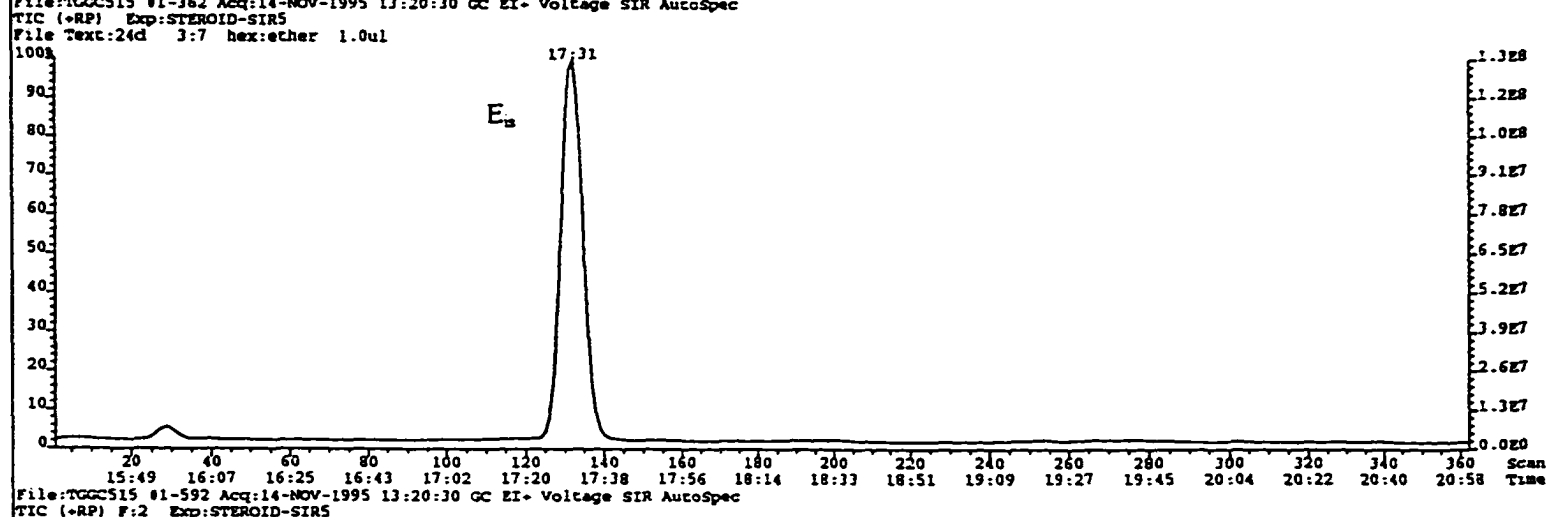


Figure 5.3.2.3: F1 and F2 SIR-TIC chromatograms of 5:5 (A&B) and 3:7 (C&D) hexane/t-butyl ether demonstrating the elution of steroids from a C18 SPE column using the 5:5 solvent. Abbreviations are as follows: D - dehydroepiandrosterone, E - native estradiol, E_{is} - deuterated estradiol as internal standard, A - androstenedione, T - testosterone, PN - pregnenolone, PR - progesterone, 17PN - 17-hydroxypregnenolone, and 17PR - 17-hydroxyprogesterone.







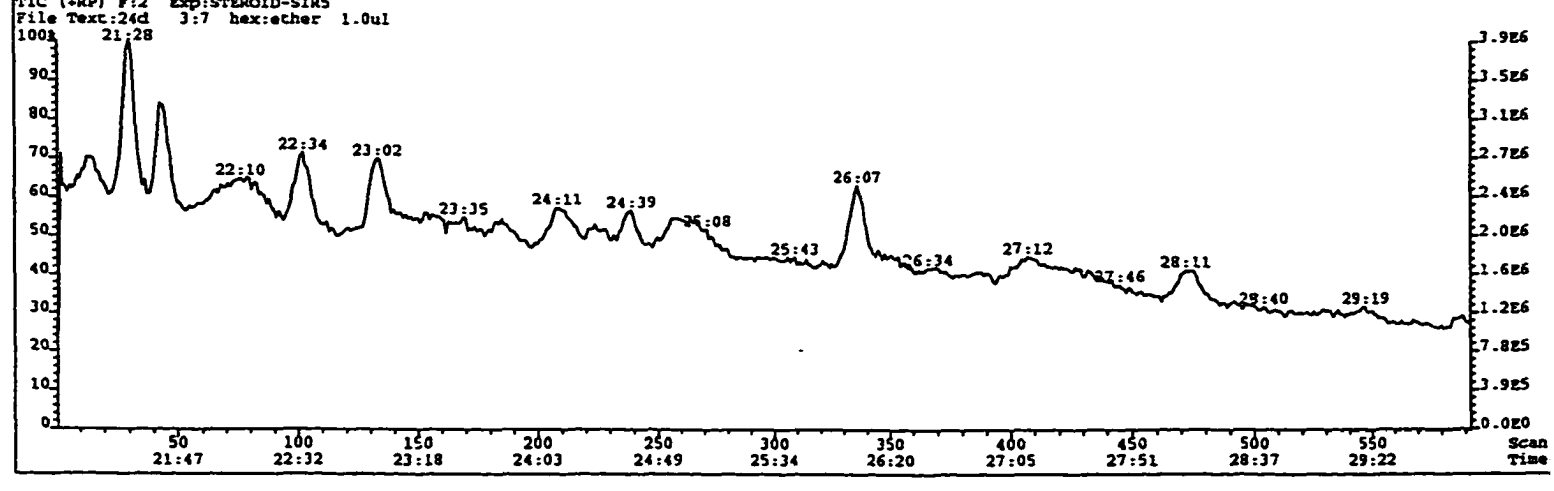
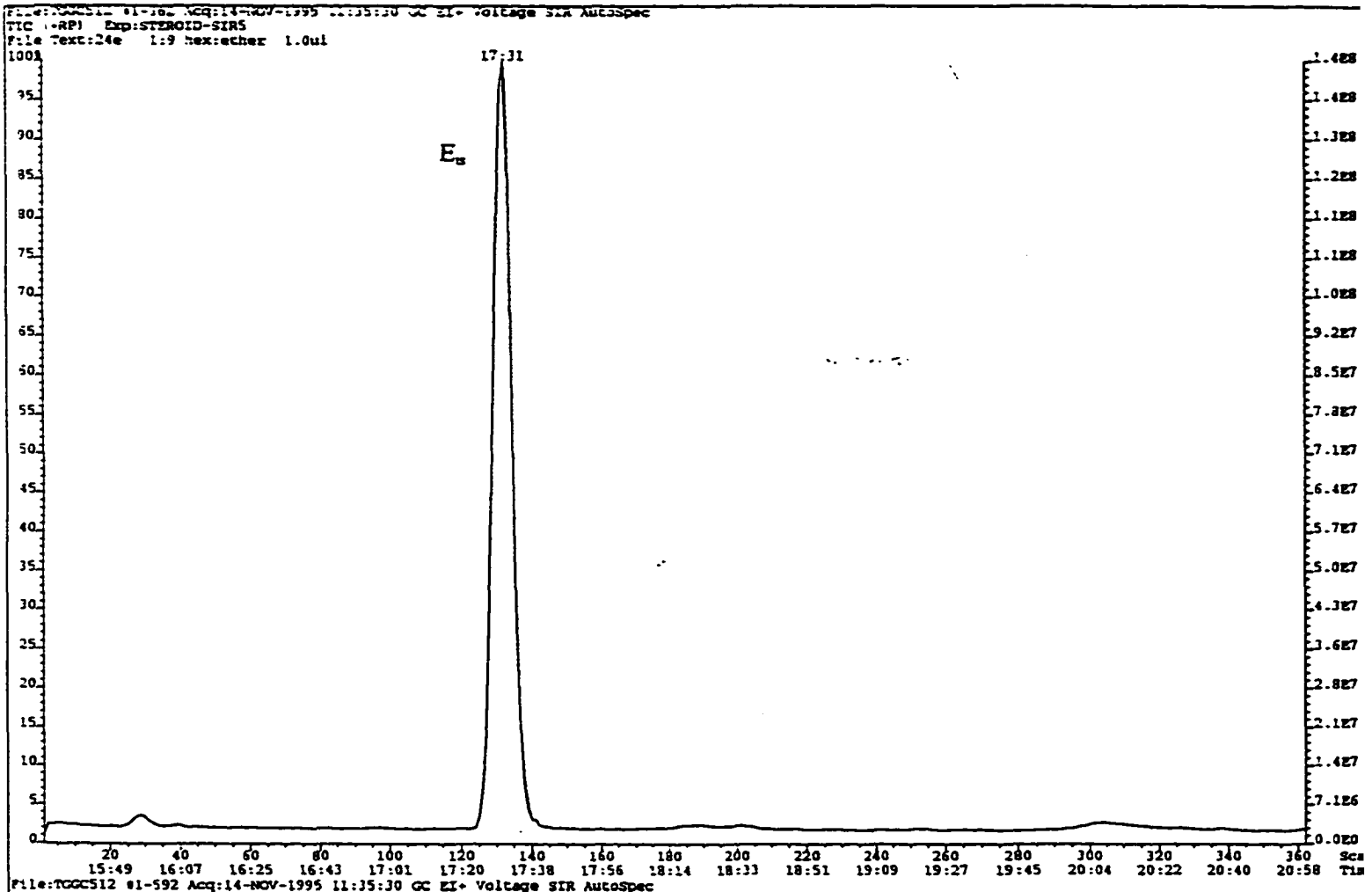
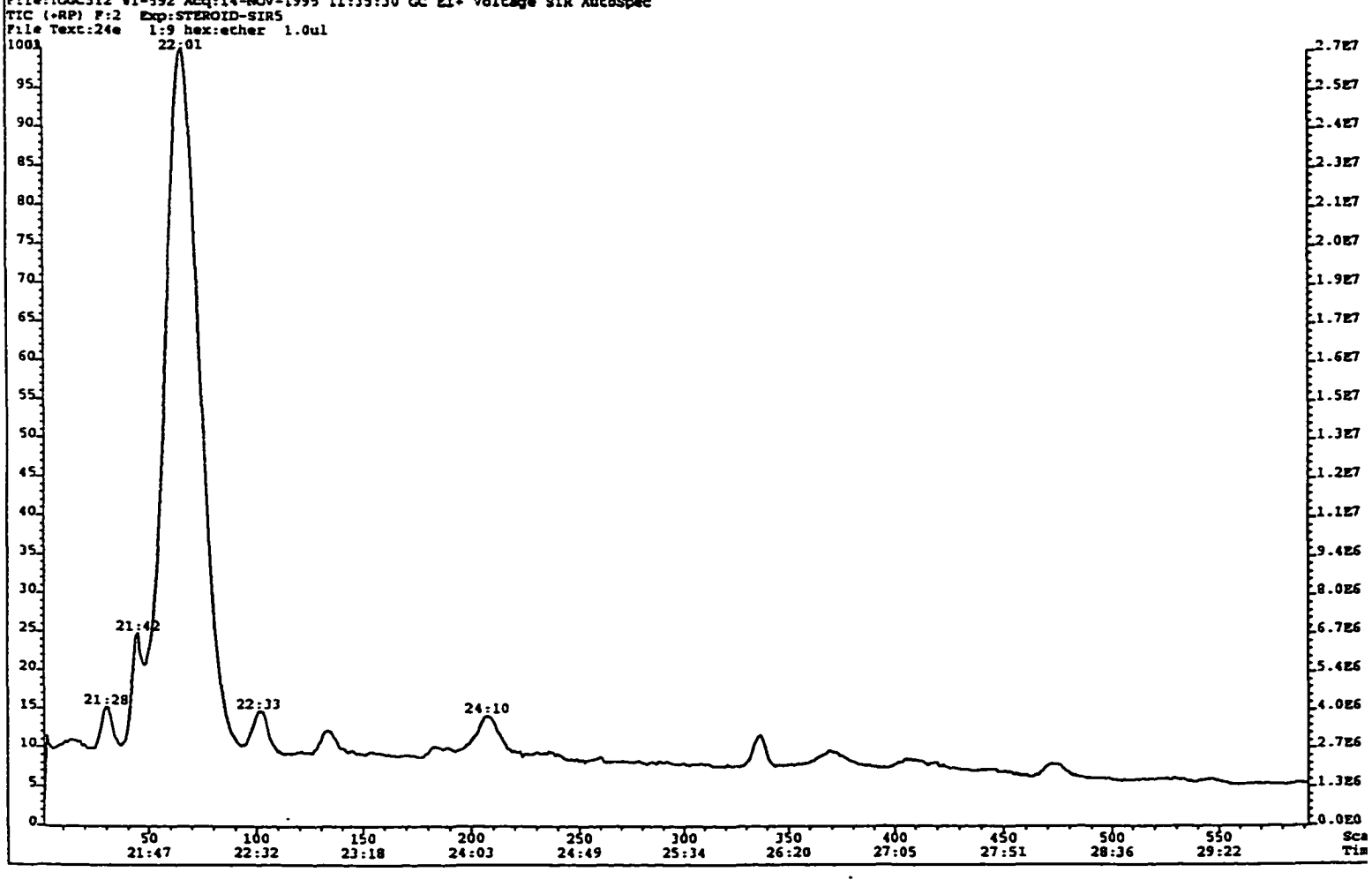


Figure 5.3.2.4: F1 and F2 SIR-TIC chromatograms of 1:9 hexane/t-butyl ether demonstrating that no steroids were eluted. Abbreviations are as follows: D - dehydroepiandrosterone, E - native estradiol, E_{is} - deuterated estradiol as internal standard, A - androstenedione, T - testosterone, PN - pregnenolone, PR - progesterone, 17PN - 17-hydroxypregnenolone, and 17PR - 17-hydroxyprogesterone.





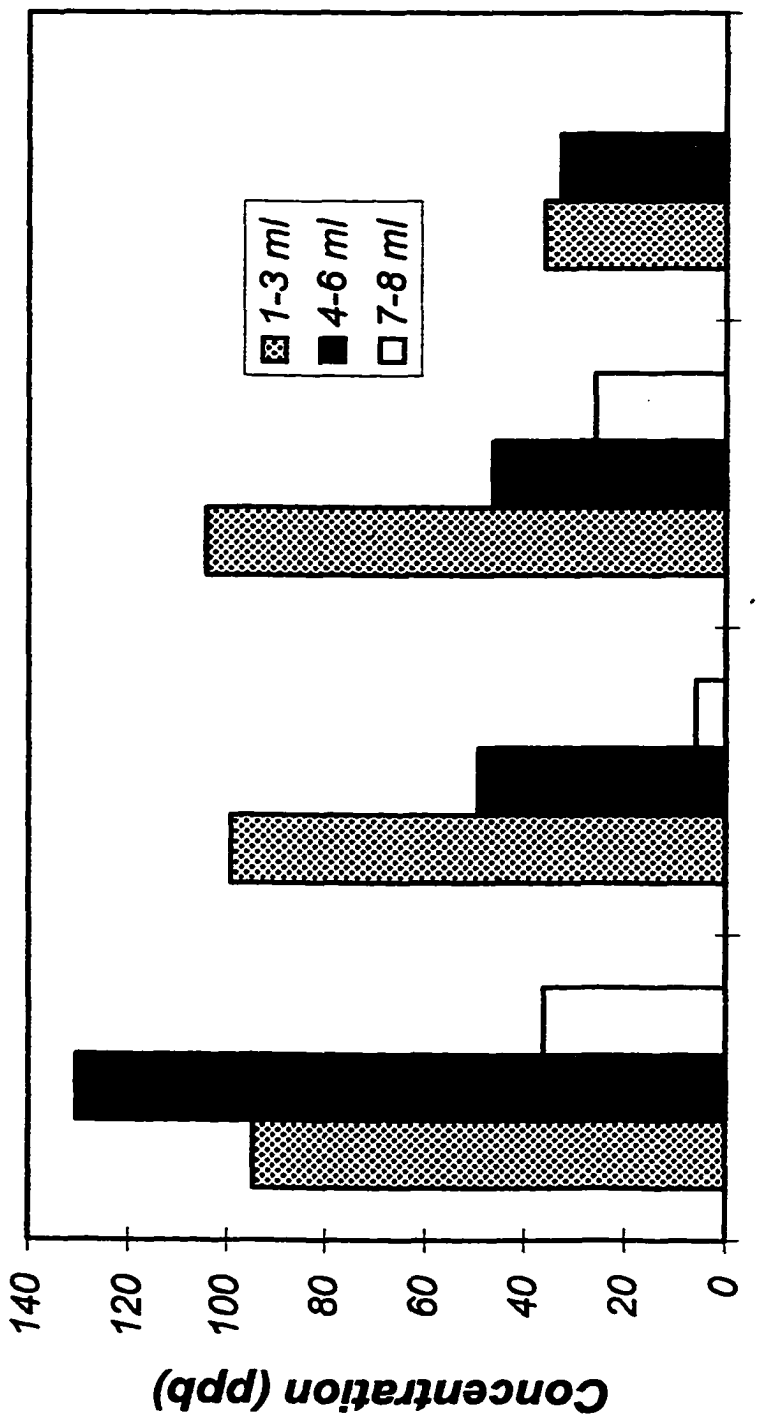


Figure 5.3.2.5: Determination of required eluant (6:3 hexane/t-butyl methyl ether) volume to completely remove testosterone (T), dehydroepiandrosterone (D), estradiol (E), and androstenedione (A) from the C18 SPE column.

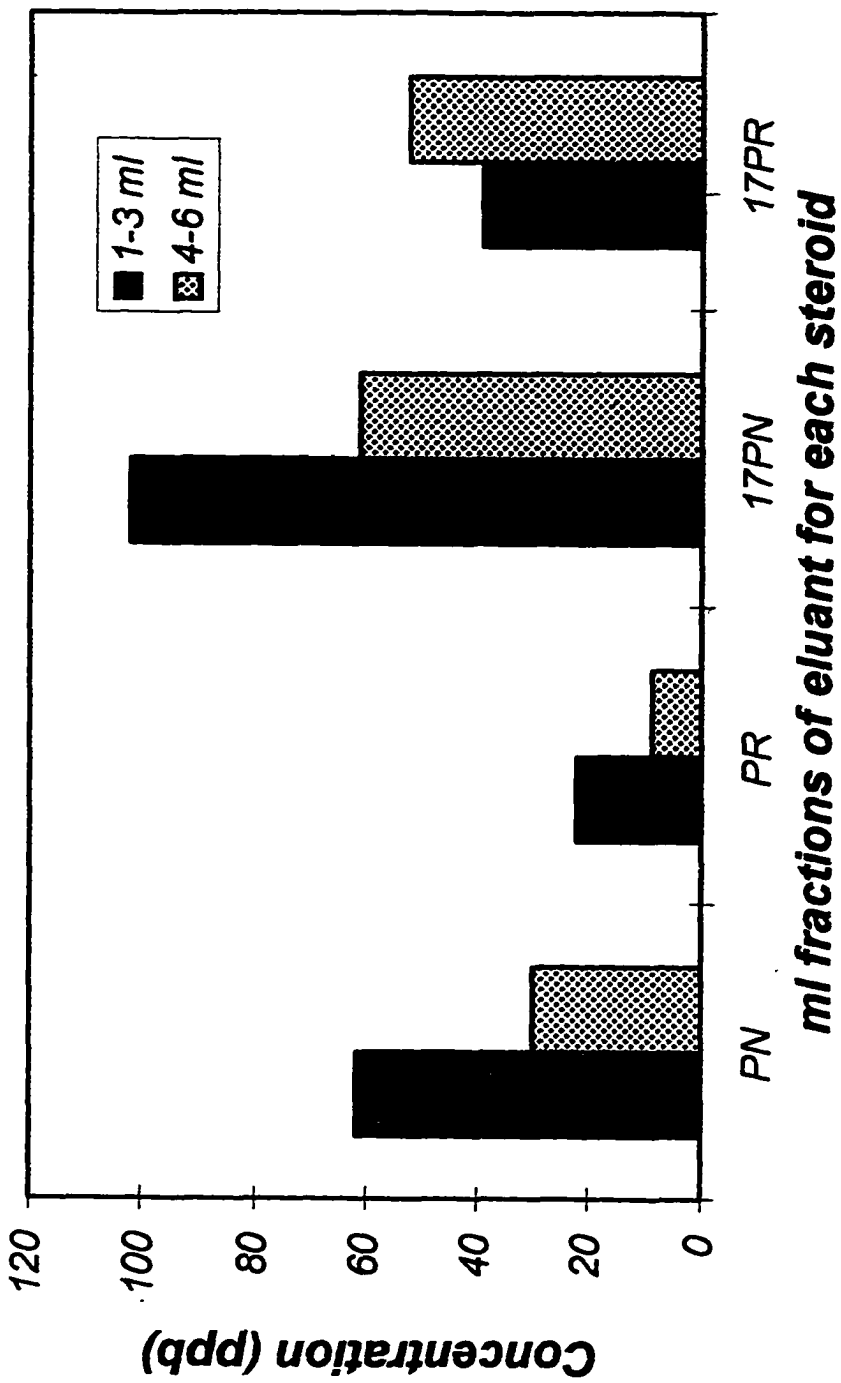


Figure 5.3.2.6: Determination of required eluant (6:3 hexane/t-butyl methyl ether) volume to completely remove pregnenolone (PN), progesterone (PR), 17-hydroxypregnenolone (17PN), and 17-hydroxyprogesterone (17PR) from the C18 SPE column.

5.3.3 GC/ECD Analysis

GC/ECD analysis of steroid profiles was performed according to the parameters outlined in Table 5.2.6 of the Methods section. Retention times were used to identify the presence of a steroid. Retention times were determined by analyzing standards of the individual steroids, combinations of 2-3 steroids, and finally the entire steroid profile. The retention times of the steroids under the above mentioned parameters are summarized in Table 5.3.3.

Table 5.3.3: Retention times of each steroid measured by GC/ECD using 15m DB-1 column of 0.1 U film thickness and 0.25 id. The carrier gas was nitrogen at a head pressure of 25psi.

Steroid	Retention time (minutes)
Estradiol	13:17
Dehydroepiandrosterone	25:27
Androstenedione	27:61
	28:83
Pregnenolone	31:67
17-Hydroxypregnenolone	36:65
Progesterone	42:46
17Hydroxyprogesterone	48:08

5.3.4 GC/MS - SCAN Analysis

GC/MS scans from 60-600 m/z were performed on both the PFB-HFB and MO-TMS steroidal derivatives. The PFB-HFB full-mass scans were performed in an attempt to identify the interfering compounds in the GC/ECD chromatograms. It was hoped that the GC/MS scans of the samples would help elucidate the nature of the interference compounds. Upon running the samples, PFB-HFB derived steroids were found to be very

unstable in the MS high energy environment. Obtaining spectra of the PFB-HFB derived steroids proved to be difficult. The suspected scan spectra of these derived steroids and their structures can be found in Appendix II. For this reason, the focus of the project switched to MO-TMS derived steroids by GC/MS-SCAN and SIR analysis. Scan spectra of the MO-TMS derived steroids examined in this thesis and their structure are in Appendix III.

A significant and unexpected consequence of the ovarian tissue scans was the discovery of a peak with a retention time of 40:30 minutes. The spectral scan of this peak matched that of sitosterol, a plant sterol, in the reference spectra, and subsequently a sitosterol standard (Fig. 5.3.4.1a). Sitosterol was found to be present in fathead minnow ovarian (Fig. 5.3.4.2a) and intestinal tissue (Fig. 5.3.4.2b), as well as the frozen brine shrimp the fathead minnows received as food (Fig. 5.3.4.1b).

5.3.5 GC/MS-SIR Analysis and Confirmation of Steroid

The ions used for selected ion monitoring were determined using spectra obtained by scan mode. A summary of the ions used for SIR analysis and the fragment of steroid they represent can be found in Table 5.3.5.1 where M1 and M2 represent the molecular mass of the two chosen fragments and M^+ is the molecular mass of the steroid.

Figure 5.3.4.1: Experimentally determined mass spectral scans of:
A) sitosterol standard (Sigma, Mississauga, Ontario, Canada) MO-TMS derivatized.
B) sitosterol present in extracted adult frozen brine shrimp (Rolf Hagen, Montreal, Quebec, Canada) used to feed fathead minnow culture.

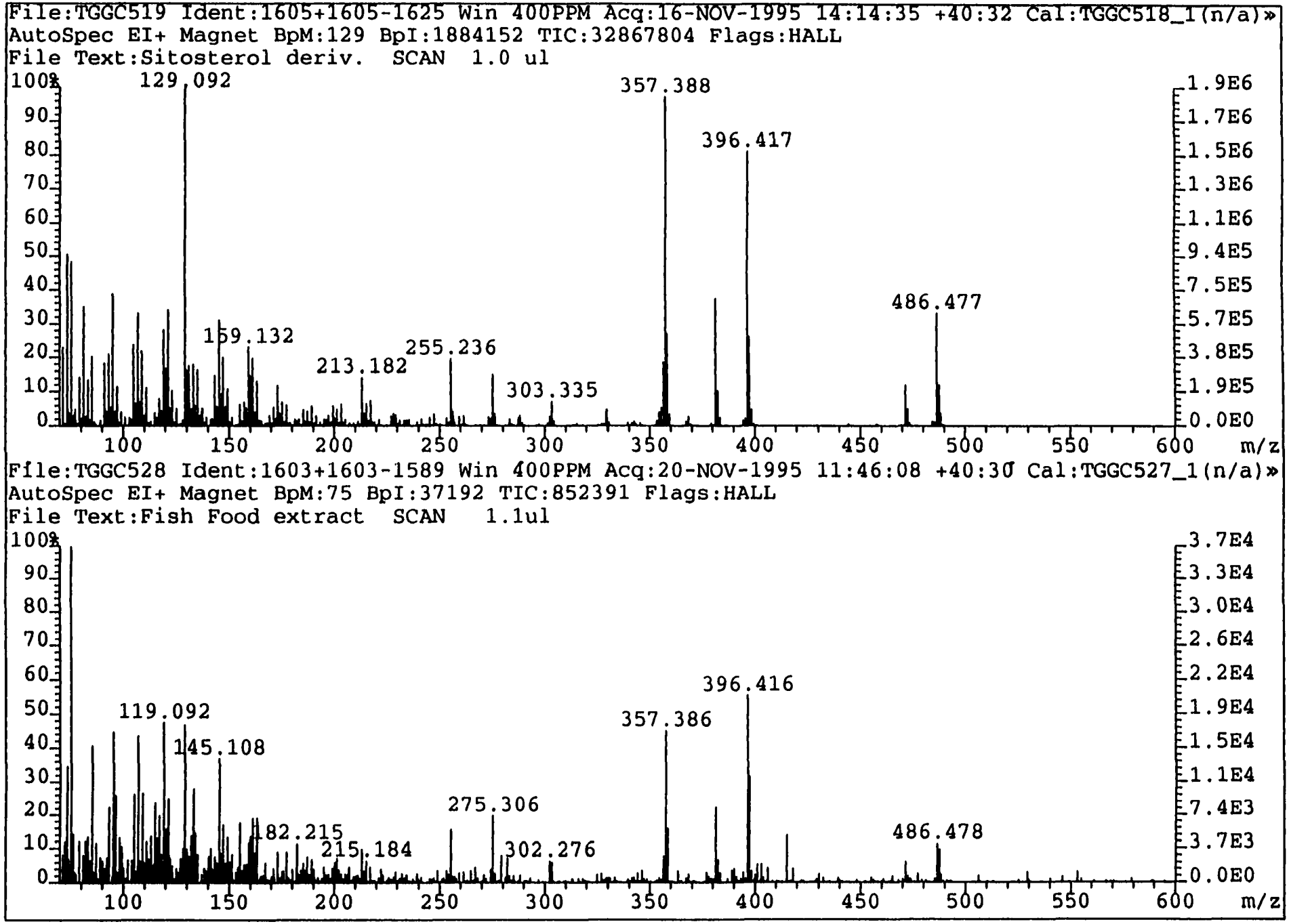


Figure 5.3.4.2: Experimentally determined mass spectral scans of:
A) sitosterol present in extracted fathead minnnow ovaries.
B) sitosterol present in extracted fathead minnow intestines.

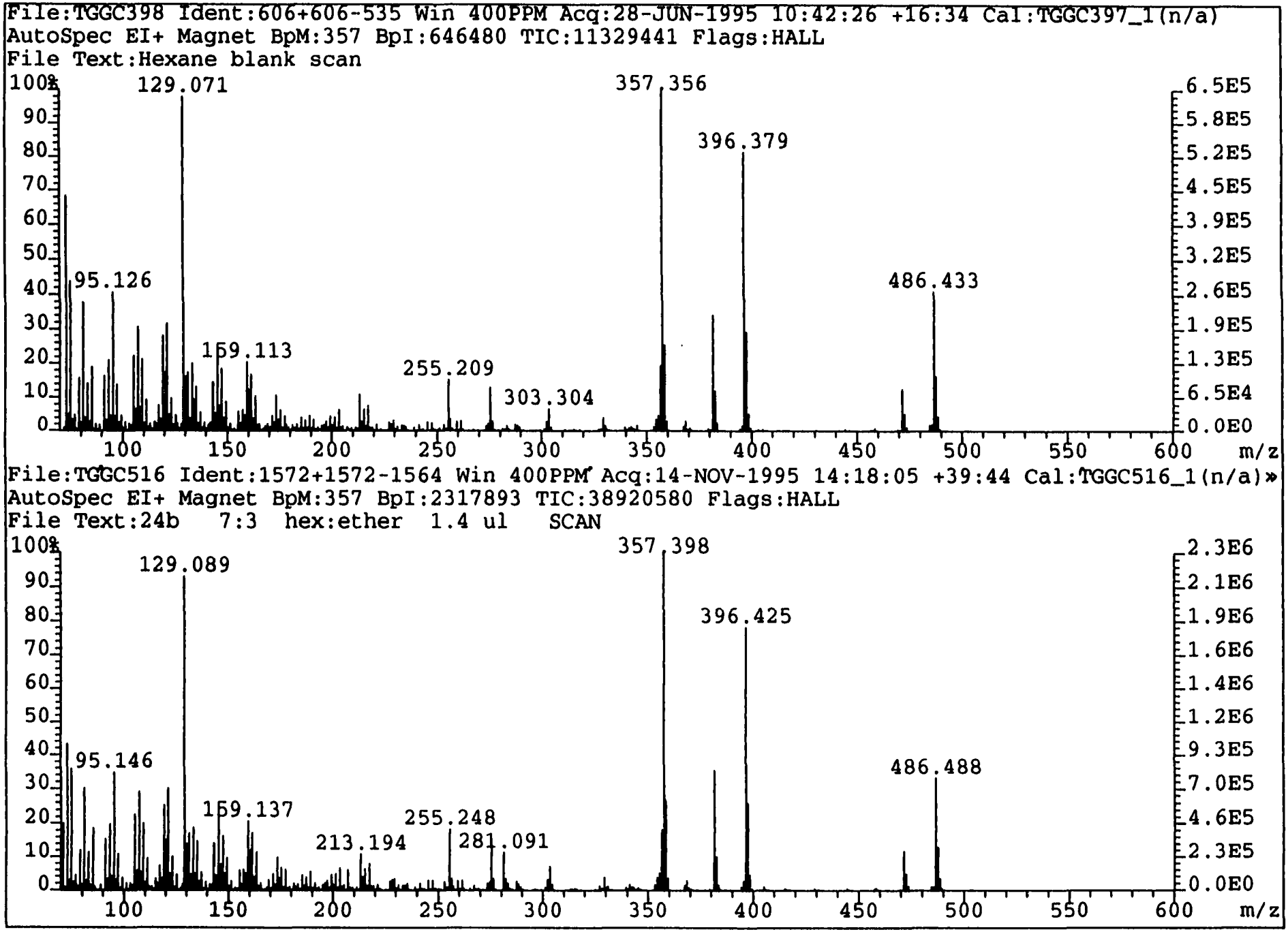


Table #5.3.5.1: Selected ions and their fragments of steroids for selected ion recording analysis by GC-MS.

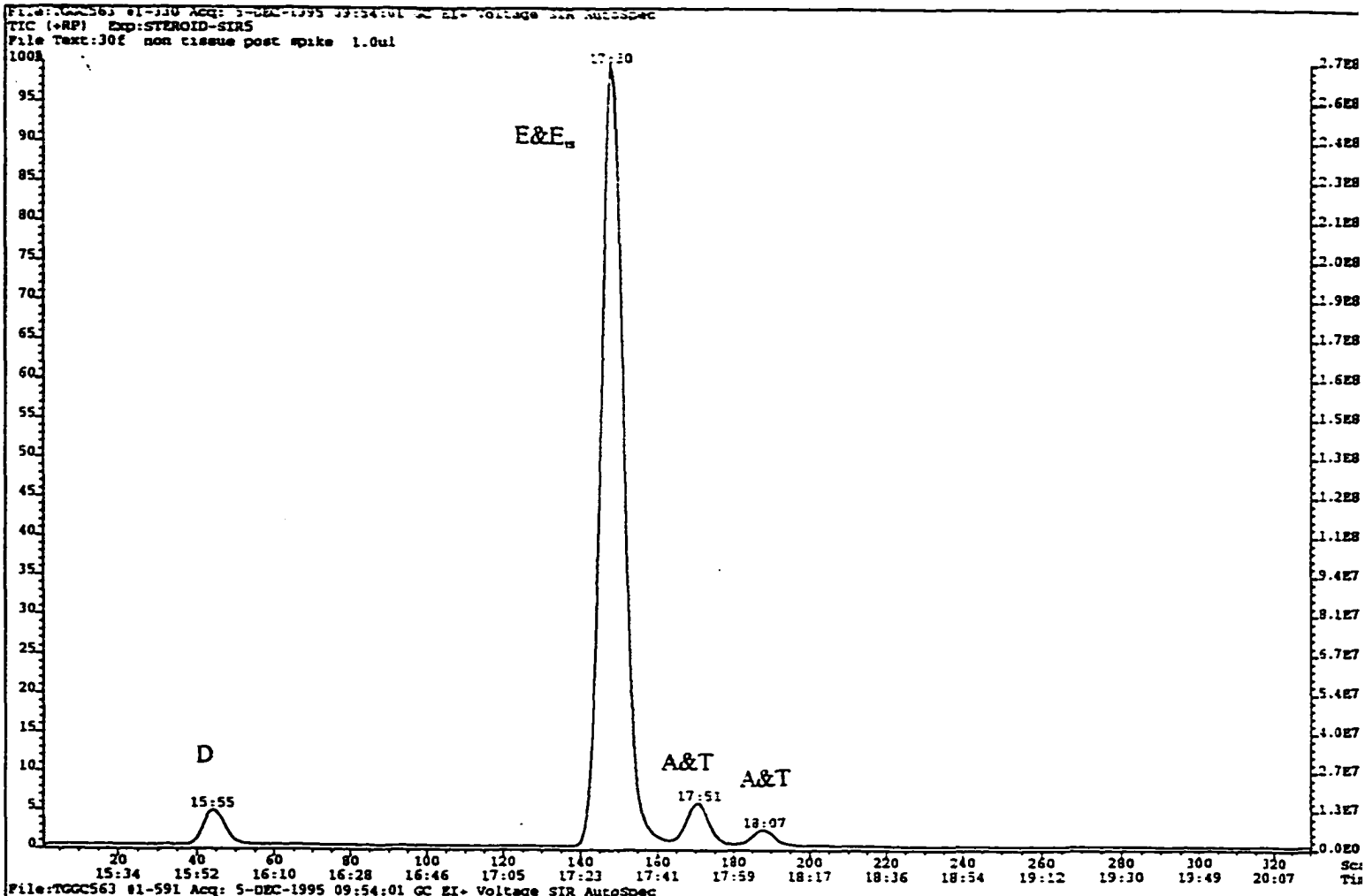
Steroid	MW(M^+)	M1	Fragment	M2	Fragment
Testosterone	389.275	358.257	M^+-OCH_3	389.275	M^+
Dehydroepiandro-sterone	389.275	358.257	M^+-OCH_3	389.275	M^+
Estradiol	416.257	285.167	M^+-C_6OSiH_{15}	416.257	M^+
Androstenedione	344.246	313.228	M^+-OCH_3	344.246	M^+
Pregnenolone	417.306	386.288	M^+-OCH_3	417.306	M^+
17-Hydroxy-pregnenolone	505.341	474.322	M^+-OCH_3	505.341	M^+
Progesterone	372.278	341.259	M^+-OCH_3	372.278	M^+
17-Hydroxy-progesterone	460.3054	429.2855	M^+-OCH_3	460.3054	M^+
Cholesterol	458.399	329.321		368.344	
D_2-Estradiol	418.267	287.1796	M^+-C_6OSiH_{15}	418.267	M^+

Two criteria were met in order to confirm the presence of a steroid in an unknown sample. The retention time and the relative intensities of the selected molecular fragments had to fall within +/- 10% of the expected value as determined by the scan spectra. A summary of the expected values is in Table 5.3.5.2. The TIC chromatogram of an SIR experiment is in Fig. 5.3.5.1 demonstrating steroid retention times. A demonstration of the selected ion chromatograms for pregnenolone (fragments 386 and 417) are in Fig. 5.3.5.2 A&B and for testosterone and dehydroepiandrosterone (fragment 358 and389) in Fig 5.3.5.2 C&D.

Table #5.3.5.2: Retention times and expected ratios of the selected ions for the steroids measured by SIR-GC/MS.

Steroid	Retention Time (min)	M1/M2
Testosterone	17.51	0.40
	18.07	
Dehydroepiandrosterone	15.55	4.00
Estradiol	17.30	0.50
Androstenedione	17.51	0.50
	18.07	
Pregnenolone	21.15	5.00
17-Hydroxypregnenolone	23.43	5.50
Progesterone	23.27	0.55
	23.43	
17-Hydroxyprogesterone	25.43	5.50
Cholesterol	32:42	1.10
D_2-Estradiol (IS)	17.30	0.50

Figure 5.3.5.1: Selected ion recording-total ion current chromatogram of a standard steroid profile indicating retention times. A is function #1 from 14.00 to 20.50 minutes, containing dehydroepiandrosterone (D), native estradiol (E), deuterated estradiol (E_{is}), androstenedione (A), and testosterone (T). B is function #2 from 20.5 to 29.00 minutes, containing pregnenolone (PN), progesterone (PR), 17-hydroxypregnenolone (17PN), and 17-hydroxyprogesterone (17PR).



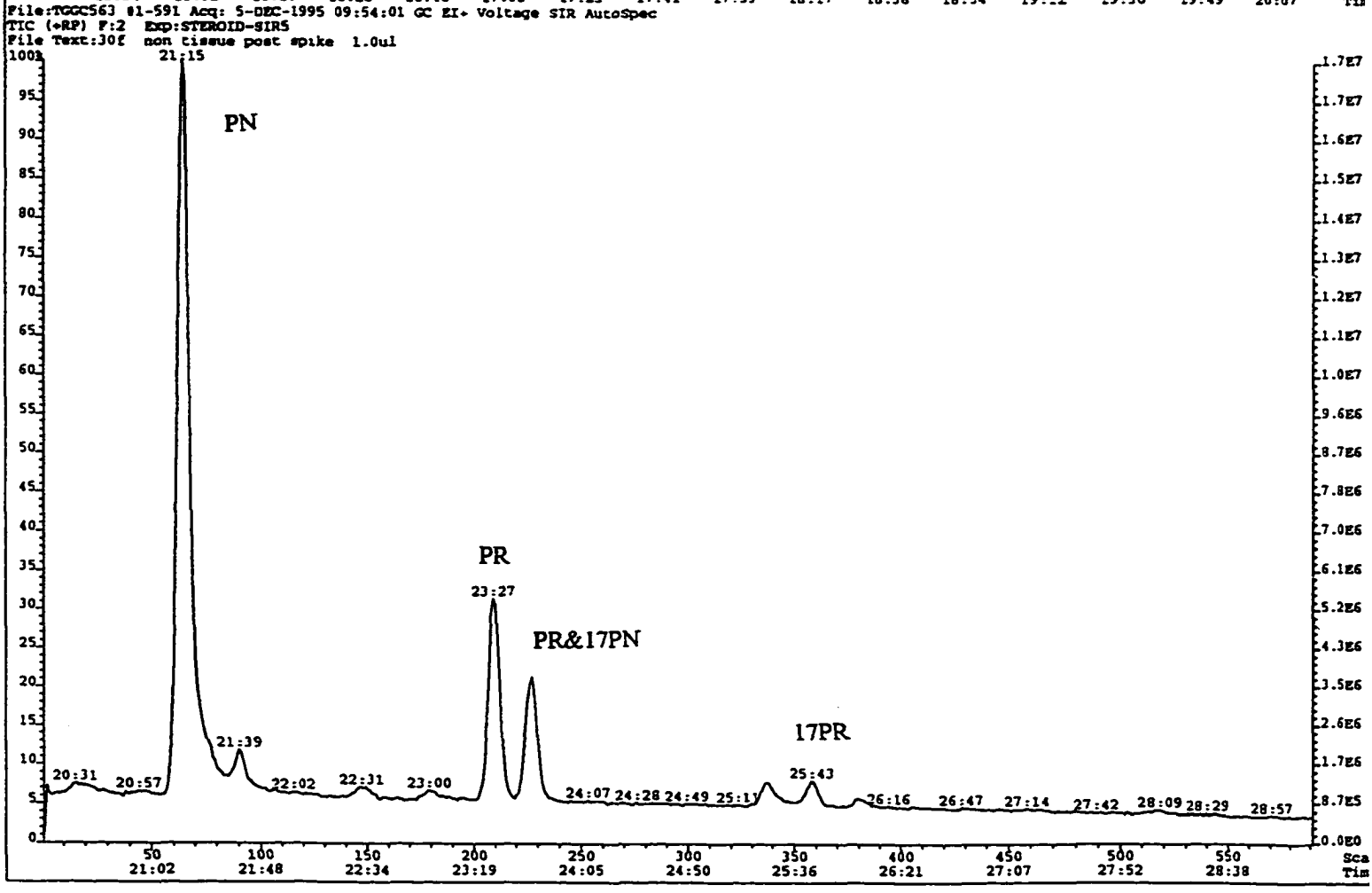
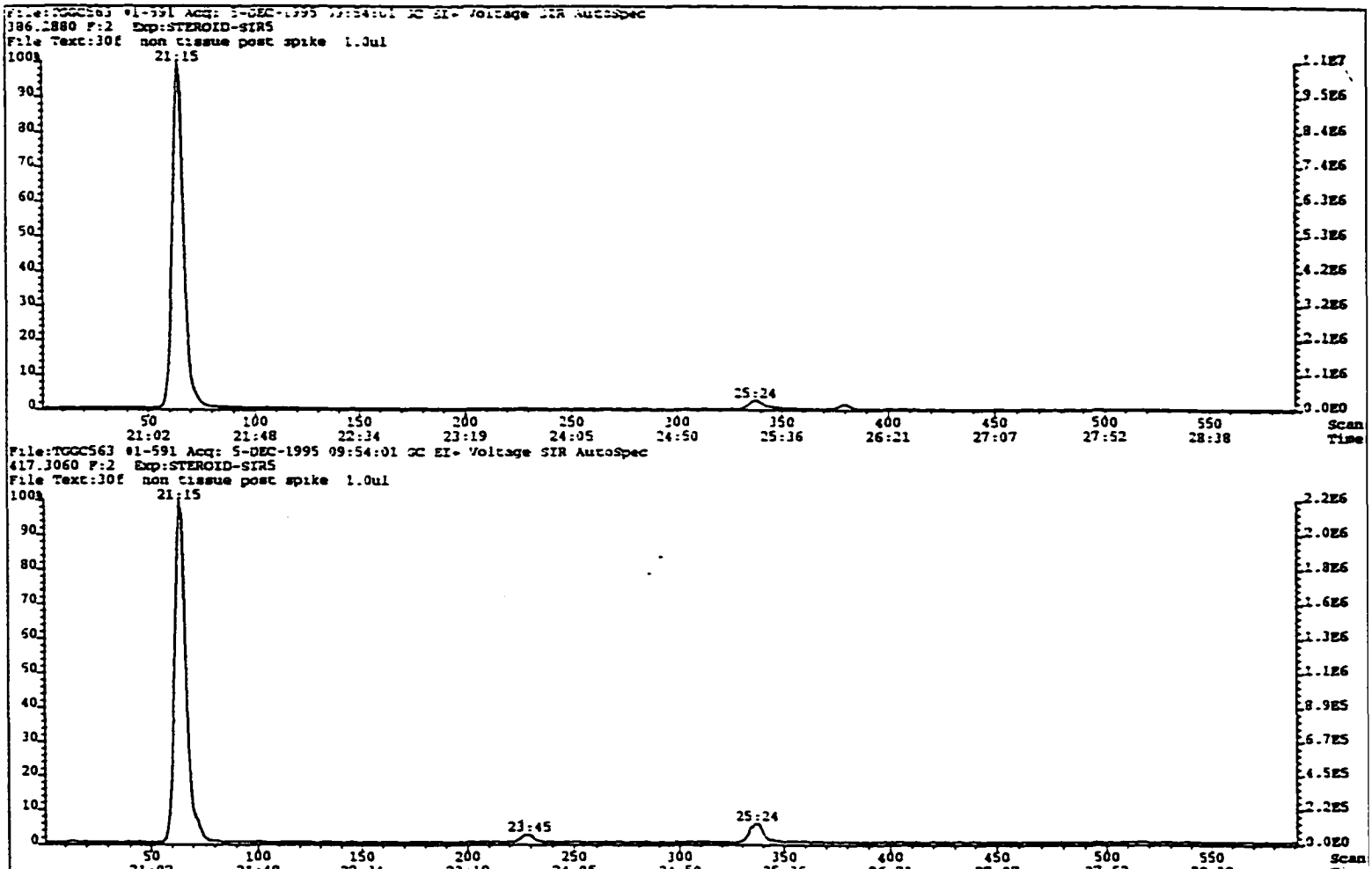
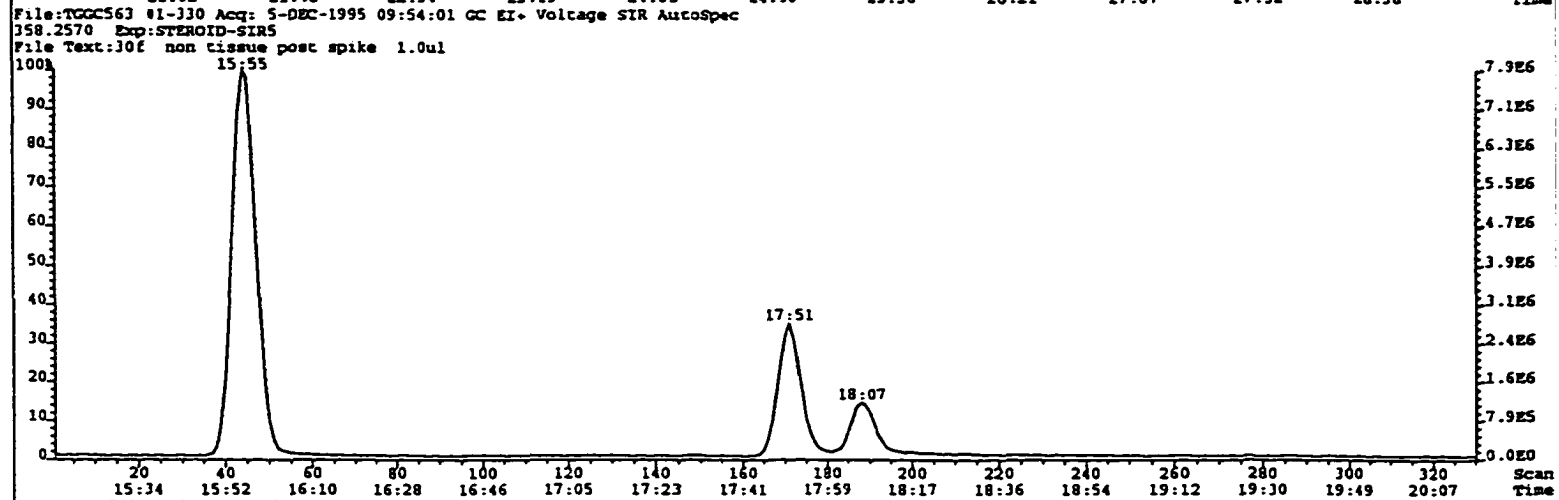
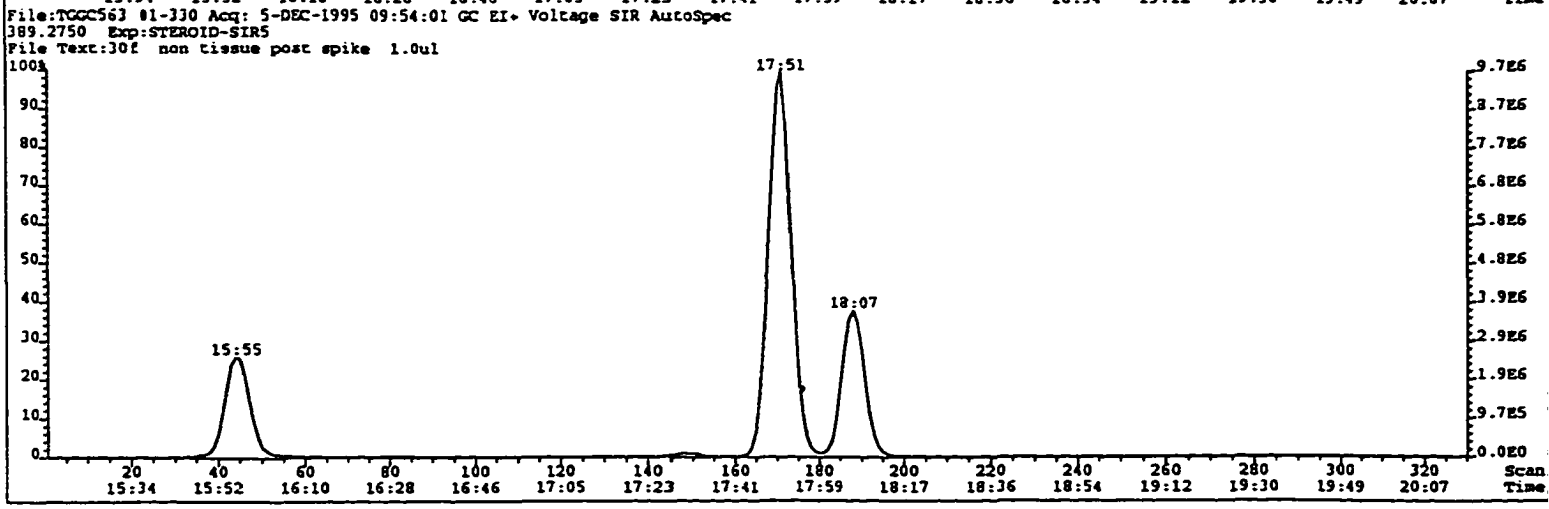


Figure 5.3.5.2: Selected ion chromatograms of M1 and M2 fragments for pregnenolone (A&B, or m/z 386/417), dehydroepiandrosterone and testosterone standards (C&D, or m/z 358/389).







5.3.6 Reproducibility

The reproducibility of the above mentioned protocol was evaluated by calculating the coefficient of variability, or the sample standard deviation expressed as a percentage of the sample mean (Steel and Torrie, 1980, p27), for both non-tissue and tissue sample matrices. The calculated coefficient of variability of the concentration for each steroid in a non-tissue sample matrix was less than 10% (Table 5.3.6.) The reproducibility of the concentration for each steroid in a tissue sample matrix was between 1.4% to 11.7% (Table 5.3.6).

Table 5.3.6: The reproducibility of steroid measurements in both non-tissue and fathead minnow ovarian tissue sample matrices as determined by the coefficient of variability (CV). *n* is the number of independent determinations.

Steroid	CV (%) for non-tissue matrix	CV (%) for tissue matrix
Androstenedione	8.65	4.44
Dehydroepiandrosterone	6.48	7.98
Estradiol	8.65	1.40
Pregnenolone	9.47	5.56
17-Hydroxypregnenolone	8.81	9.94
Progesterone	9.02	3.26
17-Hydroxyprogesterone	9.21	11.70
Testosterone	7.81	4.83
n	4	4

5.3.7 Percent Recovery

The acceptability of the clean up procedure was determined by the percent recovery. Other factors of efficiency such as reproducibility and the extent of removal of interferences to improve detection limit have also been considered. The percent recovery was calculated for both tissue and non-tissue sample matrices. Relative response factors were calculated from clean up samples spiked with the steroid profile and internal standard just prior to derivatization and used to calculate the recovery of steroids in clean up samples spiked with steroid profile prior to sample extraction. The percent recovery of the various clean up procedures for each steroid is summarized in Table 5.3.7.

Table #5.3.7. A comparison of the percent recoveries ± standard deviation between the two main clean up procedures of each steroid in both tissue and non-tissue sample matrices. Protocol #1 is the original cleanup procedure at pH 4.6 using 1 C18 and 2 NH_2 SPE columns. Protocol #2 is the modified cleanup using a 1:1 solvent to tissue extraction repeated 4 times, 1 C18 SPE column, modified wash and elution solvents and no amino SPE column. *n* is the number of independent determinations.

Steroid	% recovery protocol #1 non-tissue	% recovery protocol #1 tissue	%recovery protocol #2 non-tissue	%recovery protocol #2 tissue
Testosterone	87.5±10.8	not detected	100.1±7.8	101.4±4.9
Dehydroepiandrosterone	87.2±2.7	43.7±11.9	93.1±6.0	87.3±7.0
Estradiol	93.7±4.7	30.9±8.4	87.4±5.3	81.4±1.1
Androstenedione	92.1±5.8	not detected	93.5±8.1	74.0±3.3
Pregnenolone	97.9±2.8	37.7±14.3	83.1±7.9	99.9±5.6
17-Hydroxypregnenolone	90.8±13.9	58.4±10.6	80.9±7.1	94.2±9.4
Progesterone	99.5±6.8	not detected	82.3±7.4	95.0±3.1
17Hydroxyprogesterone	80.4±16.6	not detected	87.5±8.1	81.6±9.5
n	3	3	5	4

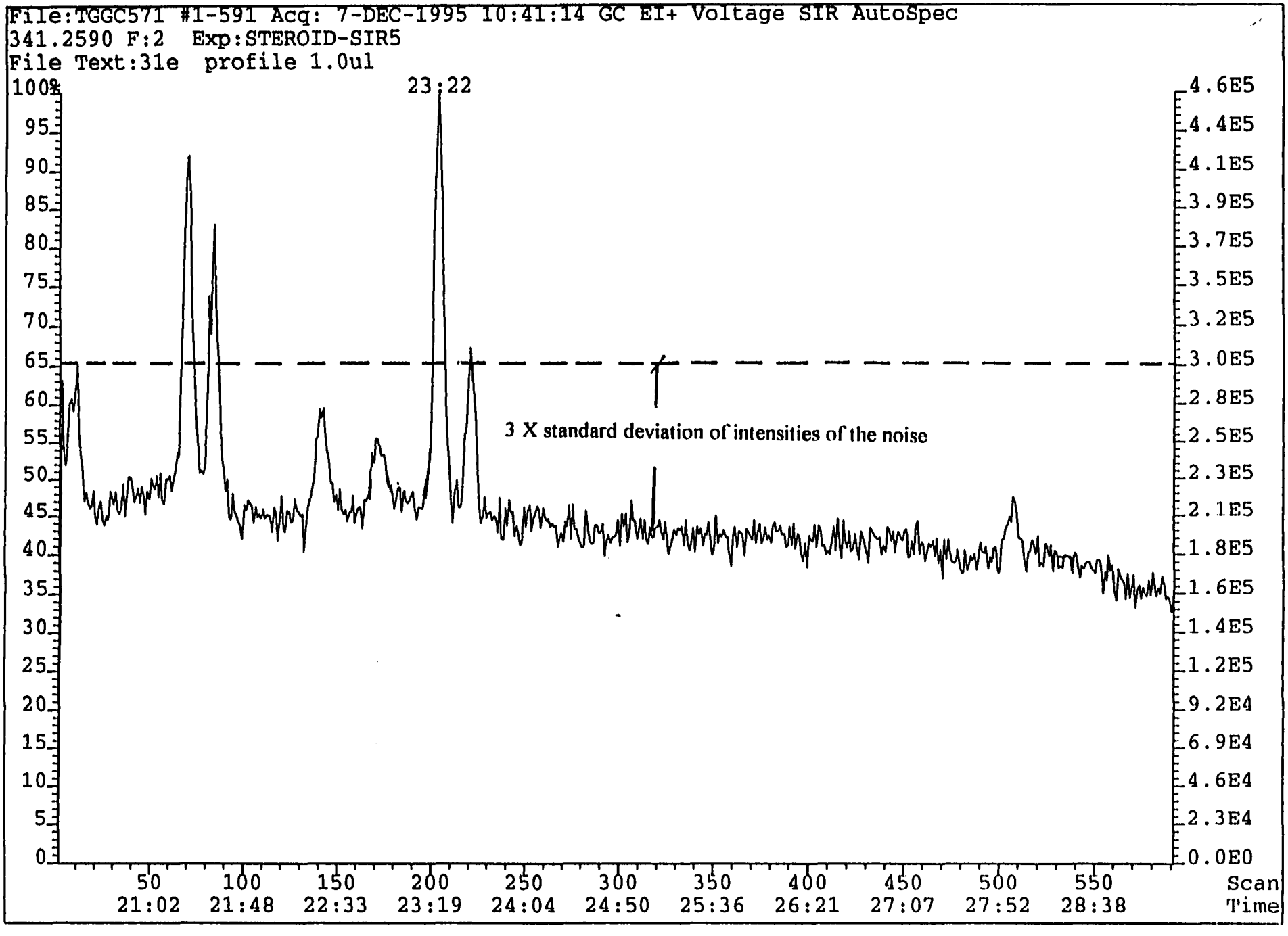
5.3.8 Detection Limit

The quantitation limit was calculated for each steroid by determining the concentration of steroid in tissue with area of three times the background noise (Fig. 5.3.8.1) by using the linear regression of increasing concentrations plotted against the signal area to noise area ratio. This level was considered the detection limit because although the peaks were observable below this limit, quantification and identification were not possible. The noise was calcuated by the standard deviation of intensities method. Since two ions were selected for each steroid, the ion with either the highest level of background, or the lowest slope in the linear regression, was chosen for the calculation. A summary of the detection limit for each steroid is in Table 5.3.8. Regression analyses for the detection limit of each steroid are in Appendix IV.

Table #5.3.8. The detection limits for steroids of interest from SIR analysis on a GC\MS.

Steroid	Detection Limit(ppb)
Testosterone	24
Dehydroepiandrosterone	1
Estradiol	1
Androstenedione	129
Pregnenolone	1
17-Hydroxypregnenolone	31
Progesterone	103
17-Hydroxyprogesterone	53

Figure 5.3.8.1: SIR-M1 chromatogram of progesterone, illustrating peaks at and above three times the standard deviation of intensities of the background noise.



5.3.9 Steroid Concentration in Ovarian Tissue

A one gram sample was prepared from fathead minnow ovarian tissue following protocol #2. Pregnenolone, androstenedione, dehydroepiandrosterone, estradiol and testosterone were all present in the sample according to the two criteria of retention time and selected ions relative abundance. Testosterone (1.60 pg steroid/mg tissue), dehydroepiandrosterone (6.43 pg steroid/mg tissue), and androstenedione (3.92 pg steroid/mg tissue) were present at levels below detection limit. Areas under the curve for these steroids were obtainable but the peak heights were less than three times the background noise. Pregnenolone (19.2 pg steroid/mg tissue) and estradiol (8.42 pg steroid/mg tissue) were present at levels above detection limit. Progesterone, 17-hydroxypregnenolone, and 17-hydroxyprogesterone were not detected.

Table 5.3.9.1: Concentration of steroid intermediates found in 1 gram of fathead minnow ovarian tissue using SIR-GC/MS detection and finalized protocol #2 clean up procedure. n=1

Steroid	Concentration (pg/mg tissue)
Pregnenolone	19.2
Progesterone	not detected
17-Hydroxypregnenolone	not detected
17-Hydroxyprogesterone	not detected
Androstenedione	3.92
Dehydroepiandrosterone	6.43
Testosterone	1.6
Estradiol	8.42

5.4 DISCUSSION

Steroid biosynthesis is catalyzed by membrane-bound enzymes which hinder the isolation of steroids from biological tissue. Biological membranes are composed mainly of a combination of protein and lipid with a weight ratio of protein to lipid ranging from 1:4 to 4:1 (Stryer, 1988, p283). Steroids belong to the large group of molecules called lipids. Although steroids are not amphiphatic, most membrane lipids are amphiphatic molecules with a hydrophilic moiety on one end and a hydrophobic moiety on the other (Stryer, 1988, p288). Other categories of lipids present in membranes include phospholipids, and glycolipids. These compounds contain long, amphiphatic fatty acid chains ranging from 14 to 24 carbon atoms in length. The most common fatty acids are palmitic (C_{16}) and oleic (C_{18}) and vary in the degree of saturation (Stryer, 1988, p285).

Lipids, such as free fatty acids and steroids, are soluble in a wide range of nonpolar solvents ranging from methanol to ether. Methanol was chosen as the extraction solvent for many reasons. It removed the more polar lipids from the tissue while still effectively extracting the neutral steroids. The more nonpolar lipids tended to remain unextracted from the tissue. Methanol is also miscible with aqueous buffers, such as acetate and phosphate, used in this protocol development. The principle by which solid phase extraction (SPE) columns function requires that the steroids be solubilized in the most polar solution possible at an optimum pH. This ensures that the steroids are more strongly attracted to the SPE sorbent than the sample

solvent. Methanol has been used as a steroid extraction solvent in preparation for SPE clean up of human urine (Schmidt *et al.*, 1985).

The methanol and aqueous buffer mixture was also effective in freeing protein-bound steroids into solution. Most proteins tend to be larger than 60 angstroms, the pore size of standard bonded SPE columns phases, and are unable to penetrate the SPE column. This could result in a loss of steroid recovery since steroids tend to be protein-bound. Steroids can be released from protein by either adjusting the pH or adding an organic solvent, such as methanol or acetonitrile (Zief and Kiser, 1994). In this case, adjusting the pH to 4.6 should ensure the precipitation of protein and the release of any protein-bound steroids into solution (Zief and Kiser, 1994). Adjustment to pH 6.5 is close to the physiological pH and may not effectively release the steroids. The combination of methanol and aqueous phosphate buffer however, ensures that the steroids are released into solution for further sample clean up.

Steroid recovery using the methanol/aqueous buffer solution, whether at pH 4.6 or pH 6.5, was low (<58% for the detected steroids) in sample matrices. Recovery in non tissue sample matrices was quite high (80.4% to 99.5% for protocol #1 at pH 4.6, and 82.3% to 100.1% for protocol #2 at pH 6.5) suggesting that either compounds in the sample matrix interfered with the clean up procedure, or the steroids were ineffectively extracted. Tissue to solvent ratios of 1:10, 1:4 and 1:1 consistently resulted in low recoveries when only one extraction was taken. Repeated extractions of the 1:1 tissue to solvent ratio resulted however in a substancial increase

in percent recovery (74% to 100% depending on the steroid). It was found the 4 removals of the 1:1 tissue to solvent ratios were most effective in increasing steroid recovery while still maintaining a low sample volume.

Steroid recovery was also affected by the method used to release steroids from the tissue. Both homogenization and sonication were evaluated as means to remove steroids from the tissue. Homogenization of the soft ovarian tissue was difficult because the tissue was easily compressed at the bottom of the homogenization tube. The method also required sample transfer steps from the homogenization tube to a centrifuge tube which had the potential for steroid loss. To minimalize such loss, methanol rinses were required and pooled resulting in enlarged sample volumes. Sonication, on the other hand, was very successful in disrupting the soft ovarian tissue. Sonication disrupted protein binding and therefore facilitated the solubilization of any protein-bound steroids. Another benefit of sonication was a decrease in sample transfer steps since the tissue could be sonicated directly in the centrifuge tube. Sonication of tissue resulted in less steroid transfer, and less solvent rinsing, and greater steroid recovery.

The overwhelming presence of lipids in the methanol extract created the difficult problem of isolating very small quantities of steroids for analysis. Two important objectives of sample preparation after extraction from tissue are clean up and concentration (Zief and Kiser, 1994). Sample clean up is required to remove the phospholipids, glycolipids, fatty acids and proteins

from the sample matrix so they do not interfere with steroid measurement. Steroid concentration is required to increase the amount of steroid per unit volume of solvent for direct measurement. The development of a protocol to isolate steroids from tissue for analysis requires knowledgeable characterization of the sample matrix and evaluation of the analytical requirements in order the fulfil the two objectives.

Sample clean up of the ovarian extracts was accomplished by using solid phase extraction columns containing C18 reverse phase, and amino ion exchange packings. C18 columns bind long chain hydrophobic molecules such as steroids and free fatty acids (Zief *et al.*, 1982). The columns have been used to isolate steroids from human urine (Schmidt, *et al.*, 1985), blood, ovarian follicle fluid, and amniotic fluid (Vanluchene and Vandekerckhove, 1988), and beef muscle (Steinhart and Hartwig, 1994). They have also been used to separate nonpolar and polar lipid classes from wheat flour (Prieto *et al.*, 1992), and to isolate free fatty acids from milk and cheese (de Jong and Badlings, 1990).

Effective sample clean up using solid phase extraction (SPE) depends on the physical and chemical nature of the steroids and coextractives in the sample matrix. Scan analyses of extracted ovarian tissue revealed that the majority of contamination arose from free fatty acids (FFAs) such as palimitic and oleic acids. Manipulation of the sample matrix pH was an effective means of eliminating these contaminants. In protocol #1, the sample matrix pH was adjusted to pH 4.6 using a boric acid-acetate buffer. This pH was unsuccessful at

eliminating the FFAs. This was not surprising considering the pKa, or the log of the dissociation constant of an acid, of FFAs is around 4.5. By definition of pKa, about 50% of the FFAs were deionized and the remaining 50% were ionized at pH 4.6. In essence, the contaminants were in two different forms with different physical properties. This made steroid isolation more difficult. In protocol #2, the sample matrix was adjusted to pH 6.5, or 2 pH units + pKa (Zief and Kiser, 1994), using a phosphate buffer. At this pH, nearly 100% of the FFAs were ionized. Ionization of the FFAs made them more water soluble and therefore less likely to be eluted with nonpolar solvents.

Subsequently, polarity of the eluant was important in optimizing the clean up procedure. In protocol #1, ethyl acetate was used as the eluant. Ethyl acetate has an eluotropic strength, or eluting solvent strength on silica, of 0.45 (Zief and Kiser, 1994). It successfully removed the steroids from the C18 packing along with many of the more polar lipids. I sought a less polar solvent to reduce impurity levels. T-butyl ether has an eluotropic strength of 0.29 and is much less polar than ethyl acetate (Zief and Kiser, 1994). 8 mls of 7:3 hexane/t-butyl ether was ideal in successfully eluting the neutral steroids while leaving the more polar interferences still adsorbed to the C18 SPE column. The selected ion recording-total ion current (SIR-TIC) chromatogram of the 7:3 hexane/ether eluant (Fig. 5.3.2.2) demonstrated the effectiveness of this clean up procedure.

With the clean up procedure optimized, steroid concentration of the sample was possible. Steroids are found at very low levels in tissue. It was therefore necessary to optimally concentrate the steroids in a sample for adequate detection. Concentration was accomplished by determining the maximal amount of tissue per sample. The amount of tissue per sample was limited by the SPE column capacity. Once column capacity was surpassed (>1.0 g), breakthrough occurred and the steroids passed through the C18 SPE column without being retained (Figs. 5.3.1.1 and 5.3.1.2). This resulted in lower recovery, increased variability, and misleading results. It was for this reason that the optimal amount of tissue per sample was set a 1.0 g ovarian tissue.

The extraction, clean up and concentration procedures were evaluated by measuring the percent recovery and reproducibility of the finalized clean up procedure. The % recovery in the final protocol (#2) was 74.4% to 101.4%, and 80.9% to 100.1% for tissue and non-tissue sample matrices. This was a big improvement over the percent recovery using protocol # 1 which ranged from 30.9% to 58.4% and 80.4 to 97.9% for tissue and non tissue sample matrices, respectively. The low recovery using this initial procedure indicated that the presence of coextracted tissue components in the sample matrix affected the clean up procedure, either during steroid extraction from tissue or from overload of column capacity. The number of tissue extractions in methanol had the largest effect on % recovery as previously discussed, improving recovery from <58%, depending on the

steroid, with one extraction (protocol #1) up to 100%, depending on the steroid, with four extractions (protocol #2).

Minimalizing sample transfer steps during the extraction and sample clean up also increased steroid recovery. The amino columns, which decreased % recovery up to 10%, depending on the steroid, were eliminated from the clean up procedure because they did not effectively remove any of the interfering contaminants. Drying the eluant with anhydrous sodium sulphate was also eliminated in order to minimalize sample transfer steps and to avoid any possible steroid loss by adsorption on the sodium sulphate. Instead, the C18 SPE columns were dried under nitrogen and washed with hexane before elution. This successfully dried the eluant and also increased the reproducibility of the protocol.

Reproducibility of the clean up procedure was also affected by numerous factors. Elution with immiscible solvents (such as water followed by ethyl acetate in protocol #1) increased variability (Zief and Kiser, 1994). By drying the column under nitrogen and washing with hexane just prior to elution with hexane/ether, reproducibility improved. Reproducibility of the clean up procedure was also improved by slowing down and stabilizing the extraction and elution flow rate (Zief and Kiser, 1994). The extraction and elution flow rate should not exceed 5 ml per minute (Zief and Kiser, 1994). A flow rate of 3.5 ml per minute was chosen which is well below the suggested maximum. This was consistently maintained throughout the sample clean up. Reproducibility of protocol #2 varied,

depending on steroid, from 6.48% to 9.47% and 1.40% to 11.7% for non-tissue and tissue sample matrices, respectively, indicating that coextracted tissue components did not affect the clean up reproducibility. Schmidt *et al.*, 1985, measured steroid metabolites in urine using protocol # 1 and the reproducibility ranged from 5.2% to 11.3%. These values are quite consistent with those found in other papers and reflect the reproducibility of this type of sample clean up.

Detection of the low levels of isolated steroids required a very sensitive analytical technique. Gas chromatography combined with an electron capture detector (GC/ECD) was the initial method chosen. A GC/ECD is very sensitive to detecting halogenated compounds. It has been used to detect fluorinated steroids at the picogram level (Walle and Ehrsson, 1970; Challis and Heap, 1970; Koshy *et al.*, 1975). The steroids were fluorinated by two different derivatization reactions. O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBA) was used to derivatize the C_3, C_{17} and C_{20} ketone groups present on the various steroids. The second derivatization involved the use of heptafluorobutyric anhydride (HFBA) which reacted with the C_3, C_{17}, and C_{20} hydroxyl groups. The combined reactions were successful in fluorinating all the steroids analysed but the resultant derivatives were thermally unstable and degraded after 24 hours. Scan analysis by GC/MS of the unstable fluorinated steroids was very difficult because they degraded within the instrument. The scan spectra that were obtained are in Appendix II. Confirmation of these spectra was not possible since the two derivatizations have never

been combined before but some of the major ions for each spectra were identified (Appendix II). The combination of these problems resulted in a search for an alternative method of chromatographic detection.

Selected ion recording on a GC/MS of MO-TMS derived steroids was the second method of steroid detection investigated. SIR-GC/MS is a powerful detection method capable of detecting certain compounds at the picogram level. MO-TMS derived steroids were ideal for this type of analysis since they are very stable. Scan spectra of the derived steroids (Appendix III) were easily obtained and verified with reference spectra. For SIR analysis, abundant, high mass ions were chosen. The selected ions' relative abundance was determined experimentally since the detector's relative sensitivity between ions varied. In most cases, the experimentally determined relative abundance was as expected from scan spectra. 17β-Estradiol and d_2-17β-estradiol were the exception, with an experimentally determined relative abundance of 0.95 and an expected relative abundance of 0.5. Schoonen *et al.*, (1989a & b) analysed TMS-derived estradiol under similar conditions and reported a similar experimentally determined relative abundance of 0.97. Overall, detection of MO-TMS derived steroids using SIR-GC/MS proved promising.

SIR-GC/MS detection limits ranged from 1 ppb for dehydroepiandrosterone, 17β-estradiol, and pregnenolone to 129 ppb for androstenedione (Table 5.3.8 of Results section), over a hundred fold difference. The high variability in detector response could

have been an artifact of varying sensitivity for the selected ions but this is unlikely because detector sensitivity for A, PR, 17PN, and 17PR was low even in SCAN analyses.

The low detector sensitivity to certain steroids seemed to be related to the structural or conformational factors associated with the MOX derivatives. GC/MS of MOX derivatized steroids indicated complete derivitization with no evidence for underivitized steroids. Nevertheless, steroids containing two methoxyamine groups, such as A and PR, had the lowest sensitivity compared to the TMS-derived steroids in the C_3 position, such as E, D, and PN. MO-derived steroids in the C_3 position however, seemed to cause cis and trans isomers of A, PR, and T. The lower sensitivity of T (detection limit 129 ppb) compared to E and D appears to arise exclusively from conformational or isomeric structures at the C_3 carbonyl MOX derivative. The additional loss in sensitivity for A (129 ppb) and PR (103 ppb) appears to arise from the addition of a second MOX derived carbonyl which may result in isomeric or confomational forms not observed in GC/MS. Support for the hypothesis is provided by the intermediate sensitivity of 17PN (31 ppb) and 17PR (53 ppb) MOX and TMS derived structures in which the 17-TMS group may block confomational or isomeric variation at the 17-MO group. Replacement of the MOX reaction may provide scope for increasing the sensitivity of A and PR around 100 fold, T 10 fold, and 17PR and 17PN 50 fold. Further investigation of the relationship between derivitization and sensitivity is required. The standard curves for detection limit analysis, on the other hand, were linear for every steroid (Appendix IV) ($0.9740<r<0.9994$, $n=6$).

Using the finalized clean up procedure, five of the eight steroid intermediates were detected in the fathead minnow vitellogenic ovarian tissue, testosterone (T), dehydroepiandrosterone (D), androstenedione (A), pregnenolone (PN), and 17β-estradiol (E) (n=1). Of the five, three, D, E and PN were at levels above the detection limit (6.34 pg/mg tissue, 8.42 pg/mg tissue and 19.2 pg/mg tissue, respectively), (Table 5.3.9.1). T and A were below detection limit (1.6 pg/mg tissue and 3.92 pg/mg tissue, respectively), (Table 5.3.9.1). Progesterone (PR), 17-hydroxypregnenolone (17PN), and 17-hydroxyprogesterone (17PR) were not detected, either due to their extremely low analytical sensitivity, their low level in the tissue, or a high intracellular turnover rate.

The fathead minnows were in the sexual stage of vitellogenesis. During this stage, the main endproduct of the steroid biosynthetic pathway is estradiol, accounting for the high level of estradiol detected in the sample. Reported basal levels of estradiol in 18 hour carp vitellogenic ovarian follicle incubations were around 50 pg/mL (Van Der Kraak *et al.*, 1990), about six times higher than the 8.42 pg/mg tissue measured here. The absolute basal levels of dehydroepiandrosterone and pregnenolone levels in vitellogenic wild-type whitefish (McMaster *et al.*, 1993) were also much higher than measured in fathead minnow ovarian tissue. One possible explanation for the difference could be the 18 hour ovarian incubation period prior to steroid analysis by RIA. Incubation of the ovaries would result in an accumulation of steroids in the incubation medium and thereby elevate steroid levels when compared to non-incubated samples. Carp and whitefish are also much

larger than fathead minnows which could result in relatively more steroid production. The relative concentrations however, of E, D, and PN in the fathead minnow ovarian tissue were very similar to the relative concentrations of E, D, and PN in the vitellogenic carp and lake whitefish incubations. The levels of these steroids (E, PN A, and T) were most effected by BKME exposure to vitellogenic ovaries of lake whitefish (McMaster *et al.*, 1993). Testosterone was also detected (below detection limit) in the vitellogenic ovarian sample at a level of 1.6 pg/mg tissue. This value is close to the value obtained by Robinson (1994) for fathead minnow ovarian incubations of 1.24 pg/mg tissue using RIA analysis. Very low levels of testosterone are expected since testosterone is the precursor of estradiol during vitellogenesis.

Delta-4 steroids, PR, 17PR, A, and T were expected to predominate in the ovarian sample. During previtellogenesis in rainbow trout, Δ-4 steroids predominate with a shift to mainly Δ-5 steroids (PN, 17PN, and D) at the end of exogenous vitellogenesis (Lambert and Van Bohemen, 1979). In the case of the fathead minnow ovarian sample, Δ-5 steroids PN and D predominated with little presence of Δ-4 steroids. The signifigance of this observation was very difficult to ascertain since A and T were below detection limit, PR, 17PN and 17PR were not detected, and data consisted of only one sample. More efficient tissue concentration, and more sample analysis would provide more relevant data.

The discovery of β-sitosterol in fathead minnow food, intestinal, and ovarian tissue was of considerable interest because sitosterol may be the (or one of the) chemical(s) in pulp and paper mill effluent that causes reproductive impairments in exposed fish (Dr. Carey, pers. comm.). Intraperitonial injections of 10, 20, and 100 ug/g dose of β-sitosterol caused a significant, dose dependent decrease in reproductive fitness in goldfish (Maclatchy and Van der Kraak, 1994). There were significant decreases in plasma testosterone and 11-ketotestosterone in males and testosterone and estradiol in females. Male goldfish exposed for 12 days to 75 to 1200 ug/l (static system) had decreased *in vitro* testosterone and pregnenolone production and reduced plasma testosterone and 11-ketotestosterone levels.

The highest level of sitosterol in fathead minnow tissue was located in the intestinal tissue at 1.35 ng/mg tissue ± 0.34 ng/mg tissue (n=4) compared to 528.7 pg/mg tissue (n=1), and 649.3 pg/mg tissue (n=1) in fathead minnow ovaries and food, respectively. The high level of sitosterol in the intestine might have resulted from the concentration of waste in the intestines since it is present in the food.

Levels of sitosterol in ovarian tissue are not as easily explained. It has been observed that phytosterols can accumulate in blood and tissue and cause a rare disease of impaired lipid metabolism called phytosterolaemia (Tvrzicka *et al.*, 1991). McMaster's research (1993) found that the acivity of the enzyme which catalyzes cholesterol to pregnenolone,

cytochrome P-450$_{scc}$, is unaffected by BKME. Low endogenous stores of cholesterol were the cause of the observed biosynthetic lesion. Phytosterols, such as sitosterol, are known as effective inhibitors of cholesterol absorption in the gut. Potentially the presence of sitosterol in fish ovaries could affect endogenous cholesterol availability and thereby cause the observed reproductive impairments in fish.

It should be noted that cholesterol and sitosterol were also found in non-tissue blanks at a range of 3.36 pg/sample to 194.8 pg/sample. Laboratory materials made of natural rubber, such as gloves and pasteur pipette bulbs used in this experiment were the source of contamination (Banner, 1991). It is unknown how indirect contact between the bulb and the solvent resulted in cholesterol and sitosterol contamination but it has been postulated that it may have been mediated by solvent vapour or by fragments of material falling into the pipette (Banner, 1991). Obviously the level of sitosterol found in the tissue and food were much greater than the level of contamination but in the future, the sources of this contamination should be eliminated when evaluating levels of sitosterol in fish tissue.

Other researchers measuring steroid levels in fish should also be aware of the potential for contamination of their experiments with sitosterol contaminated food. San Francisco Bay Brand was the type containing β-sitosterol. These brine shrimp are wild-type naturally occurring shrimp, harvested from salt ponds in San Francisco. They are foragers feeding

on algae, bacteria and any other organic particles less than 50 microns (San Francisco Bay Brand, pers. comm.).

The steroid extraction, clean up and detection procedures established in this thesis are capable of identifying and quantifying dehydroepiandrosterone, estradiol, and pregnenolone in healthy fathead minnow ovarian tissue. Modification of the carbonyl derivitization procedure may improve sensitivity of A and PR by up to 100 fold. Tissue concentration, by increasing column capacity, will also be required to assist the quantification of other intermediates such as androstenedione and testosterone. With that in mind, this protocol could profile the steroid biosyntehetic intermediates of healthy and exposed fish and thereby be used in BKME-exposure studies to determine the effects of BKME and its components on steroid biosynthesis. The protocol can also be used to monitor β-sitosterol levels in the ovaries of healthy and exposed fathead minnows if the use of natural rubbers are eliminated from the procedure, and providing the percent recovery and reproducibility of sitosterol are evaluated.

Ultimately, the development of a protocol for profiling steroids from body tissue may assist in evaluating chronic effects leading to the observed physiological changes in fish exposed to pulp mill effluents. Furthermore, the use of such a profiling technique with exposures to individual components or fractionated effluents may identify the troublesome (compound "x") components in effluents. To do so would provide greater focus for

efforts to reduce the problems associated with pulp mill effluents. To test the validity of using steroid profiling to observe changes leading to physiological disturbances, the profiling technique should be first used after exposure to a substance for which these changes are well documented.

With minor modification, the profiling technique may be used to probe the activity of $P450_{scc}$, 17-ketosteroid reductase/17-hydroxysteroid dehydrogenase, and aromatase in biological tissue since the steroids before and after the reactions these enzymes catalyze may be detected. Potentially a first step in this direction would be to evaluate cholesterol and pregnenolone levels upon exposure to sitosterol. This method may be useful to other researchers involved in the study of estrogen mimics, organochlorines or other xenobiotics which effect reproductive physiology.

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Appendix I: Nomenclature and abbreviations of steroids.

Trival name and abbreviation	Systematic name
Androstenedione (A)	4-androstene-3,17-dione
Dehydroepiandrosterone (DHEA)	3beta-hydroxy-5-androstene-17-one
17-Estradiol (E)	3,17beta-dihydroxy-1.3,5(10)-estratriene
Estrone	3-hydroxy-1,3,5(10)-estratriene-17-one
Pregnenolone (PN)	3beta-hydroxy-5-pregnene-20-one
17-Hydroxypregnenolone (17PN)	3beta,17alpha-dihydroxy-5-pregnene-20-one
Progesterone (PR)	4pregnene-3,20-one
17-Hydroxyprogesterone (17PR)	17alpha-hydroxy-4-pregnene-3,20-one
17,20-Dihydroxyprogesterone	17alpha, 20beta-dihydroxy-4-pregnene-3,20-one
Testosterone (T)	17beta-hydroxy-4-androstene-3-one

Appendix II: Mass spectral scans and structures of suspected FLOROX and HFBA derived steroids where pentafluorobenzyl-derived is represented as PFB, and heptafluorobutyric-derived is represented as PFB:

a) Androstenedione-PFB
b) Cholesterol
c) Dehydroepiandrosterone-PFB-HFB
d) 17β- Estradiol-HFB
e) Pregnenolone-PFB-HFB
f) 17-Hydroxypregnenolone-PFB-HFB
g) Progesterone-PFB

Appendix II A: Mass spectral scan (opposite page) and structure of androstenedione-PFB.

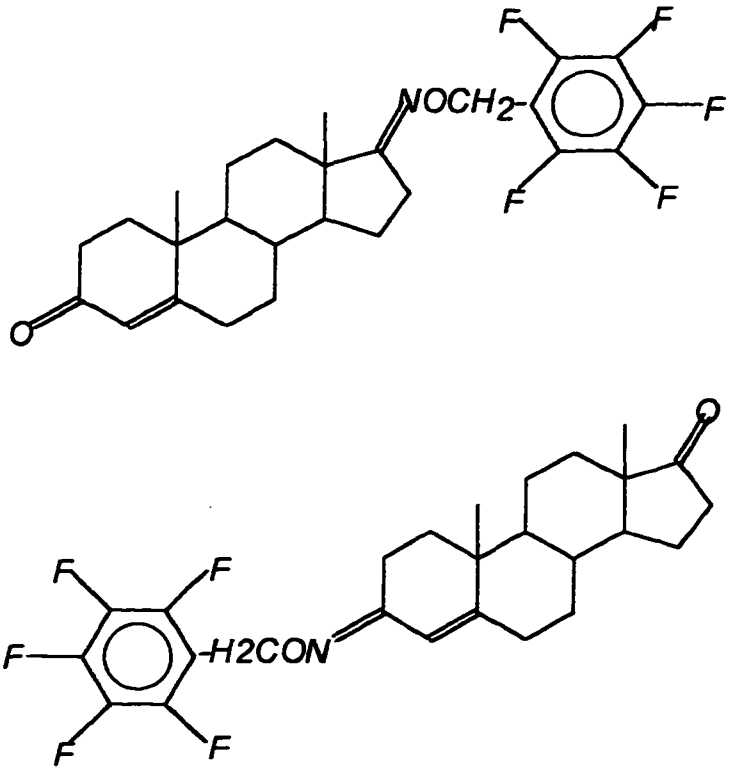
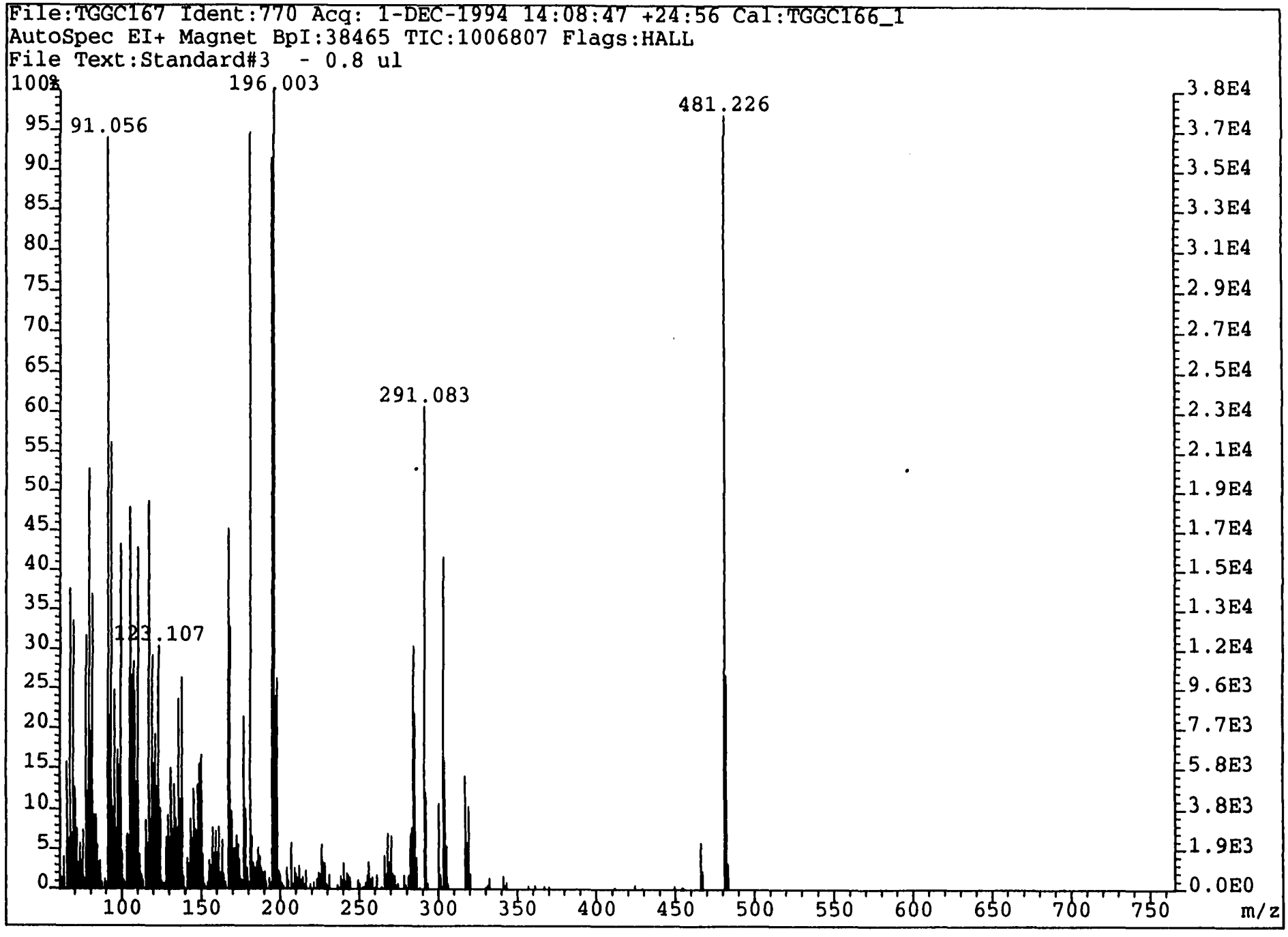


Figure A.II.A: The suspected structures of androstenedione-PFB as determined by mass spectral scan. One pentafluorobenzyl group is attached to either one of the two ketone groups.

The identified ions include:

481 = M^+ (molecular mass)

181 = CH_2-C_6F_5



Appendix II B: Mass spectral scan (opposite page) and structure of HFBA-derivatized cholesterol. This derivatizing reagent causes cholesterol to dehydrate at the C3 position. The spectrum was confirmed by the software library.

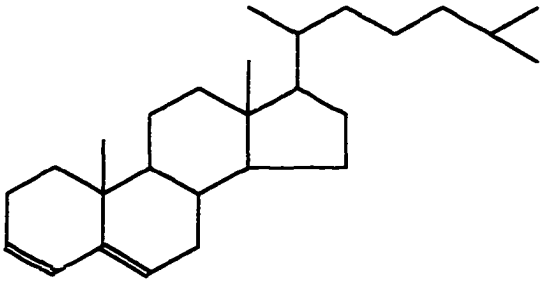
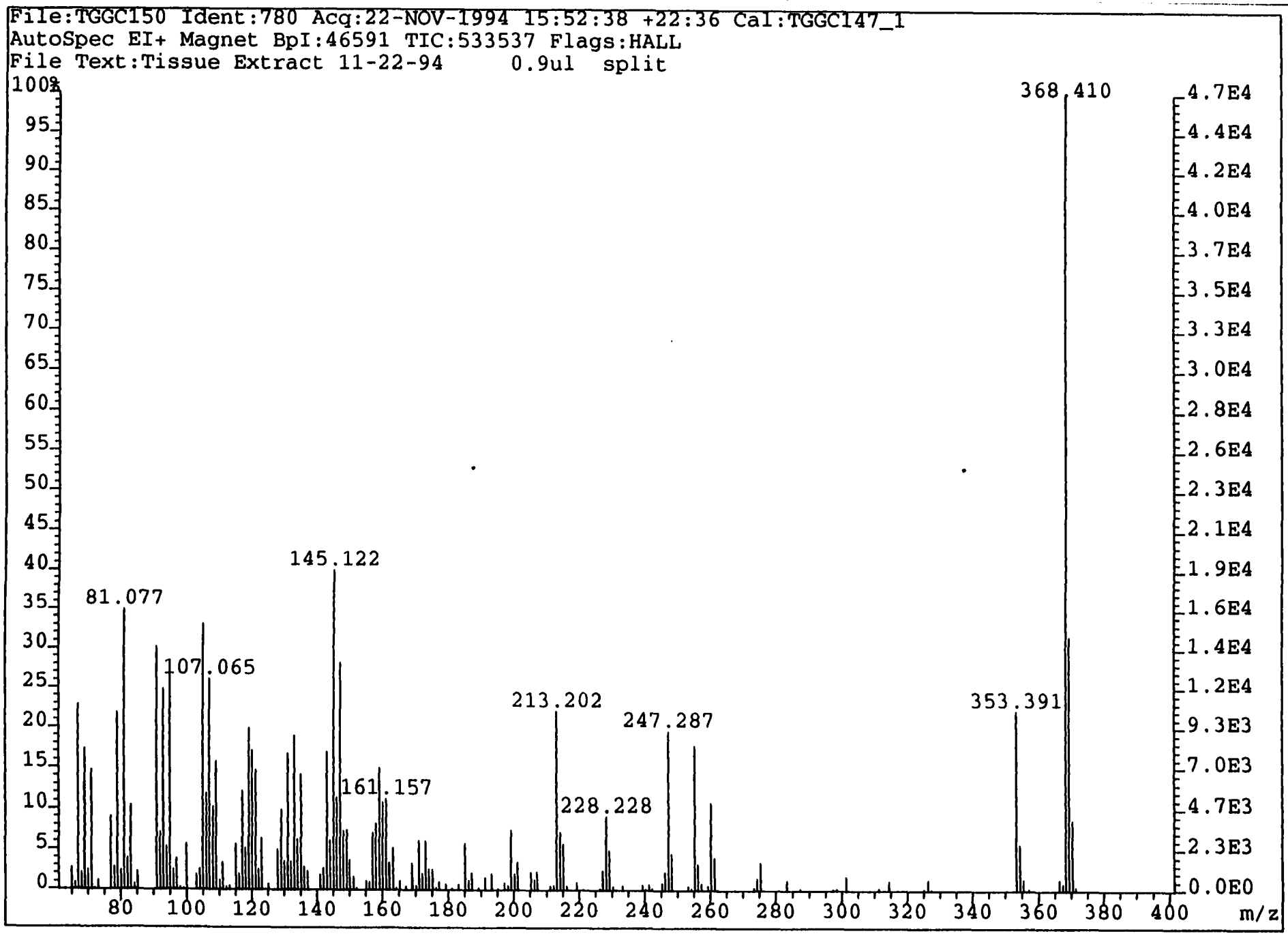


Figure A.II.B: The structure of dehydrated cholesterol, created by reaction with HFBA.



Appendix II C: Mass spectral scan (opposite page) and structure of dehydroepiandrosterone-HFB-PFB.

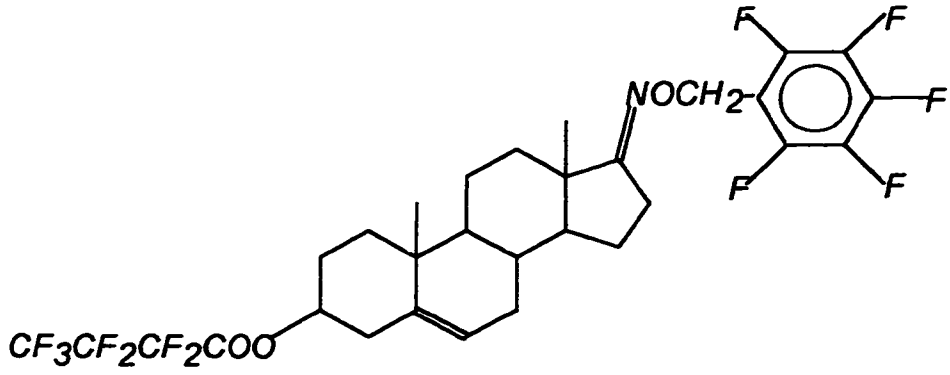


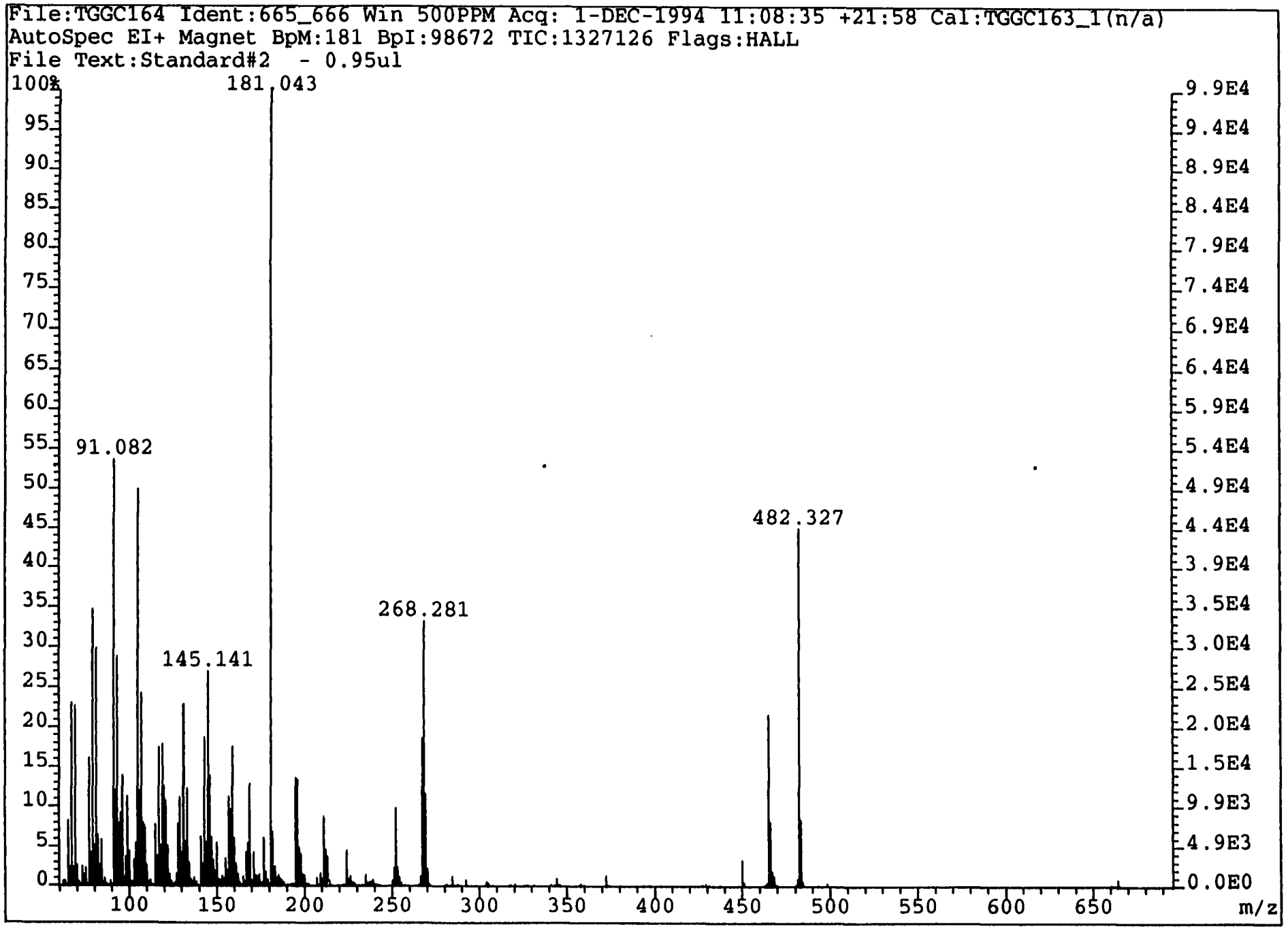
Figure A.II.C: The suspected structure of dehydroepiandrosterone-HFB-PFB as determined by mass spectral scan.

The identified ions include:

679 = M^+ (molecular mass)

482 = M^+-(CO-CF_2-CF_2-CF_3)

181 = CH_2-C_6F_5



Appendix II D: Mass spectral scan (opposite page) and structure of 17β-estradiol-HFB.

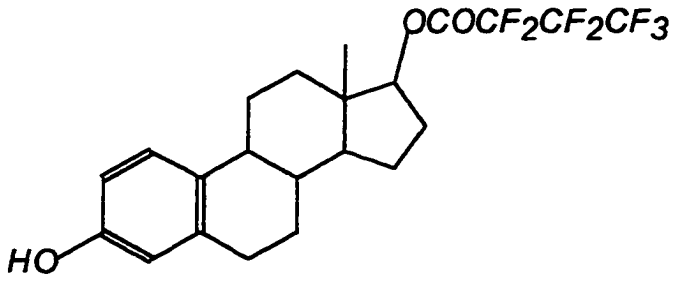


Figure A.II.D: The suspected structure of 17β-estradiol-HFB as determined by mass spectral scan.

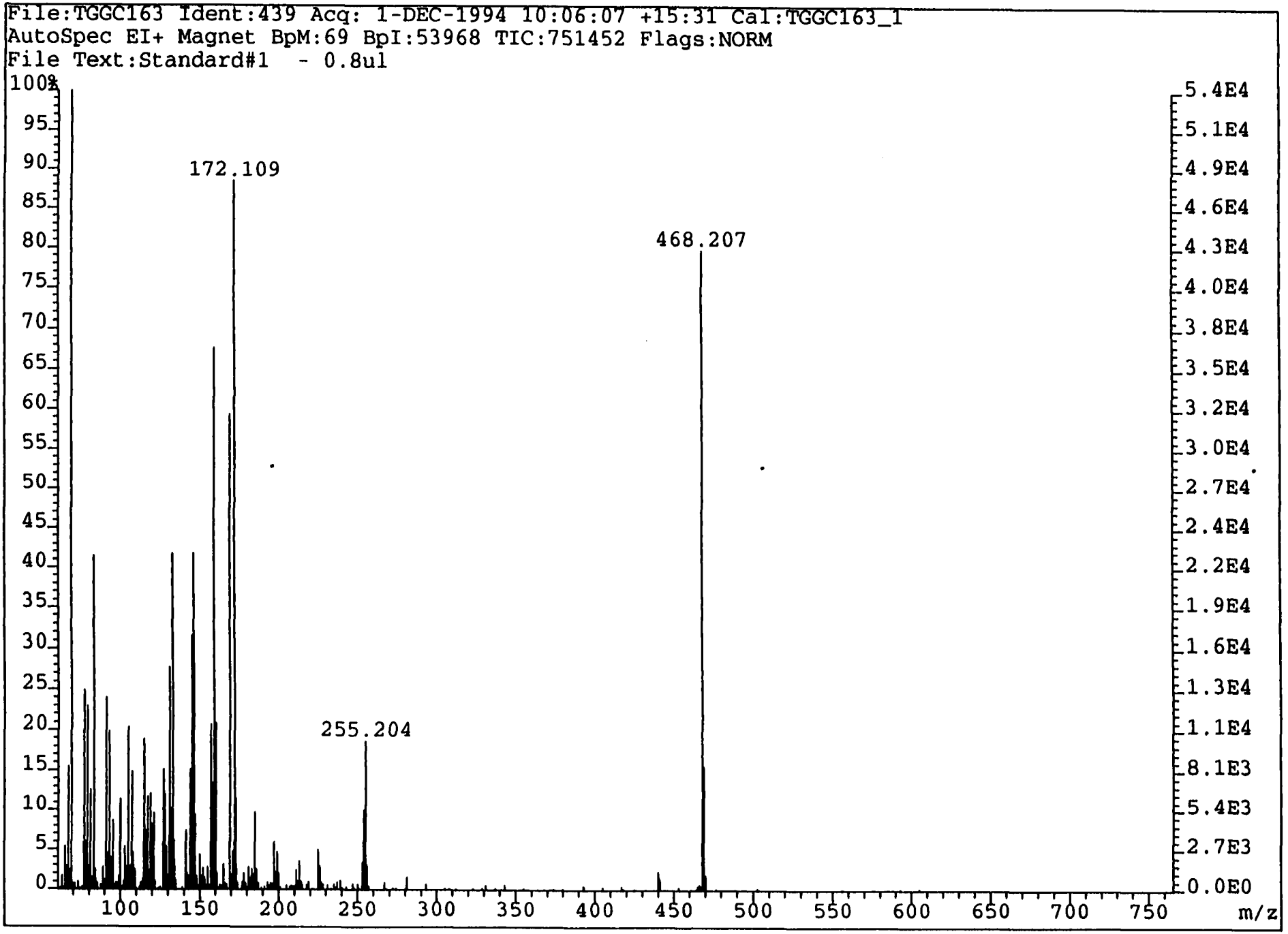
The identified ions include:

468 = M^+ (molecular mass)

\ 255 = M^+-(O-CO-CF_2-CF_2-CF_3)

133 = HO-C_6H_4-CH_2-CH_2-CH_2

83 = C_5H_8-CH_3



Appendix II E: Mass spectral scan (opposite page) and structure of pregnenolone-HFB-PFB.

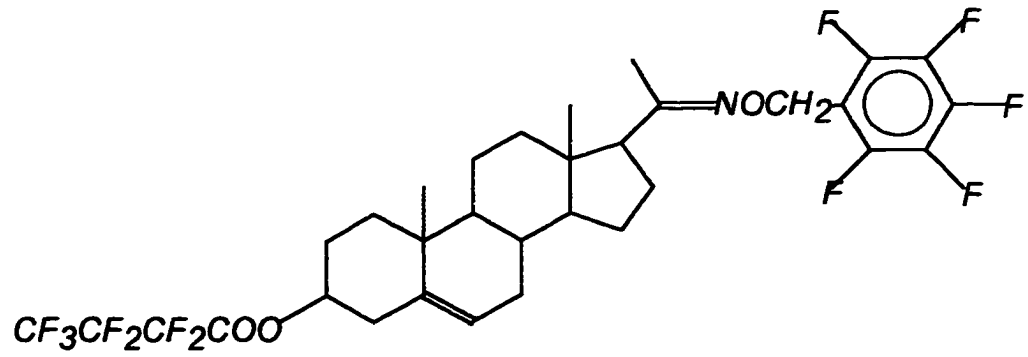
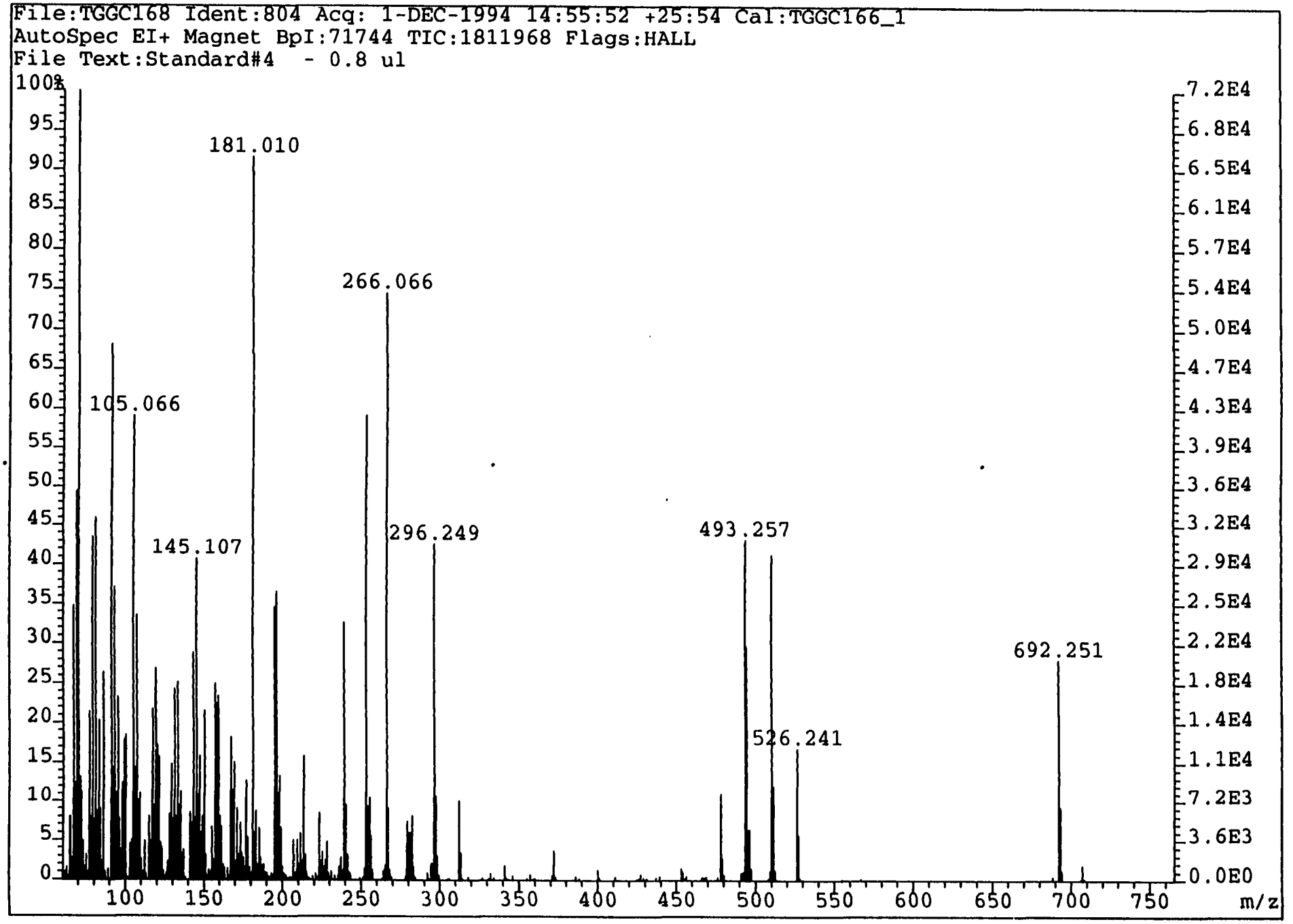


Figure A.II.E: The suspected structure of pregnenolone-HFB-PFB as determined by mass spectral scan.

The identified ions include:

707 = M^+ (molecular mass)
511 = M^+-(CH_2CH_2-C_6F_5)
493 = M^+-(CH_3)-(CO-CF_2-CF_2-CF_3)-(2H)
312 = M^+-(CO-CF_2-CF_2-CF_3)-(2H)-(CH_2-C_6F_5)
181 = CH_2-C_6F_5



Appendix II F: Mass spectral scan (opposite page) and structure of 17-hydroxypregnenolone-HFB-PFB.

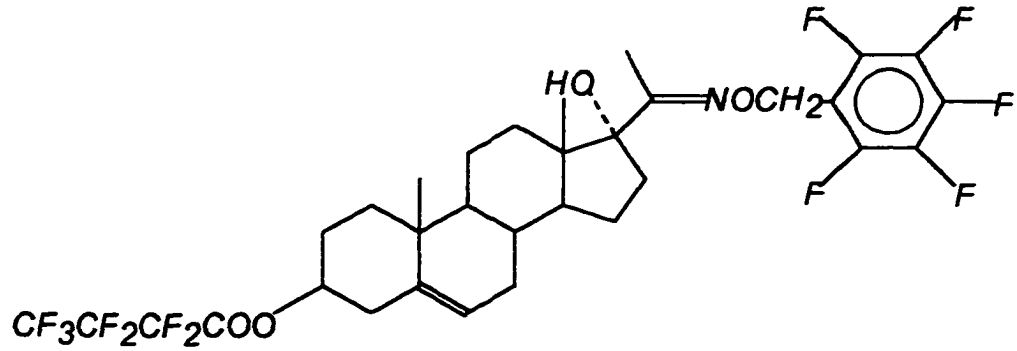


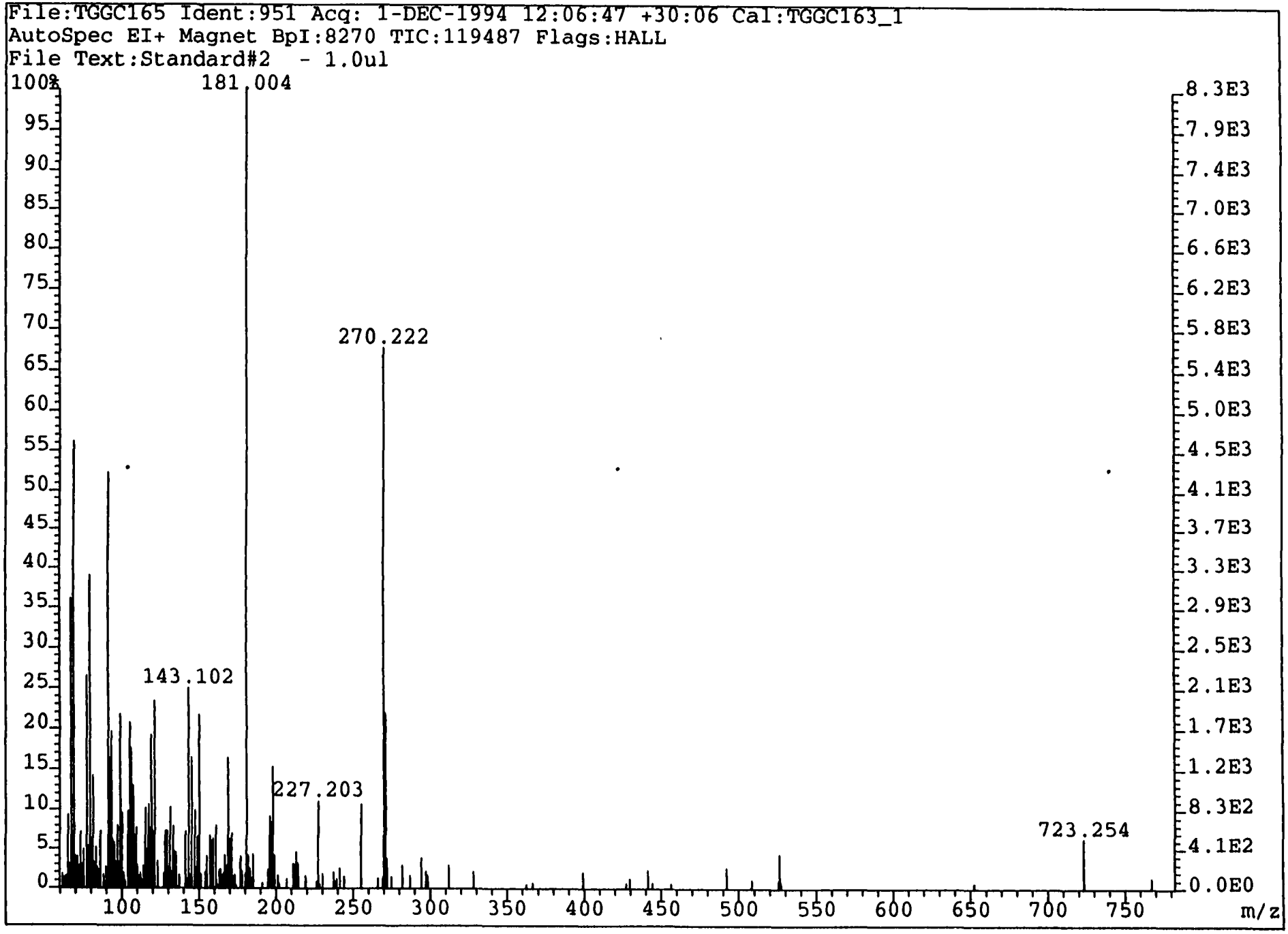
Figure A.II.F: The suspected structure of 17-hydroxypregnenolone-HFB-PFB as determined by mass spectral scan.

The identified ions include:

723 = M^+ (molecular mass)

526 = M^+-(O-CF_2-CF_2-CF_3)

181 = CH_2-C_6F_5



Appendix II G: Mass spectral scan (opposite page) and structure of progesterone-PFB.

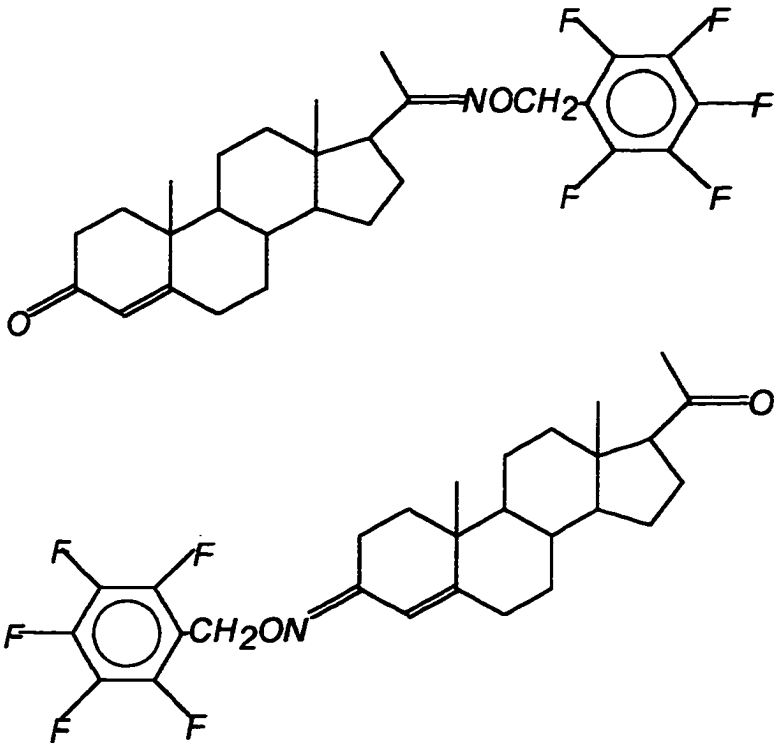
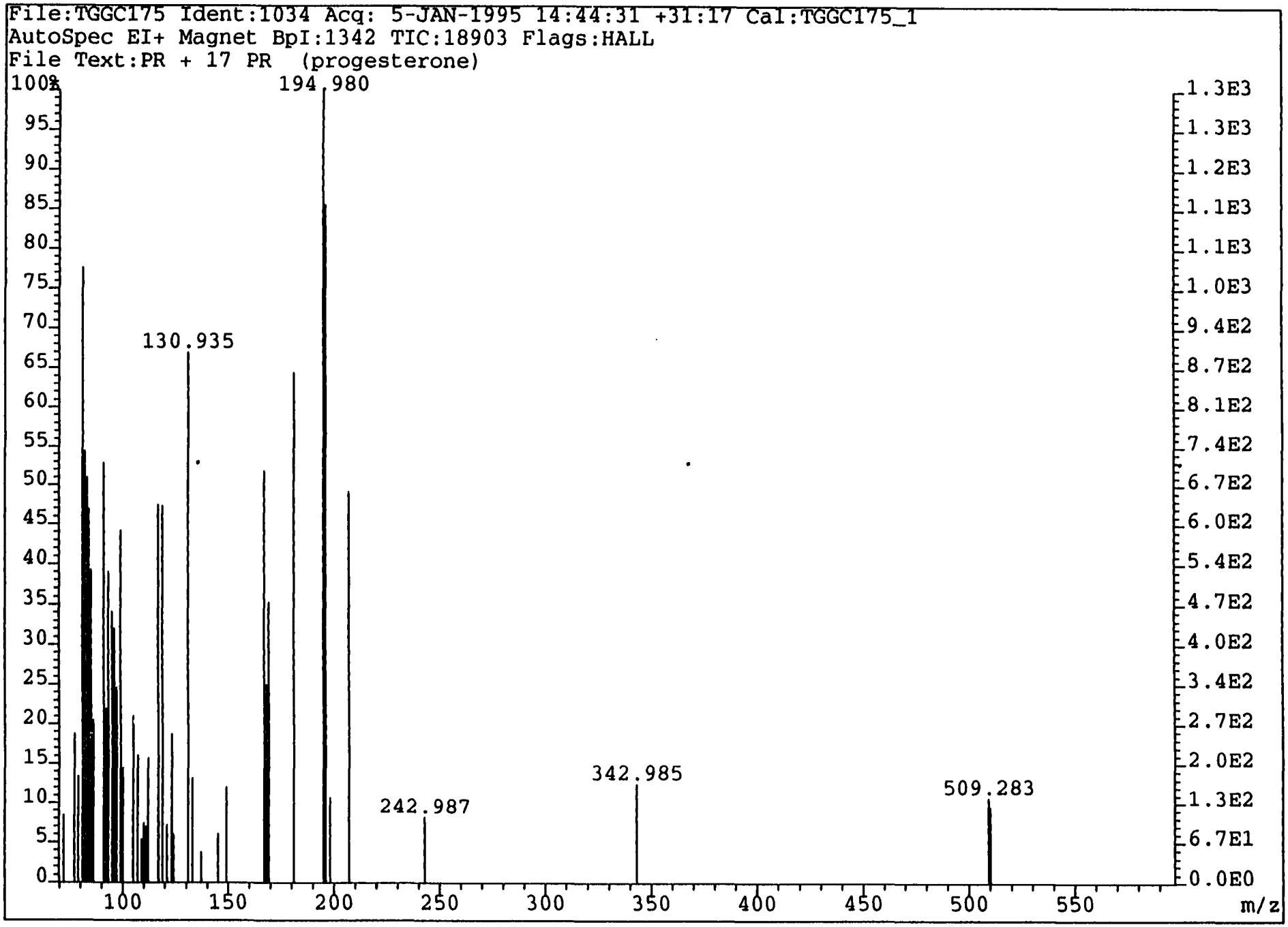


Figure A.II.G: The suspected structure of progesterone-PFB as determined by mass spectral scan. One pentafluorobenzyl group is attached to either of the two ketone groups.

The identified ions include:

509 = M^+ (molecular mass)

181 = CH_2-C_6F_5



Appendix III: Mass spectral scans and structure of methoxiamine-trimethylsilyl (MOX-TMS)-derived steroids:

a) Androstenedione
b) Cholesterol
c) Dehydroepiandrosterone
d) 17-β-estradiol
e) 17-β-d_2-estradiol
f) Pregnenolone
g) 17-Hydroxypregnenolone
h) Progesterone
i) 17-Hydroxyprogesterone
j) Testosterone

Appendix III.A: The mass spectral scan (opposite page) and structure of androstenedione-2MOX.

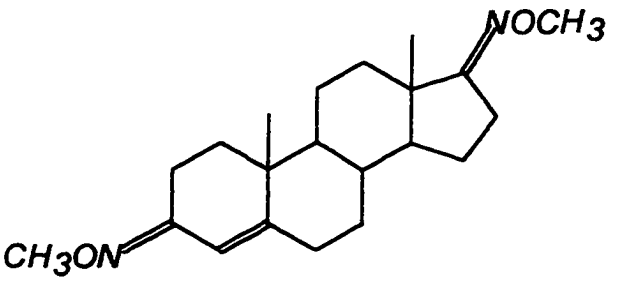
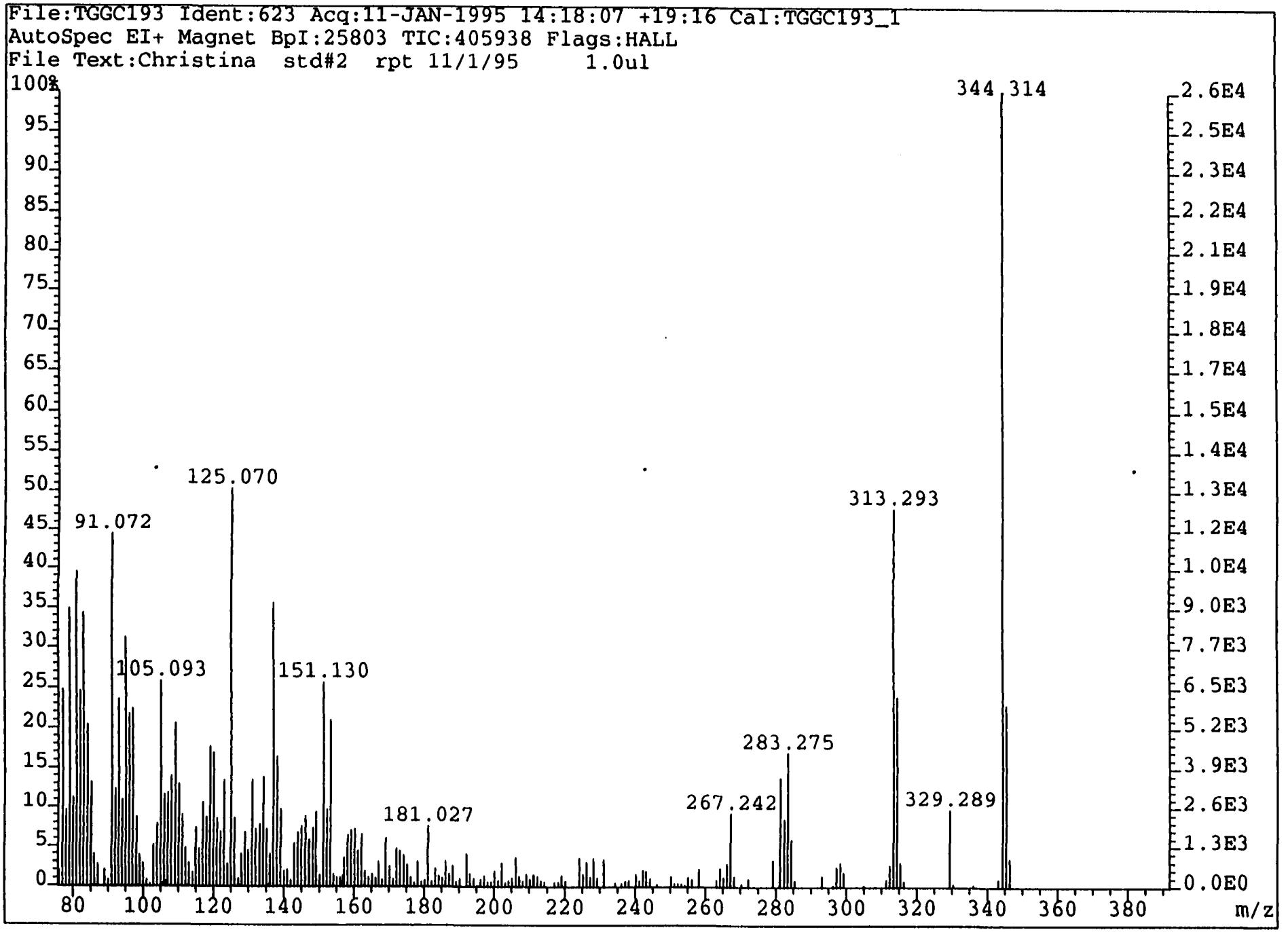


Figure A.III.A: The structure of androstenedione-2MOX.



Appendix III.B: The mass spectral scan (opposite page) and structure of cholesterol-TMS.

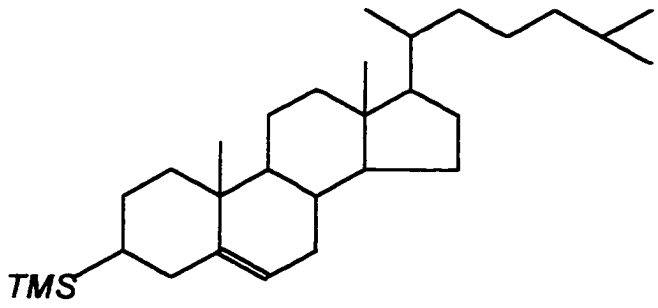
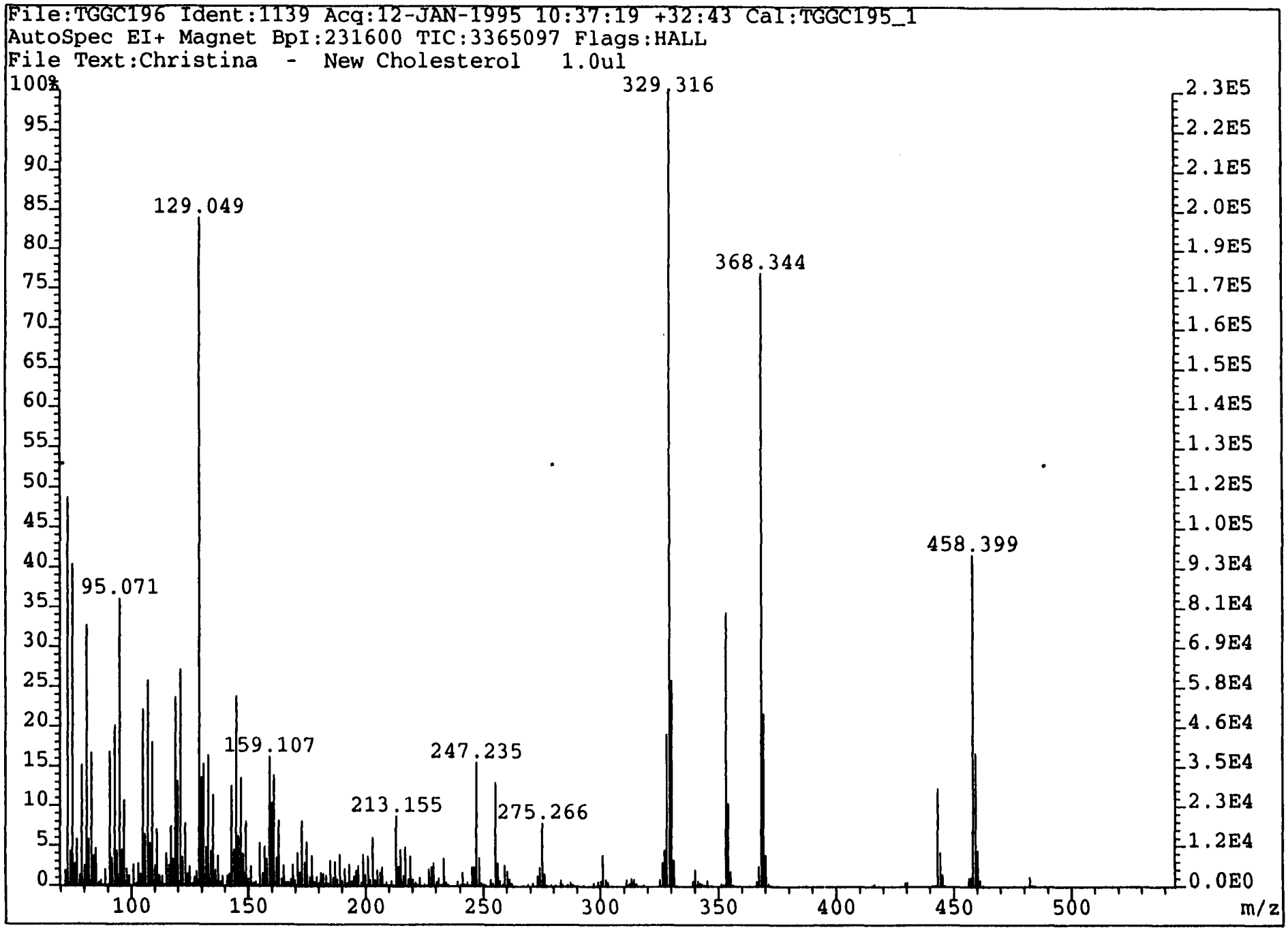


Figure A.III.B: The structure of cholesterol-TMS.



Appendix III.C: The mass spectral scan (opposite page) and structure of dehydroepiandrosterone-MO-TMS.

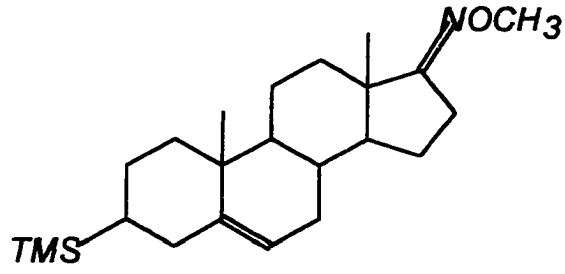
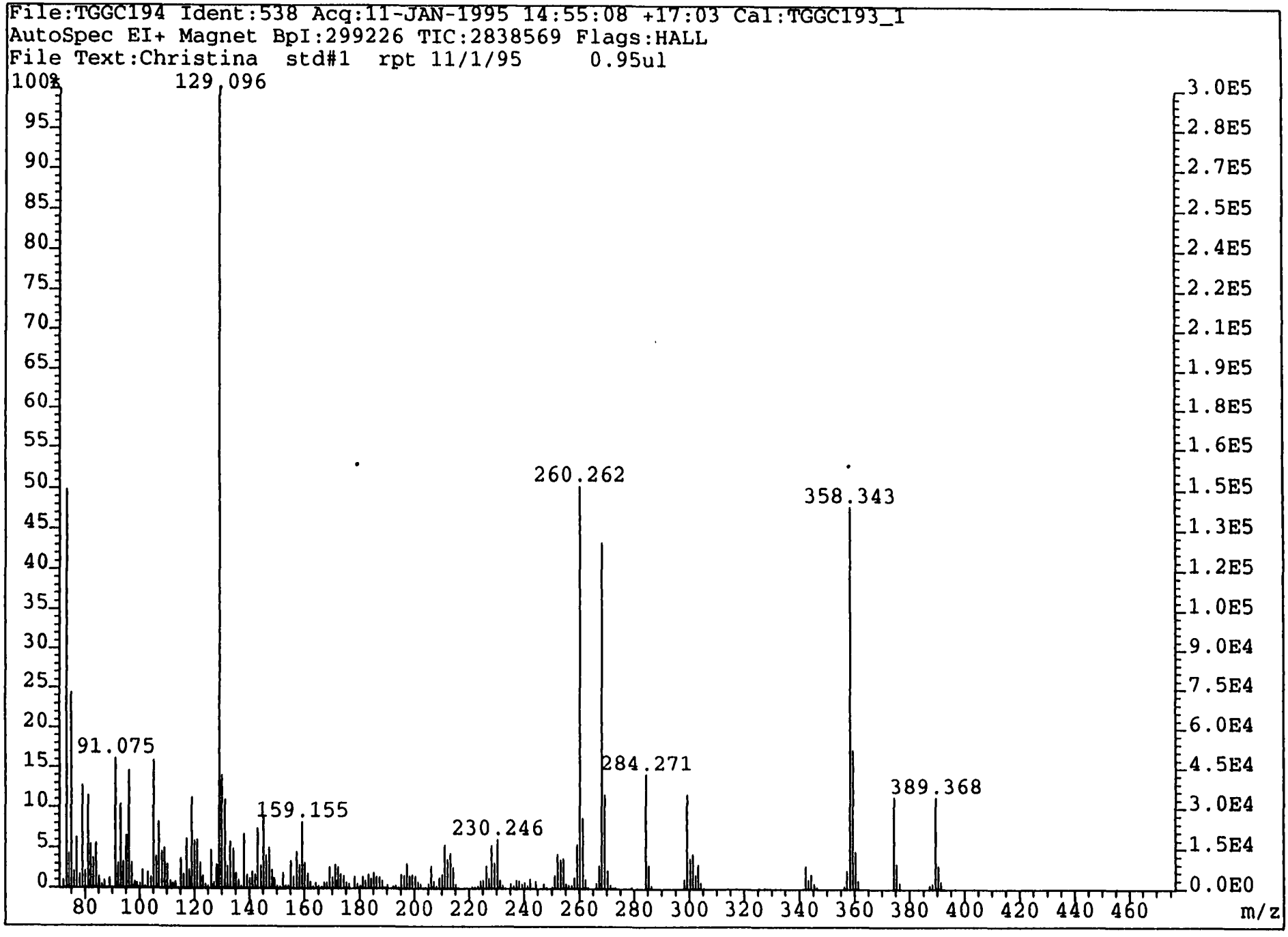


Figure A.III.C: The structure of dehydroepiandrosterone-MO-TMS.



Appendix III.D: The mass spectral scan (opposite page) and structure of 17β-estradiol.

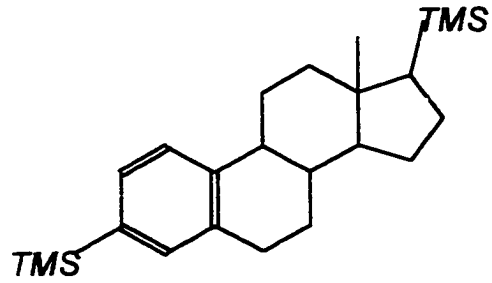
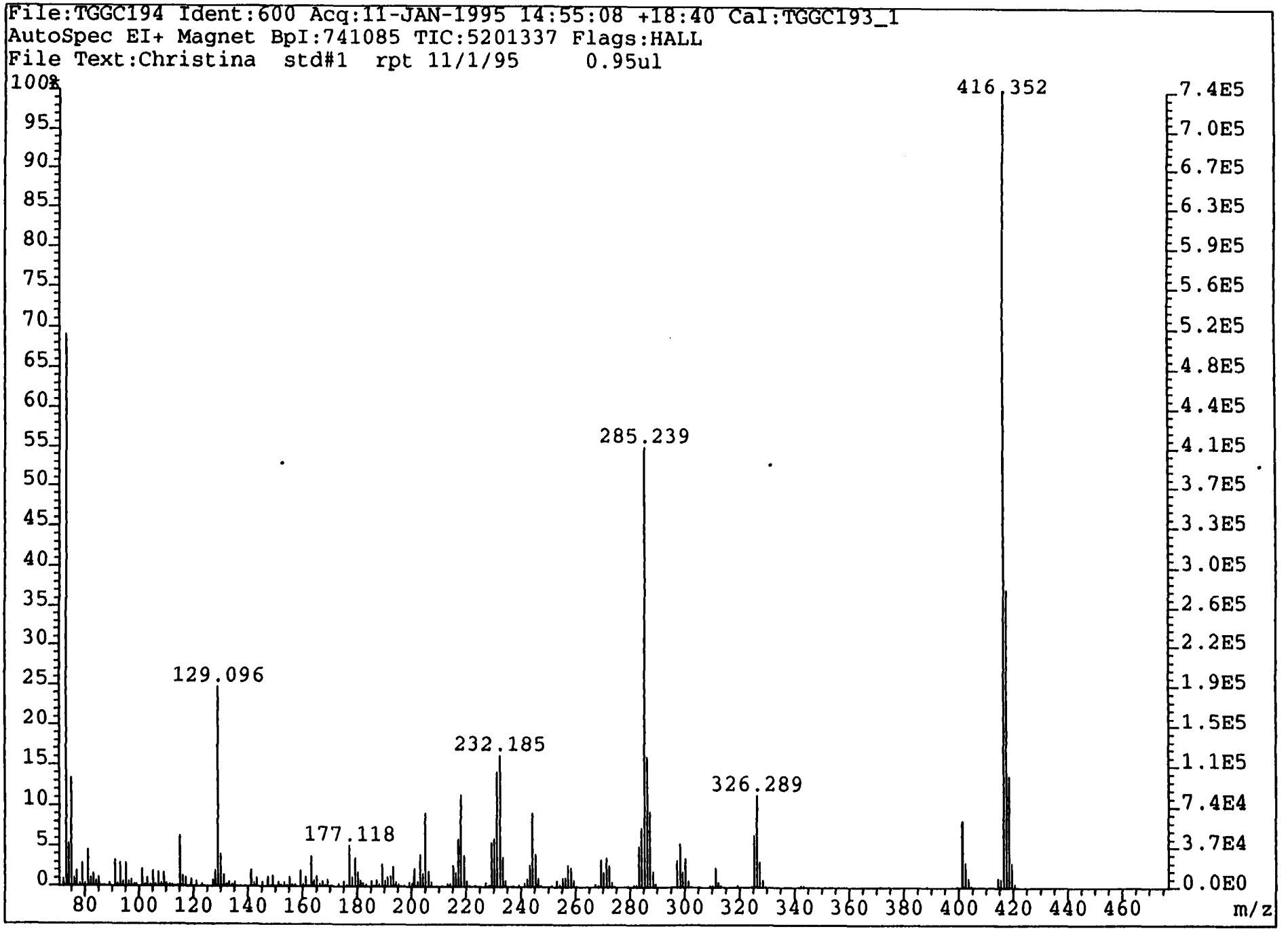


Figure A.III.D: The structure of 17β-estradiol-2TMS.



Appendix III.E: The mass spectral scan (opposite page) and structure of 17β-d_2-estradiol-2TMS.

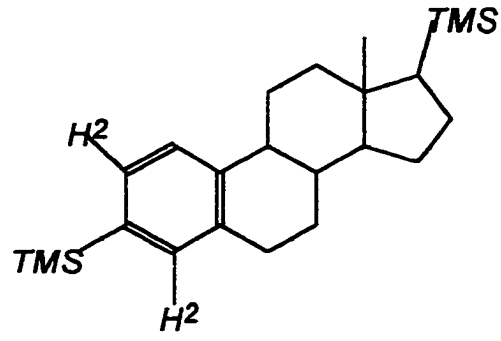
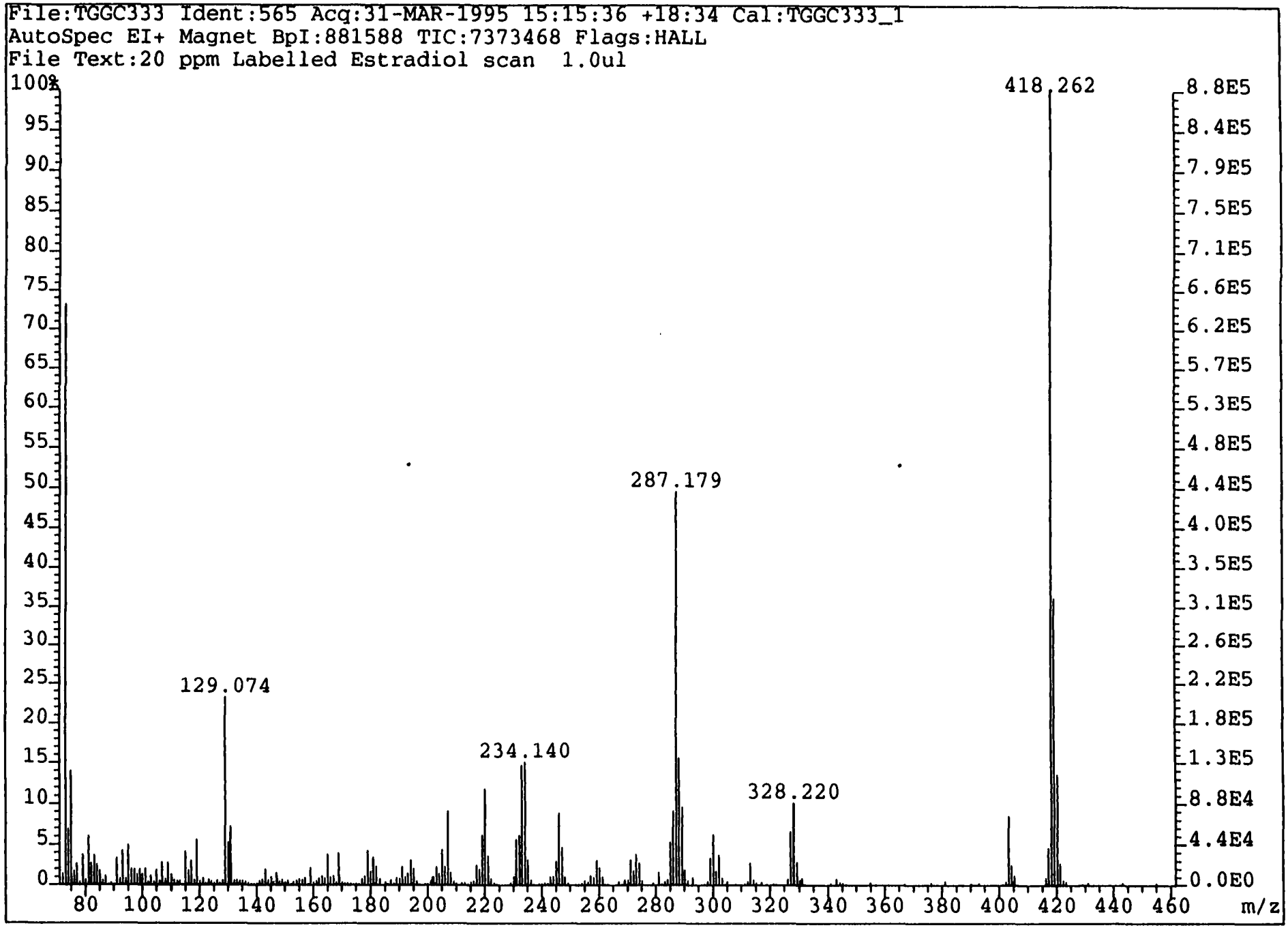


Figure A.III.E: The structure of 17β-d_2-estradiol-2TMS.



Appendix III.F: The mass spectral scan (opposite page) and structure of pregnenolone-MO-TMS.

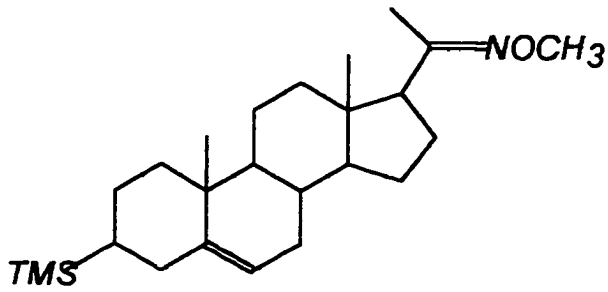
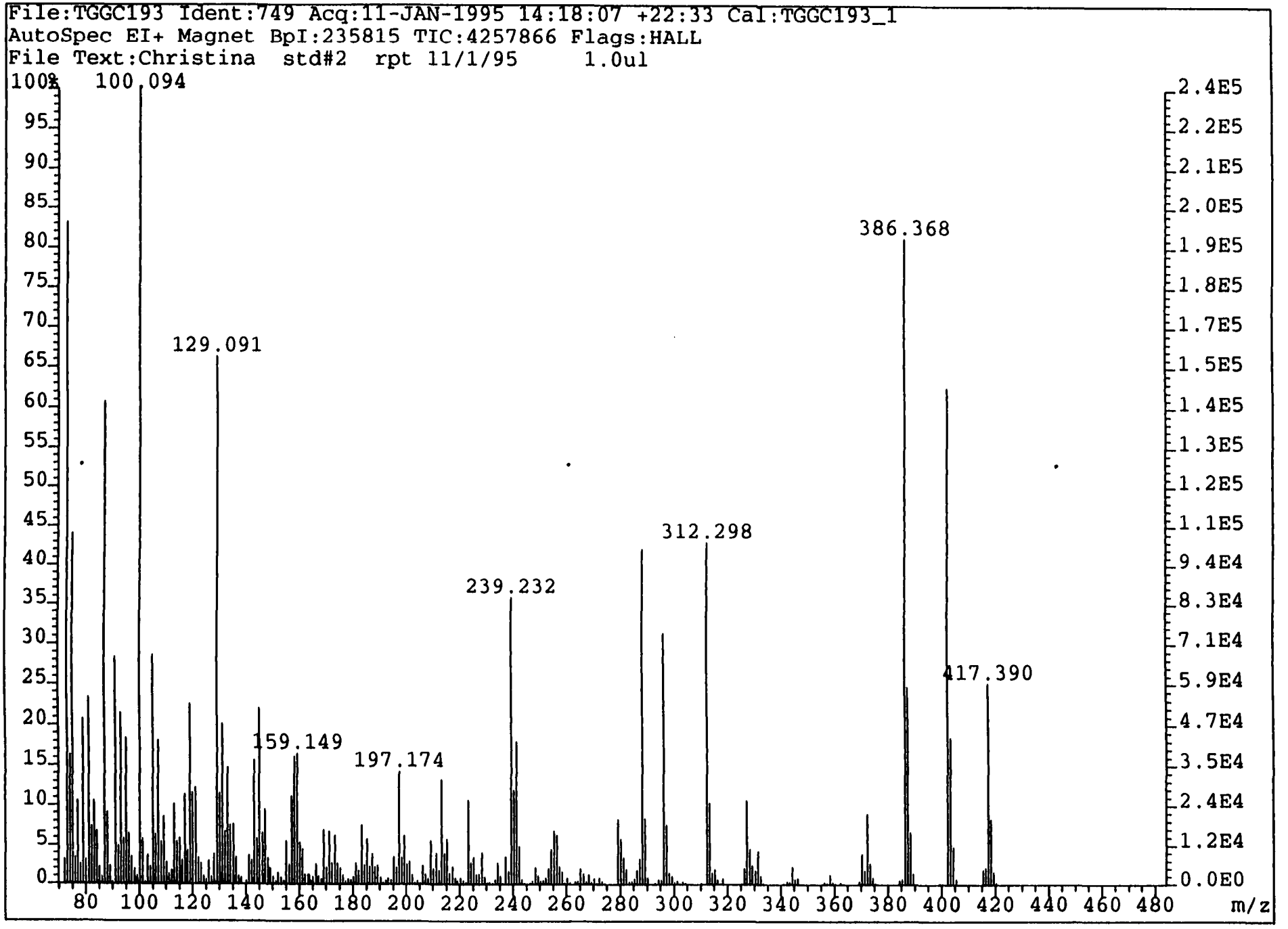


Figure A.III.F: The structure of pregnenolone-MO-TMS.



Appendix III.G: The mass spectral scan (opposite page) and structure of 17-hydroxypregnenolone-MO-2TMS.

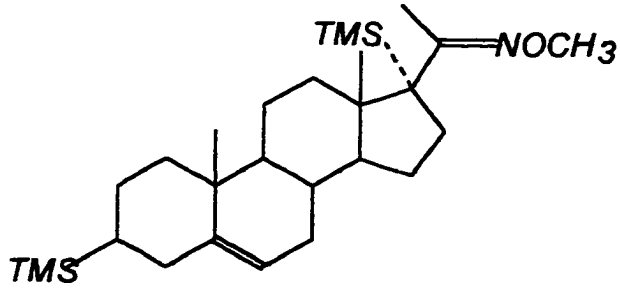
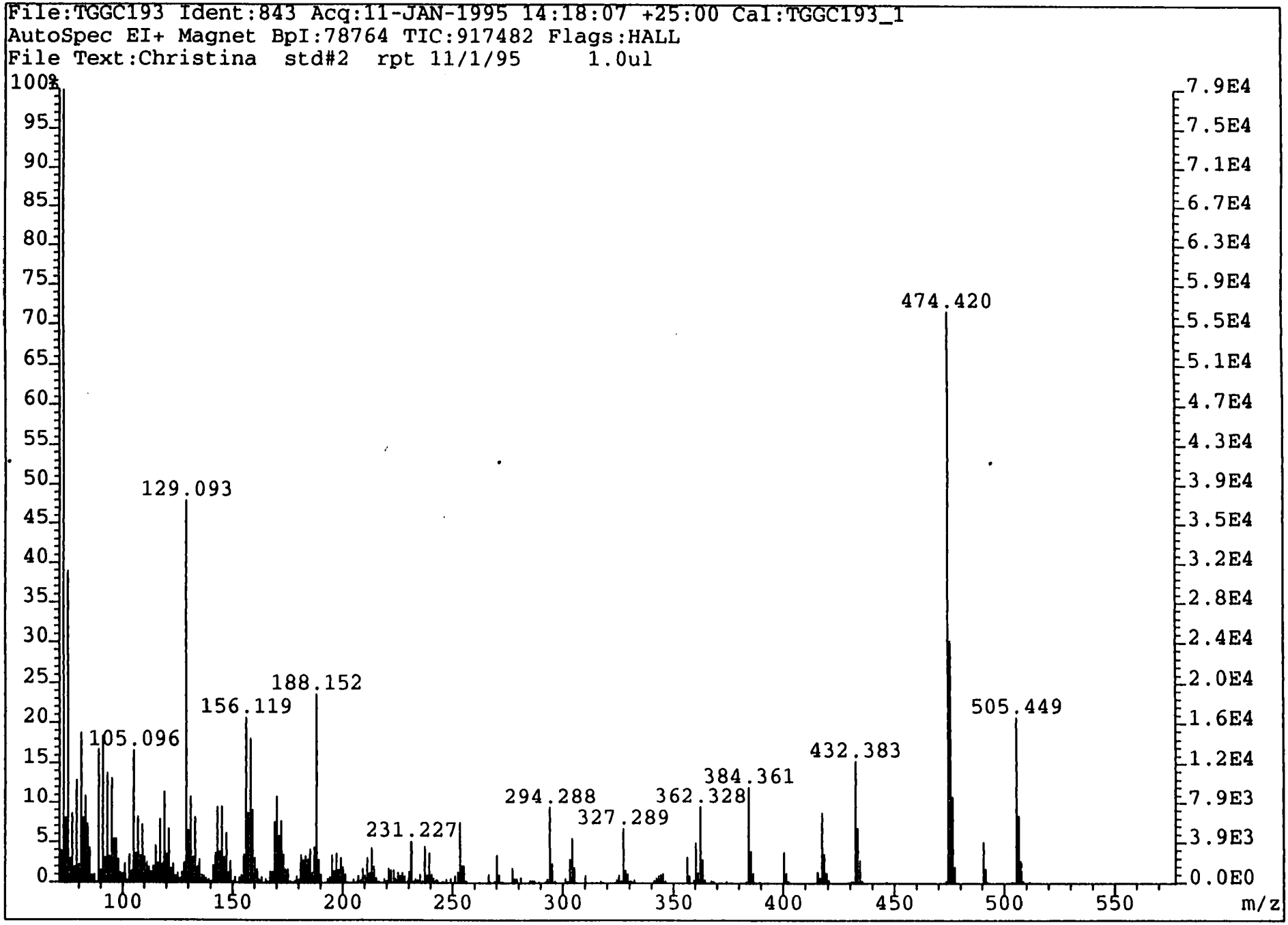


Figure A.III.G: The structure of 17-hydroxypregnenolone-MO-2TMS.



Appendix III.H: The mass spectral scan (opposite page) and structure of progesterone-2MOX.

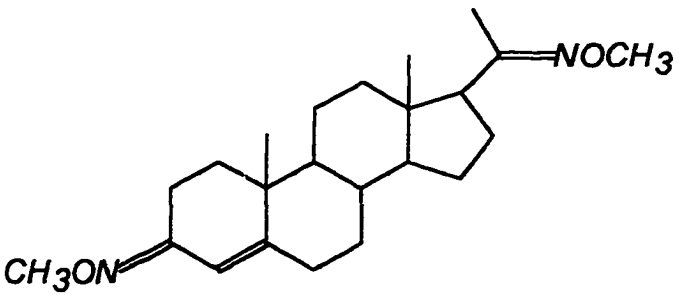
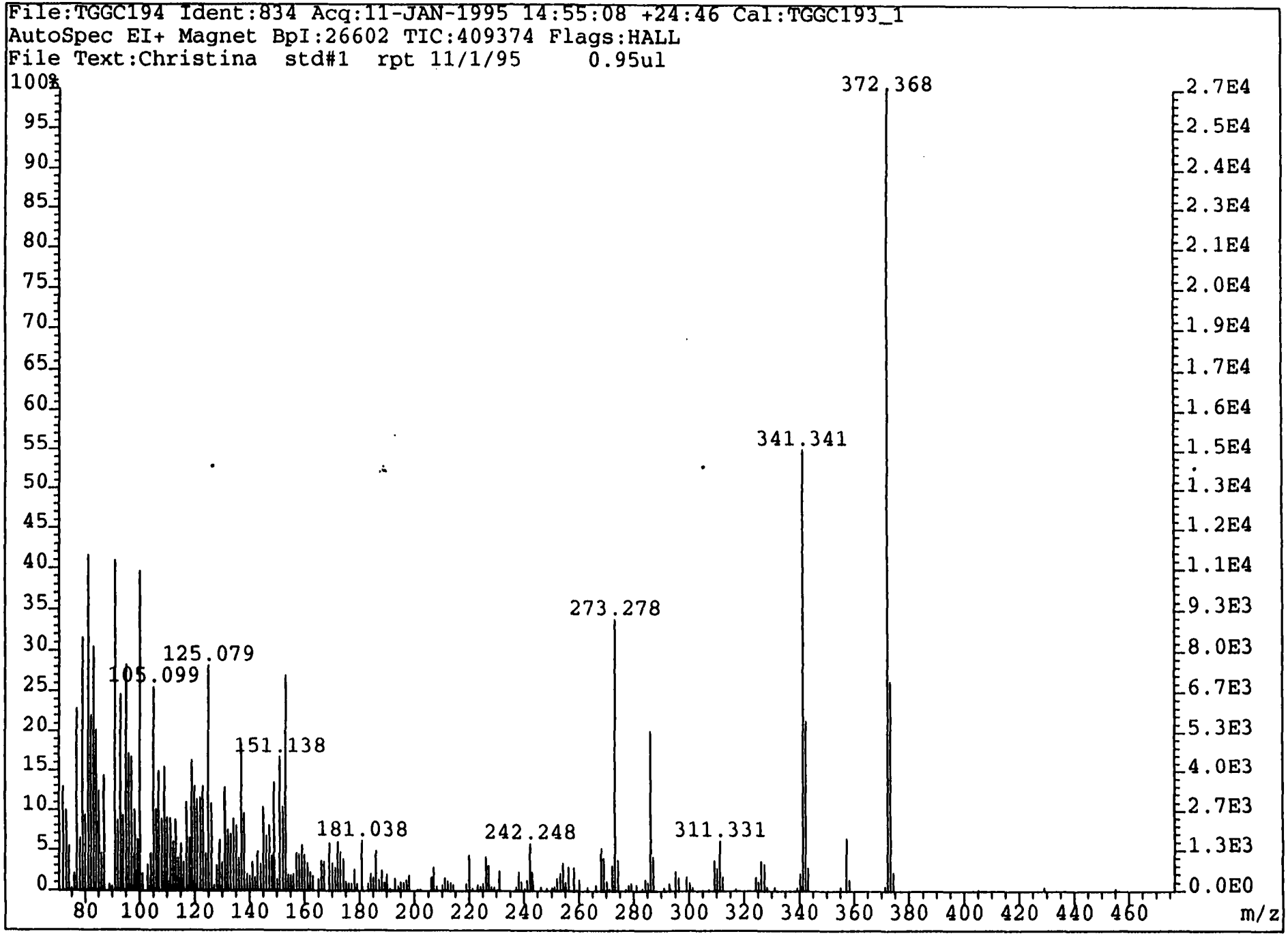


Figure A.III.H: The structure of progesterone-2MOX.



Appendix III.I: The mass spectral scan (opposite page) and structure of 17-hydroxyprogesterone-2MO-TMS.

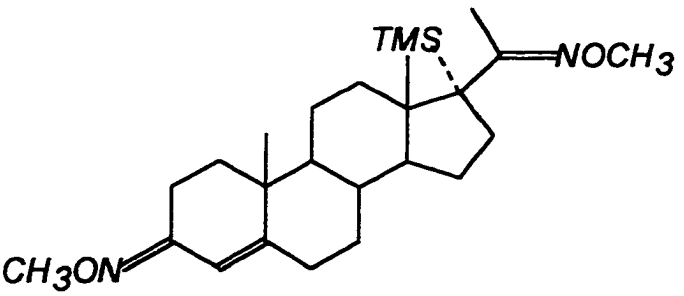
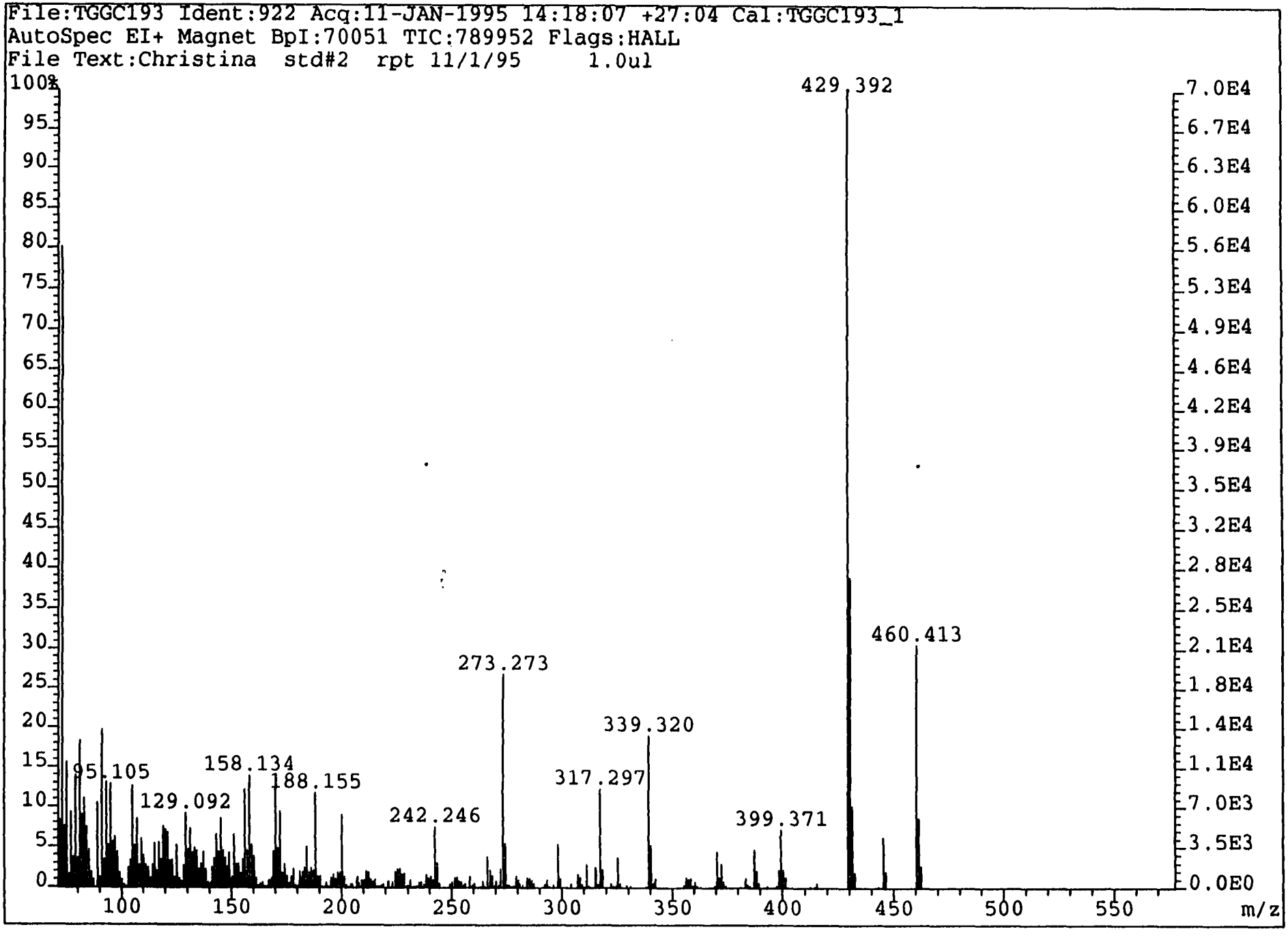


Figure A.III.I: The structure of 17-hydroxyprogesterone-2MO-TMS.



Appendix III.J: The mass spectral scan (opposite page) and structure of testosterone-MO-TMS.

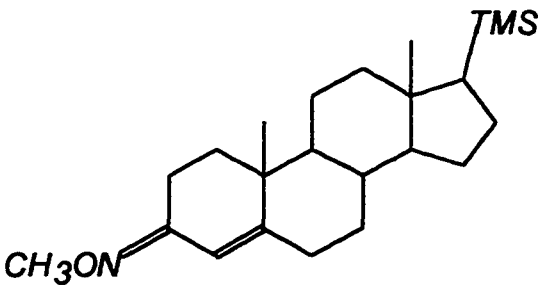
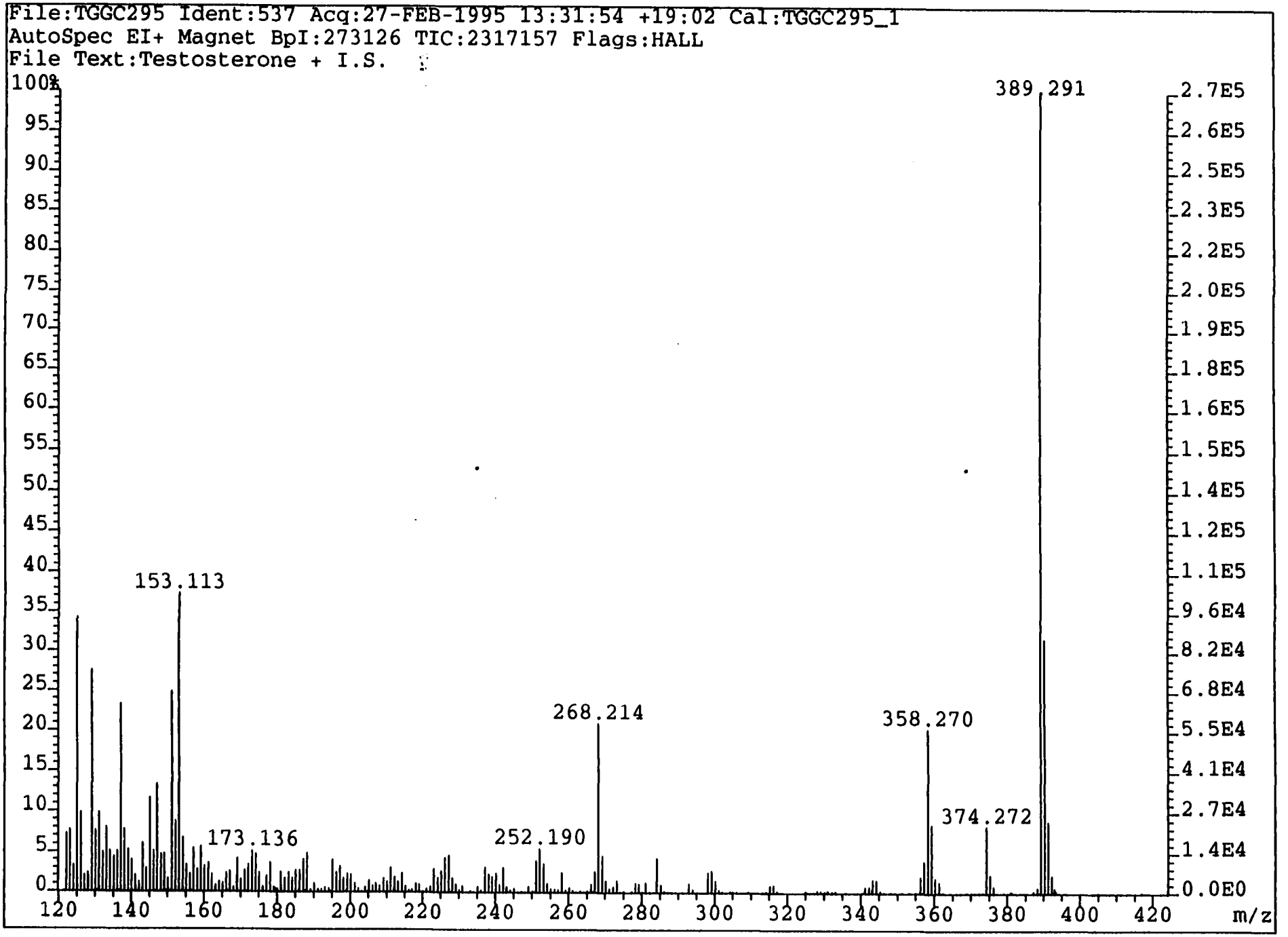


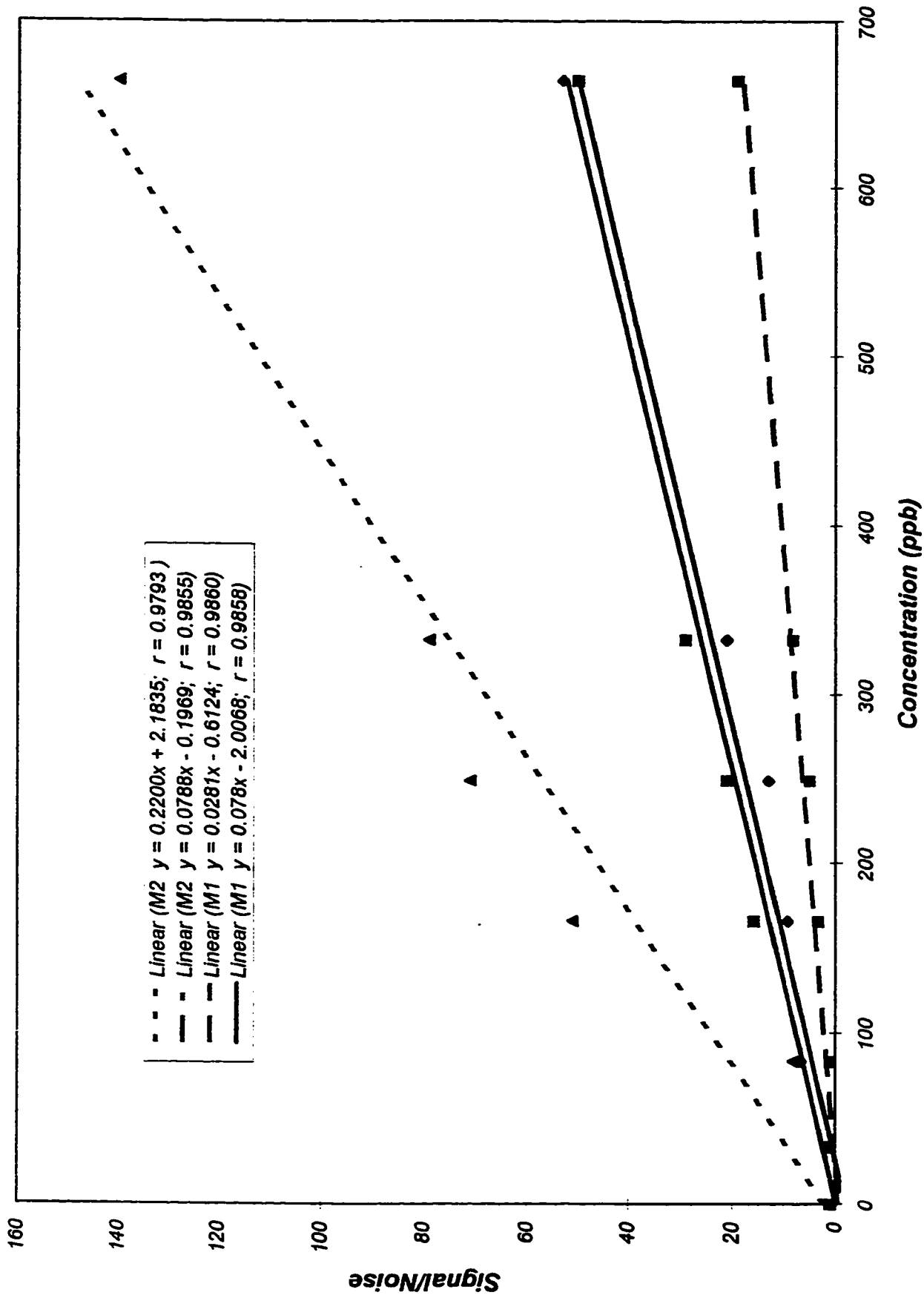
Figure A.III.J: The structure of testosterone-MO-TMS.



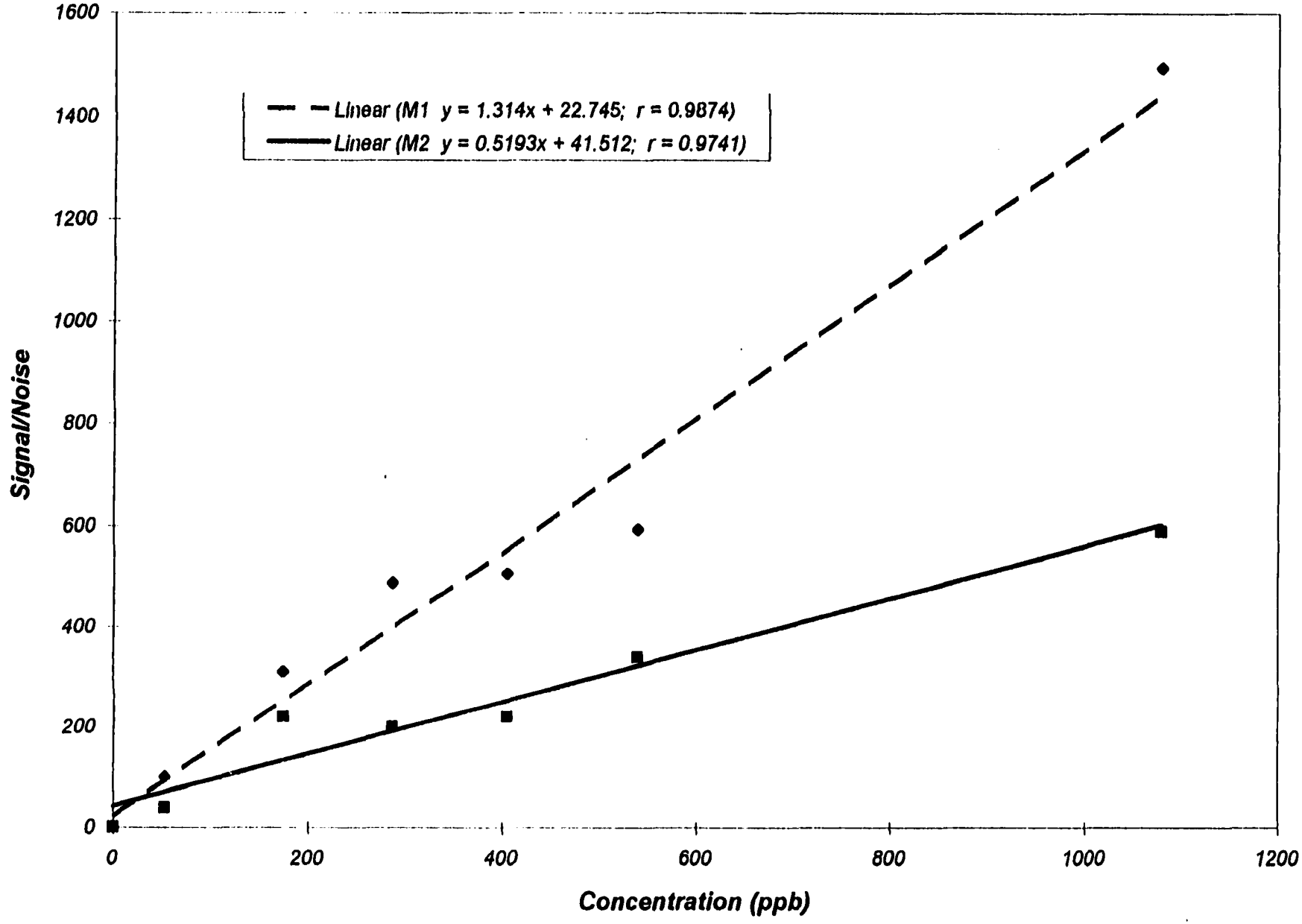
Appendix IV: Linear regressions of the signal response to noise standard deviation of intensities ratio versus concentration in determining the detection limit of the following steroids:

a) Androstenedione
b) Dehydroepiandrosterone
c) 17β-estradiol
d) Pregnenolone
e) 17-Hydroxypregnenolone
f) Progesterone
g) 17-Hydroxyprogesterone
h) Testosterone

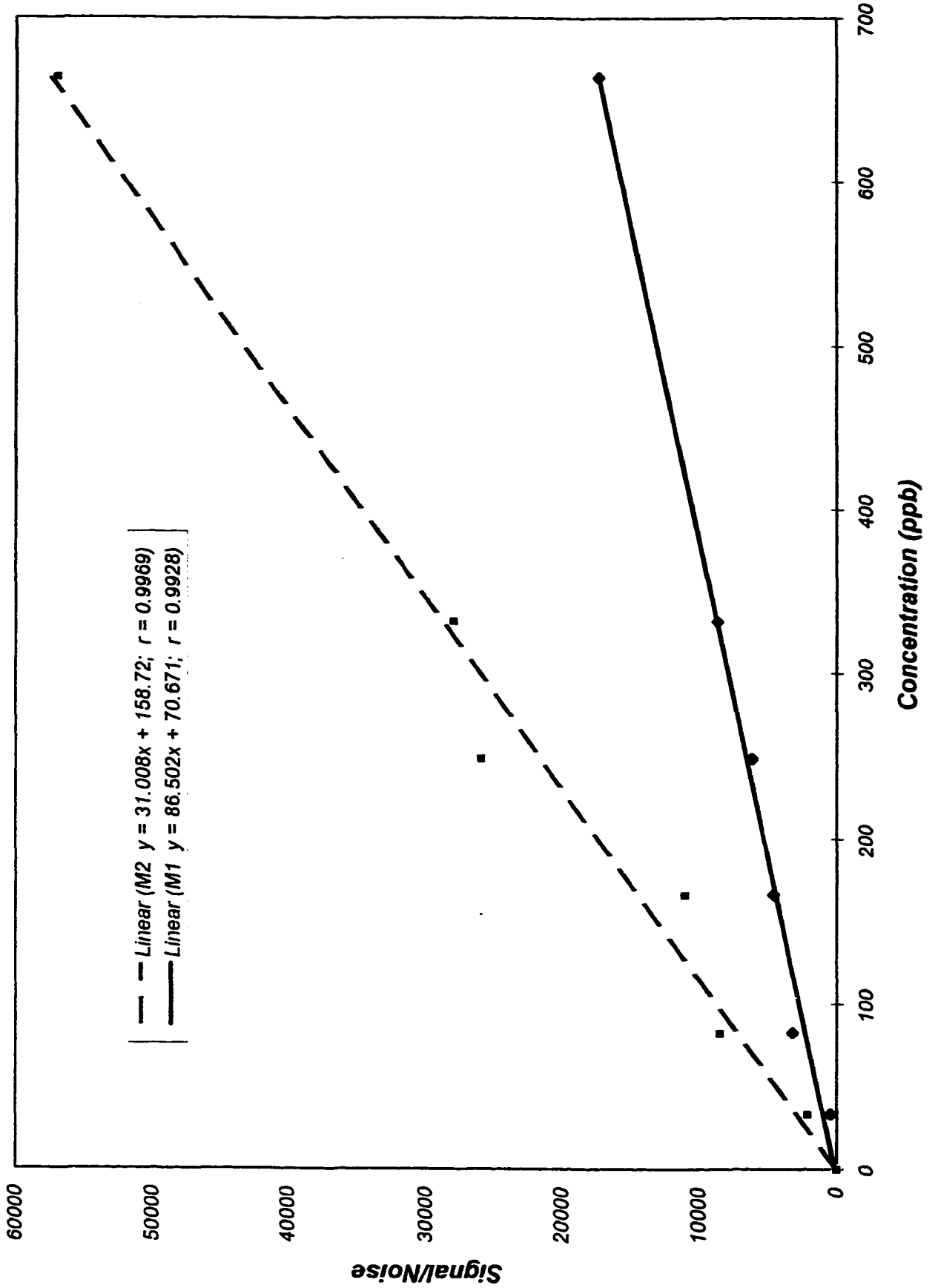
Appendix IV.A: Regression analysis (opposite page) to determine the analytical detection limit for androstenedione, where 'x' represents the signal/noise ratio. The detection limit is calculated from the regression using a value of 3 for 'x' which represents a signal to noise ratio of 3:1.



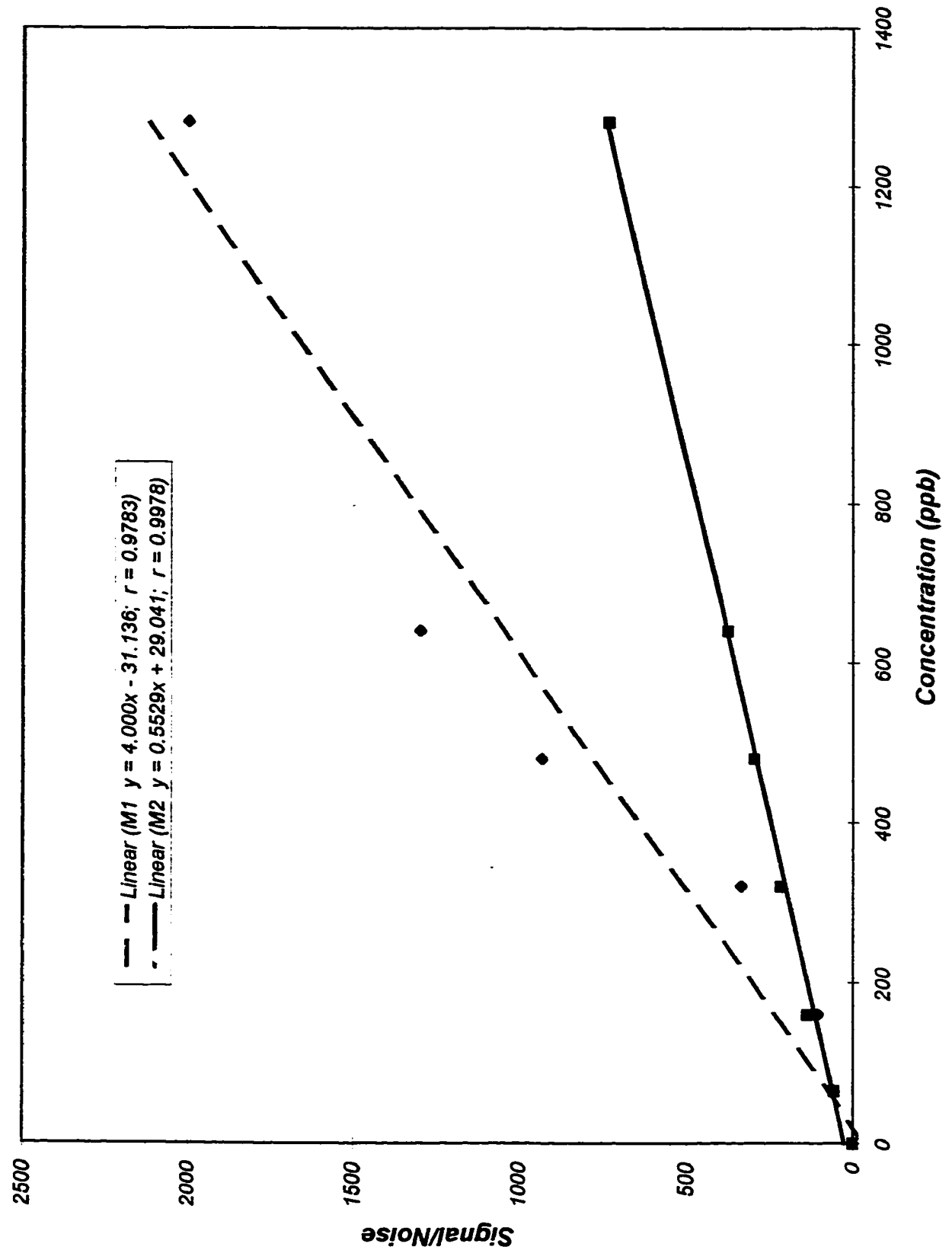
Appendix IV.B: Regression analysis (opposite page) to determine the analytical detection limit for dehydroepiandrostenedione, where 'x' represents the signal to noise ratio. The detection limit is calculated from the regression using a value of 3 for 'x' which represents a signal to noise ratio of 3:1.



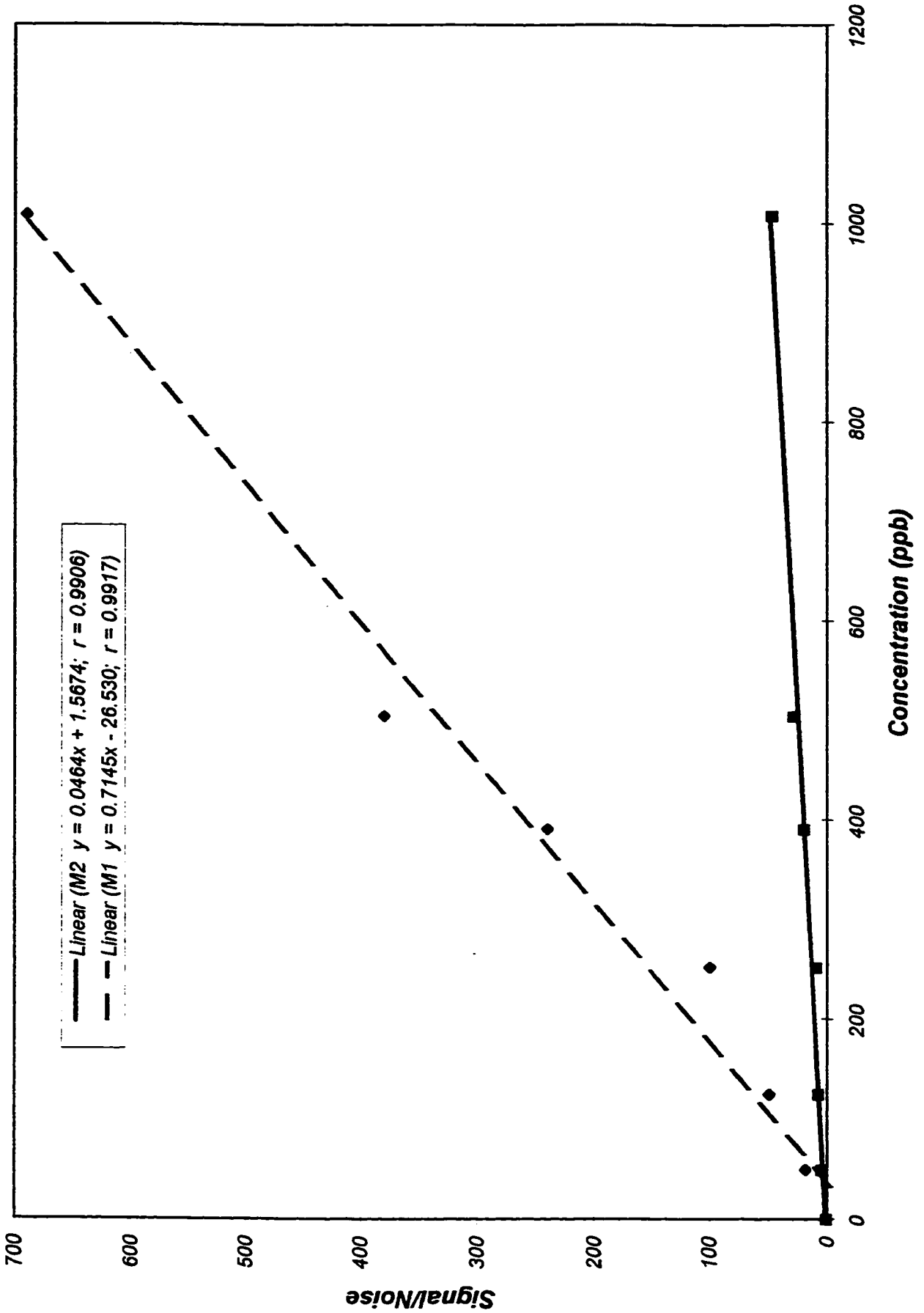
Appendix IV.C: Regression analysis (opposite page) to determine the analytical detection limit for 17β-estradiol, where 'x' represents the signal to noise ratio. The detection limit is calculated from the regression using a value of 3 for 'x' which represents a signal to noise ratio of 3:1.



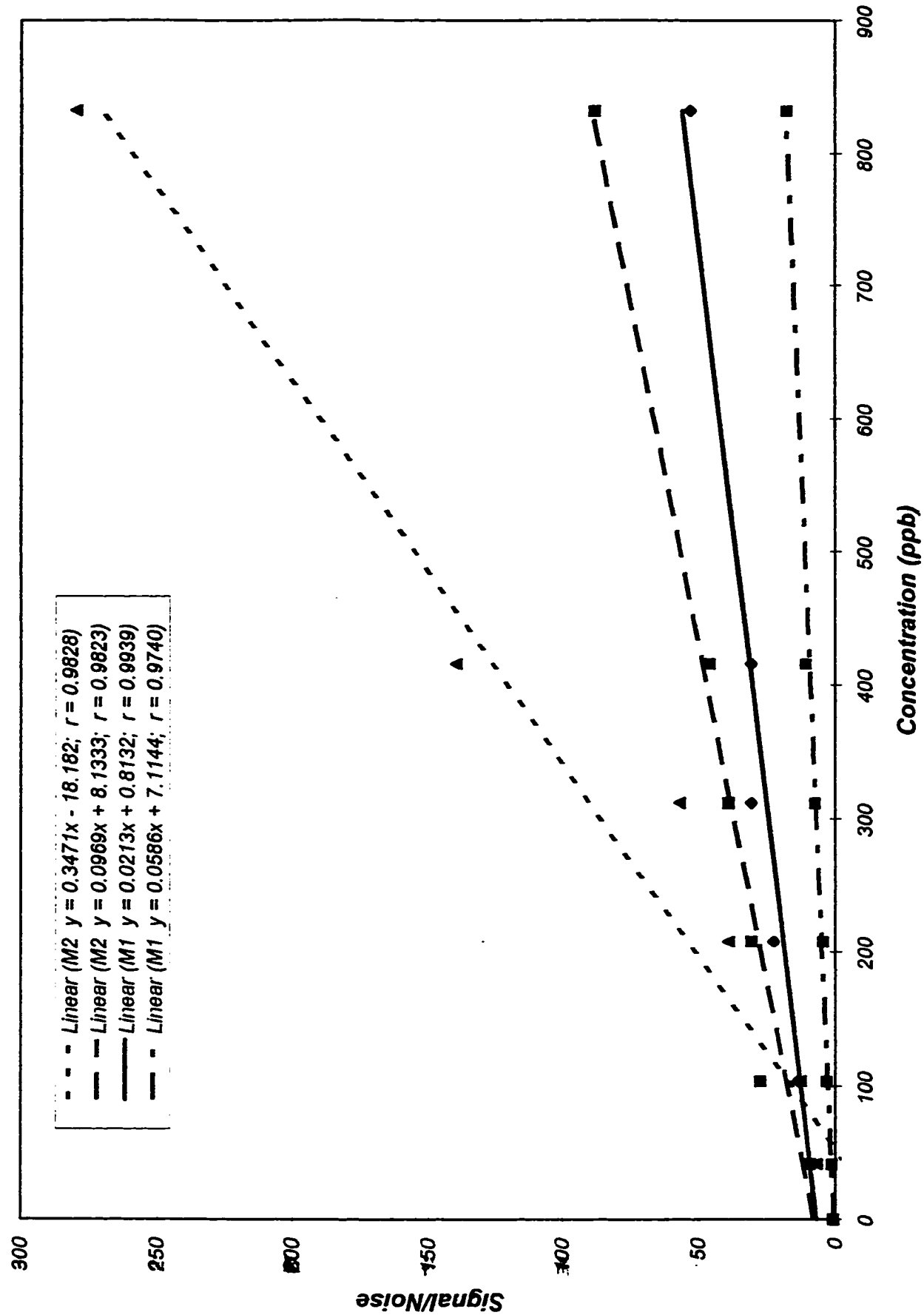
Appendix IV.D: Regression analysis (opposite page) to determine the analytical detection limit for pregnenolone, where 'x' represents the signal to noise ratio. The detection limit is calculated from the regression using a value of 3 for 'x' which represents a signal to noise ratio of 3:1.



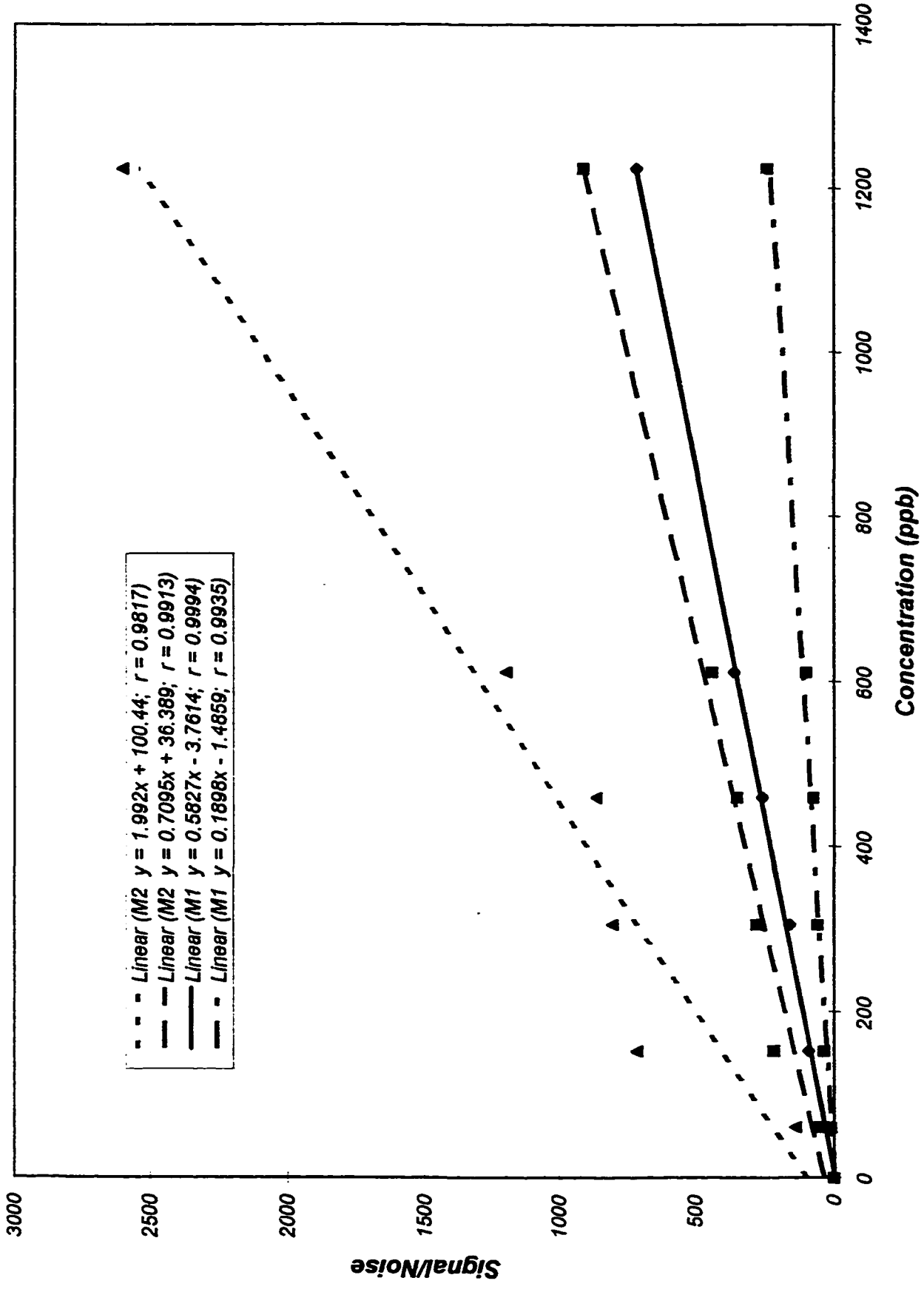
Appendix IV.E: Regression analysis (opposite page) to determine the analytical detection limit for 17-hydroxypregnenolone, where 'x' represents the signal to noise ratio. The detection limit is calculated from the regression using a value of 3 for 'x' which represents a signal to noise ratio of 3:1.



Appendix IV.F: Regression analysis (opposite page) to determine the analytical detection limit for progesterone, where 'x' represents the signal to noise ratio. The detection limit is calculated from the regression using a value of 3 for 'x' which represents a signal to noise ratio of 3:1.



Appendix IV.G: Regression analysis (opposite page) to determine the analytical detection limit for 17-hydroxyprogesterone, where 'x' represents the signal to noise ratio. The detection limit is calculated from the regression using a value of 3 for 'x' which represents a signal to noise ratio of 3:1.



Appendix IV.H: Regression analysis (opposite page) to determine the analytical detection limit for testosterone, where 'x' represents the signal to noise ratio. The detection limit is calculated from the regression using a value of 3 for 'x' which represents a signal to noise ratio of 3:1.