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# Isolation and Characterization of Ligands for the Goldfish Testicular Androgen Receptor from Kraft Mill Chemical Recovery Condensates

Philip David Scott
*Wilfrid Laurier University*

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# Isolation and Characterization of Ligands for the Goldfish Testicular Androgen Receptor from Kraft Mill Chemical Recovery Condensates

By

Philip David Scott
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Thesis

submitted to the Department of Chemistry
in partial fulfillment of the requirements for
the degree of Master of Science

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Waterloo, Ontario, Canada
2010

## AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Philip Scott

## Abstract

An investigation of cause was started in the 1990s at a bleached Kraft mill (BKM), located in Saint John NB, Canada. The 5$^{th}$ effect chemical recovery condensates waste stream (derived from wastewater generated during the pulping process) was identified as having the greatest potential for affecting mummichog (*Fundulus heteroclitus*), compared to the rest of the in-mill waste streams. A solid phase extraction (SPE) protocol was utilized to isolate the causative chemicals in the condensate stream. Goldfish testicular androgen receptors (ARs) were exposed to five extracts: filter paper non-polar/polar, solid phase extraction non-polar/polar, and residual condensates. The filter paper non-polar (FP-NP) extract contained a large quantity of ligands for the AR. A normal phase high performance liquid chromatography (HPLC) method was then developed to fractionate the FP-NP extract based on polarity, further isolating the androgenic compounds present in the condensates. Most of the androgenic activity ($p \leq 0.05$) was isolated in one HPLC fraction, with a smaller amount of activity identified in 3 additional fractions, demonstrating the presence of multiple ligands. Gas chromatography mass spectrometry (GC-MS) analysis of the active HPLC fractions identified four different families of diterpenoid compounds probably responsible for most of the androgenic activity. The naturally occurring cyclic diterpene manool was identified as a novel androgenic compound. Manool accounted for 25 and 14 % of the androgenicity in the FP-NP extract and the main HPLC fraction, respectively. To investigate if the AR ligands identified in condensates were present in mill discharges, final effluent from the Saint John mill and 10 other mills from Canada (3), Brazil (5) and New Zealand (2) were analyzed for manool and androgenic activity. It was determined that final effluent from all mills exhibited

androgenic activity in vitro. The highest levels of activity were consistently associated with solid phase extraction non-polar (SPE-NP) extracts of each effluent (with the exception of two mills), which ranged from ~100-800 ng testosterone /L condensate (equivalent). Of the two excepted mills, one was a thermomechanical pulp mill that treated effluent with activated sludge, while the other was a kraft pulp mill, which utilized reverse osmosis (RO) to treat effluent. All other mills used biological treatment to treat effluent. The highest androgenic activity detected was 3500 ng/L, from the Saint John mill, which was also the only mill effluent that had detectable levels of manool (115 ng/L).

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# Chapter 1

# Thesis Synopsis and Objectives

## 1.1 Synopsis

An investigation of cause was started in the 1990s as part of the environmental effects monitoring program at a bleached Kraft pulp and paper mill, located in Saint John NB, Canada. A study using artificial stream exposures and laboratory bioassays was used to identify the in-mill waste stream responsible for the presence of endocrine disrupting chemicals (EDCs). 5$^{th}$ Effect Chemical recovery condensates (derived from wastewater generated during the pulping process) showed the greatest potential for affecting mummichog (*Fundulus heteroclitus*) via decreases in circulating sex steroid levels. In order to isolate the causative chemicals in the chemical recovery condensate stream, a solid phase extraction (SPE) protocol was developed and utilized as part of a toxicity identification evaluation approach. In order to produce less chemically complex solutions, high performance liquid chromatography (HPLC) was used to fractionate SPE extracts. Mummichog were exposed to these HPLC fractions during a seven-day bioassay. An exposure concentration of 4 % (v/v) condensates elicited a significant depression in circulating sex hormones ($p \leq 0.05$), but 4 % (v/v) HPLC fractions of the same condensate sample did not. Recent studies determined that activity was lost during sample handling. A new SPE protocol was developed to generate condensate extracts, ensuring complete chemical recovery and retention of biological activity. The goldfish testicular androgen receptor (AR) binding assay was employed to examine the androgenic potential of condensate extracts generated with the new protocol. It was also used to guide HPLC

fractionation of active extracts. Final effluent and wood feedstock samples were also examined using the AR binding assay in order to compare androgenic activity of condensate, final effluent and wood feedstock from the same mill.

## 1.2 Thesis Objectives

The primary objective of this thesis was to characterize to the maximum possible extent the chemicals in Kraft mill chemical recovery condensates affecting fish reproduction. This objective was addressed according to the following steps:

1. To use the goldfish androgen receptor (AR) binding assay to determine the androgenic activity of condensate extracts generated with a new SPE protocol.
2. To develop and apply chemical methods for the isolation and identification of androgen receptor ligands in condensate extracts.
3. To compare the condensate extracts/fractions androgenically and chemically to wood and final effluent extracts to determine the relevance of the results beyond an in-mill waste stream.

This thesis is outlined as follows: Chapter 2, "General Introduction", contains a literature review and background information related to the research reported in this thesis. Chapter 3, "Manool Identified as a Novel Androgenic Compound Found in Bleached Kraft Pulp and Paper Condensate", has been written as a separate paper and will be submitted for

publication. It examines the androgenic activity and chemical analysis of chemical recovery condensate extracts and subsequent HPLC fractions. Chapter 4, "Quantifying Ligands for the Goldfish Testicular Androgen Receptor in Effluents and Wood Feedstocks from Pulp and Paper Mills in Canada, South America, and New Zealand" is this authors' contribution to an international study "Evaluating the Potential of Effluents and wood feedstock from Pulp and Paper Mills in Canada, South America and New Zealand to Affect Fish Reproduction". Chapter 4 will be used in a forthcoming manuscript to be submitted for publication in Fall 2010. It examines the androgenic activity of final effluent and wood feedstock extracts. Chapter 5, "General Discussion" provides conclusions based on information acquired in Chapters 3 and 4. It also suggests future studies that will allow the project to advance based on the discoveries presented in this thesis. Appendix A contains additional mass spectra to accompany Chapter 3. Finally, a collective bibliography (Chapter 6) can be found at the end of the thesis.

# Chapter 2
# General Introduction

## 2.1 Historical Overview

The Canadian pulp and paper industry is one of the largest industries in North America. Canada is the world's largest newsprint manufacturer and the primary exporter of wood pulp (Natural Resources Canada, 2002). In 1997, the industry employed 103,446 workers and recorded profits of $3.4 billion (Statistics Canada). 179 pulp mills and 129 paper mills produce 23 million tonnes of pulp and 17 million tonnes of paper, respectively (Smook, 1994; Langlois et al., 1997). Final effluent from these mills has the potential to harm fish in receiving waters by way of decreased gonad and egg size, increased age of sexual maturation, increased mixed function oxygenase (MFO) induction (an indicator of contaminant uptake in fish), altered expression of secondary sex characteristics, sexual dimorphism and decreased levels of reproductive sex hormones (Denton et al., 1985; McMaster et al., 1991; Munkittrick et al., 1991; Servos et al., 1996).

One of the first studies to examine the potential for ecological consequences of discharges of pulp and paper effluents was performed in Scandinavia between 1982 and 1985 (Södergren, 1989). This study showed that fish collected close to the effluent outfall of a bleached Kraft mill showed delayed sexual maturity, reduced gonad growth, liver enlargement, enhanced growth, induced ethoxyresorufin-O-deethylase enzyme (EROD) activity, affected

carbohydrate metabolism, and opercular deformities (Andersson et al., 1988). Induced mixed function oxygenase (MFO) enzyme activity is commonly measured as EROD activity in the livers of many fish species. It is generally considered an indication of exposure to a wide variety of planar organic contaminants. It is a highly sensitive indicator of contaminant uptake in fish (Munkittrick et al., 1994).

Effects were found further than 10 km from the pulp mill where the effluent was diluted more than 1000 fold. Impacts on biomass and distribution of invertebrates and plants were also reported. Soon after, Canadian studies were carried out to examine if the same impacts were observed at North American mills; similar effects were in fact observed (Munkittrick et al., 1998). Fish exposed to effluent exhibited decreased gonad and egg size, increased age of sexual maturation, increased MFO induction, altered expression of secondary sex characteristics, sexual dimorphism and decreased levels of reproductive sex hormones (Denton et al., 1985; McMaster et al., 1991; Munkittrick et al., 1991; Servos et al., 1996).

Before 1971 in Canada, pulp and paper effluents were discharged directly into aquatic environments with few regulations. Besides the effects that effluent had on fish physiology, aquatic environments were also greatly altered by effluent via habitat degradation (smothering of spawning beds and reduced oxygen concentrations in the water column). Fish populations near pulp and paper mills decreased substantially due to high levels of total suspended solids (TSS) and biochemical oxygen demand (BOD) of effluents (Mcleay, 1987). In 1971, government regulations were implemented under the Canadian Pulp and Paper

Effluent Regulation (CPPER) of the Fisheries Act (FA). Daily and monthly mass-based limits were implemented for TSS and BOD. These regulations were only applicable to mills built after 1971, which in the 1980s amounted to fewer than 10 % of the mills in Canada. Therefore, despite the 1971 federal government regulations, the majority of Canadian mills still produced effluent with high TSS and BOD that was harmful to aquatic life (Munkittrick et al., 1998).

In 1992, the federal government introduced the Canadian Environmental Protection Act (CEPA) whose purpose was to improve environmental performance of the forestry sector. CEPA introduced effluent limits for discharge of polychlorinated dibenzo-p-dioxins (PCDD) and dibenzofurans (PCDF), which were created by the use of elemental chlorine in pulp bleaching. CEPA further reduced acceptable levels of biological oxygen demand (BOD), chemical oxygen demand (COD), and acute toxicity of final effluents (Munkittrick et al., 1998). Throughout the 1990s, mills eliminated the use of elemental chlorine, implemented secondary treatment systems, and improved bleaching technologies and delignification processes to meet these new regulations. The Environmental Effects Monitoring (EEM) program was also created with the 1992 regulations. It required mills to monitor benthic and fish populations and thereby provide a feedback loop to determine if the 1992 regulations were adequate in protecting the environment. Canadian mills spent $5 billion implementing new mill processes to ensure their effluents met these regulations (Munkittrick et al., 1998). The EEM program is currently in its $6^{th}$ cycle, which began in 2010.

## 2.2 Effects of pulp mill effluent on fish reproduction

Pulp mill effluent (PME) has been known to negatively affect fish reproduction via endocrine disruption. Effects on fish reproduction were identified in Scandinavia (Södergren, 1989, 1992), and have since been documented in other pulp producing countries such as New Zealand (van den Heuvel and Ellis, 2002; van den Heuvel et al., 2002; Ellis et al., 2003), the U.S.A. (Drysdale and Bortone, 1989; Cody and Bortone, 1997; Bortone and Cody, 1999; Sepulveda et al., 2001) and Canada (McMaster et al., 1991; Munkittrick et al., 1991; MacLatchy and Van der kraak, 1995; Munkittrick et al., 1998; Couillard and Nellis, 1999; McMaster et al., 2006).

In Canada, the pulp and paper industry, academia and the federal government are working together to eliminate the toxicity of PME on fish reproduction in a cost-effective manner as part of overall sustainability objectives. Studies were initially undertaken in Canada to determine whether or not the effects in Scandinavian PME were present in Canadian PME. The first studies were done at Terrace Bay, ON, Canada during the late 1980s (McMaster et al., 1991). It was found that wild fish living downstream of a bleached Kraft mill (BKM) exhibited an increased age of maturity and smaller gonads in both males and females (McMaster et al., 1991). Males also showed a depression in secondary sex characteristics and females exhibited lower fecundity with age. The studies performed by McMaster et al. (1991) and Munkittrick et al. (1992) provided the first evidence that Canadian PMEs could disrupt the hormone system of fish as decreased levels of major circulating sex hormones

were found. The mill at Terrace Bay, ON, Canada installed a secondary treatment system in 1989 and switched its bleaching from molecular chlorine to chlorine dioxide in the mid 1990s. It was found that despite mill modernization, depression of circulating sex steroids persisted along with the induction of liver MFO (Munkittrick et al., 1992). This suggested that although ECF bleaching and secondary treatment improved effluent quality, they did not eliminate causative compounds responsible for steroid hormone depression from PME. Interestingly, impacts from causative compounds were found to be short-lived as populations recovered promptly after mill shutdowns in 1990 and more recently in 2006 (Bowron et al., 2009).

The toxic chemicals in PME responsible for the effects seen in fish are collectively termed endocrine-disrupting chemicals (EDCs) because they elicit their effects through endocrine-mediated pathways. The source of EDCs remains a mystery mostly due to the complexity of effluent (which consists of thousands of chemical compounds), and the large variation in responses of different fish species exposed to PME. This makes it difficult to identify mechanisms in which causative compounds affect fish (Van Der Kraak et al., 1998). Because of this, chemical fractionation techniques (filtering, solid-phase extraction; SPE, HPLC) and characterization techniques (gas chromatography-mass spectrometry, liquid chromatography-mass spectrometry) have been developed (Hewitt et al., 2002; Hewitt and Marvin, 2005; Belknap et al., 2006). Mechanistically linked bioassays have also been established to provide new insights as to the identities of the causative compounds as well as their source (MacLatchy et al., 2005).

Although estrogenic effects have been seen many times in PME, they have not been consistent, even within a single effluent (Van den Heuvel, 2010). The majority of estrogenic evidence has been based on the induction of vitellogenin, the egg yolk precursor protein. HPLC fractionation isolated MFO inducing chemicals that were relatively non-polar (Burnison et al., 1996) and later a chlorinated pterostilbene structure was postulated for an unknown compound associated with MFO induction (Burnison et al., 1999). This study performed on final PME was crucial as it linked a mill-altered natural compound with biological activity. More consistent responses have been associated with androgenic effects of PME compared to estrogenic effects.

### 2.2.1 Androgenic Effects of Mill Effluents

A significant body of evidence regarding the androgenicity of mill effluents has accumulated since the early 1980s. Effects have been measured in the New Zealand, USA, and Canada. The earliest research reporting androgenic properties of PME was published three decades ago by Howell et al. (1980). A more recent study indicated that 90 % of female mosquitofish (*Gambusia affinis*) exposed to BKME exhibited partial masculinization of the anal fin, while the remaining 10 % were completely masculinized (Cody and Bortone, 1997). Larson et al. (2002; 2003) reported male-biased sex ratios of fish embryos near a bleached Kraft pulp mill in Sweden. A temporary recovery was observed with a mill shutdown suggesting effects are not bioaccumulative.

Androgenicity has also been measured using in vitro methods, and these studies have been used to identify compounds associated with androgenic activity. For instance, Parks et al. (2001) reported that three BKME extracts from Florida contained ligands for human AR. An androgen-dependent gene expression was also observed. Hewitt et al. (2000; 2002; 2005) exposed fish to BKME from three Canadian mills. Liver extracts from all three groups of fish were then exposed to goldfish AR and found to contain ligands for the goldfish AR; these ligands were determined to be non-polar in nature. Final effluent from each mill elicited an androgenic response as well, however, primarily treated effluent showed higher concentrations of androgenic ligands (Hewitt et al., 2005).

Other recent studies have reported moderately polar compounds present in bioactive androgenic fractions. Carson et al. (2008) found that progesterone, androstenedione, and androstadienedione were present in water and sediment downstream from a bleached Kraft mill that caused masculinization of mosquitofish (*Gambusia affinis*). Androstenedione and progesterone are both intermediates in the biosysnthesis of testosterone, estrone, and estradiol (Carson et al., 2008). The authors proposed that progesterone and other androgens present in bioactive fractions could have been byproducts generated by the biodegradation of plant sterols, such as ß-sitosterol. Effluent samples from the same mill had masculinizing effects on eastern mosquitofish (*Gambusia holbrooki*) as evidenced by elongation of the anal fin (Jenkins et al., 2001; 2003; 2004). Ellis et al. (2003) reported that PME had androgenic

properties in *in vitro* experiments (using goldfish AR) as well as *in vivo* experiments (21 d exposure using mosquitofish; *Gambusia affinis*). Glass-fibre filtration reportedly eliminated the androgenic activity, suggesting that suspended solids contained the ligands for the goldfish AR.

## 2.3 Investigation of Cause

With numerous published articles noting the effects of PME on fish reproduction and growth, it was clear that government regulations were inadequate in protecting Canadian receiving environments. The Environmental Effects Monitoring (EEM) program was implemented nationwide in 1992, forcing mills to monitor their effluents more strictly using biomonitoring, to determine what improvements needed to be made. The EEM program focuses on effluent toxicity, benthic community structure, fish populations, fish consumption, and fish contamination (Munkittrick, 2004). The investigation of cause (IOC) approach was implemented as part of the larger EEM program, to address the lack of knowledge regarding the group of EDCs present in PME. Its objective is to identify causative compounds affecting fish reproduction and determine if mill processes and regulations need to be changed to eliminate EDCs from PME (Hewitt et al., 2003a).

### 2.3.1 Investigating Causes of Endocrine Disruption at Irving Pulp and Paper

Among the first mills to implement the IOC program was Irving Pulp and Paper (IPP) Ltd., a bleached Kraft pulp mill located in Saint John, NB, Canada. Ongoing studies at this mill started in 1997 and have been used to drive the development of IOC tools and approaches (MacLatchy et al., 2010). The objectives of the IOC work at IPP have been to: 1) Confirm the mill process/waste stream responsible for the introduction of EDCs into the effluent; 2) Identify the causative chemicals within the waste stream(s); and 3) Determine what technological changes can be made to eliminate EDCs from final effluent, and thus eradicate the negative effects on fish and the receiving ecosystem (MacLatchy et al., 2010).

Initial research at the mill used mesocosms to expose mummichog to multiple in-mill waste streams (condensates, post-oxygen washer filtrates) and final mill effluent for 21-57 d at environmentally relevant concentrations of 0.05 to 5 % v/v in order to identify the mill process responsible for the introduction of causative compounds (Dube and MacLatchy, 2001). The exposure to condensates caused females to have increased liver size and decreased *in vitro* production of plasma 17ß-estradiol, indicating that the condensates waste stream was contributing to nonlethal fish responses (Dube and MacLatchy, 2001). Both males and females also exhibited reduced plasma testosterone levels at 1 % (v/v) combined effluent.

In order to meet government regulations, a reverse osmosis (RO) system was installed at the mill in 1998 to decrease acute toxicity and BOD. This time, circulating hormone levels of mummichog exposed to 1 % (v/v) final effluent were unaffected. Thus the RO system significantly improved final effluent quality. However, mummichog exposed to 50 % (v/v) final effluent did experience decreased levels of plasma testosterone. It is evident that although the implementation of RO did reduce toxic effects, it did not eliminate EDCs from combined mill effluent. These results further confirmed that the 5$^{th}$ effect waste stream (RO feed) was a source of EDCs (Dube and MacLatchy, 2000). This study was the first of its kind to identify an in-mill waste stream responsible for endocrine disruption in fish, and show that RO treatment successfully reduced effluent toxicity. To date, IPP is the only known mill in Canada to use RO as a treatment technique for wastewater treatment.

Chemical complexity of PME has made it difficult to isolate and identify EDCs. For instance, the presence of residual lignin makes studying smaller bioactive compounds difficult (Hewitt et al., 2008). Condensates on the other hand, do not contain any lignin and are less chemically complex than PME. Dube and MacLatchy (2001) determined that the majority of EDCs were present in the 5$^{th}$ effect chemical recovery condensate waste stream (which is also the RO feed) and in the RO retentate, but not in the RO permeate. Therefore, focus shifted to biological and chemical analysis of the 5$^{th}$ effect chemical recovery condensates (MacLatchy et al., 2001).

As a result, Hewitt et al. (2002) developed a two-stage solid phase extraction (SPE) method to separate 5$^{th}$ effect chemical recovery condensates into chemically distinct extractives. Their endocrine-disrupting abilities were then assessed using a mummichog fish exposures (7 d static exposures with daily renewal). Mummichog were exposed to whole condensates, extracts from suspended solids (>1 μm), two fractions from the first SPE (styrene divinylbenzene); SPE-1 ethyl acetate and SPE-1 methanol, and one fraction from the second SPE (reversible graphitized carbon); SPE-2 extract, as well as residual condensates after SPE. Plasma testosterone levels were significantly depressed from SPE-1 methanol, SPE-2 extract, and suspended solids. No depression was observed with fish exposed to residual condensates after SPE and so it was concluded that the SPE method completely recovered activity from whole condensates (Hewitt et al., 2002).

A reverse-phase HPLC method was developed in order to fractionate the most potent fraction (SPE-2) on a preparative scale for a 7 d mummichog *in vivo* exposure (Shaughnessy et al., 2007), and to isolate the EDCs responsible for decreased circulating testosterone levels so they could be analyzed and identified. The first exposure tested 6 HPLC fractions of SPE-2 at 1 % (v/v) condensate equivalents. The fish response to the positive control (unfractionated SPE-2) was not as pronounced as the original exposure in Hewitt et al. (2002). A repeat exposure with 1.5 % (v/v) was inconsistent as well (Shaughnessy et al., 2007).

Mummichog were re-exposed to 0.5, 1, 2 and 4 % SPE-2, in order to determine if SPE-2 did in fact contain EDCs. Although females did not show any response, male fish exposed to 4 % SPE-2 showed a significant depression in plasma testosterone. Since it was confirmed that SPE-2 contained EDCs, a third fish exposure was performed with 4 % v/v HPLC fractions of SPE-2. Males experienced a significant reduction of plasma testosterone when exposed to 4 % v/v $5^{th}$ effect condensates. HPLC fractions showed no decrease in plasma testosterone. In fact, two HPLC fractions showed an increase in plasma testosterone. Females again showed no significant response to any fractions (Shaughnessy et al., 2007).

Despite the loss of activity reported by Shaughnessy et al. (2007), Belknap et al. (2006) was able to confirm the existence of 9 compounds in SPE extracts using GC-MS, quantified against authentic standards. Phenolic guaiacyl-based lignin degradation products, sulfur ($S_8$), three diterpenoids, and a dimethoxy pinosylvin stilbene were all confirmed (Belknap et al., 2006). Out of a total of 39 unique compounds in bioactive SPE extracts of condensates associated with hormone activity, there were 6 potential EDCs. Classes of chemicals included associated with reduction of plasma testosterone levels included hydroxylated diterpenoids, sesquiterpenois, and a lignin-derived stilbene. Candidate compounds associated with plasma testosterone depressions were identified by using a set of predetermined criteria based on previous experiments in which RO feed and RO retentate depressed testosterone (Dube and MacLatchy, 2001; MacLatchy et al., 2001; Belknap et al., 2006).

A study was then performed to ascertain why SPE-2 exhibited endocrine disrupting abilities, but HPLC fractions of SPE-2 did not. It was determined that the EDCs were lost when the mobile phase (a mixture of acetontitrile and water) was evaporated in preparation for solvent exchange. As it turns out, a large portion of the known compounds in condensates were partially volatilized at conditions similar to those required to evaporate water. Furthermore, samples dried beyond "just dryness" using nitrogen evaporation showed decreased concentrations of phenolics and diterpenes (such as manool and geranyl linalool; MacLatchy et al., 2010).

Normal-phase HPLC was tried unsuccessfully as an alternative to reverse-phase HPLC in an effort to implement a non-aqueous method for the fractionation of SPE-2. A different approach was taken and the graphitized carbon SPE-2 was eluted with a variety of solvents with decreasing polarity, but this resulted in a washing effect. These problems along with the fact that graphitized carbon SPE has the potential to bind organic compounds irreversibly (Hennion, 2000) shifted the focus to SPE-1. Experimentation with SPE-1 revealed that many compounds were being left on the cartridge using the original method, and therefore, not assessed in the *in vivo* fish exposures. It was therefore essential to develop a novel method of condensate extraction that produced chemically distinct fractions (MacLatchy et al., 2010).

A completely new SPE method was implemented for use with the RO-feed (5th effect evaporator condensates) thus eliminating the problems associated with the previous protocol

(Hewitt et al., 2002; Belknap et al., 2006). A total of 5 chemically distinct fractions (analyzed using GC-MS) were created (Figure 3-1; Milestone et al. 2010). Condensates were first glass-filtered and filter paper was extracted with dichloromethane to yield a non-polar fraction, followed by methanol giving a polar fraction. Filtrate was loaded onto Oasis HLB SPE cartridges, which were subsequently dried and eluted with dichloromethane to produce a non-polar fraction, and methanol to yield a polar fraction. Residual condensates were collected as the final fraction. Mass balance was used to ensure the complete recovery of a variety of compounds (Milestone et al., 2010). Given the robustness of this method, it was now possible to undertake new bioassay-directed fractionation experiments of compounds capable of affecting fish reproduction. It was hypothesized that androgenic substances are present in condensates and these compounds are affecting fish reproduction.

## 2.4 Description of the Kraft Pulping Process

### 2.4.1 Chemical Composition of Wood

Wood can be categorized into two main types: hardwood and softwood. Hardwood comes from angiosperms (meaning flowering plant) and is made up of deciduous trees; ash, beech, cherry, maple and oak are all examples of hardwood trees. Softwood comes from gymnosperms (meaning seed-bearing plants) and is made up of coniferous trees such as cedar, cypress, fir, pine, and spruce. Hardwood and softwood alike consist primarily of three chemical structures: cellulose, hemicellulose, and lignin. Structures for these three compounds are presented in Figure 2-1. Cellulose is a straight chain polymer with a rigid

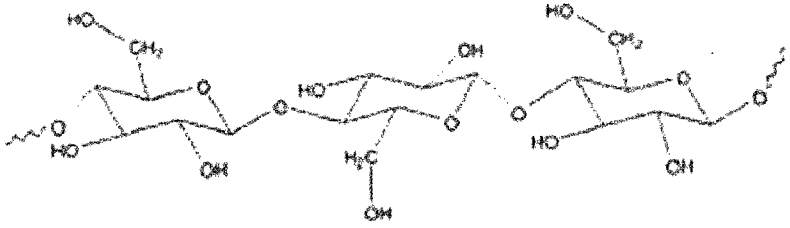
conformation. It is made solely from D-glucose units that are linked through β(1→4) glycosidic bonds (Fengel and Wegener, 1984). Glucose has many hydroxyl groups, which can form hydrogen bonds with other hydrogens in the same molecule, as well as neighboring molecules, adding strength to the overall structure. Cellulose chains consist of 7000 to 15000 monomers. Dried wood consists of roughly 40 to 50 % cellulose by mass (Fengel and Wegener, 1984).

Unlike cellulose, hemicellulose is a branched polymer that is made from several sugars, such as xylose, galactose, aribinose and glucose. Due to branching, hemicellulose is unable to sustain as many hydrogen bonds as cellulose. It has a lower tensile strength compared to cellulose, and its polymer chains consist of only 500 to 3000 sugar monomers. Hemicellulose accounts for 20 to 30 % of dried wood by mass (Fengel and Wegener, 1984).

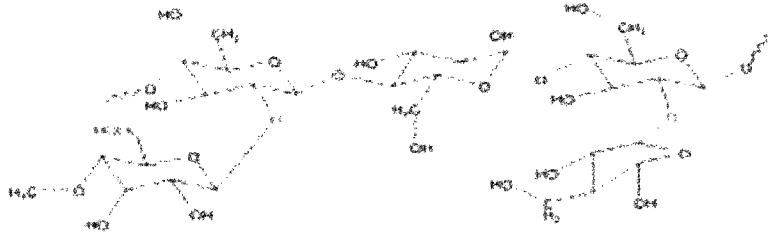
Lignin is a three-dimensional hydrophobic polymer, which gives wood its mechanical strength by covalently bonding to hemicellulose and therefore creating numerous crosslinks. Lignin has molecular masses greater than ten thousand amu and is constructed randomly thus lacking ordered repeating units, unlike cellulose and hemicellulose. Lignin is comprised of coniferyl alcohol (softwood) or a mixture of coniferyl and sinapyl alcohol (hardwood) (Figure 2-2). The mode of polymerization of these alcohols in the cell wall leads to a heterogeneous branched and cross-linked macromolecule (Fengel and Wegener, 1984; Dence and Reeve, 1996). During the bleaching process, lignin can undergo a large number of reactions including electrophilic substitution and oxidation of aromatic structures, coupling

of lignin free radicals, and free radical-initiated cleavage of ortho-quinonoid rings (Dence and Reeve, 1996). Lignin makes up 16-24 % of dried softwood by mass, and 24-33 % of dried hardwood by mass (Fengel and Wegener, 1984).

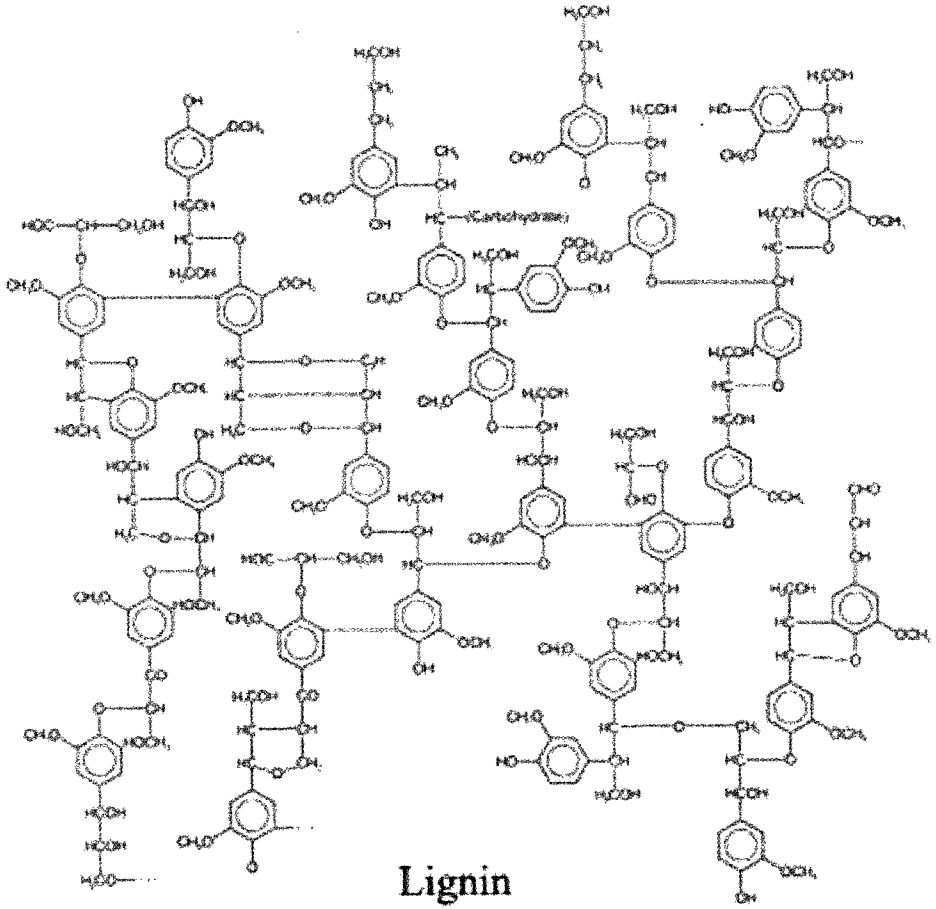
Figure 2-1: Structures of the Three Primary Chemical Components Present in Wood (Fengel and Wegener, 1984)



Cellulose

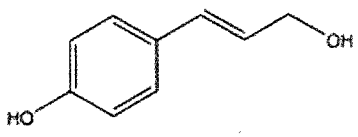


Hemicellulose

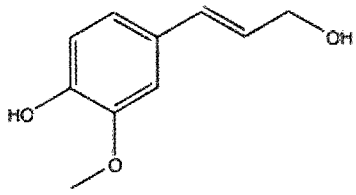


Lignin

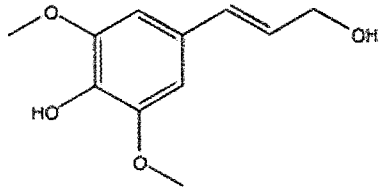
Figure 2-2: Structures of the Three Main Components of Lignin (Dence and Reeve, 1996)




p-Coumaryl alcohol




Coniferyl alcohol




Sinapyl alcohol

### 2.4.2 Recovery

The main objective of a Kraft pulp and paper mill is to separate the useable cellulose and hemicellulose fibres by dissolving lignin. White liquor (NaOH, $Na_2S$, $Na_2CO_3$) is added to woodchips in a digester that is heated to temperatures between 130 and 180 °C and pressure of 90 to 100 psi. Under these conditions the white liquor dissolves the lignin along with a small amount of hemicellulose. Digested pulp, or "brownstock", remains and is rinsed of spent cooking chemicals that take the form of weak black liquor (WBL). The pulp is then sent for removal of residual lignin via oxygen delignification and/or bleaching with chlorine dioxide ($ClO_2$).

The WBL (also known as "brownstock wash"), is a mixture of dissolved and degraded carbohydrates (from cellulose), hemicellulose, macromolecular lignin, total reduced sulfur (TRS) byproducts (methyl mercaptan, dimethyl sulfide, dimethyl disulfide), organic wood-derived coextractives (turpentine, soap, dissolved pitch), and spent inorganic cooking chemicals (US EPA, 1997). In order to recycle cooking chemicals from the WBL, it is first heated in a series of evaporators for dewatering (Figure 2-3). WBL (18 % oven dried solids; ODS) is concentrated across 6 evaporators (known as a multiple effect evaporator system; MEE) under vacuum to yield strong black liquor (SBL; 70 % ODS), which is then sent to the recovery boiler. The MEE uses a countercurrent flow of steam to carry vaporized components between a series of evaporators in sequence. A different vapour consisting of low molecular weight volatile and semi-volatile black liquor compounds is produced at each effect, which is subsequently used to heat the liquor at the next effect. The vapours consist

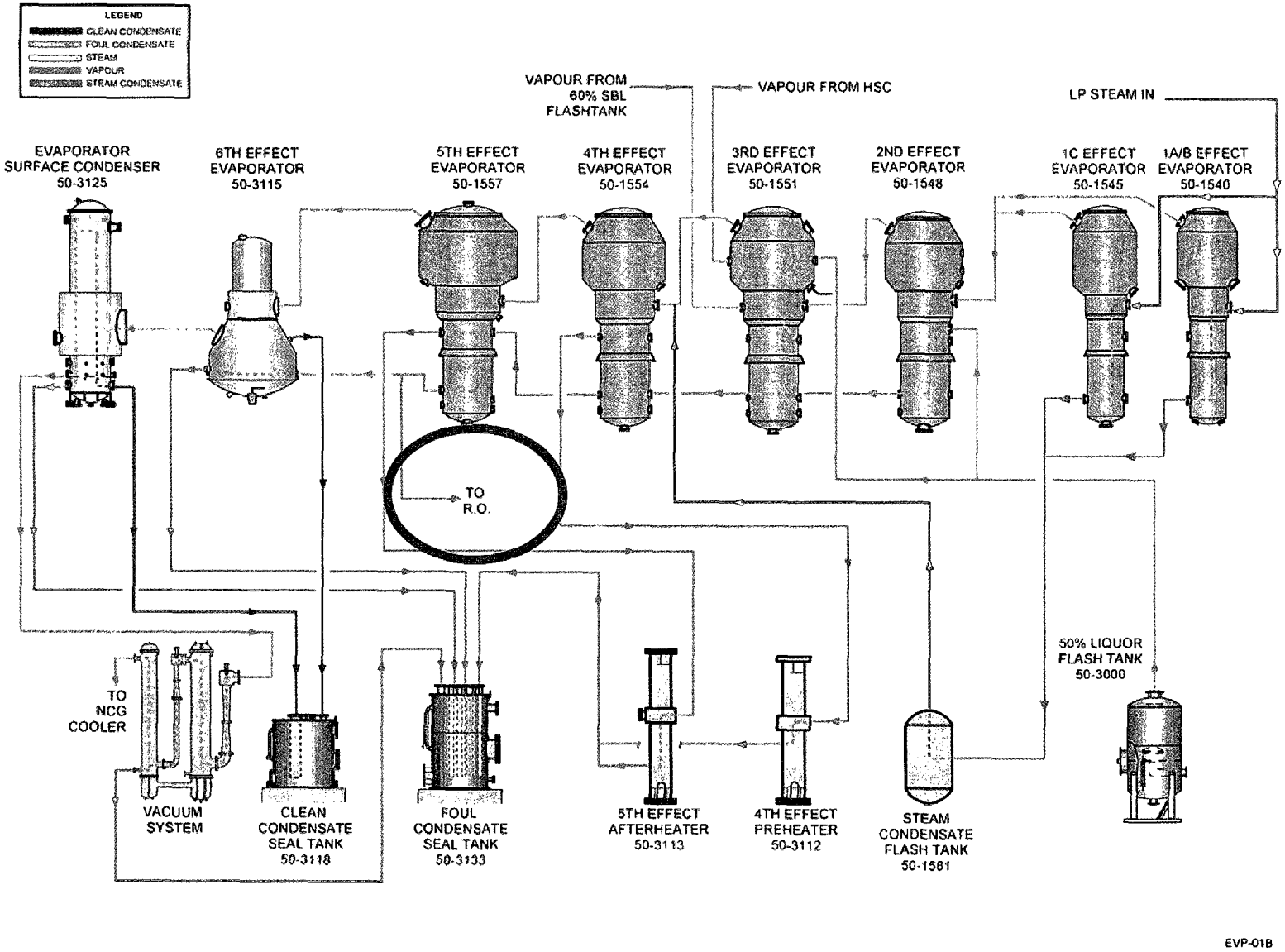
of many aldehydes, ketones, terpenes, alcohols (mainly methanol), sulfur-containing compounds, phenolics, and dissolved gases (Blackwell et al., 1979; Lafleur et al., 1995). As the vapour heats up the liquor at the next effect, it loses its energy and condenses. The liquor then produces a new vapour mixture. The cycle repeats itself at the each effect.

A vacuum is applied to the 3$^{rd}$, 4$^{th}$, 5$^{th}$, and 6$^{th}$ effects to facilitate in the flow of the vapour, which enters as low pressure steam from the 1$^{st}$ effect, and to reduce the boiling point of the liquor. The pressure at the 1$^{st}$ effect is 20 psi, so only the more readily volatile compounds evaporate (e.g. methanol). The 5$^{th}$ effect condensate is comprised of gases produced from the liquor at the 4$^{th}$ effect, which has an inlet temperature of 90 °C and an outlet temperature of 78 °C. The 5$^{th}$ effect condensate therefore consist of compounds with boiling points greater than 90 °C, but due to the vacuum applied to the 5$^{th}$ effect, many compounds with much higher boiling points are present. The temperatures of each effect can therefore only give an estimate as to the boiling points of compounds found in each effect condensate. The 6$^{th}$ effect condensate is made up of vapour from the 5$^{th}$ effect, which is condensed and eventually goes into a foul condensate tank where it is typically steam stripped before being sent to the combined chemical sewer. It should be noted that the major sources of BOD and final effluent toxicity within a Kraft mill are from condensates as well as the bleach plant. A series of studies at a bleached Kraft pulp and paper mill in Saint John NB, Canada led to the implementation of a condensate waste treatment system within the mill for the primary purpose of reducing final effluent BOD for regulatory compliance (Dubé et al., 2000). This treatment employs a reverse osmosis (RO) treatment of the 5$^{th}$ effect condensates.

### 2.4.3 Reverse Osmosis

RO is a filtration method that removes many dissolved ions (e.g. salts) and molecules from water. It works by applying an external pressure across a semi-permeable membrane in the opposite direction of the osmotic gradient. The solute is retained on the pressurized side of the membrane, while the solvent passes to the other side. In the Saint John mill, 5$^{th}$ effect condensate (RO-feed) is pressurized and forced through the RO system at an average rate of 4164 L/min. The RO-permeate ("clean condensates" that have passed through the membrane; 99 % of the flow) consists of low molecular weight molecules and water. The RO-retentate (compounds that did not make it across the membrane; 1 % of the flow) is made of large ions and molecules that were too big to travel across the membrane. The membrane removes most low molecular weight dissolved salts, inorganic compounds and organic compounds with particle diameters less than 0.001 μm (Dubé et al., 2000). RO-retentate is burned in the bark-boiler, while RO-permeate is used in the bleaching section of the mill to dilute pulp stock. Irving Pulp and Paper Ltd. is the only known example of a pulp and paper mill to implement a large-scale RO system to treat its condensates. It has replaced the need for secondary treatment and studies performed before and after installation indicated that effects on fish reproduction (measured as plasma hormone depressions) were significantly decreased. A significant effect was observed at concentrations of 50 % (v/v) final effluent or higher following RO implementation (Dubé et al., 2000).

Figure 2-3: Diagram of the Multiple Effect Evaporator (MEE) at a Mill Located in Saint John, NB, Canada. WBL enters the MEE at the 1st effect evaporator. As the WBL travels through each effect it is concentrated. The blue circle represents the location where 5th effect evaporator condensates (RO-feed) were sampled for this study. The sampling takes place prior to the condensates entering the reverse osmosis (RO) system (JD Forest Product, Ltd.). 5th Effect vapour is sent to the 6th and final effect evaporator. The discharge from the $6^{th}$ effect is then sent to a foul condensate storage tank.


LEGEND
CLEAN CONDENSATE
FOUL CONDENSATE
STEAM
VAPOUR
STEAM CONDENSATE
VAPOUR FROM 60% SBL FLASHTANK
VAPOUR FROM HSC
LP STEAM IN
EVAPORATOR SURFACE CONDENSER 50-3125
6TH EFFECT EVAPORATOR 50-3115
5TH EFFECT EVAPORATOR 50-1557
4TH EFFECT EVAPORATOR 50-1554
3RD EFFECT EVAPORATOR 50-1551
2ND EFFECT EVAPORATOR 50-1548
1C EFFECT EVAPORATOR 50-1545
1A/B EFFECT EVAPORATOR 50-1540
TO R.O.
TO NCG COOLER
VACUUM SYSTEM
CLEAN CONDENSATE SEAL TANK 50-3118
FOUL CONDENSATE SEAL TANK 50-3133
5TH EFFECT AFTERHEATER 50-3113
4TH EFFECT PREHEATER 50-3112
STEAM CONDENSATE FLASH TANK 50-1581
50% LIQUOR FLASH TANK 50-3000
EVP-01B

# Chapter 3

# Isolation and Identification of Ligands for the Goldfish Testicular Androgen Receptor in Chemical Recovery Condensates from a Canadian Bleached Kraft Pulp and Paper Mill

## 3.1 Abstract

An investigation of cause was started in the 1990s at a bleached Kraft pulp and paper mill, located in Saint John NB, Canada. The 5$^{th}$ effect chemical recovery condensates waste stream was identified as having the greatest potential for effecting mummichog (*Fundulus heteroclitus*), compared to the rest of the in-mill waste streams. A solid phase extraction (SPE) protocol was utilized to isolate the causative chemicals in the condensate stream. Goldfish testicular androgen receptors (ARs) were exposed to five extracts: filter paper non-polar/polar, solid phase extraction non-polar/polar, and residual condensates. The filter paper non-polar (FP-NP) extract contained a large quantity of ligands for the AR. A normal phase high performance liquid chromatography (HPLC) method was then developed to fractionate the FP-NP extract based on polarity, further isolating the androgenic compounds present in the condensates. Most of the androgenic activity ($p \leq 0.05$) was isolated in one HPLC fraction, with a smaller amount of activity identified in 3 additional fractions, demonstrating the presence of multiple ligands. Gas chromatography mass spectrometry (GC-MS) analysis of the active HPLC fractions identified four different families of diterpenoid compounds likely responsible for most of the androgenic activity. The naturally occurring cyclic diterpene

manool was identified as a novel androgenic compound. Manool accounted for 25 and 14 % of the androgenicity in the FP-NP extract and the main HPLC fraction, respectively.

## 3.2 Introduction

Bleached Kraft pulp and paper mill effluents (BKMEs) are known to contain endocrine disrupting compounds (EDCs) that negatively affect fish reproduction. The first evidence of this came out of Scandanavia (Södergren, 1989), and has since been reported in the New Zealand (van den Heuvel and Ellis, 2002; van den Heuvel et al., 2002; Ellis et al., 2003), U.S.A (Drysdale and Bortone, 1989; Cody and Bortone, 1997; Bortone and Cody, 1999; Sepulveda et al., 2001), Chile (Orrego et al., 2005; 2006), and Canada (McMaster et al., 1991; Munkittrick et al., 1991; Couillard and Nellis, 1999). Regulations implemented under the Canadian Environmental Protection Act and the amended Canadian Pulp and Paper Effluent Regulation of the Federal Fisheries Act in 1992 placed restrictions on biochemical oxygen demand (BOD), acute toxicity, and dioxin and furan concentrations in whole effluents (Munkittrick et al., 1998). Because of this, Canadian pulp and paper mills have invested an estimated $5 billion implementing secondary treatment and eliminating chlorine in bleaching techniques (Munkittrick et al., 1998). Effluent quality was greatly improved, but unfortunately, sublethal effects are still observed in fish living near pulp and paper mills (Lowell et al., 2002).

The Environmental Effects Monitoring (EEM) program was implemented nationwide in 1992, forcing mills to monitor their effluents more strictly by using biomonitoring techniques to determine whether or not current regulations were sufficient in protecting the environment. The EEM program focuses on effluent toxicity, benthic community structure, fish populations, fish consumption, and fish contamination (Munkittrick, 2004). Since its creation, the EEM program has found that wild fish exposed to PMEs suffer from decreased gonad and egg size, increased age of sexual maturation, increased MFO induction, altered expression of secondary sex characteristics, sexual dimorphism and decreased levels of reproductive sex hormones (Denton et al., 1985; McMaster et al., 1991; Munkittrick et al., 1991; Servos et al., 1996). The source of EDCs remains a mystery due mainly to the complexity of effluent (which consists of thousands of compounds), and the large variation in responses of different fish species exposed to PME; this makes it difficult to identify the mechanisms by which causative compounds affect fish (Van Der Kraak et al., 1998). Chemical fractionation techniques (filtering, solid-phase extraction, HPLC) and characterization techniques (GC-MS, LC-MS) have been developed (Hewitt et al., 2002; Hewitt and Marvin, 2005; Belknap et al., 2006) along with mechanistically linked bioassays (MacLatchy et al., 2005) to facilitate in the identification of causative compounds.

The Investigation of Cause (IOC) was implemented as part of the EEM program, in order to identify causative compounds affecting fish reproduction, and determine if current mill processes and regulations are adequately protecting the aquatic receiving environments (Hewitt et al., 2003a). One of the first mills to use the IOC approach was a bleached Kraft

pulp mill located in Saint John NB, Canada, in 1997. The objectives of the IOC study have been to: 1) Confirm the mill process/waste stream(s) responsible for the introduction of EDCs into the effluent; 2) Identify the causative chemicals within the waste stream(s); and 3) determine what technological changes can be made to eliminate EDCs from final effluent, and thus eradicate the negative effects pulp mills have on fish and the receiving ecosystems (MacLatchy et al., 2010).

Initial research at the mill used mesocosms to expose mummichog to multiple in-mill waste streams (condensates, post-oxygen washer filtrates, and final mill effluent) for 21-57 d at environmentally relevant concentrations of 0.05 to 5 % v/v (Dube and MacLatchy, 2000). Both males and females exhibited reduced plasma testosterone levels at 1 % (v/v) combined effluent. The exposure to condensates caused females to have increased liver size and also decreased *in vitro* production of plasma 17ß-estradiol. This indicated that the condensate waste stream was contributing to nonlethal fish responses (Dube and MacLatchy, 2000). As a result of these findings, the Saint Johns mill installed a reverse osmosis (RO) system (described later) in 1998 to treat 5$^{th}$ effect condensates (RO-feed) for biochemical oxygen demand (BOD) and acute toxicity removal. Experiments performed before and after installation of the RO system showed that RO treatment reduced the potential of condensates and combined mill effluent to depress circulating sex steroids (Dube and MacLatchy, 2000; MacLatchy et al., 2001).

Toxicity identification evaluation (TIE) framework was used to aid in the identification of causative compounds. By using this framework, bioactive compounds can be isolated into less complex mixtures using iterative extractions and fractionations, thus facilitating in the identification of causative chemicals. Once chemicals are confirmed, mechanisms of effect can then be deduced (Hewitt et al., 2003a). The TIE was implemented in 2001 in order to characterize the bioactive compounds in the RO-feed. A extraction method was developed, which created 5 chemically distinct RO-feed extracts. An androgen receptor (AR) binding assay guided fractionation of androgenic extracts to facilitate in the isolation of androgenic compounds.

The objectives of this study were to: 1) Assess the androgenic variance in multiple temporal samples of $5^{th}$ effect condensates. 2) Develop chemical methods for fractionation of the most androgenic condensate extract. 3) Isolate those fractions responsible for androgenic activity and identify candidate compounds using GC-MS analysis.

## 3.3 Methods and Materials

### 3.3.1 Chemicals

Dichloromethane, hexane, methanol, toluene and water (all Optima grade) as well as geranyl linalool were supplied by Fisher Scientific (Nepean, ON, Canada). Chemical standards of 4-ethyl guaiacol, isoeugenol, squalene, and veratraldeyhde were supplied by Pfaltz and Bauer (Waterbury, CT, USA). Manool was supplied by Industrial Research (Wellington, New

Zealand). Vanillin, glycerol, Trizma hydrochloride, Ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate, activated charcoal, and dextran from *leuconostoc mesenteroides*, stigmasterol, cholesterol, and testosterone were supplied by Sigma-Aldrich Chemical (Milwaukee, WI, UA). Tritium labeled ($^3$H) testosterone was provided by Perkin Elmer (Waltham, Massachusetts, USA).

### 3.3.2 Mill Process Description

The bleached Kraft mill selected for this study was located in Saint John NB, Canada, where condensates have previously been studied (Dubé et al., 2000; Dube and MacLatchy, 2000, 2001; MacLatchy et al., 2001; Hewitt et al., 2002; Belknap et al., 2006; Shaughnessy et al., 2007). The mill uses a 5-stage $D_{100}E_{op}DED$ bleaching sequence ($D_{100}$ = chlorine dioxide, oxygen bleaching; $E_{op}$ = caustic, peroxide, and oxygen extraction; D = chlorine dioxide bleaching; E = caustic extraction) to produce pulp from alternating batch runs of hardwood (maple and birch) and softwood (spruce, pine, and fir). The mill uses primary clarifiers to remove suspended solids before discharge of final effluent. Approximately 990 air-dried tonnes of pulp are produced daily.

Weak black liquor (also known as "brownstock") is generated from rinsing digested pulp. Initially, it consists of 18 % oven dried solids (ODS) but it is concentrated across 6 evaporators (known as a multiple effect evaporator system; MEE) under vacuum to yield strong black liquor (SBL; 50 % ODS). SBL is dried further in the MEE to 70 % ODS and

sent to the recovery boiler and bark burner for combustion in order to generate electricity for the mill. The MEE uses a countercurrent flow of steam to carry vaporized components between a series of evaporators in sequence. A different vapour, consisting of low molecular weight volatile and semi-volatile black liquor compounds, is produced at each effect, which in turn is used to heat the liquor at the next effect. The vapours consist of many aldehydes, ketones, terpenes, alcohols (mainly methanol), sulfur-bearing compounds, phenolics, and dissolved gases (Blackwell et al., 1979; Lafleur, 1996). As the vapour heats up the liquor at the next effect, it loses its energy. Once the condensates are sufficiently heated, a new vapour mixture is produced. The cycle repeats itself at the next effect. The $5^{th}$ effect condensates are comprised of gases produced form the liquor at the $4^{th}$ effect, which has an inlet temperature of 90 °C and an outlet temperature of 78 °C. $5^{th}$ effect vapours are segregated from the condensed vapors from evaporators 1 and 3, which progress to the $6^{th}$ effect evaporator, and on to the surface condenser and stripper. Condensates from the surface condenser are stripped with steam to liberate the volatile compounds and then reused in the mill for post-oxygen wash dilution and shower water in the bleach plant.

RO-feed ($5^{th}$ effect condensates) is sent to the RO system and separated into 2 product streams. The RO-permeate ("clean condensates" that have passed through the membrane; 99 % of the flow), which consists of low molecular weight molecules and water, is reused in the mill for brownstock washing and post-oxygen wash/dilution water. The RO-retentate (compounds that did not make it across the membrane; 1 % of the flow), which consists of large ions and molecules that were too big to travel across the membrane, is incinerated in the bark burner (Dubé et al., 2000). To the best of our knowledge, this mill is the only of its

kind to use a large-scale RO system to treat its condensates. It has replaced the need for secondary treatment and studies performed before and after installation indicate that sub-lethal plasma hormone depressions are no longer apparent in fish belonging to the receiving environment (Dubé et al., 2000).

### 3.3.3 Condensate sampling and preparation

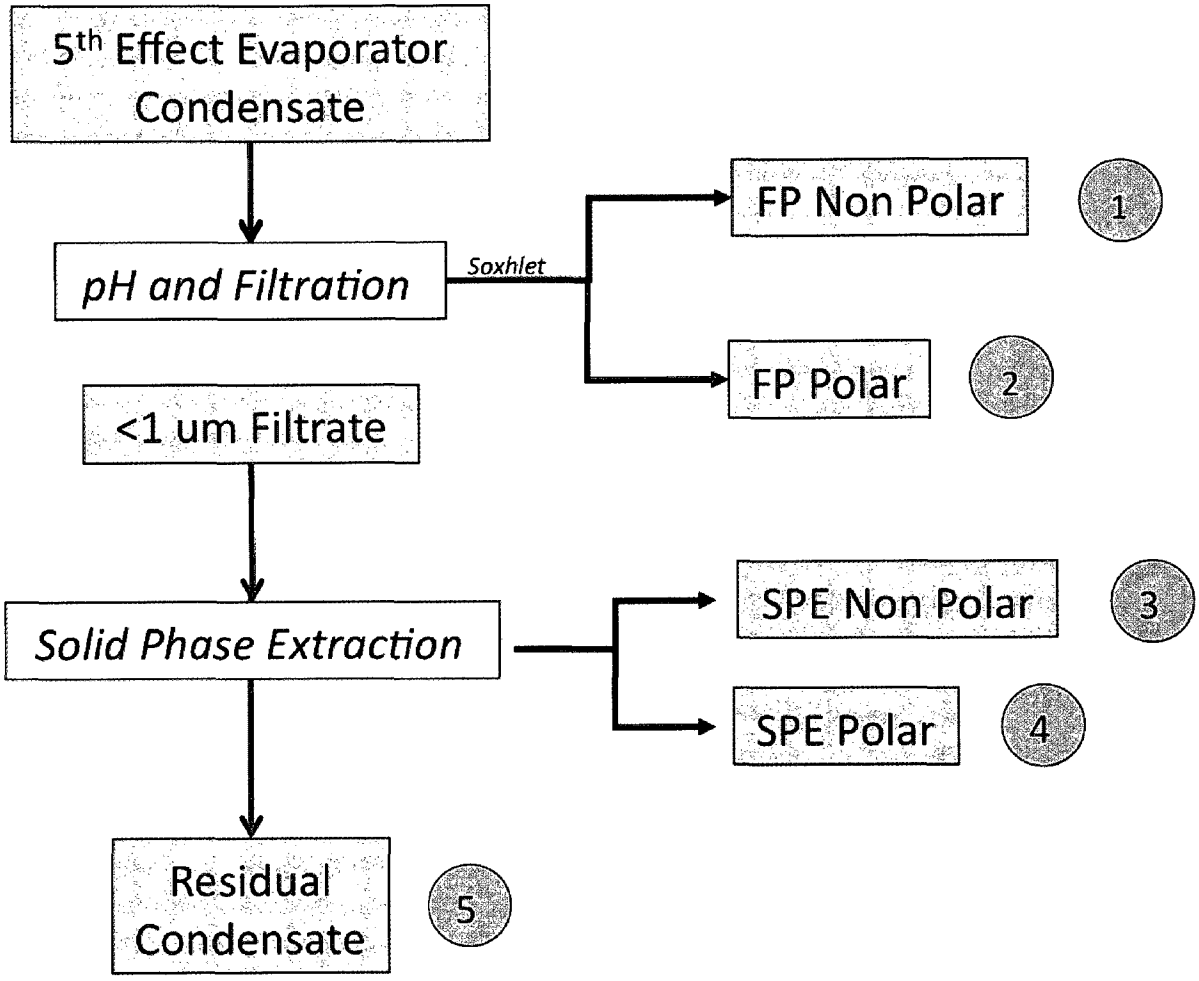
On 3 different occasions throughout a 1-year period (May 2009, August 2009, April 2010), condensates were collected in 20 L stainless steel containers and shipped overnight to the National Water Research Institute (Burlington, ON, Canada). Condensates were collected from the $5^{th}$ effect evaporator stage within the evaporator train of chemical recovery. All sampling for this study was from softwood production, and condensates were sampled 5 days after switchover from hardwood to ensure condensates were representative of softwood production (D. Embley, JD Irving Forestry, personal communication). Upon arrival, condensates were adjusted to pH 4 using 3 M hydrochloric acid (HCl) at room temperature. Subsamples were submitted to Environment Canada's Wastewater Technology Centre (WTC) Analytical Laboratory (Burlington, ON, Canada) for evaluation of alkalinity, anions, colour, conductivity, total and dissolved metal analysis, total organic carbon (TOC) analysis, and total and volatile suspended solids (TSS) according to established protocols. Condensate batches 1, 2, and 3 were sampled to determine what variations, if any, exist in alkalinity, anions, colour, conductivity, total and dissolved metal analysis, total organic carbon (TOC)

analysis, total and volatile suspended solids (TSS), concentrations of known compounds, and androgenicity. Condensate 3 was subjected to full fractionation.

### 3.3.4 Condensate Extraction and Fractionation

Condensate extraction occurred upon arrival (within 24 h) to minimize the risk of chemical modifications and were conducted according to Milestone et al. (2010). After pH adjustment, condensates were fortified with 2 % (v/v) methanol and filtered using 1.0 μm glass fiber filters (45 mm, GF/C; Whatman, Clifton, NJ, USA). Fouled filters were air dried, cut into 1 $cm^2$ sections and non-polar compounds extracted with approximately 300 mL dichloromethane using a Soxhlet apparatus for 24 h (Figure 3-1: Fraction 1; FP-NP). Methanol was then used to recover polar compounds (Figure 3-1: Fraction 2; FP-P). Condensate filtrates were then subjected to solid phase extraction to recover androgen receptor ligands (Milestone et al., 2010). Prior to solid phase extraction, Oasis HLB cartridges (Waters Ltd., Mississauga, ON, Canada) were conditioned with 2 bed volumes each of water, methanol and dichloromethane. Then cartridges were loaded at 100 mL condensate/500 mg packing material at a flow rate of 5 mL/min. Residual condensate that had passed through solid phase extraction was also collected (Figure 3-1: Fraction 5) for in vivo studies. Cartridges were dried using an air vacuum of 20 mm Hg for 1 hr/g and then eluted with dichloromethane (40 mL/g) to obtain a non-polar fraction (Figure 3-1: Fraction 3; SPE-NP). This was followed by 40 mL/g methanol to yield a polar fraction (Figure 3-1: Fraction 4; SPE-P).

Figure 3-1: The Condensate Solid Phase Extraction Protocol (Milestone et al., 2010). Condensates are separated into 5 chemically distinct extracts for biological and chemical analysis, as well as further fractionation. A mass balance approach using GC-MS quantification was used to ensure full chemical recovery.


5th Effect Evaporator Condensate
pH and Filtration
Soxhlet
FP Non Polar
1
FP Polar
2
<1 um Filtrate
Solid Phase Extraction
SPE Non Polar
3
SPE Polar
4
Residual Condensate
5

### 3.3.5 High Performance Liquid Chromatography (HPLC)

HPLC fractionations of condensate extracts were performed using an Agilent 1200 series HPLC system complete with diode array detector (DAD), fluorescence light detector (FLD), evaporative light scattering detector (ELSD) and a fraction collector. A Phenomenex Luna Silica 5μ 4.6 x 250 mm normal phase analytical column was used for HPLC fractionation. The system was optimized for maximum resolution of detectable components. 10 μL of sample (1 mL sample = 500 mL condensate equivalent) was injected onto the column with a flow rate of 1 mL/min and a 50/50 hexane/dichloromethane mobile phase, held for 13 min then linearly programmed to 100 % dichloromethane over 37 min and held for 15 min. A mobile phase consisting of 50/50 dichloromethane/isopropyl alcohol was used to verify polar compounds in FP-NP, if present, were eluted, and this fraction was tested in the AR binding assay. All fractions generated by HPLC were collected manually based on retention time. A total of 4 injections were combined for all AR binding assays and chemical analyses. Fractions from each injection were combined to produce a single sample of each fraction.

### 3.3.6 Gas Chromatography-Mass Spectrometry (GC-MS)

Fractions were first evaporated under a gentle stream of nitrogen to just dryness and then reconstituted in toluene. Aliquots of each fraction (1 μL) were injected onto a GC-MS triple quadrupole system (7890A/7000 series, Agilent Technologies, Mississauga, ON, Canada) using a HP-5MS capillary column (30 m; inner diameter, 0.25 mm; film thickness, 0.25 μm; Agilent Technologies, Mississauga, ON, Canada) with a He carrier gas.

Condensates 1 and 2 were analyzed using the following parameters: injector temperature, 300 °C in splitless mode; oven temperature, 90 °C for 30s; increased at 40 °C/min to 300 °C; held for 10 min. Concentrations of reported compounds were obtained by select ion monitoring (SIM) GC-MS analysis operating at unit resolution.

Condensate 3 and HPLC fractions were analyzed using the following parameters: injector temperature, 300 °C in splitless mode; oven temperature, 90 °C for 30s; increased at 5 °C/min to 300 °C; hold for 10 min. Concentrations of reported compounds (Figure 3-3) were obtained by SIM GC-MS analysis operating at unit resolution.

For all 3 condensate experiments, compounds were quantified against standard calibration curves using commercially available authentic standards previously identified in condensates (Belknap et al., 2006).

### 3.3.7 Androgen Receptor (AR) Binding Assay

The contents of androgen receptor ligands for condensate fractions were evaluated using a competitive binding assay with goldfish testicular androgen receptors (Wells and Van Der Kraak, 2000). Goldfish (*Carassius auratus*, 50-100 g) were obtained from Aelong's Aquarium (Burlington, ON, Canada) and transported to the National Water Research Institute (Environment Canada, Burlington, ON, Canada) where they were euthanized by

spinal severance for recovery of gonadal tissues the same day. Two pools consisting of 8 g of testes, each pool contributing from 5 to 7 fish, were obtained. Tissues were chopped into a fine paste and homogenized with 3 volumes of homogenization buffer (50mM Tris-HCl, 1mM EDTA disodium salt, 30 % glycerol; pH 7.5 at 4 °C) using a Potter-Elvehjem Teflon–glass homogenizer. Homogenized tissue was centrifuged at 1000 g for 15 min at 4°C. The supernatant was removed and centrifuged at 100 000 g for 60 min at 4 °C using an Optima Max centrifuge (Beckman Coulter, Mississauga, ON, Canada). The supernatant was then charcoal stripped: A charcoal pellet was obtained by spinning charcoal buffer (50mM Tris-HCl, 1 mM NaEDTA, 10 % glycerol; pH 7.5 at 4 °C) at 10 000 g for 15 min at 4°C using an Avanti J 301 (Beckman Coulter, Mississauga, ON, Canada). The supernatant was discarded and the gonad cytosolic fractions were added to the charcoal pellet with a concentration of 0.35 % charcoal. The combination was then vortexed and incubated for 5 min on ice. The samples were finally centrifuged at 10 000 g for 15 min at 4 °C and the supernatant was divided into aliquots, flash frozen in liquid nitrogen and placed in a -80 °C freezer.

3.3.7.1 Characterization of Androgen Receptors (ARs)

Gonad cytosolic fractions were incubated with increasing concentrations of [$^3$H]testosterone (0.05-20 nM) in the presence or absence of 100-fold excess unlabeled testosterone to obtain Scatchard plots. Cytosolic fractions were diluted 10-fold with TEG buffer (10 mM Tris HCl, 10 mM NaEDTA and 10 % glycerol). Each sample contained 150 µL of diluted cytosolic fractions, 50 µL [$^3$H]testosterone standard, and 50 µL of TEG buffer (total binding) or 50 µL

unlabeled testosterone standard (nonspecific binding). Samples were incubated for 18 h at 4 °C. Charcoal buffer (10 mM Tris HCl; 10 mM NaEDTA; 10 % glycerol; 0.5 % Norit A charcoal; 0.05 % Dextran T-70) was added to the samples, which were incubated for 5 min on ice before being centrifuged at 2000 g for 15 min at 4°C. 5 mL of scintillation cocktail was added to the supernatant and finally samples were counted using a LS6500 scintillation counter (Beckman Coulter, Mississauga, ON, Canada). $K_d$ (dissociation constant) and $B_{max}$ (maximum binding capacity) were calculated using the procedure found in Wells and Van Der Kraak (2000) to assess quality of cystolic fractions.

3.3.7.2 Competitive Binding Studies

Condensate fractions were first reduced under a gentle stream of nitrogen to just dryness, and then reconstituted in methanol for the androgen receptor-binding assay (500 µL of sample was equivalent to 50 mL of condensates). Scintillation tubes contained 1 % methanol since samples were stored in methanol. 1 % methanol did not affect binding of the sample, tracer, or testosterone standard to the androgen receptor (Wells and Van Der Kraak, 2000).

Competition studies were performed to determine the relative affinity of various pulp and paper mill condensate fractions for ARs in the gonad cytosolic fractions. Cytosolic fractions were diluted in TEG buffer to 25 fmol binding sites/mg protein. Increasing amounts of unlabeled testosterone (50 µL) and samples (50 µL) were incubated with 50 µL of 6.25 nM [$^3$H]testosterone and 150 µL of diluted cytosol for 18 h at 4 °C. A series of blank tubes

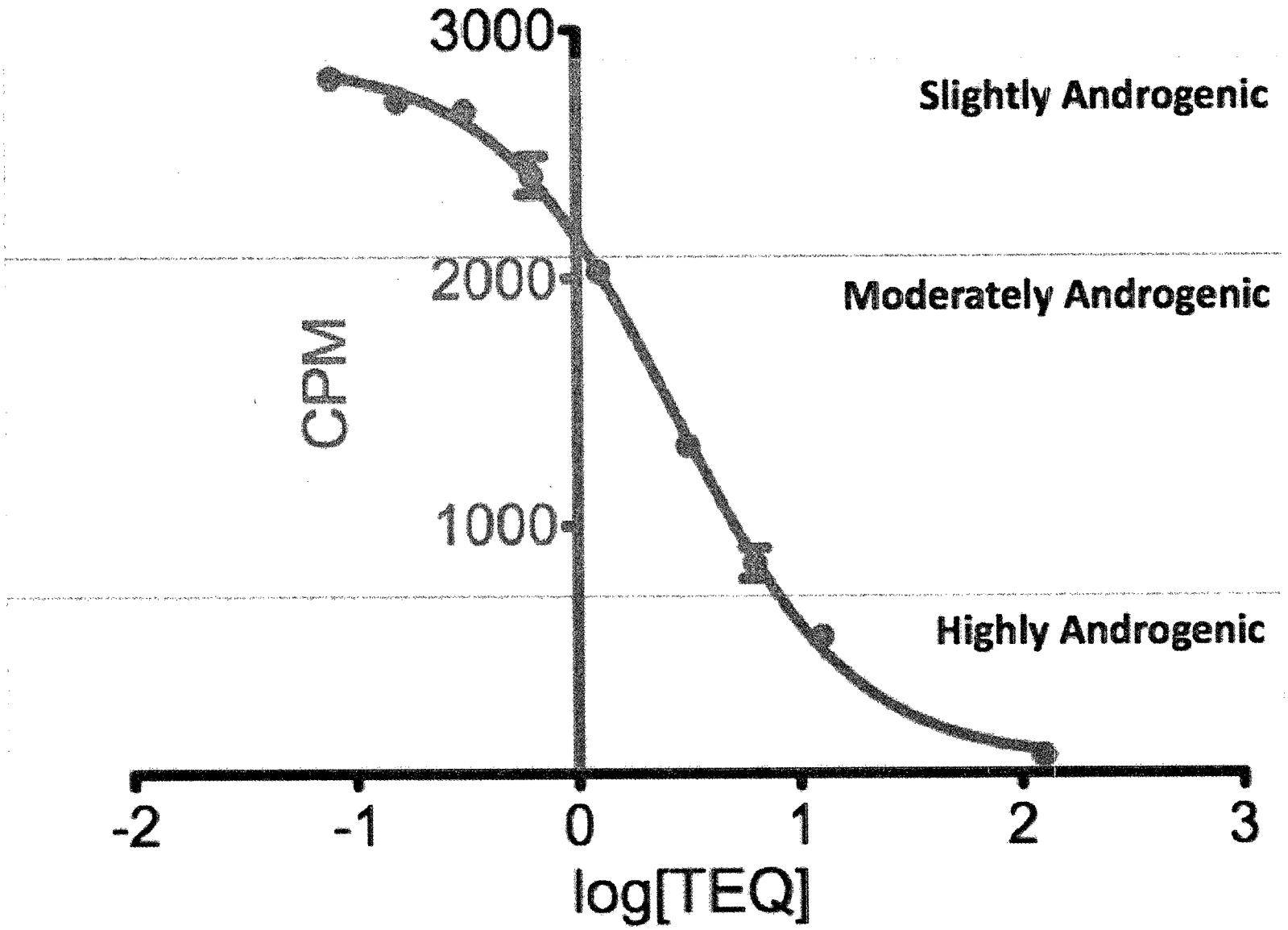
consisting of 50 μL methanol lab blank, 50 μL tracer and 150 μL TEG buffer was incubated as above to obtain non-specific binding (radioactivity trapped in the absence of receptor). Another series of tubes consisting of 50 μL methanol blank, 50 μL tracer and 150 μL diluted AR prep was prepared and incubated as above to measure total binding (total amount of tracer bound to the AR prep without competition). 50 μL tracer and 700 μL TEG buffer were prepared in a scintillation vial in order to measure total counts (total radioactivity of tracer without receptor). Percent specific binding of [$^3$H]testosterone was calculated at given concentrations of competitor by subtracting the blank value and dividing by the total binding, from which the lab blank value was also subtracted. In order to account for possible variability in binding ability and increase statistical power, two different AR preps were used per experiment with three sample repetitions each.

Samples were first tested using the AR binding assay at stock concentration (ranging from 50 mL condensate sample/500 μL sample to 200 mL condensate sample/500 μL sample). Samples with values at the top of the testosterone standard curve (Figure 3-2) contained very few (if any) ligands for AR. TEQ, or testosterone equivalents, is defined as the theoretical testosterone concentration in a sample: the higher the value, the more androgenic the sample (all values herein are reported as amount of testosterone /L condensates). TEQ values of samples in the linear section of the standard curve were considered accurate, whereas TEQ values of samples in the bottom portion of the standard curve were considered to have high concentrations of ligands for AR. The highly androgenic samples were diluted in series and

re-tested using the AR binding assay until a result on the linear portion of the standard curve was obtained. Dilution factors were taken into account when back-calculating TEQ values.

A manool displacement curve was generated using concentrations of 0.445 μM – 1000 μM. The median inhibition concentration (IC50) of manool was then determined using a least squares fit and reported as the relative binding potency at 50 % inhibition (Figure 3-11).

Figure 3-2: Standard Curve Generated with the AR Binding Assay. The top section is considered slightly androgenic. The linear portion is considered moderately androgenic and TEQ values obtained from this portion of the graph are accurate. The bottom section of the testosterone standard curve is considered highly androgenic.


3000
2000
1000
CPM
Slightly Androgenic
Moderately Androgenic
Highly Androgenic
-2
-1
0
1
2
3
log[TEQ]

### 3.3.8 Data analysis

Data generated from the AR binding assay was imported into Prism (GraphPad Software, La Jola, CA, USA) and a non-linear regression (a sigmoidal curve fit) of dose-response vs. inhibition with a variable slope was applied to the testosterone standards. TEQ values for samples were then interpolated from the standard curve and subsequent TEQ values were assigned to samples based on interpolation of sample radioactivity via the testosterone standard curve. Sample TEQ values located outside the linear portion of the testosterone standard curve were considered inaccurate and so series dilutions were performed until the sample was in the linear portion of the standard curve.

AR binding assay data was transformed and tested for normality using the D'Agostino and Pearson omnibus normality test. A Ln transformation resulted in a normalized data set. Normalized data was analyzed using a one-way analysis of variance (ANOVA; $P<0.05$) to determine whether or not there were significant differences in androgenic responses of samples and blanks. Tukey's post hoc test made pair wise comparisons to determine where these differences occurred. All statistic analysis was conducted using Prism.

## 3.4 Results

### 3.4.1 Condensate concentrations of confirmed chemicals

In order to assess temporal variability of condensates, the three condensate samples were analyzed using GC-MS chemical analysis. Seven compounds previously identified in

condensates from this mill (Belknap et al., 2006) were confirmed with authentic standards in the three samples of condensates of this study (Figure 3-3). The average detected concentrations for 3 phenolic compounds were 2.5, 2.3, and 0.04 mg/L, for 4-ethylguaiacol, isoeugenol, and veratraldehyde respectively. Vanillin, another phenolic, was not analyzed for in either Condensates 1 or 2, but was present in Condensate 3 at a concentration of 1.4 mg/L. Based on pKa values and hydrophobicity, diterpenes exhibited a higher affinity for filter paper fractions compared to phenolics, which were primarily isolated in the SPE fractions. Levels of confirmed compounds detected in each of the 3 condensate samples were similar, with the exception of geranyl linalool, which was 5.9, 9, and 14.7 mg/L in Condensates 1, 2 and 3, respectively. Progesterone, androstenedione, and androstadienedione (previously identified by Carson et al. (2008) in water and sediment samples from a river with a population of masculinized female mosquitofish; a result of upstream PME discharge) were not found in any condensate samples using GC-MS analysis.

Figure 3-3: Concentrations of Previously Identified Compounds in BKM Condensates sampled in this study. Condensate 1 was sampled on in May 2009, Condensate 2 in August 2009 and Condensate 3 in April 2010.

| | Condensate 1 | | | Condensate 2 | | | Condensate 3 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Filter Paper (mg/L) | SPE (mg/L) | Total (mg/L) | Filter Paper (mg/L) | SPE (mg/L) | Total (mg/L) | FP-NP (mg/L) | FP-P (mg/L) | SPE-NP (mg/L) | SPE-P (mg/L) | Total (mg/L) |
| Manool | 40.66 | 1.16 | 41.82 | 47.14 | 2.01 | 49.15 | 36.40 | 0.029 | 1.660 | 0.036 | 38.129 |
| Geranyl linalool | 5.64 | 0.26 | 5.9 | 8.61 | 0.35 | 8.96 | 13.129 | 0.018 | 1.417 | 0.153 | 14.717 |
| 4-ethylguaiacol | nd[a] | 2.18 | 2.18 | nd | 3.59 | 3.59 | nd | nd | 1.748 | 0.026 | 1.774 |
| Vanillin | na[b] | na | 0 | na | na | 0 | nd | nd | 1.333 | 0.028 | 1.362 |
| Isoeugenol | nd | 4.36 | 4.36 | nd | 1.18 | 1.18 | nd | nd | 1.242 | nd | 1.242 |
| Veratraldehyde | nd | nd | 0 | nd | 0.03 | 0.03 | nd | nd | 0.089 | nd | 0.089 |
| Squalene | 0.16 | nd | 0.16 | 0.25 | 0.02 | 0.27 | 0.900 | nd | nd | nd | 0.900 |

[a] nd = not detected (but analyzed for)
[b] na = not analyzed

### 3.4.2 Androgenic activity of condensates

Condensates 1, 2, and 3 were extracted into 5 chemically distinct extracts (Figure 3-1). Androgenic activity was measured in all of the extracts except for the residual condensates fraction. They were not compatible with the AR binding assay because they were in water. Solvent exchanging to methanol posed the risk of losing certain volatile and semi-volatile compounds that may have been present. In all three condensates experiments, FP-NP was the most androgenic, followed by SPE-NP (Figure 3-4). All FP-P and SPE-P fractions showed minimal androgenic activity relative to non-polar fractions. Condensate 1 was sampled during sequential softwood production runs (as were Condensates 2 and 3) in order to establish a benchmark for androgenicity in BKM condensates. Condensate 1: FP-NP had a TEQ value of ~115,000 ng/L and SPE-NP had a value of ~4500 ng/L. Condensate 2 was sampled to determine whether or not levels of androgenicity were consistent among different samples. TEQ of Condensate 1: FP-NP (~115,000 ng/L) was ~6 times lower compared to Condensate 2: FP-NP (~705,000 ng/L). Condensate 2: SPE-NP (~11,500 ng/L) was ~2.5 times more androgenic than its counterpart in Condensate 1 (~4,500 ng/L).

Since 5$^{th}$ effect condensates were found to contain ligands for the goldfish AR, condensate 3 was sampled and tested for androgenic activity to determine if it was a good candidate for further studies. Condensate 3: FP-NP (~518000 ng/L) was ~4.5 times more androgenic than Condensate 1: FP-NP, but Condensate 2: FP-NP was ~1.5 times more androgenic than Condensate 3: FP-NP. Condensate 3: SPE-NP (~21000 ng/L) was ~5 and ~2 times more androgenic than its counterparts in Condensates 1 and 2, respectively. The amount of

androgenic activity in Condensate 3: FP-NP made it a viable candidate for chemical fractionation to further characterize androgenic compounds present in $5^{th}$ effect condensates.

### 3.4.3 HPLC Fractionation of Active SPE Extract

FP-NP of Condensate 3 was subjected to fractionation by normal phase HPLC, producing ~12 distinct, reproducible peaks. 11 fractions were then collected as designated and GC-MS chemical analysis took place along with an assessment of androgenic activity using the AR binding assay. HPLC chromatograms of Condensate 3: FP-NP, fractionation times, and androgenic activity of subsequent HPLC fractions are presented in Figure 3-5.

Figure 3-4: Testosterone equivalents measured with the goldfish testicular androgen receptor competitive binding assay incubated with extracts from 3 batches of BKM condensates. Alphabetical superscripts indicate treatments different than each other ($p<0.05$, $n=6$). Error bars represent standard error of the mean.

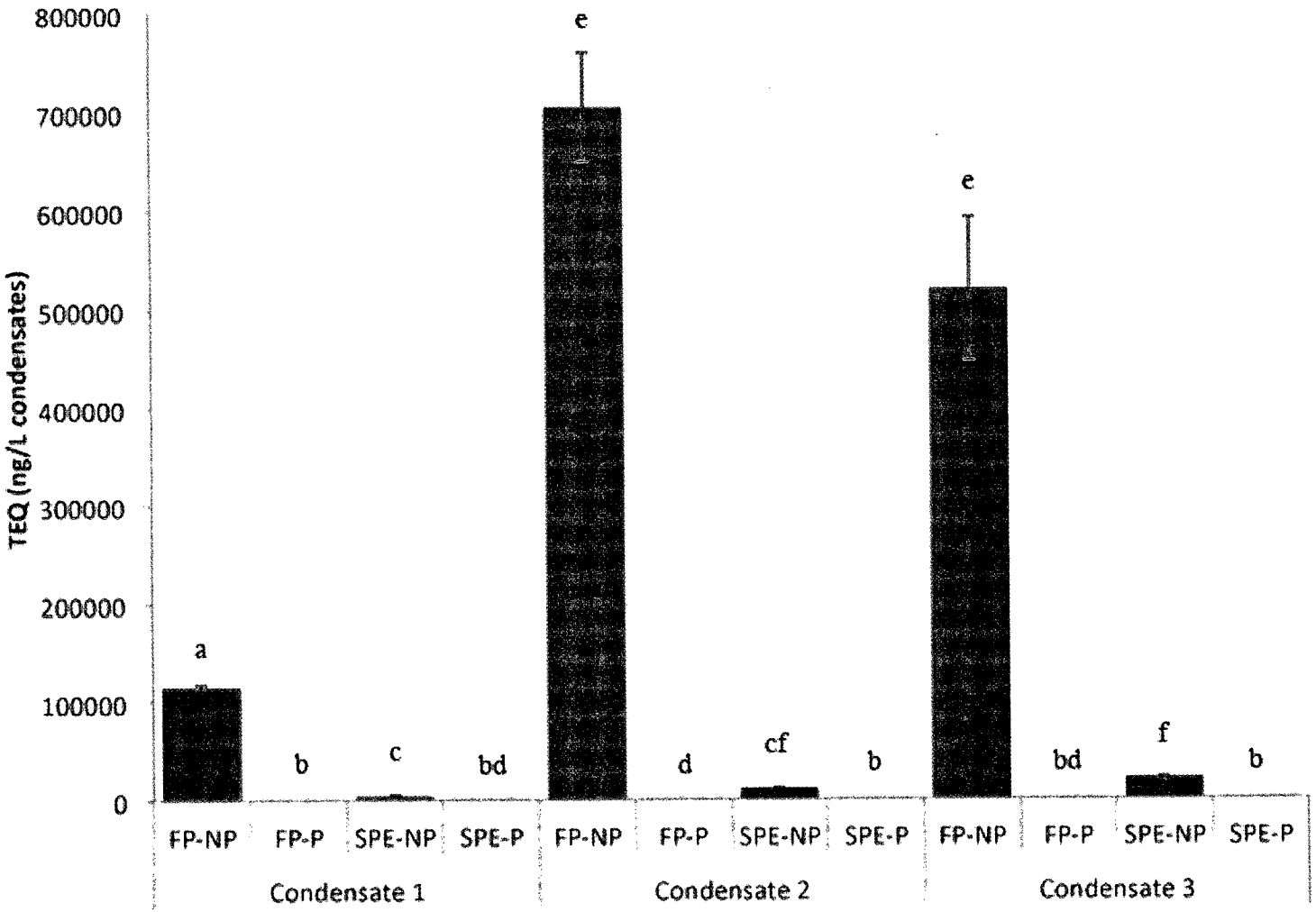
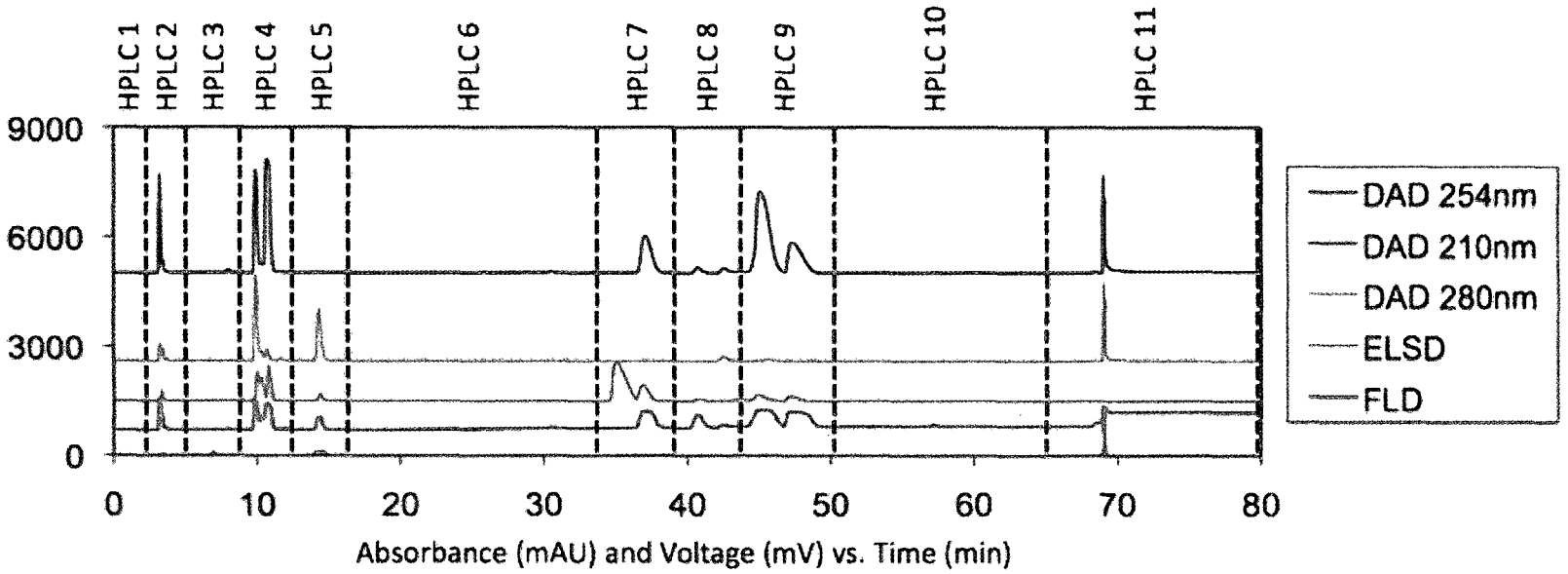
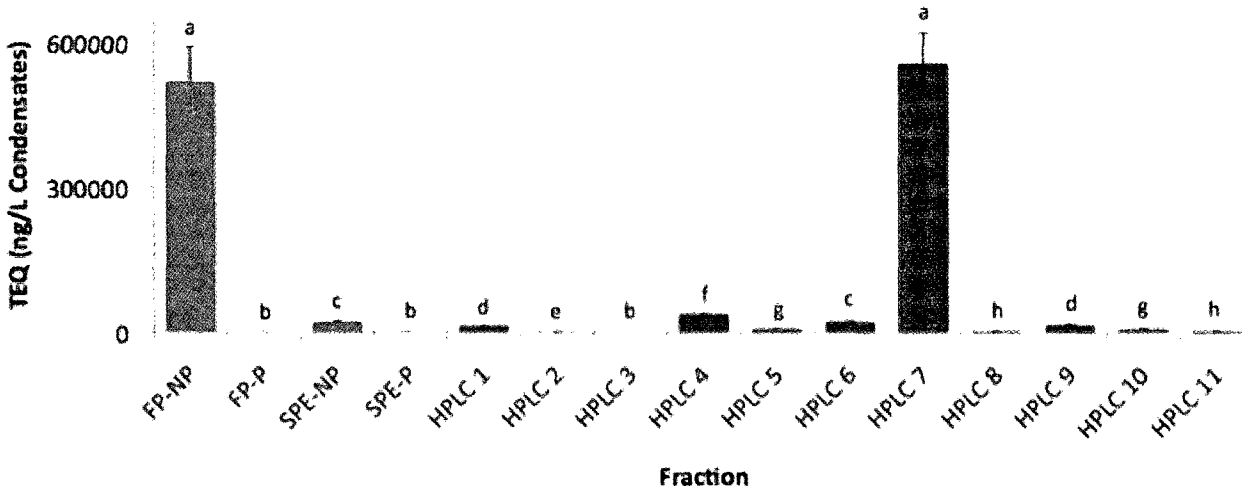

800000
700000
600000
500000
400000
300000
200000
100000
0
TEQ (ng/L condensates)
a
b
c
bd
e
d
cf
b
e
bd
f
b
FP-NP
FP-P
SPE-NP
SPE-P
FP-NP
FP-P
SPE-NP
SPE-P
FP-NP
FP-P
SPE-NP
SPE-P
Condensate 1
Condensate 2
Condensate 3

Figure 3-5: HPLC chromatograms of Condensate 3: FP-NP and Subsequent Androgenic Activity of HPLC Fractions. TOP: Dotted lines in chromatograms indicate start/end times of HPLC fractions collected. BOTTOM: Testosterone equivalents (theoretical ng testosterone/L condensate equivalents) generated from the goldfish AR binding assay, associated with FP, SPE condensate extracts and HPLC fractions generated from FP-NP. Alphabetical superscripts indicate treatments different than each other ($p<0.05$, $n=6$). Error bars represent standard error of the mean. DAD=diode array detector; ELSD=evaporative light scattering detector; FLD=fluorescence light detector.


HPLC 1
HPLC 2
HPLC 3
HPLC 4
HPLC 5
HPLC 6
HPLC 7
HPLC 8
HPLC 9
HPLC 10
HPLC 11
9000
6000
3000
0
0
10
20
30
40
50
60
70
80
DAD 254nm
DAD 210nm
DAD 280nm
ELSD
FLD
Absorbance (mAU) and Voltage (mV) vs. Time (min)


TEQ (ng/L Condensates)
600000
300000
0
a
b
c
b
d
e
b
f
g
c
a
h
d
g
h
FP-NP
FP-P
SPE-NP
SPE-P
HPLC 1
HPLC 2
HPLC 3
HPLC 4
HPLC 5
HPLC 6
HPLC 7
HPLC 8
HPLC 9
HPLC 10
HPLC 11
Fraction

### 3.4.4 GC-MS Analysis of HPLC Fractions

HPLC fraction 7 had the most androgenic activity, which did not differ from the activity of unfractionated FP-NP ($p<0.05$; Figure 3-5). Other fractions also demonstrated androgenic activities that ranged from 15-1500 fold less active than HPLC 7. The total ion chromatogram (TIC) from a full scan GC-MS analysis of HPLC 7 is given in Figure 3-6 with the major detectable constituents numbered. The chromatogram is dominated by a large peak (Figure 3-6: Peak 6) with an apparent molecular ion of m/z 272 and fragment ions consistent with a diterpene. For example, m/z 272 represents loss of $H_2O$ from a molecular ion of m/z 290, while m/z 257 represents a subsequent loss of $-CH_3$ (McLafferty and Turecek, 1992). The mass spectra closely resembled that of bicyclic diterpene manool, previously identified in condensates by Belknap et al. (2006). A positive confirmation of manool was obtained through mass spectral and GC retention time matches with an authentic standard (Industrial Research, Wellington, New Zealand). The presence of manool in HPLC 7 and FP-NP condensate extracts is consistent with its hydrophobicity, as this compound would be expected to partition to solids in the condensates (log $K_{ow}$=7.09) and is not readily soluble in water (0.0395 mg/L) (Luebke, 2010).

Closer inspection of the remaining 7 major peaks evident in the TIC of HPLC 7 reveal mass spectra consistent with a series of diterpenes. All have apparent molecular ions of 272 m/z and prominent fragment ions at m/z 257, 137, 95, and 81, similar to manool. Since the molecular ion for manool is weak to nonexistent, this is likely also the case for these unknowns and their molecular ions are not evident in these EI spectra. Peak 5 was

subsequently confirmed with an authentic standard as geranyl linalool, an acyclic diterpene. While library searches of the remaining peaks gave poor quality spectral matches, they nevertheless were consistent with diterpenoid structures. It is therefore likely that these unidentified diterpenes are involved with the androgenicity observed with HPLC 7 and the condensates as a whole.

HPLC 4 was the second most androgenic fraction. The TIC from a full scan GC-MS analysis is presented in Figure 3-7 with the major detectable constituents numbered. Inspection of the 7 major peaks evident in the TIC revealed mass spectra consistent with a different series of diterpenes. With the exception of peak 5, all peaks have apparent molecular ions of m/z 286. Peaks 1 to 4 also have prominent fragment ions at m/z 271, 243, 91, and 79. Although peak 6 lacked many common prominent fragment ions, a library search suggested a cyclic diterpene structure. Likewise, library searches of peaks 1 to 4 yielded poor quality spectral matches, however they were also consistent with diterpenoid structures. In comparing spectra from peaks 1 and 2 it appears they are the same compound with slightly different retention times, suggesting the presence of two isomers. A library search of peak 5 provided a strong correlation with the spectrum of dehydroabietal (86 %), a cyclic diterpene previously identified in softwood (Nikolic et al., 2009). The presence of dehydroabietal in HPLC 4 and FP-NP condensate extracts is consistent with its hydrophobicity, as this compound would be expected to partition to solids in the condensates and is not readily soluble in water. Besides an apparent molecular ion at m/z 286, peak 7 was substantially different from the others. It had a prominent fragment ion at m/z 135, and small fragment ions with m/z 187 and 148 that

were not consistent with other spectra in HPLC 4. Despite this, a library search suggested that peak 7 has a diterpene structure. Given the results from HPLC 7, the diterpenes present in this fraction are also likely involved with the androgenicity observed with HPLC 4 and the condensates as a whole.

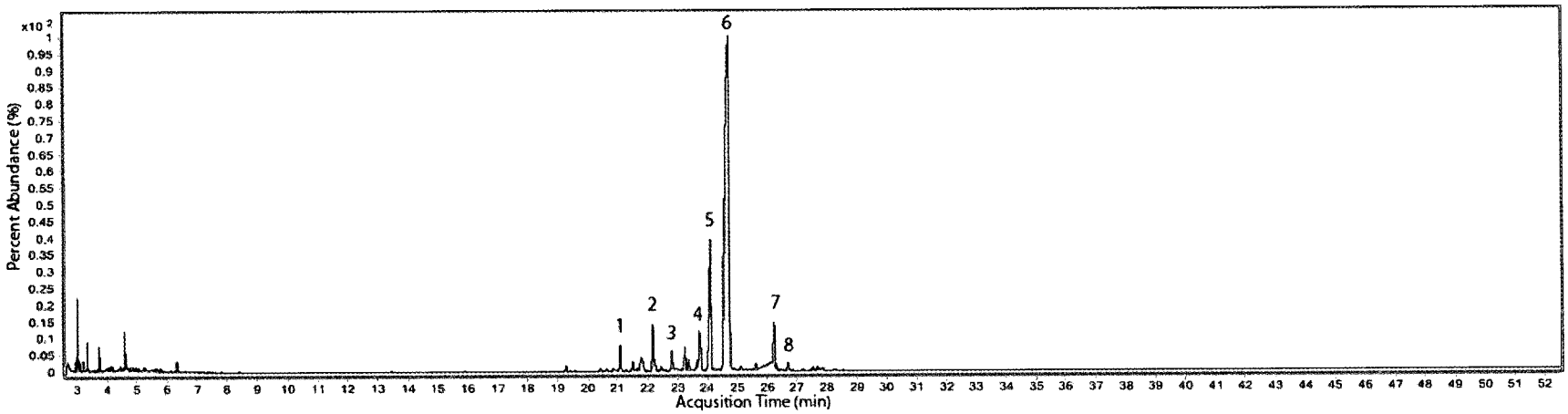
A TIC of HPLC 6 (the 3rd most androgenic HPLC fraction) revealed 12 major detectable constituents (Figure 3-8), many of which had mass spectra consistent with a series of diterpenes. Peaks 1 to 4, 6, and 8 had apparent molecular ions of m/z 272 and prominent fragment ions at m/z 257, representing the loss of $-CH_3$ (McLafferty and Turecek, 1992). Although library analyses of these peaks gave poor quality spectral matches, they were consistent with diterpenoid structures. Peaks 3 and 4 had nearly identical spectra and are likely isomers, given their different retention times. Peaks 9 and 12 had different apparent molecular ions (m/z 291 and 308, respectively) compared to previously discussed peaks (m/z 272), as well as unique fragmentation patterns. Although library analyses did not offer strong structural correlations, diterpene structures were consistently suggested. The mass spectrum of peak 8 closely resembled that of the bicyclic diterpene manool, which was confirmed with an authentic standard. A small amount of manool was expected to be in HPLC 6 as mentioned previously. Upon comparison, it was determined that peaks 1, 6 and 7 from HPLC 6 were the same as peaks 1, 3, and 4, respectively, in HPLC 7. Fragmentation patterns of compounds represented by peak 5 and 10 are unique compared to other numbered peak. Presently, these compounds are unknown.

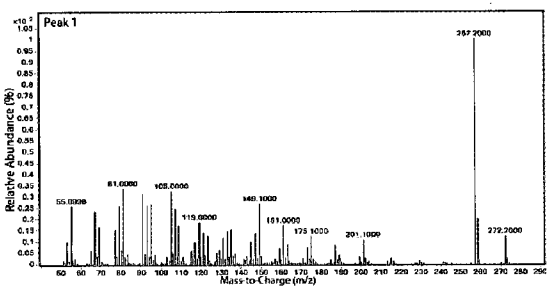
With a TEQ value of 16,000 ng/L, HPLC 9 was the 4$^{th}$ most androgenic fraction. A TIC from a full scan GC-MS analysis is presented in Figure 3-9 with 14 major detectable constituents numbered. Peaks 1 to 7 had apparent molecular ions of m/z 272, while peaks 1, 5, and 7 also had prominent fragment ions at m/z 257, representing a loss of $-CH_3$ (McLafferty and Turecek, 1992). A close comparison of peaks 2, 3, 4, and 6 suggests that they are 4 different isomers of the same compound, given their spectra and retention times. While library searches of these peaks gave poor quality spectral matches, they nevertheless were consistent with diterpenoid structures. Spectral and retention time comparisons revealed that peaks 8 and 9 are likely different isomers of the same compound. Comparing the spectra and retention times of peaks 8 and 9 suggest that they are isomers of the same compound. Presently these compounds are unknown. Peaks 10, 11, 13, and 14 had an apparent molecular ion at m/z 288, and library analyses revealed that their spectra are consistent with diterpene structures, with the exception of peak 14. The spectra for peak 14 has an apparent molecular ion at m/z 139, and prominent fragment ions at m/z 125, 111, 97, 83, and 69 and looks similar to a "picket fence", typical of linear alkanes (McLafferty and Turecek, 1992). Peak 12 had an apparent molecular ion of m/z 286, and prominent fragment ions at m/z 271, 253,173, among others. A library search revealed that the mass spectra closely resembled that of the cyclic diterpene dehydroepiabietal (86 % spectral match), a compound previously found in softwood (Carman and Deeth, 1967; Oh et al., 2007). Given this body of evidence, it is also likely that unidentified diterpenes are contributing the androgenicity in HPLC 9, and FP-NP.

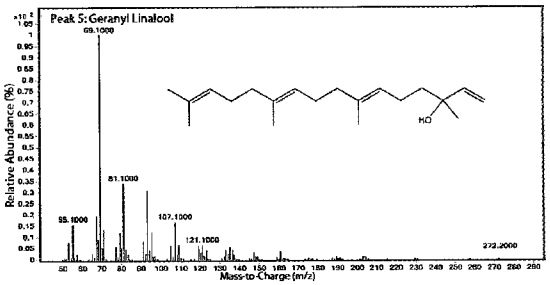
HPLC 1, which was not statistically different than HPLC 9 ($p<0.05$), was the 5$^{th}$ most androgenic fraction. The TIC in Figure 3-10 shows only one prominent peak. Peak 1 had an apparent molecular ion at m/z 167 and a major ion peak at m/z 149, which is diagnostic of phthalate esters typical in laboratory blanks (McLafferty and Turecek, 1992). The ligands binding to the AR in HPLC 1 are not known at this time, it is possible that they are not detectable by GC-MS.

Appendix A (page 116) contains a TIC of Condensate 3: FP-NP, as well as a complete set of TICs for HPLC 1-11, and spectra of all major peaks.

Figure 3-6: Total Ion Chromatogram (TIC) from a full scan positive ion electron impact ionization analysis of HPLC 7, with the major detectable components labeled. Peak 5 was confirmed as the acyclic diterpene geranyl linalool (inset 1) and peak 6 was confirmed as the cyclic diterpene manool (inset 2), which was found to account for 14 % the androgenic activity associated with HPLC 7. Interpretation of mass spectra of the other main components indicated that they are cyclic diterpenes, a representative spectra is provided (inset, peak 1).


Percent Abundance (%)
Acqusition Time (min)
1
2
3
4
5
6
7
8


Peak 1
Relative Abundance (%)
Mass-to-Charge (m/z)


Peak 5: Geranyl Linalool
Relative Abundance (%)
Mass-to-Charge (m/z)

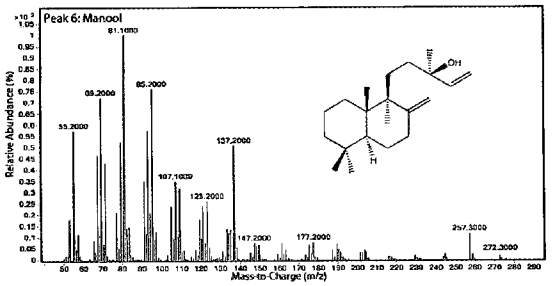
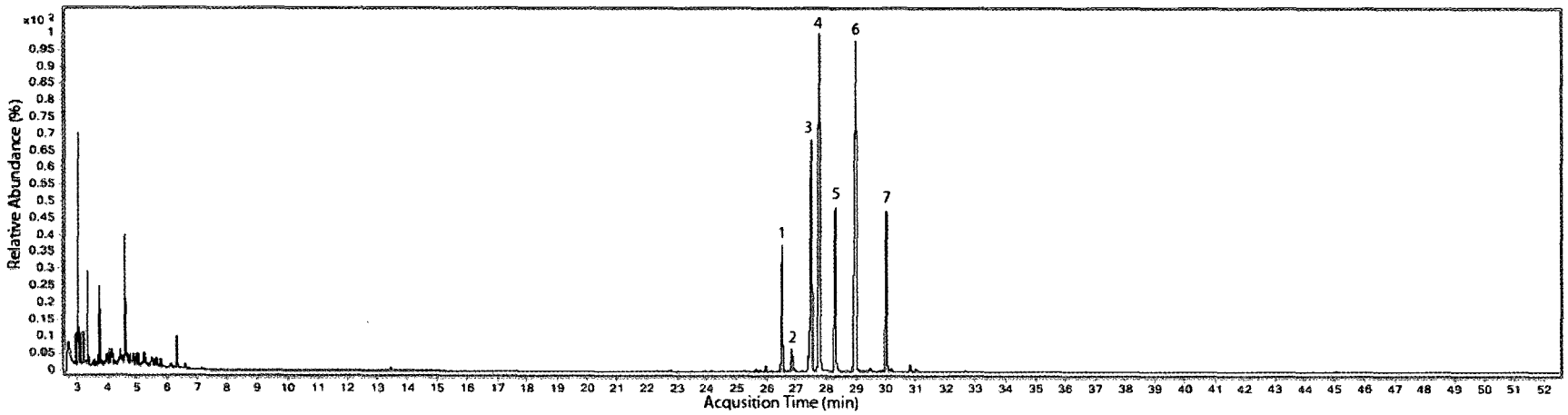

Peak 6: Manool
Relative Abundance (%)
Mass-to-Charge (m/z)

Figure 3-7: Total Ion Chromatogram (TIC) from a full scan positive ion electron impact ionization analysis of HPLC 4 with the major detectable components labeled. HPLC 4 is comprised of 7 major detectable constituents. Peak 5 was tentatively identified as dehydroabiatal and library searches indicated that many of the other constituents were cyclic diterpenes as well. Peak 7 does not appear to be a diterpene, its mass spectra is depicted (inset).


Relative Abundance (%)
Acquisition Time (min)
1
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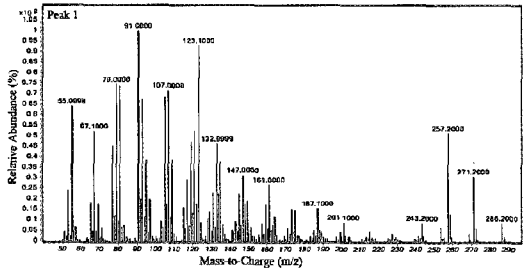
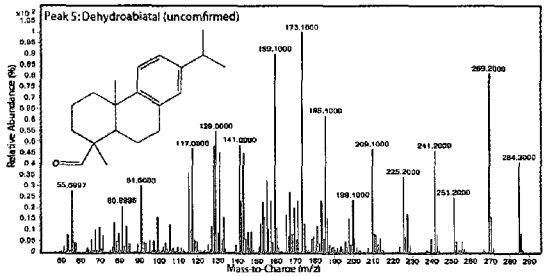
4
5
6
7


Peak 1
Relative Abundance (%)
Mass-to-Charge (m/z)


Peak 5: Dehydroabiatal (uncomfirmed)
Relative Abundance (%)
Mass-to-Charge (m/z)

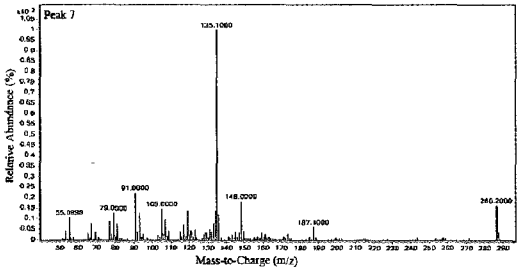
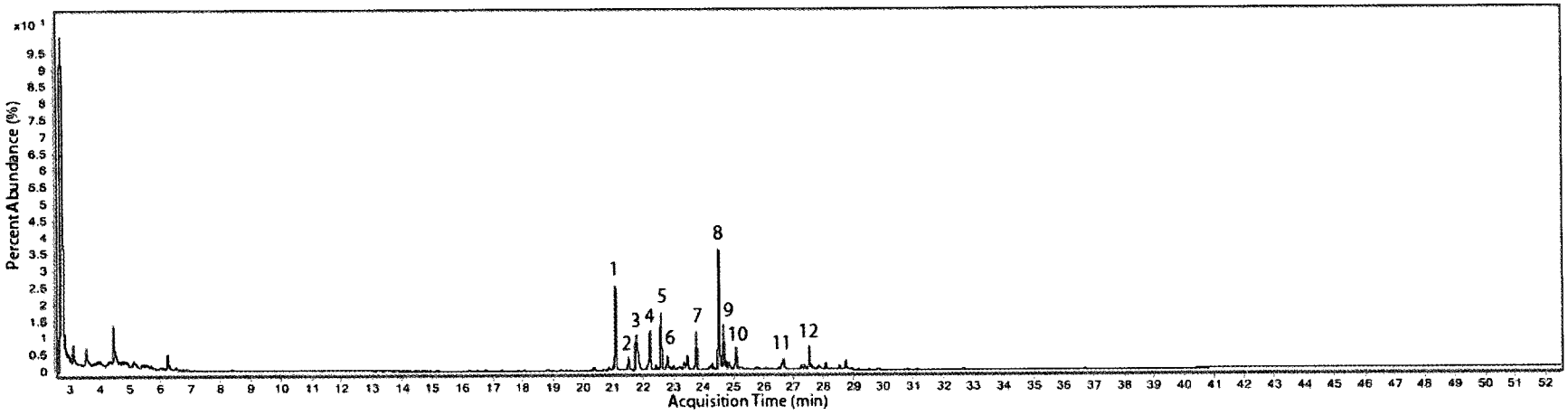

Peak 7
Relative Abundance (%)
Mass-to-Charge (m/z)

Figure 3-8: Total Ion Chromatogram (TIC) from a full scan positive ion electron impact ionization analysis of HPLC 6, with the major detectable components labeled. Peak 8 was confirmed to be manool (3 mg/L), which accounted for 43 % of the androgenic activity associated with this fraction. Library searches indicated that many of the other constituents were cyclic diterpenes as well. The mass spectra for peaks 5 and 10 are shown as representative of additional non-diterpene components of HPLC 6.


Percent Abundance (%)
Acquisition Time (min)
1
2
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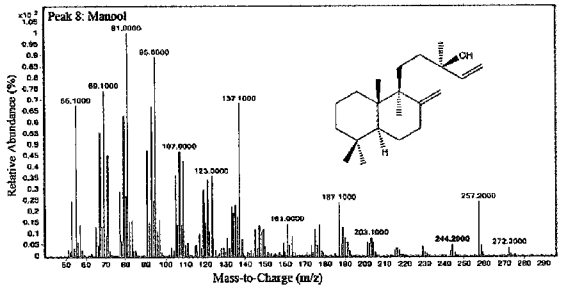
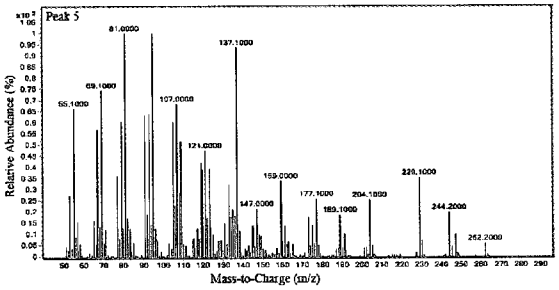
10
11
12


Peak 8: Manool
Relative Abundance (%)
Mass-to-Charge (m/z)
OH


Peak 5
Relative Abundance (%)
Mass-to-Charge (m/z)

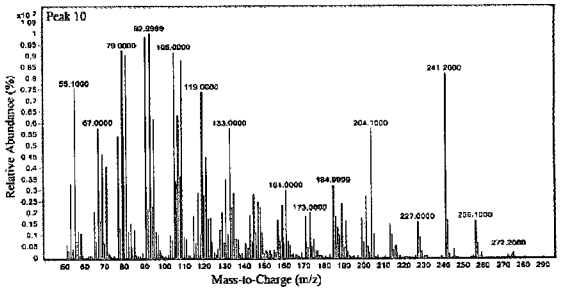
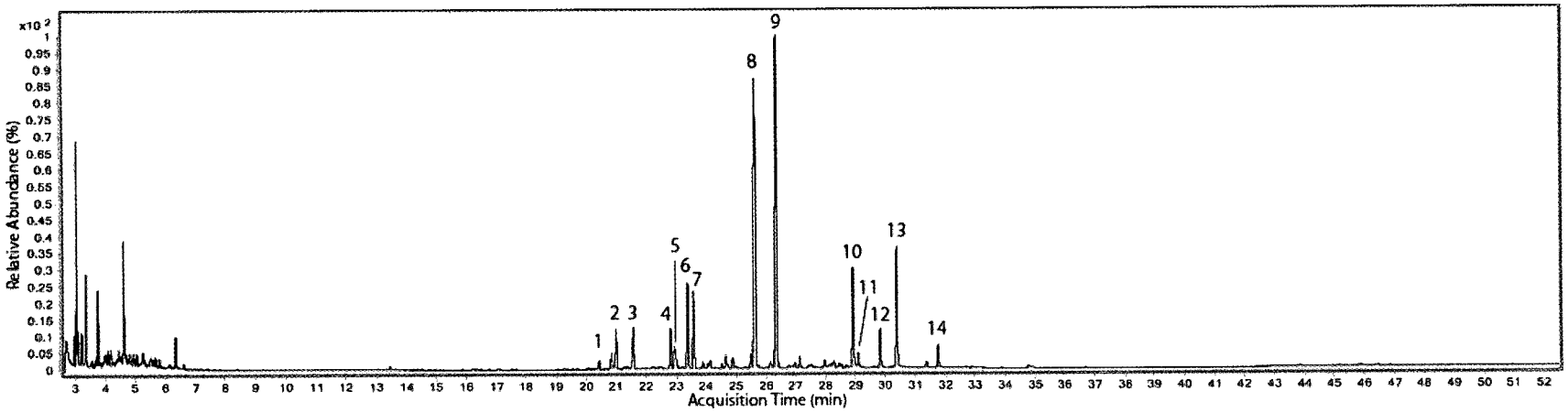
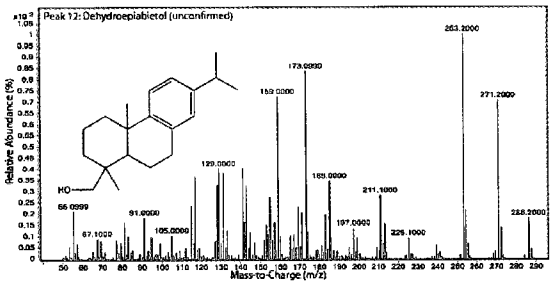
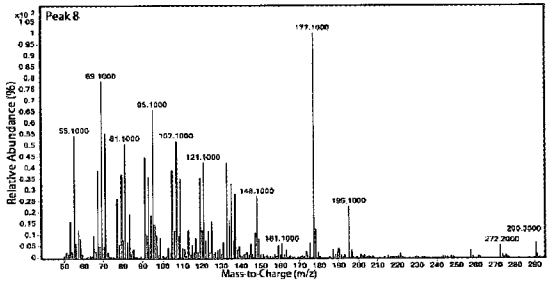

Peak 10
Relative Abundance (%)
Mass-to-Charge (m/z)

Figure 3-9: Total Ion Chromatogram (TIC) from a full scan positive ion electron impact ionization analysis of HPLC 9, with the major detectable components labeled. Peak 12 was tentatively identified as dehydroepiabietol, a cyclic diterpene. Library searches indicated that many of the other constituents were cyclic diterpenes as well. The mass spectra for peaks 8 and 14 are shown as representative of additional non-diterpene components of HPLC 9.


Relative Abundance (%)
Acquisition Time (min)
1
2
3
4
5
6
7
8
9
10
11
12
13
14


Peak 12: Dehydroepiabietol (unconfirmed)
Relative Abundance (%)
Mass-to-Charge (m/z)


Peak 8
Relative Abundance (%)
Mass-to-Charge (m/z)

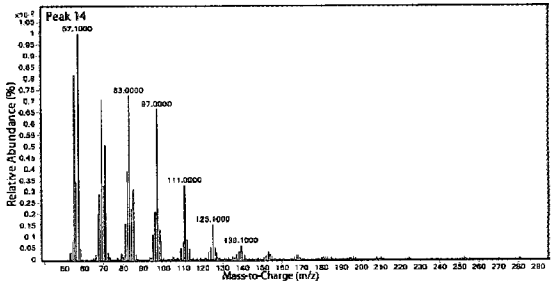
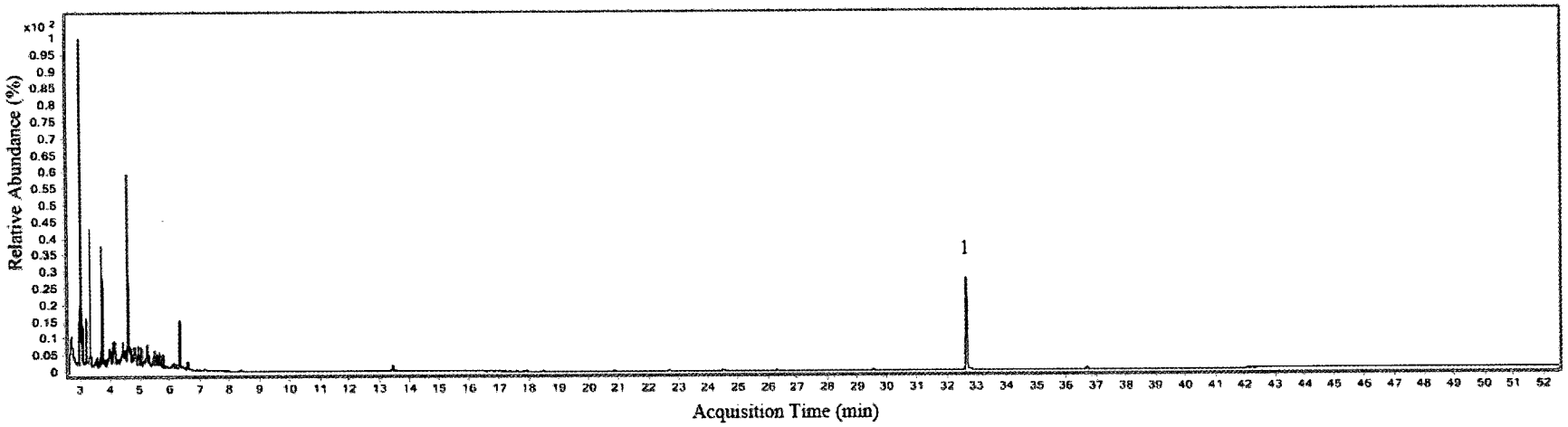
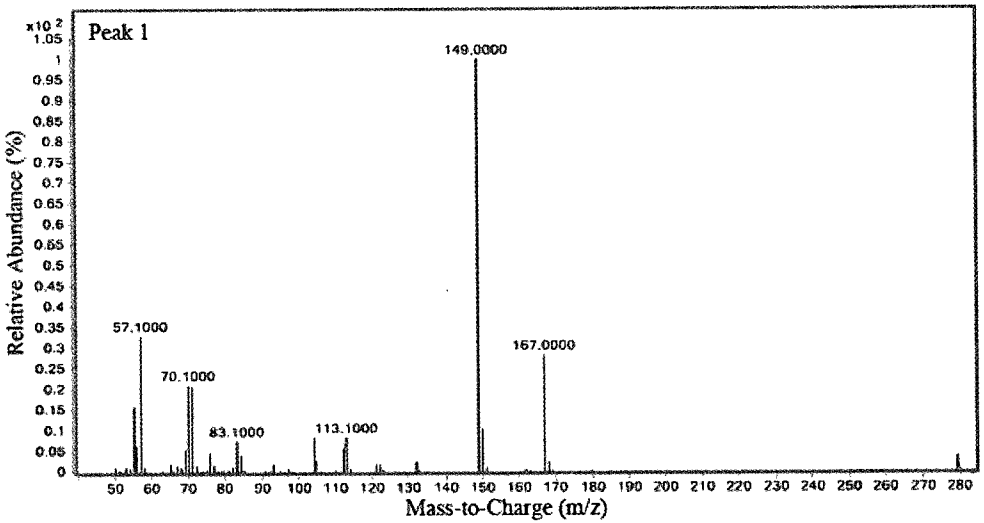

Peak 14
Relative Abundance (%)
Mass-to-Charge (m/z)

Figure 3-10: Total Ion Chromatogram (TIC) from a full scan positive ion electron impact ionization analysis of HPLC 1, with the major detectable component labeled. Peak 1 is likely a phthalate and it was also found in the lab blank. At this time the compounds contributing to the androgenic activity of HPLC 1 are unknown.

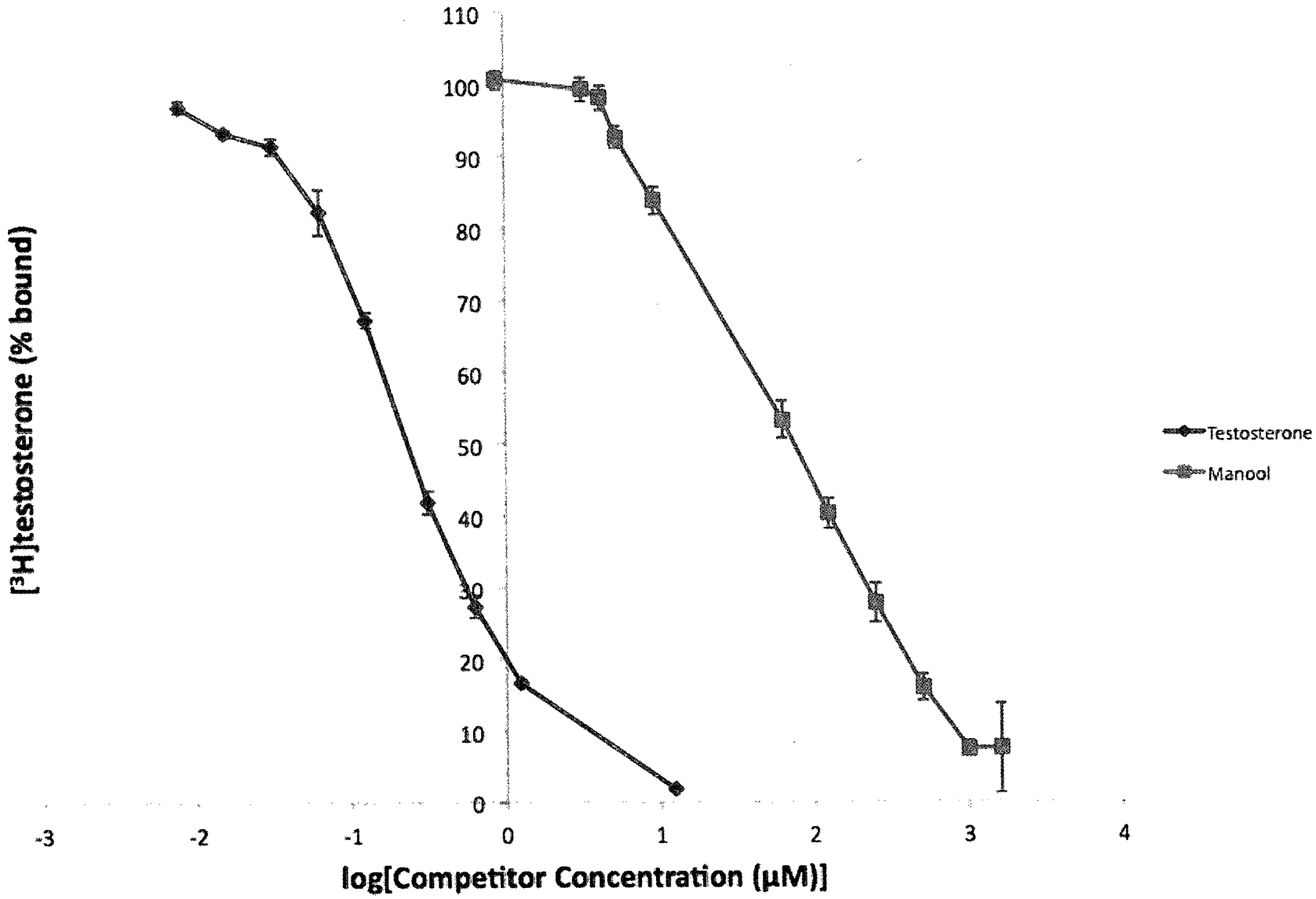

x10 2
Relative Abundance (%)
1
Acquisition Time (min)


Peak 1
x10 2
Relative Abundance (%)
149.0000
57.1000
70.1000
83.1000
113.1000
167.0000
Mass-to-Charge (m/z)

### 3.4.5 Androgenic Activity of Confirmed Compounds

A total of 7 compounds previously found in condensates (Belknap et al., 2006) were confirmed in condensate samples in this experiment (Figure 3-3). These compounds were assessed for androgenic activity using the AR binding assay. 4-ethylguaiacol, geranyl linalool, isoeugenol, veratraldehyde, squalene, and vanillin showed no androgenic activity at 1600 μM. Manool was highly androgenic at the same concentration. A dilution series of manool was used to make a standard curve that was then compared to a testosterone standard curve (Figure 3-11) to obtain a value for the median inhibition concentration (IC50 value). The IC50 values for testosterone and manool were 0.23 μM and 71.61 μM, respectively, placing Manool as 306 times less potent than testosterone. This value was used to determine the amount of androgenic activity resulted from manool, in extracts and fractions that contained manool. Manool accounted for ~29, ~22, and ~28 % of the total androgenic activity in FP-NP extracts from Condensates 1, 2, and 3, respectively. Out of the total androgenicity of HPLC 6 and 7, manool accounted for ~44 and ~14 %, respectively. A dilution series of geranyl linalool produced a standard curve with slope that was not parallel to that of testosterone. It was therefore concluded that geranyl linalool was not interacting with the AR.

Figure 3-11: AR binding Assay Standard Curves of Testosterone and Manool. The parallel slopes indicate that manool was binding to the AR. The testosterone IC50 value was 0.23 μM and the manool IC50 value was 71.61 μM. Testosterone was 306 times more androgenically potent than manool. Error bars represent standard error of the mean.


110
100
90
80
70
60
50
40
30
20
10
0
-3
-2
-1
0
1
2
3
4
[3H]testosterone (% bound)
log[Competitor Concentration (µM)]
Testosterone
Manool

## 3.5 Discussion

In this study, androgenic effects of chemical recovery condensates from a BKM were studied in detail. A relatively potent novel ligand for the goldfish testicular AR was isolated and identified as the cyclic diterpene manool. Manool was subsequently found to account for approximately 25 % of the activity observed in FP-NP extracts. Three separate batches of softwood Kraft condensates were obtained during a one-year period and fractionated using a newly defined fractionation protocol (Milestone et al., 2010). Confirmed compounds previously identified in condensates were used for quantitative analysis of the condensate samples (Belknap et al., 2006). Condensate androgenic activities were assessed and were found to be temporally variable, varying from 4-6 fold. Conversely, with the exception of geranyl linalool, all measured condensate extractives were temporally consistent in each of the three condensate samples. Progesterone, androstenedione, and androstadienedione (previously identified by Carson et al. (2008) in water and sediment samples from a river with a population of masculinized female mosquitofish; a result of upstream PME discharge) were not detected in any condensate samples

Normal phase HPLC was used to fractionate FP-NP extracts, which contained the majority of androgenic activity. Condensates 1 and 2 demonstrated that BKM condensates were androgenic and the majority of this androgenic activity was isolated in FP-NP. The third condensate sample was obtained and extracted using the SPE protocol. The AR binding assay showed that Condensate 3: FP-NP was a good candidate for further fractionation with normal phase HPLC due to its high androgenic activity.

Condensate 3: FP-NP was separated into 11 distinct fractions, where the activity was essentially isolated in one fraction (HPLC 7) that exhibited activity no different than FP-NP ($p<0.05$). Interestingly, if one sums the androgenic activity of the HPLC fractions the total is greater than that of FP-NP (~671000 ng/L vs. ~519000 ng/L). This is potentially due to matrix effects contributing to the activity of FP-NP.

The total ion chromatogram of HPLC 7 is presented in Figure 3-6. The major constituent observed in this fraction by GC-MS was confirmed with an authentic standard as the wood-derived bicyclic diterpene manool (inset, Figure 3-6; 24 mg/L). A small amount of manool (3 mg/L) was also found in HPLC 6 due to fraction collection carryover. Peak 5 in HPLC 7 was confirmed with an authentic standard to be the acyclic diterpene geranyl linalool (12 mg/L). Both of these diterpenes were previously identified in BKME condensates (Belknap et al., 2006). Condensate 3: FP-NP contained 36 mg/L manool compared to the 24 mg/L in HPLC 7. This was likely due to sample handling, as steps were taken to ensure no compounds were left on the HPLC column. Furthermore, if time permitted to run multiple HPLC fractionations, an average concentration could have been calculated thus reducing error.

Standards for both compounds were tested for androgenic activity using the AR binding assay and it was found that manool was highly androgenic but geranyl linalool was not. Since geranyl linalool was not androgenic, it can be inferred that other acyclic compounds present

in BKM condensates are not capable of binding to the AR. A displacement curve was created using a series dilution of manool that was tested for androgenic activity and then compared to a testosterone standard curve. From this, an IC50 value of manool was calculated to be 71.61 μM (Figure 3-11), ~300 times less potent than testosterone. Application of this relative potency to the condensates sampled showed manool accounted for 29, 22 and 23 % of the total androgenicity of Condensates 1, 2, and 3, respectively. Manool accounted for 43 and ~14 % of the androgenicity in HPLC 6 and 7, respectively. To our knowledge, this is the first evidence that manool has exhibited androgenic activity. It can be concluded that the remaining 86 % of activity in HPLC 7 must be derived from additional compounds present. Library analysis suggested that the remaining peaks in HPLC 7 all had mass spectra consistent with a series of cyclic diterpenes. It is possible that these compounds may be more potent ligands than manool, considering GC-MS analyses suggests they are present in much lower amounts.

HPLC 4 accounted for 6 % of the cumulative HPLC androgenic activity with a value of ~38,000 ng/L condensates; more than double that of Condensate 2: FP-NP. GC-MS analysis indicated that many of the peaks had common spectra (Figure 3-7). For instance, with the exception of peak 5, all major peaks had an apparent molecular ion at m/z 286. Peaks 1 to 4 had numerous fragment ions in common as well. Although library searches yielded poor quality spectral matches, they were consistent with diterpenoid structures. Peak 5 closely resembled dehydroabietal (86 %), a cyclic diterpene previously identified in softwood (Nikolic et al., 2009). The presence of dehydroabietal provides further support for the

existence of diterpenes in HPLC 4. It is likely that at least some of the diterpenoid compounds in this fraction are responsible for the androgenic activity.

HPLC 6 was the 3rd most androgenic HPLC fraction. GC-MS analysis revealed 12 major detectable constituents (Figure 3-8). Many of these peaks had spectral similarities to one another. Library searches of 9 of these compounds consistently suggested cyclic diterpenoid structures. Some of these diterpene compounds are probably acting as ligands for the AR. Similarly, this was the case with HPLC 9 as well. GC-MS analysis of HPLC 9 revealed 14 major detectable constituents (Figure 3-9). Although library searches yielded poor quality spectral matches for 10 of these peaks, they were consistent with diterpenoid structures. A library search of peak 12 revealed that the mass spectra closely resembled that of the cyclic diterpene dehydroepiabietal, which has previously been identified in softwood (Carman and Deeth, 1967; Oh et al., 2007). The body of evidence suggests that HPLC 9 contains a fourth group of diterpenes present in FP-NP, which are potentially accountable for at least some of the androgenicity observed.

This experiment is the first of its kind to identify manool, a wood-derived cyclic diterpene, as an androgenic compound. Manool was previously found in BKM condensate at concentrations of approximately 40 mg/L condensate (Belknap et al., 2006). Manool accounted for 29, 22, and 28 % of the androgenic activity of Condensate 1: FP-NP, Condensate 2: FP-NP, and Condensate 3: FP-NP, respectively. Geranyl linalool did not

interact with the AR and so it is likely that other acyclic diterpenes are also non-androgenic. Four different families of diterpenes were successfully isolated in 4 HPLC fractions and evidence presented in this paper suggests that some of these compounds have the potential to act as ligands for the goldfish AR.

# Chapter 4

# Quantifying Ligands for the Goldfish Testicular Androgen Receptor in Effluents and Wood Feedstocks from Pulp and Paper Mills in Canada, South America, and New Zealand

## 4.1 Abstract

There is a large body of evidence dating back to the 1980s reporting that pulp and paper effluents from numerous countries have the potential to affect fish reproduction by way of endocrine disruption. However, despite this evidence, a clear relationship between industrial manufacturing process and/or effluent treatment type and specific reproductive effects remains unclear. This study compares the ability of final mill effluent and wood feedstocks from 11 pulp and paper mills located in Brazil (5), Canada (4), and New Zealand (2) to affect fish reproduction. This chapter in particular focuses on the author's contribution to the project, which was to examine the androgenic potential of final mill effluent and wood feedstock extracts using the goldfish testicular androgen receptor (AR) binding assay. A solid phase extraction (SPE) protocol was utilized to separate particulate and dissolved polar and non-polar extracts. All mill effluents exhibited androgenic activity, ranging from 423-940 ng/L testosterone equivalents (TEQ). Activity was not related to mill process, effluent treatment type, or location. Two Canadian mills exhibited the highest activities found in this study; ~3.5 and ~9.5 fold higher than the average of the other effluents sampled. Overall, polar extracts contributed very little to androgenic activity. Causative compounds in wood

feedstocks were isolated into three extracts ranging from non-polar to polar and in every case, androgenicity decreased with increasing polarity. In general, hardwood species had minimal androgenic activity (~50 ng/L TEQ), while softwood species and mixtures of the two showed high levels of androgenicity (~4000 ng/L TEQ). The results of this study indicate that effluent biotreatment systems are effective in removing compounds functioning as ligands for the goldfish AR.

## 4.2 Introduction

It has been well documented since the 1980s that pulp and paper effluents can potentially affect fish reproduction by way of endocrine disruption (Kringstad and Lindstrom, 1984), In vivo experiments with white sucker (McMaster et al., 1991), mummichog (Dubé and MacLatchy, 2000; Hewitt et al., 2002) and other fish species have found this to be true in Canadian, American and New Zealand effluents. In vitro competitive binding assays testing for the existence of ligands for goldfish testicular androgen receptors (AR) (Wells and Van Der Kraak, 2000; Ellis et al., 2003), goldfish plasma sex steroid binding proteins (SSBP; Hewitt et al., 2003b), and retinoic acid receptors (Alsop et al., 2003) have all reported endocrine disrupting effects in pulp and paper effluents. Recent in vivo experiments in Chile have identified a predominantly estrogenic response in fish caged below BKMs and fish exposed to downstream sediment (Orrego et al., 2005; 2006). These are some of first studies to come out of Chile (and South America) regarding the reproductive effects of mill effluent.

Therefore further studies in South America are needed to assess the environmental impact of these mills.

Despite the large body of evidence, a clear relationship between industrial manufacturing process and/or effluent treatment type and specific reproductive effects remains unclear. A comprehensive evaluation of these studies has not been possible due to the numerous approaches involving different fish species, experimental conditions, responses evaluated, and various in vitro assays. Adding to the difficulties is the fact that effluent composition is chemically complex and is greatly dependent on mill processes, bleaching sequences, effluent treatment type, and wood feedstock. An increasing amount of evidence is being collected indicating that wood feedstocks may be the source of endocrine disrupting chemicals (Denton et al., 1985; MacLatchy and Van der kraak, 1995; Hewitt et al., 2002; Belknap et al., 2006).

The objective of this study was to compare the ability of final mill effluent and wood feedstocks from 11 pulp and paper mills located in Brazil, Canada, Chile and New Zealand to affect fish reproduction. Effluents and wood feedstocks were assessed using in vivo and in vitro methods. Methods include a rainbow trout in vivo injection protocol (molecular biomarkers, genomic assays), two neuro-endocrine assays, an AR binding assay and an estrogen receptor (ER) binding assay. Chemical analysis focused on candidate compounds associated with hormonal activities (e.g. phytosterols) with analyses by GC-MS.

This study is the first of its kind to compare the ability of mill effluents and wood feedstocks from different countries to affect fish reproduction. In doing so it will address a large research gap by comparing mill effluents and wood feedstocks to not only determine global trends of endocrine disruption, but also determine what links exist between wood feedstock and mill effluent toxicity. Comparisons of effluents between mills from pulp producing countries will provide information on the differences and similarities in which effluents from other countries affect fish reproduction. These comparisons can be normalized to Canadian mill effluents, where extensive information on effluent effects in receiving water fish has been generated via the Environmental Effects Monitoring (EEM) Program. Chemical and biological characterizations of extracts from wood feedstocks at each mill were conducted based on the hypothesis that wood is the ultimate source of endocrine disrupting compounds in mill effluents.

## 4.3 Methods and Materials

### 4.3.1 Mill Process Description

There were 11 mills used in this study from Canada, South America, and New Zealand. Of the 5 Canadian mills, 3 were elemental chlorine free (EFC) Kraft mills and the remaining 2 were thermomechanical pulp (TMP) mills. There were 4 Brazilian mills, 3 of which were ECF Kraft mills and another Kraft mill, which did not use EFC. All of the Brazilian mills are

state-of-the-art and were built within the last decade. Both New Zealand mills were ECF Kraft mills. Figure 4-1 provides detailed information on each mill involved in this study.

Figure 4-1: Mill Process Description. This table summarizes all known details about each of the 11 pulp and paper mills taking part in this study.

| Location | Assigned Letter | Mill Type | Bleaching Sequence | Wood Type at sampling | Woodtype Switch? | Stormwater? | Other wastewaters? | Treatment Type | Wastewater Flow | Efficiency | | TSS | Tertiary? |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | $m^3$/ADMT | BOD removal | COD removal | | |
| Canada | Mill A | ECF Kraft | $DE_{op}D$ | Hardwood | Yes | Yes | | AS | 44.5 | 98 | 74.3 | 94.1 | No |
| Canada | Mill B | TMP | None | Softwood (75% spruce/25% Pine) | No | No | No | AS | 45.9 | 99 | 93 | 91 | No |
| Canada | Mill C | TMP | None | Softwood (Spruce 85%/15% Balsam fir) | No | Small | Yes [3] | ASB | 30.6 | 74.6 | ~75 | | |
| Canada | Mill D | ECF Kraft | $DE_{p/o}DED$ | Softwood | Yes | No | No | MBBR and RO[4] | 23.8 | 92 | 41 | | No |
| Brazil | Mill E | ECF Kraft | $D_oE_{op}DD$ $D_oE_pPPP$ | Hardwood | No | Yes | Yes | ASB | 28.8 | 89.9 | 62.5 | 75 | |
| Brazil | Mill F | Kraft | Unbleached | Softwood | No | No | No | AS | 52.4 | 93.8 | 88 | 93.2 | No |
| Brazil | Mill G | ECF Kraft | $D_{hot}O_pD$ | Soft/hard | No | No | No | AS | | 94 | 80 | | Yes [1] |
| Brazil | Mill H | ECF Kraft | $OADE_{op}D$ | Hardwood | No | No | No | AS | 30.6 | >99 | 88.3 | 95 | |
| Brazil | Mill I | ECF Kraft | $D_{hot}E_{op}DP$ | Hardwood | No | No | Yes [2] | AS | 21.8 | 97.2 | 81 | 86 | |
| NZ | Mill J | ECF Kraft | $ODE_{op}E_pD$ | Softwood | No | | | ASB | | | | | No |
| NZ | Mill K | ECF Kraft | ODEDED | Softwood | Yes | | | ASB | | | | | No |

[1] Mill uses ultrafiltration on 40 % of flow prior to discharge, sample for study was taken before tertiary treatment
[2] Sanitary sewer enters in to treatment system
[3] Small volume from nearby oil refinery
[4] MBBR=Moving Bed Bio-Reactor treats bleaching effluent, RO=Reverse Osmosis treats black liquor condensates

### 4.3.2 Effluent Sampling and Preparation

In order to avoid chemical and legal complications in shipping effluent internationally, each mill extracted 6 L of final effluent according the extraction method previously detailed in Section 3.3.4 on page 49. Filter paper and SPE cartridges were then shipped frozen to Environment Canada (Burlington, ON, Canada) where elution was performed for *in vivo* and *in vitro* experiments.

### 4.3.3 Woodchip Sampling and Preparation

Wood feedstock samples were sent to Environment Canada (Burlington, ON, Canada) from certain mills that participated in the study. Woodchips samples were extracted using hexane, dichloromethane, and acetone so that three extracts were obtained based on increasing polarity. Woodchips were put into a Soxhlet extraction apparatus with 300 mL of hexane, followed sequentially by dichloromethane and finally acetone. Each solvent was cycled for 24h. Extracts were reduced in volume and solvent exchanged into methanol for androgenicity evaluations and toluene for GC-MS analysis.

### 4.3.4 Gas Chromatography-Mass Spectrometry

Fractions were first evaporated under a gentle stream of nitrogen to just dryness and then reconstituted in toluene. Aliquots of each fraction (1 μL) were injected onto a GC-MS triple quadrupole system (7890A/7000 Series, Agilent Technologies, Mississauga, ON, Canada) using a HP-5MS capillary column (30 m; inner diameter, 0.25 mm; film thickness, 0.25 μm;

Agilent Technologies, Palo Alto, CA, USA) with a He carrier gas.

All extracts were analyzed using the following parameters: injector temperature, 300 °C in splitless mode; oven temperature, 90 °C for 30s; increased at 40 °C/min to 300 °C; held for 10 min. Concentrations of manool were obtained by select ion monitoring (SIM) GC-MS analysis operating at unit resolution.

### 4.3.5 Androgen Receptor Binding Assay

The AR binding assay was performed using the methods described in the previous chapter. Please refer to Section 3.3.7 on page 53.

### 4.3.6 Data Analysis

AR binding assay data was transformed and tested for normality using the D'Agostino and Pearson omnibus normality test. A Ln transformation yielded a normalized data set. Normalized data was analyzed using a one-way analysis of variance (ANOVA; $P \leq 0.05$) to determine whether or not there were significant differences in androgenic responses of samples and blanks. Tukey's post hoc test made pair wise comparisons to determine where these differences occurred. All statistic analysis was conducted using Prism (GraphPad Software, La Jola, CA, USA).

## 4.4 Results

Levels of ligands for the goldfish testicular AR in final mill effluents were similar for all mills except Canadian mills C and D, which exhibited the highest level of testosterone equivalents found in all the mills surveyed in this study (Figure 4-2). SPE-NP fractions from mills A, B and E to K were more androgenic than their respective FP-NP fractions. However the reverse was found with mills C and D. In both instances, FP-NP was significantly higher than that of SPE-NP ($p<0.05$; data not shown); graphical inspection revealed that overall effluent androgenicity of mills C and D was higher than all other mills studied. Mills A and B had total androgenic activities >525 ng/L. Mills C and D had total androgenic activities of ~2000 ng/L and ~5700 ng/L, respectively. Overall, polar fractions contributed very little to androgenic activity.

All Brazilian mills contained a moderate amount of androgenic activity with mill E being highest overall, followed by mill I. Furthermore, SPE-NP was significantly more androgenic than FP-NP in all Brazilian mill effluent fractions. Despite geographic differences and wood feedstock variation, levels of androgenic activity in New Zealand mill effluents were similar to those of Brazilian mills.

Excluding Canadian mills C and D, androgenicity was similar between all samples. However, wood feedstock androgenic activity varied widely between wood feedstock samples. Despite androgenic variation between wood feedstocks, androgenicity consistently

decreased with increasing polarity of extract (with the exception of Canadian aspen, which had minimal androgenic effects to begin with).

The general trend seen in the wood feedstocks sampled was that hardwood (HW) species had minimal androgenic activity, while softwood (SW) species and mixtures of the two showed high levels of androgenicity. For example, all extracts of eucalyptus (HW) from Brazil and Chile had almost no androgenic activity, whereas pine (SW) from either country was highly androgenic (~80 fold that of eucalyptus). Brazilian pine was roughly twice as androgenic as Chilean pine. New Zealand pine had the highest levels of androgenic activity out of all the wood feedstock samples (~6300 μg/g dry weight) followed by a Canadian mixture of HW and SW (~5900 μg/g). Similar to eucalyptus, Canadian aspen (HW) showed minimal androgenic activity.

A recent study (Chapter 2; page 16) discovered that a natural cyclic diterpene found in BKM condensates called manool, acted as a ligand for the goldfish AR. This study analyzed effluent and woodchip extracts for manool. Manool was identified in Mill D FP/SPE-NP fractions in small quantities, compared to concentrations in condensates reported in Scott et al. (*in press*). Mill D: FP-NP and SPE-NP contained 18.78 and 10.45 μg/L manool, respectively.

Figure 4-2: Testosterone equivalents measured with the goldfish testicular androgen receptor competitive binding assay incubated with extracts of final effluent sampled from Canadian Brazilian and New Zealand mills. Error bars represent standard error of the mean. Wood feedstock used at each mill during sampling is indicated by: HW=hardwood, SW=softwood, Mix=hardwood and softwood.

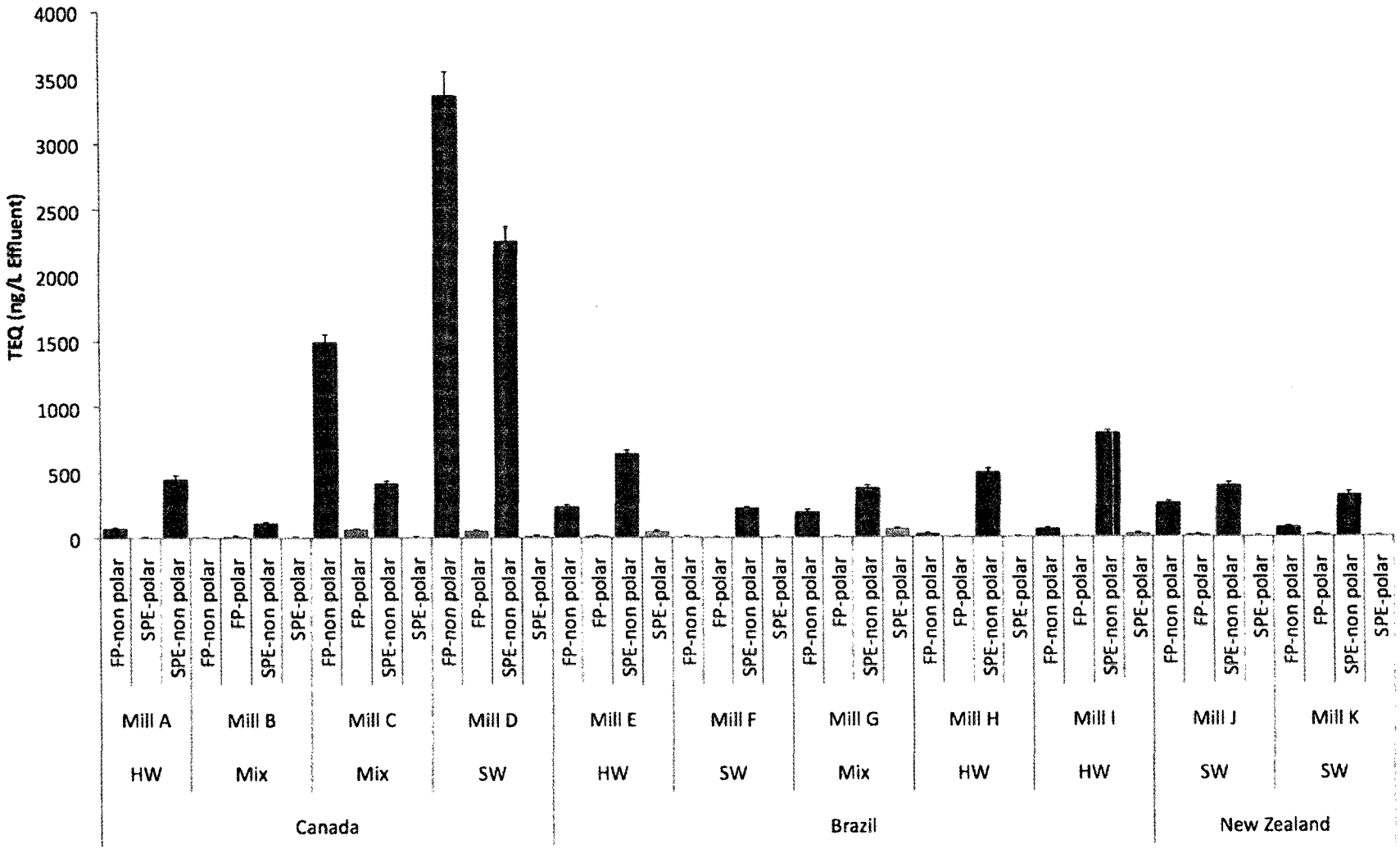
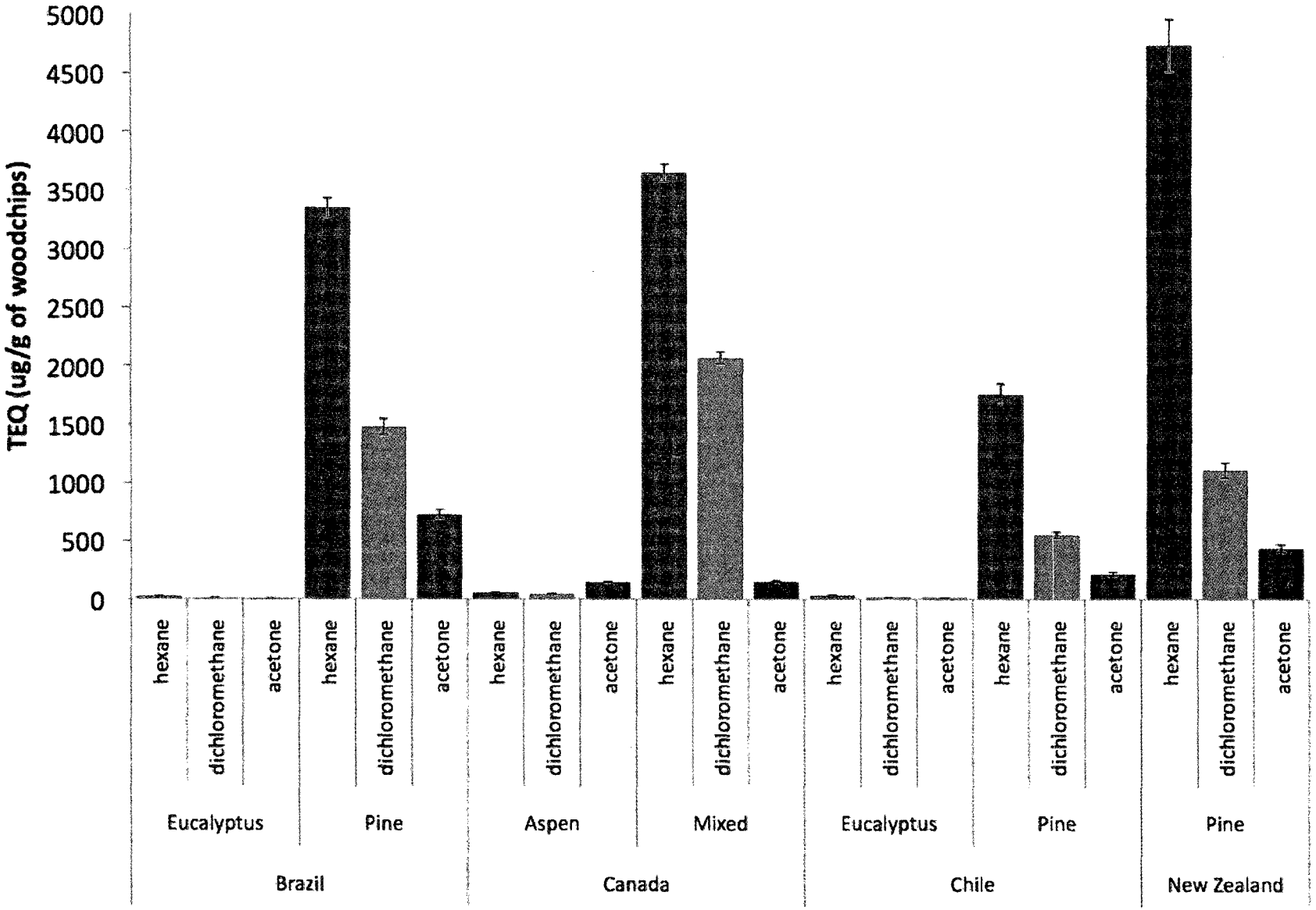

TEQ (ng/L Effluent)
4000
3500
3000
2500
2000
1500
1000
500
0
FP-non polar
SPE-polar
SPE-non polar
Mill A
HW
Mill B
Mix
Mill C
Mix
Mill D
SW
Mill E
HW
Mill F
SW
Mill G
Mix
Mill H
HW
Mill I
HW
Mill J
SW
Mill K
SW
Canada
Brazil
New Zealand

Figure 4-3: Testosterone equivalents measured with the goldfish testicular androgen receptor competitive binding assay incubated with extracts of woodchips sampled in Brazil, Canada, Chile, and New Zealand. Error bars represent standard error of the mean.


TEQ (ug/g of woodchips)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
hexane
dichloromethane
acetone
hexane
dichloromethane
acetone
hexane
dichloromethane
acetone
hexane
dichloromethane
acetone
hexane
dichloromethane
acetone
hexane
dichloromethane
acetone
hexane
dichloromethane
acetone
hexane
dichloromethane
acetone
hexane
dichloromethane
acetone
Eucalyptus
Pine
Aspen
Mixed
Eucalyptus
Pine
Pine
Brazil
Canada
Chile
New Zealand

## 4.5 Discussion

This study demonstrated that ligands for the goldfish testicular androgen receptor are present in final mill effluents and wood feedstocks from different pulp producing countries. To our knowledge, this is the first study of its kind to compare the potential of effluents from different countries for their potential to affect fish reproduction. With the exception of two mills, all effluents examined in this study yielded similar androgenic responses to the AR binding assay, regardless of geographic location or wood feedstock. Canadian mills C and D exhibited the highest activities found in this study; ~3.5 and ~9.5 fold higher than the average of the other effluents sampled. They are further set apart by the fact that both of their respective FP-NP extracts were more androgenic than SPE-NP extracts; the opposite was seen in all other mill effluents. Effluents from mills A (Canada), E to I (Brazil) and J to K (New Zealand) had similar TEQ values (423-940 ng/L); mill B effluent extracts were only slightly androgenic. These mills treated their effluent using settling tanks and activated sludge (AS), which potentially reduced effluent toxicity. Settling tanks are used to separate insoluble particulate from effluent. Many toxic mill effluent constituents have low water solubilities (e.g. resin acids) and so settling tanks are an effective way to decrease concentrations of these chemicals in final effluent. Bacteria in AS treatment breakdown potential environmentally harmful compounds such as terpenes and diterpenes (e.g. resin acids are diterpenes and are known to be degraded into retene through a well established pathway). As a result of these two treatments, many of the potential EDC are eliminated from final effluent (Schnell et al., 2000) and therefore FP-NP/P extracts. Canadian mill D does not

use settling tanks or AS. Instead, mill D uses an in-plant reverse osmosis system (RO; described in Chapter 2) to treat the primary source of effluent BOD and toxicity and an additional bioreactor to treat bleach plant effluent. RO successfully reduces effluent toxicity, but many potential EDCs that in other mills would biodegrade do not do so because of the lack of bacteria. The higher androgenicity in mill D: FP-NP could be due to the absence of these two treatment types. It could also be the reason FP-NP is more androgenic than SPE-NP, in contrast to all other effluents sampled. Higher levels of insoluble compounds would be present in mill D effluent and thus FP-NP/P extracts would likely contain greater concentrations of potential non-polar endocrine disrupting compounds. A previous study (Scott et al. *in press*; Chapter 3) reported high levels of androgenic activity in FP-NP extracts of chemical recovery condensates prior to RO treatment from the same mill. This study identified the cyclic diterpene manool, a causative compound that, in other mills, would be removed from final effluent, as a ligand for the AR that accounted for ~25 % of the androgenic activity measured in condensates. FP/SPE-NP fractions from Canadian Mill D had concentrations of manool ~2,000 to ~7,000 times less than the concentrations reported in chemical recovery condensates in the original study. Manool accounted for less than 0.05 % of the measured androgenic activity in each final effluent fraction that contained manool. RO treatment did not eliminate manool from final effluent, but it did reduce manool concentrations to levels that had almost no contribution to total androgenic activity of final effluent. The biodegradation products of manool are unknown so although low levels were measured in final effluent, it is possible that manool biodegradation products present in final effluent are androgenic. The fact that manool concentrations are greatly reduced in final

effluents illustrates the benefits of RO treatment. The presence of manool in final effluent from mill D implies that other cyclic diterpene compounds must be also present in final effluent. Some of these compounds may have a stronger affinity for the AR ligand compared to manool, and therefore be responsible for at least some of the androgenicity reported.

It was initially proposed that effluent androgenicity could be partially explained by the androgenic activity of the wood feedstocks used within the mills, but interestingly the opposite was found to be the case. Although Brazilian eucalyptus (and Chilean eucalyptus) woodchips had minimal androgenic activity, effluents from Brazilian mills that processed eucalyptus (mills E, H and I) had higher amounts of androgenic activity than Brazilian mill G, which processed a mix of eucalyptus and pine, which in turn was higher than Brazilian mill F, which processed only pine. Pine extracts were found to contain some of the highest amounts of androgenic activity. Both New Zealand mills using pine feedstock (mills J and K), which was the most androgenic out of woodchip samples, had similar levels of androgenicity. These amounts were higher than Brazilian mills using eucalyptus, but were similar to Brazilian mills using a eucalyptus/pine mix or pine. Canadian mill B, which used a mixture of hardwood and softwood (which was the most androgenic wood sample), consisted of the lowest levels of effluent androgenicity. Canada mill A, which used hardwood (specifically aspen and balsam poplar with very little androgenic activity), had the $4^{th}$ most androgenic effluent. This data strongly indicates that effluent hormonal activities are derived from pulp digestion and effluent treatment processes, which are not obtained through direct solvent extraction of wood feedstocks. Previous work has suggested the role of biological

treatment in generating androgenic compounds capable of masculinizing female mosquitofish (Denton et al., 1985; Bortone et al., 1989).

As mentioned previously, wood species androgenicity varied greatly according to the type of wood (HW vs. SW as well as specific tree species). However, androgenic activity of the woodchip extracts (hexane, dichloromethane, acetone) decreased with increasing solvent polarity in every wood type studied. What is consistent between effluent and wood extracts are that the non-polar extracts of both contained the majority of androgenic activity. This indicates that ligands for the AR are non-polar, which was consistent with previous studies (Ellis et al., 2003).

# Chapter 5
# General Discussion

## 5.1 Summary

The primary objective of this thesis was to characterize to the maximum possible extent the chemicals in Kraft mill chemical recovery condensates affecting fish reproduction. The goldfish androgen receptor (AR) binding assay was used to determine the androgenic activity of condensate extracts generated with a new SPE protocol. A normal phase HPLC method was developed for the isolation and identification of androgen receptor ligands in condensate extracts. The results were then compared to wood and final effluent extracts from different mills to determine their relevance beyond an in-mill waste stream.

In Chapter 3, normal phase HPLC fractionation of Condensate 3: FP-NP isolated the majority of androgenic activity seen in FP-NP into one HPLC fraction: HPLC 7. In fact, androgenicity of HPLC 7 did not differ from the activity of unfractionated FP-NP ($p \leq 0.05$). The TIC from a full scan GC-MS analysis showed that it was comprised of 8 main detectable constituents. The chromatogram is dominated by a large peak that was confirmed to be the bicyclic diterpene manool, previously identified in condensates by Belknap et al. (2006). A dilution series of manool was used to make a standard curve, from which the median inhibition concentration ($IC_{50}$ value) was obtained. The $IC_{50}$ values for testosterone and manool were 0.23 μM and 71.61 μM, respectively, placing Manool as 306 times less potent than

testosterone. Manool accounted for ~29, ~22, and ~28 % of the total androgenic activity in FP-NP extracts from Condensates 1, 2, and 3, respectively. Out of the total androgenicity of HPLC 6 and 7, manool accounted for ~43 and ~14 %, respectively.

HPLC 7 also contained the linear diterpene, geranyl linalool, which was also confirmed with an authentic standard. Incubation with the goldfish AR revealed that geranyl linalool did not interact with the AR, suggesting that other acyclic diterpenes do not either. GC-MS analysis indicated that HPLC 4, 6, 7, and 9 consisted primarily of 4 different groups of tentatively identified cyclic diterpene compounds. Given that manool had a strong affinity to bind to the AR, it is plausible that these compounds have androgenic properties as well.

Chapter 4 demonstrated that ligands for the goldfish testicular androgen receptor are present in final mill effluents and wood feedstock from different pulp producing countries (Canada, Brazil, New Zealand), but in much lower doses than those in condensate extracts. With the exception of two mills, all effluents examined in the international study yielded similar androgenic responses to the AR binding assay, regardless of geographic location or wood feedstock. Canadian mills C and D exhibited the highest activities out of all the mills; ~3.5 and ~9.5 fold, higher than the average of the other effluents sampled. They are further set apart by the fact that both of their respective FP-NP extracts were more androgenic than SPE-NP extracts; the opposite was seen in all other mill effluents. Condensates in the first study (Chapter 3) were sampled from Canadian Mill D, allowing for comparisons between condensates and final effluent from the same mill.

RO treatment was successful in removing the majority of manool from final effluent in Mill D. The concentration of manool was 36 mg/L in Condensate 3: FP-NP, (which accounted for 23 % of the androgenic activity), and 113 μg/L in final effluent (Mill D: FP-NP), which accounted for less than 0.05 % of the androgenic activity associated with that fraction. The fact that manool concentrations are greatly reduced in final effluents illustrates the benefits of both RO and AR treatments (data for other mills not shown). However, the presence of manool in final effluent implies that other cyclic diterpene compounds may be present. Some of these compounds may have a stronger affinity for the AR compared to manool, based on the high concentration of manool relative to other compounds in HPLC 7 and the fact that 86 % of the androgenic activity was unaccounted for. The biodegradation products of manool are unknown so although low levels were measured in final effluent, it is possible that manool biodegradation products, or biodegradation products of the other 4 groups of diterpenes identified in HPLC 7, are present in final effluent and are androgenic. Further work to confirm these suspicions is required.

Initially, it was proposed that effluent androgenicity could be partially explained by the androgenic activity of the wood feedstock used within the mills, as previous studies have found wood extractives to be androgenic (Carson et al., 2008). Progesterone, androstenedione, and androstadienedione (natural products derived from wood) were previously identified as androgenic compounds found in water and sediment downstream of a pulp and paper mill (Carson et al., 2008). None of these compounds were detected in any

condensate samples with GC-MS. Furthermore, no correlation with wood type and androgenic activity was found in this study. This suggests that effluent treatment likely has more influence on final effluent androgenicity, compared to type of wood feedstock used in the mill. Effluent treatment has been implicated in the biotransformation of plant sterols into androgenic compounds causing mosquitofish masculinization (Denton et al., 1985; Howell and Denton, 1989). It is also possible that the wood extractives liberated by the direct solvent extraction techniques employed in Chapter 3 are not representative of the digestion conditions of pulp and paper mills, such that even though wood feedstocks may be the ultimate source of androgens, it is only through the pulping and effluent treatment processes that these compounds are released to the environment.

Interestingly, non-polar extracts of effluent and woodchips contained the majority of androgenic activity, likewise with non-polar condensate extracts. This indicates that ligands for the AR are non-polar, which was consistent with previous studies (Ellis et al., 2003). It also indicates that digestion of wood is the source of these materials.

## 5.2 Future Work

TIC chromatograms of the 4 most androgenic HPLC fractions (4, 6, 7, and 9) suggest that there are 4 families of diterpene compounds potentially contributing to overall androgenicity of their respective fractions. The body of evidence presented in this thesis suggests that diterpenes found in HPLC fractions are likely contributing to the androgenicity of their

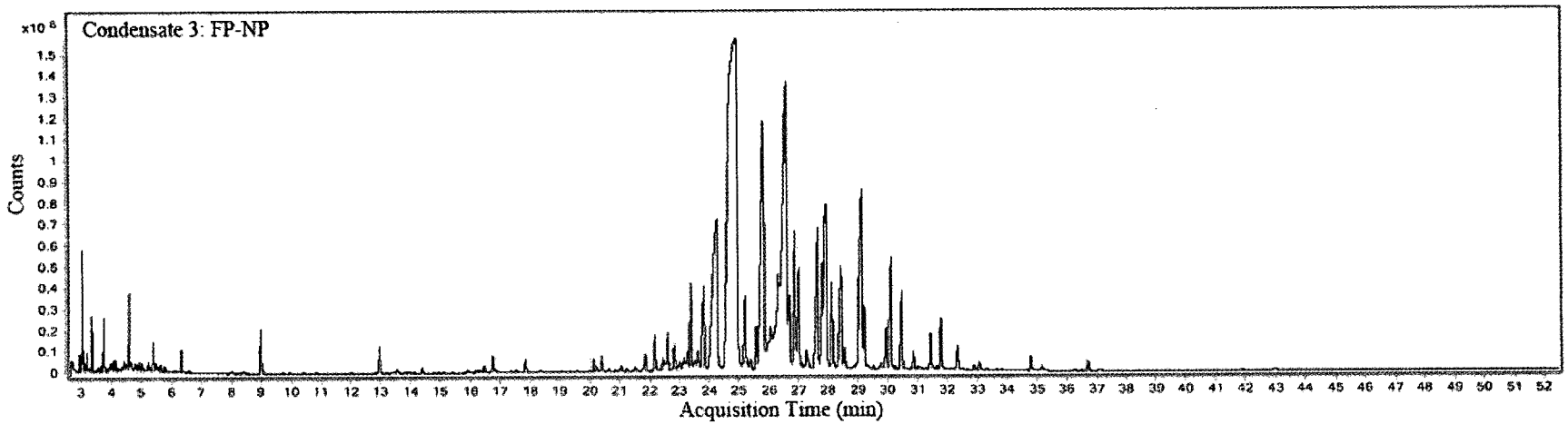
respective HPLC fractions, as well as Condensate 3: FP-NP. More research is necessary in order to identify these other diterpenoid compounds in FP-NP. Once positive confirmations are obtained with pure standards, the standards can be incubated with the AR to determine their androgenic potential. GC-MS analysis comparing condensates, final effluent, and wood extracts would be helpful in determining if there are any common potentially androgenic compounds.

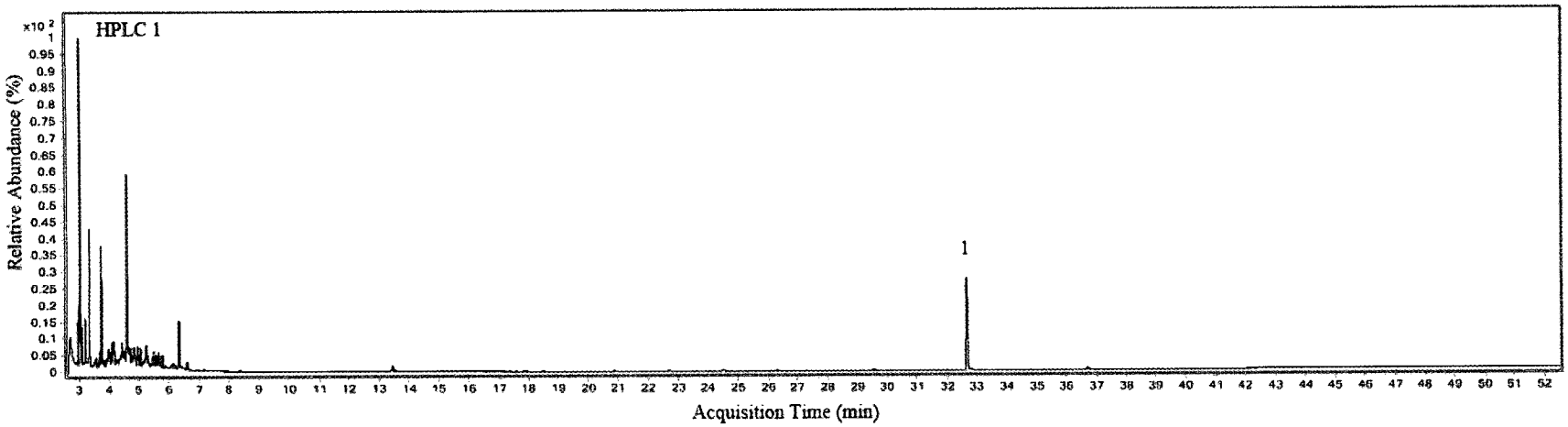
Another important piece of information that needs to be determined is whether or not AR binding is at all linked to hormone depression. An in vivo bioassay exposing mummichog to the 5 main fractions generated by the SPE protocol would help deduce whether or not a correlation between AR binding and hormone depression in fish exists.

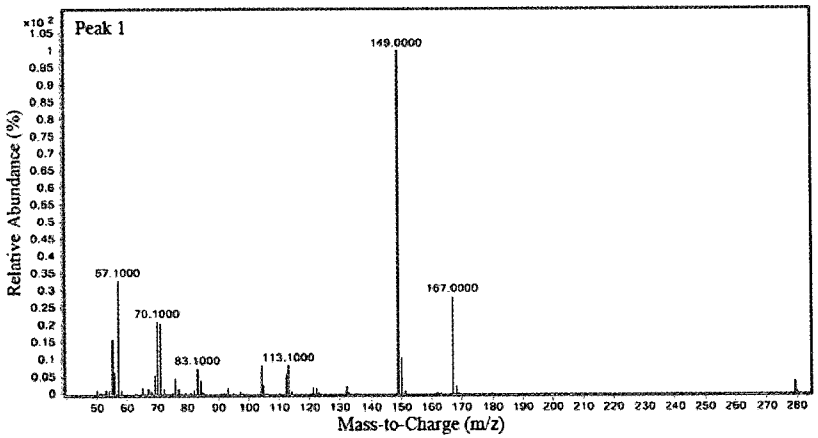
It also needs to be determined whether or not the androgenic properties of manool translate to hormone depression in fish. Therefore, an *in vivo* experiment exposing mummichog to various doses of manool is essential. This will determine if the *in vitro* response translates into an *in vivo* response. If the AR binding assay identifies other significantly androgenic compounds in FP-NP, an *in vivo* fish exposure would be beneficial in determining their endocrine disrupting potential. Furthermore, an experiment investigating the effects of manool on egg production would be beneficial in determining whether or not manool affects fish reproduction. Using egg production in short-term tests is proving to be beneficial in advancing the environmental performance of the Canadian pulp and paper sector (Kovacs et al., 2010).

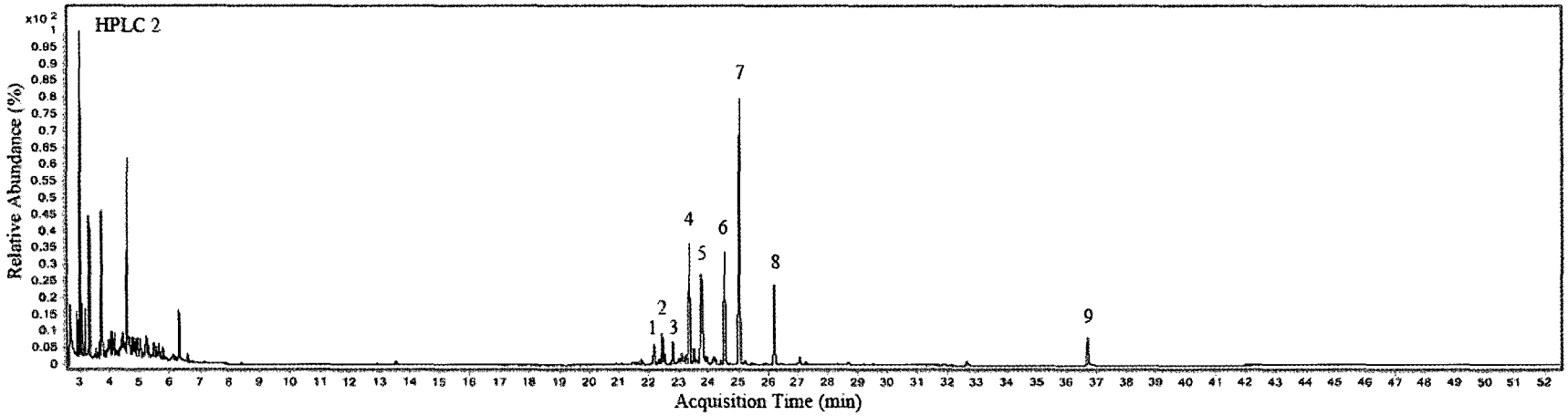
# Appendix A
# Additional Mass Spectra

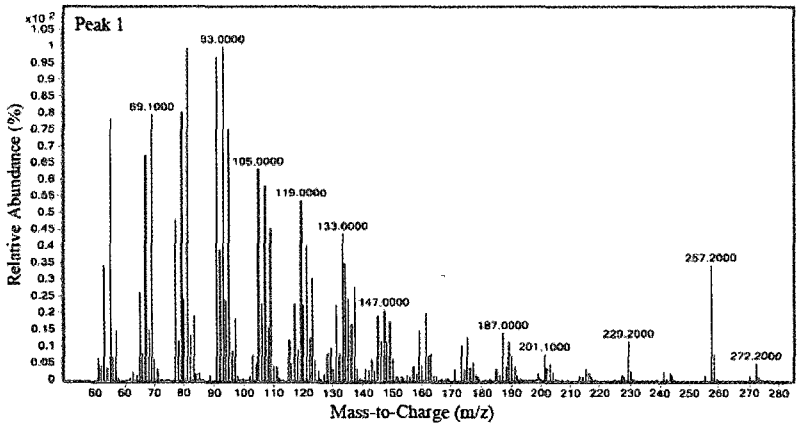
The GC-MS total ion chromatograms (TICs) and spectra in this appendix are supporting material for Chapter 2 of this thesis. Included herein is a TIC for Condensate 3: FP-NP, as well as TICs for HPLC 1-11, and spectra for each major peak.

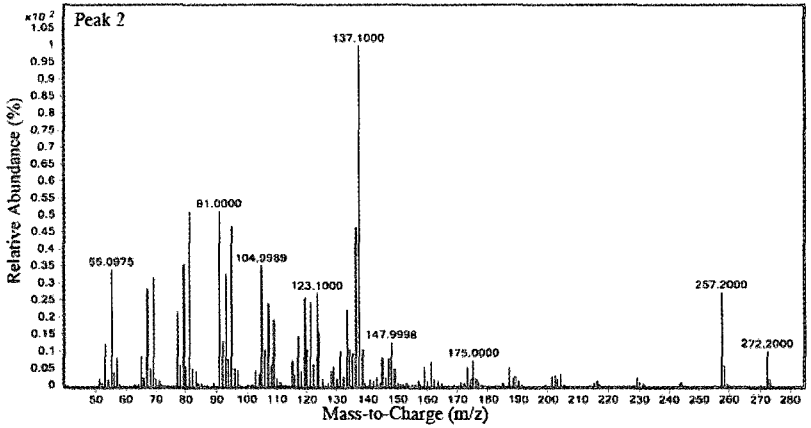

Condensate 3: FP-NP
x10 6
Counts
Acquisition Time (min)

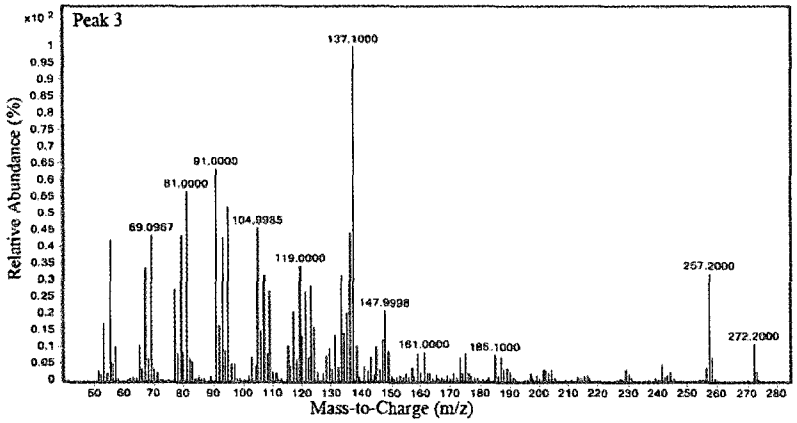

HPLC 1
Relative Abundance (%)
Acquisition Time (min)
1

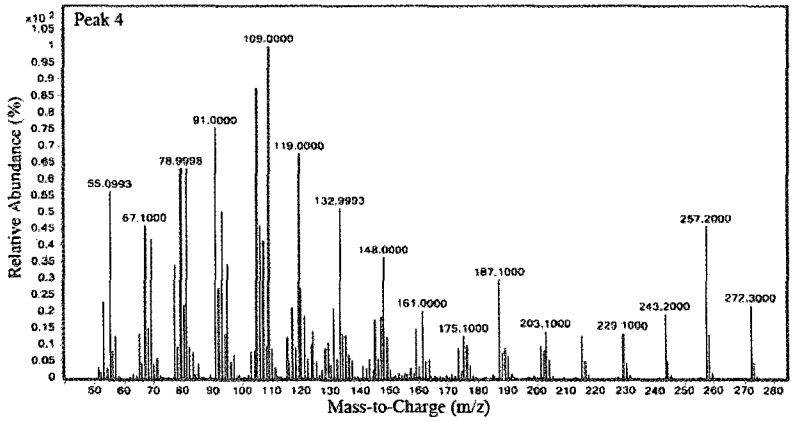

Peak 1
Relative Abundance (%)
Mass-to-Charge (m/z)
149.0000
57.1000
70.1000
83.1000
113.1000
167.0000

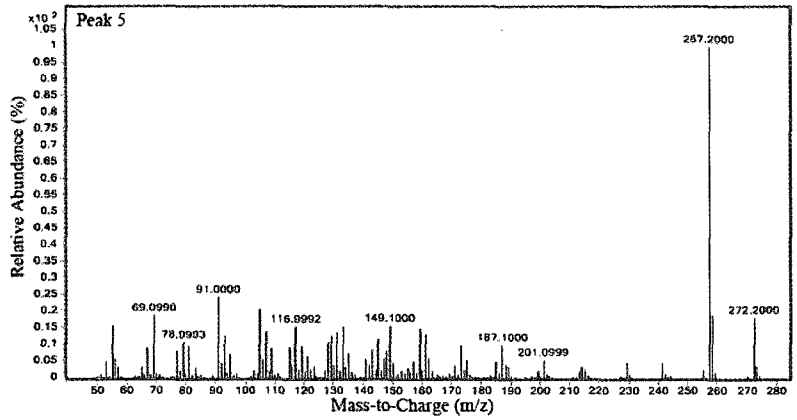

HPLC 2
Relative Abundance (%)
Acquisition Time (min)
1
2
3
4
5
6
7
8
9

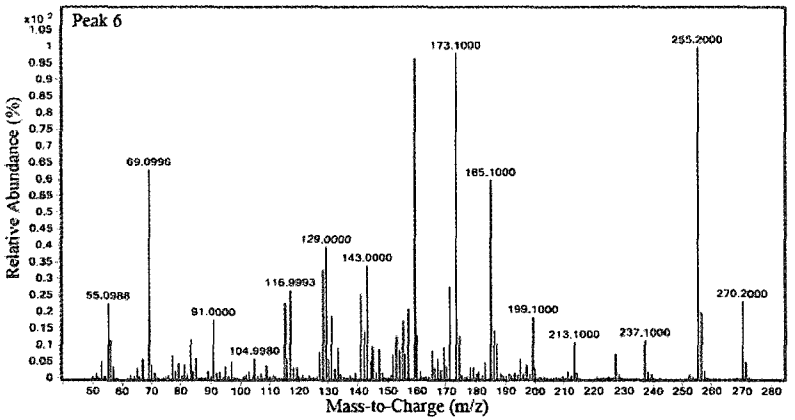

Peak 1
Relative Abundance (%)
Mass-to-Charge (m/z)
93.0000
69.1000
105.0000
119.0000
133.0000
257.2000
147.0000
187.0000
201.1000
229.2000
272.2000

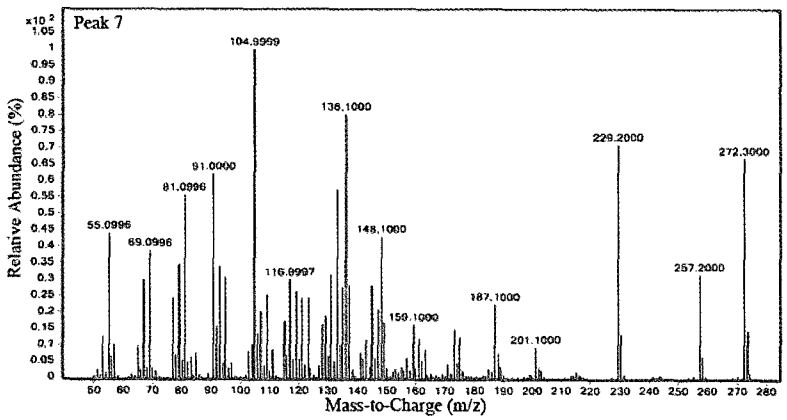

Peak 2
Relative Abundance (%)
Mass-to-Charge (m/z)
137.1000
81.0000
55.0975
104.0989
123.1000
257.2000
147.9998
175.0000
272.2000

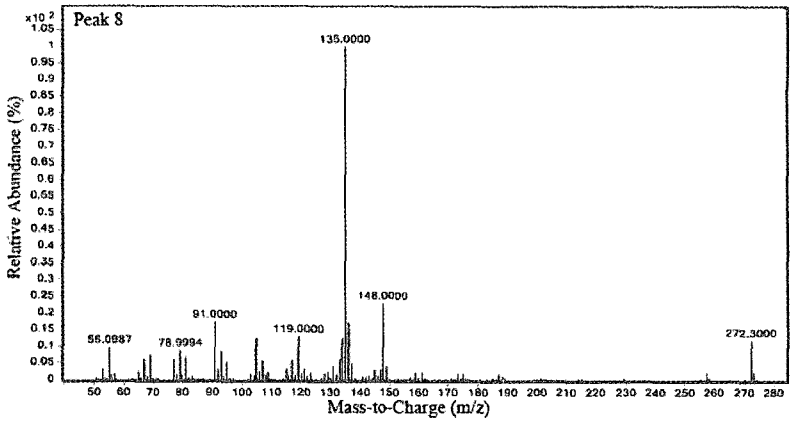

Peak 3
x10 2
Relative Abundance (%)
137.1000
91.0000
81.0000
69.0967
104.9985
119.0000
257.2000
147.9998
272.2000
161.0000
185.1000
Mass-to-Charge (m/z)

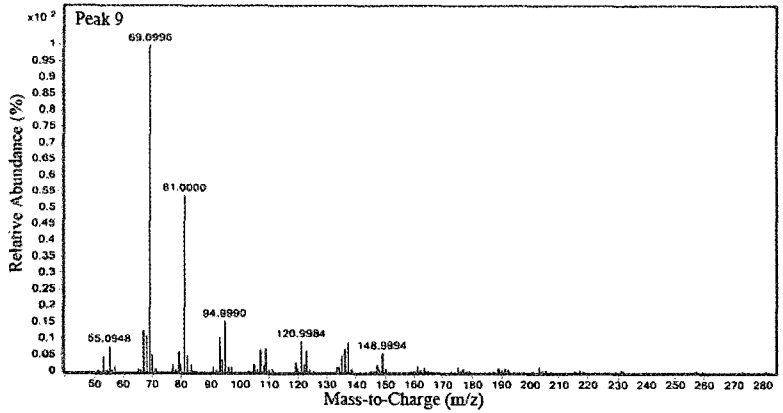

Peak 4
x10 2
Relative Abundance (%)
109.0000
91.0000
119.0000
78.9998
55.0993
67.1000
132.9993
257.2000
148.0000
187.1000
161.0000
272.3000
175.1000
203.1000
229.1000
243.2000
Mass-to-Charge (m/z)

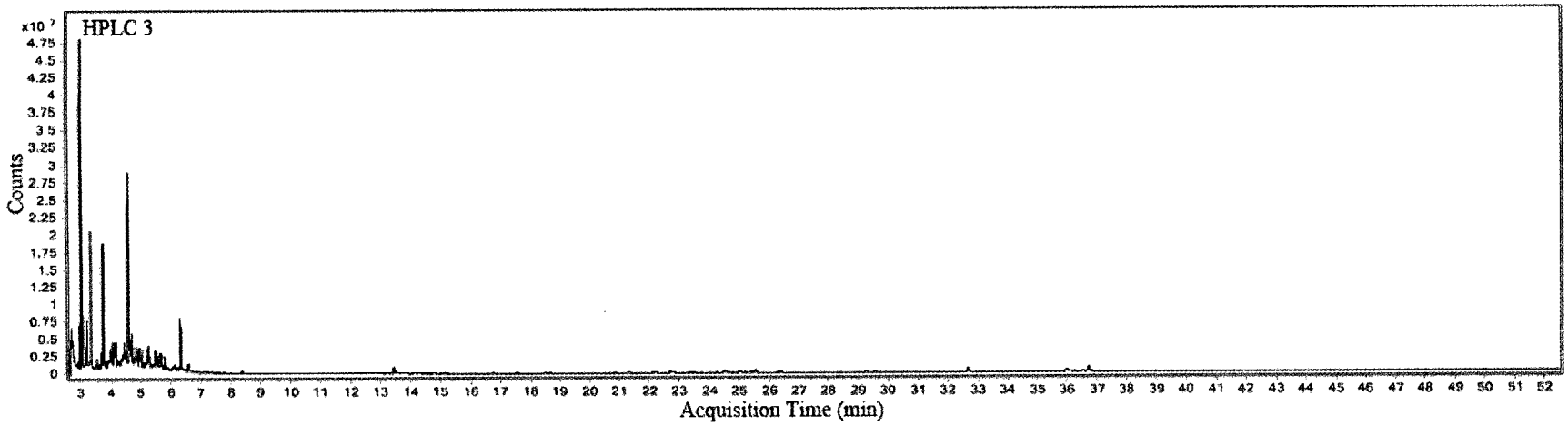

Peak 5
x10 2
Relative Abundance (%)
257.2000
91.0000
69.0990
116.9992
149.1000
272.2000
78.0993
187.1000
201.0999
Mass-to-Charge (m/z)

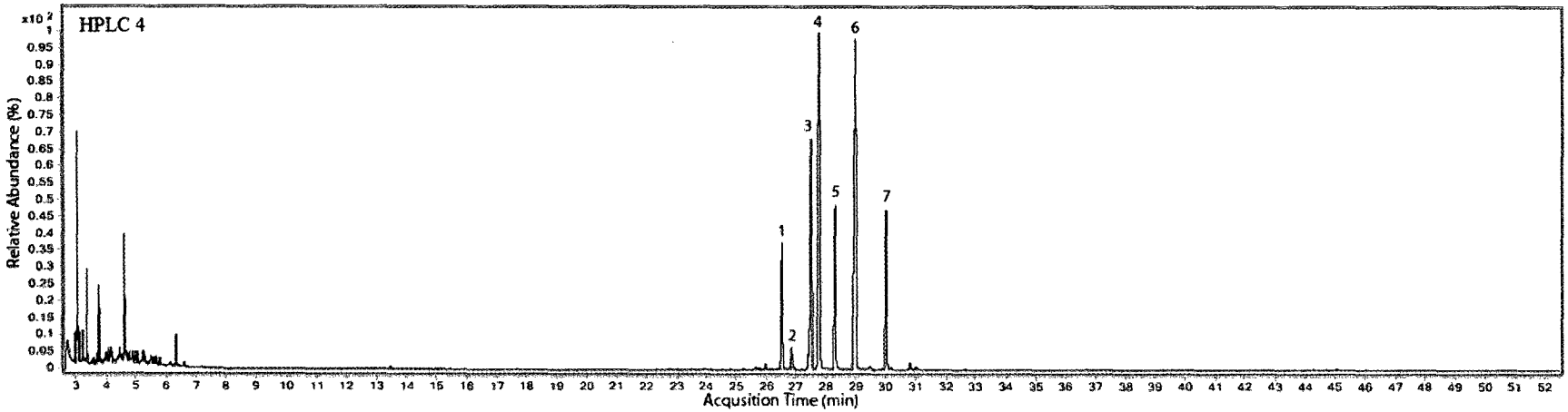

Peak 6
x10 2
Relative Abundance (%)
173.1000
255.2000
69.0990
185.1000
129.0000
143.0000
116.9993
55.0988
270.2000
91.0000
199.1000
213.1000
237.1000
104.9980
Mass-to-Charge (m/z)

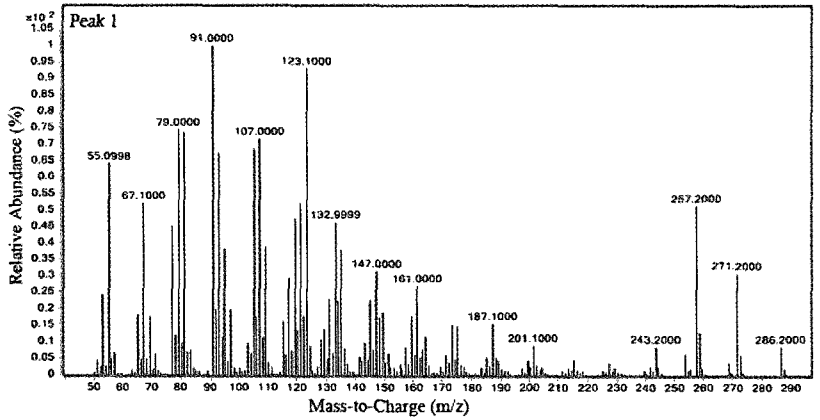

Peak 7
Relative Abundance (%)
Mass-to-Charge (m/z)
104.9999
136.1000
229.2000
272.3000
91.0000
81.0996
55.0996
69.0996
148.1000
116.0997
257.2000
159.1000
187.1000
201.1000

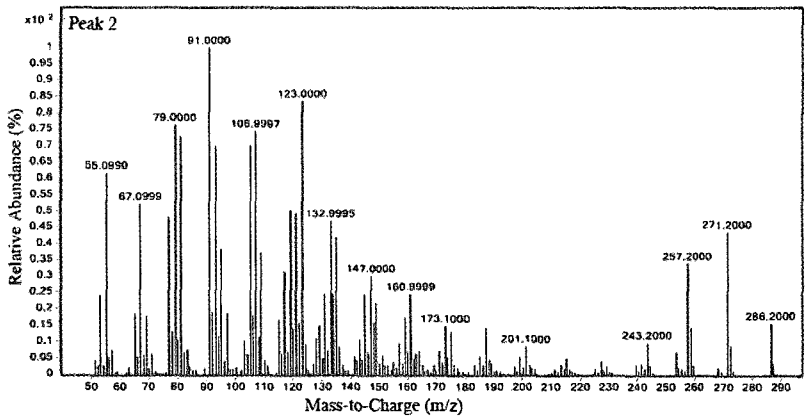

Peak 8
Relative Abundance (%)
Mass-to-Charge (m/z)
135.0000
148.0000
91.0000
119.0000
55.0987
78.9994
272.3000

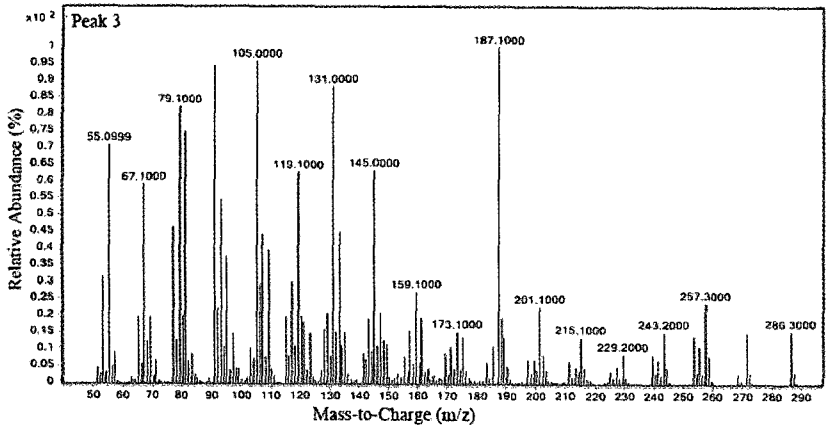

Peak 9
Relative Abundance (%)
Mass-to-Charge (m/z)
69.0996
81.0000
84.9990
120.9984
148.9994
55.0948


HPLC 3
x10 7
Counts
Acquisition Time (min)


HPLC 4
Relative Abundance (%)
Acquisition Time (min)
1
2
3
4
5
6
7


Peak 1
Relative Abundance (%)
Mass-to-Charge (m/z)
91.0000
123.1000
79.0000
107.0000
55.0998
67.1000
132.9999
147.0000
161.0000
187.1000
201.1000
243.2000
257.2000
271.2000
286.2000


Peak 2
Relative Abundance (%)
Mass-to-Charge (m/z)
91.0000
123.0000
79.0000
106.9997
55.0999
67.0999
132.9995
147.0000
160.9999
173.1000
201.1000
243.2000
257.2000
271.2000
286.2000


Peak 3
Relative Abundance (%)
Mass-to-Charge (m/z)
55.0999
67.1000
79.1000
105.0000
119.1000
131.0000
145.0000
159.1000
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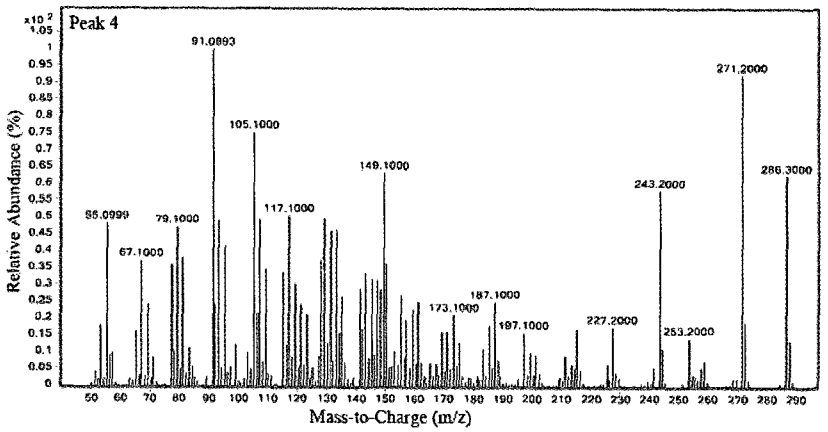
187.1000
201.1000
215.1000
229.2000
243.2000
257.3000
286.3000


Peak 4
Relative Abundance (%)
Mass-to-Charge (m/z)
55.0999
67.1000
79.1000
91.0893
105.1000
117.1000
149.1000
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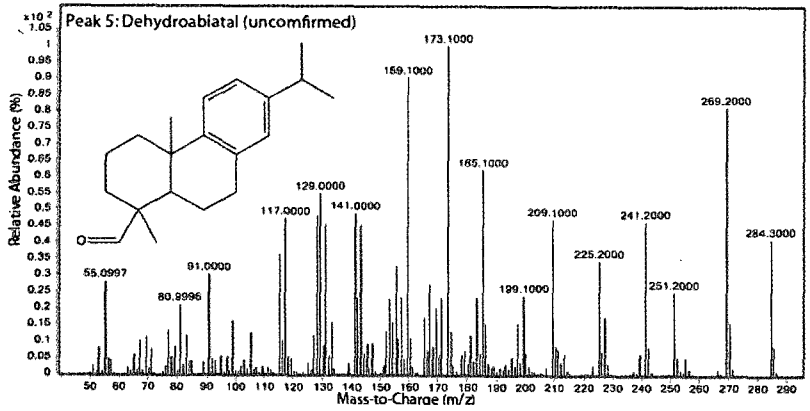
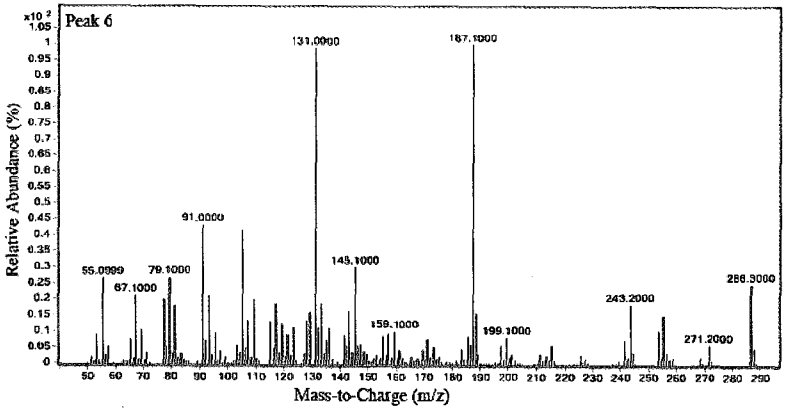
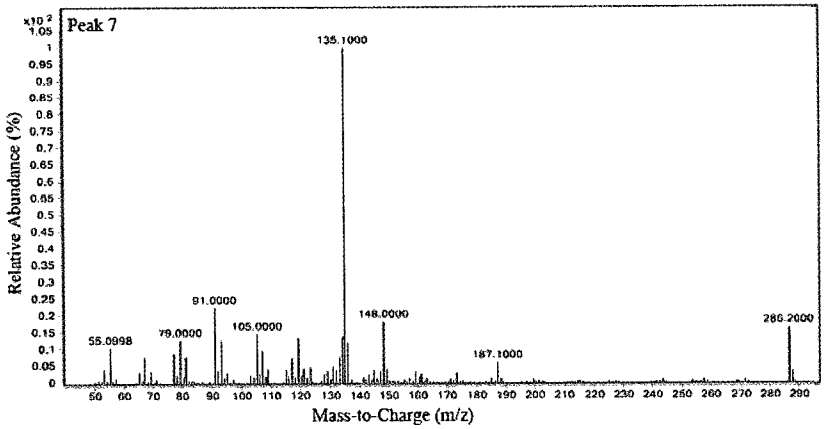
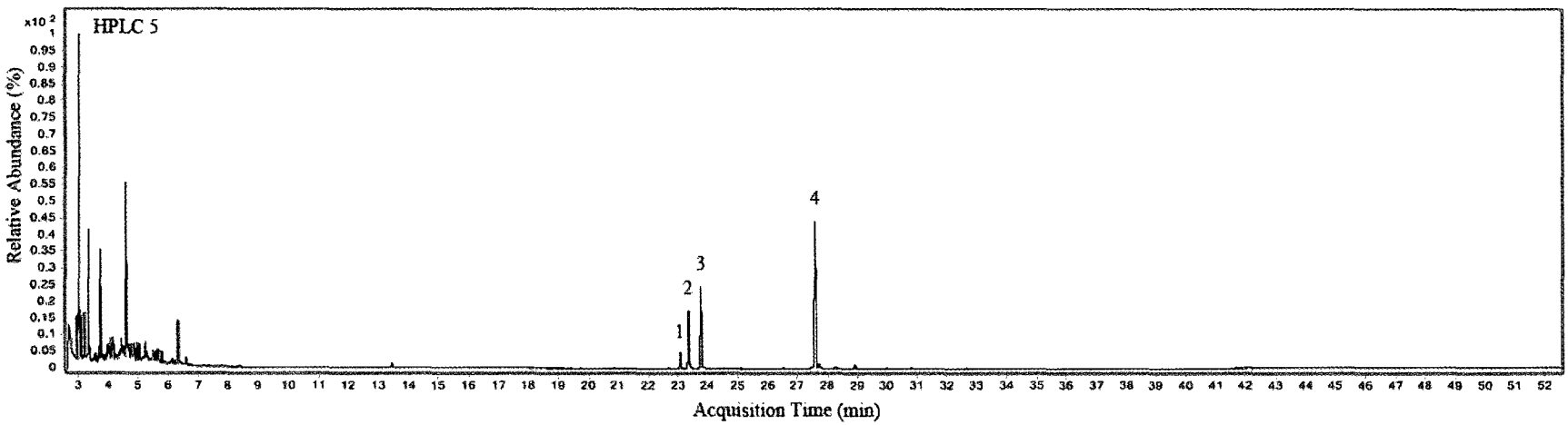
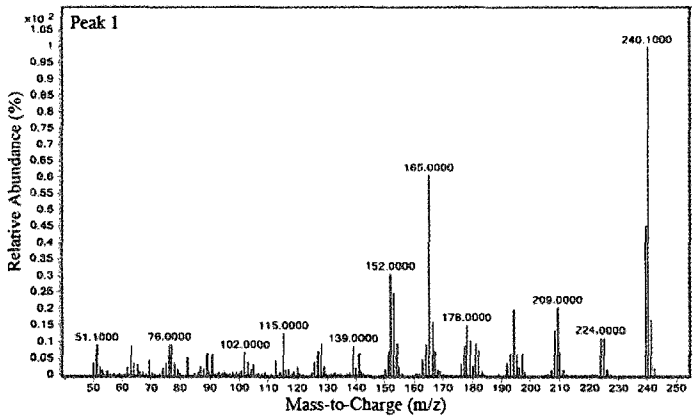
187.1000
197.1000
227.2000
243.2000
253.2000
271.2000
286.3000

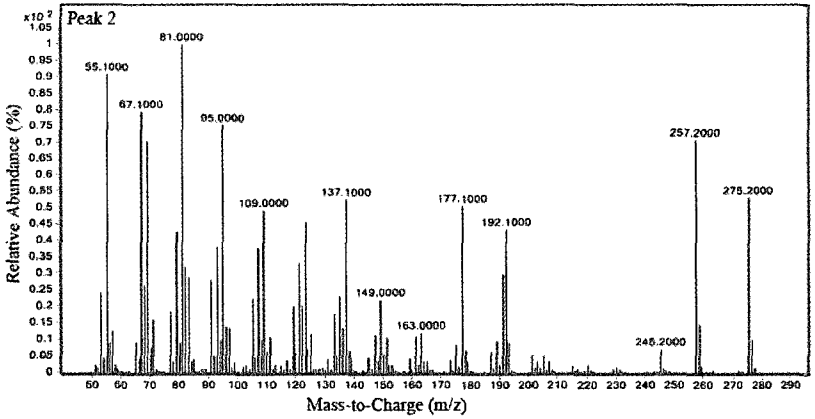

Peak 5: Dehydroabiatal (uncomfirmed)
Relative Abundance (%)
Mass-to-Charge (m/z)
55.0997
80.9996
91.0000
117.0000
129.0000
141.0000
159.1000
173.1000
185.1000
199.1000
209.1000
225.2000
241.2000
251.2000
269.2000
284.3000

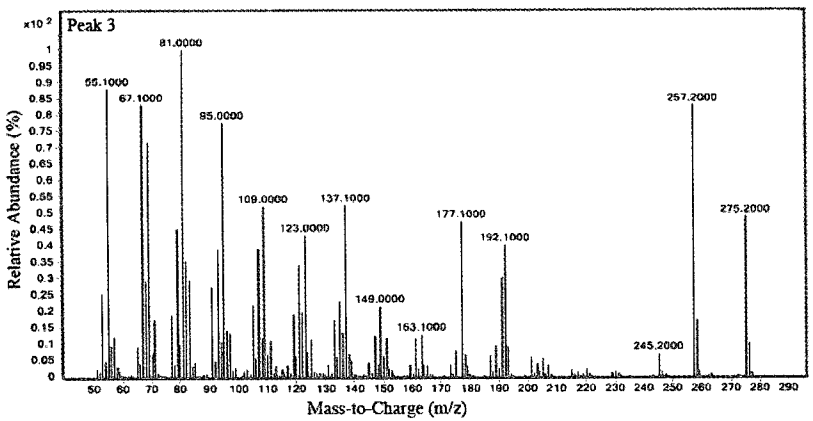

Peak 6
Relative Abundance (%)
Mass-to-Charge (m/z)
55.0999
67.1000
79.1000
91.0000
131.0000
149.1000
159.1000
187.1000
199.1000
243.2000
271.2000
286.3000

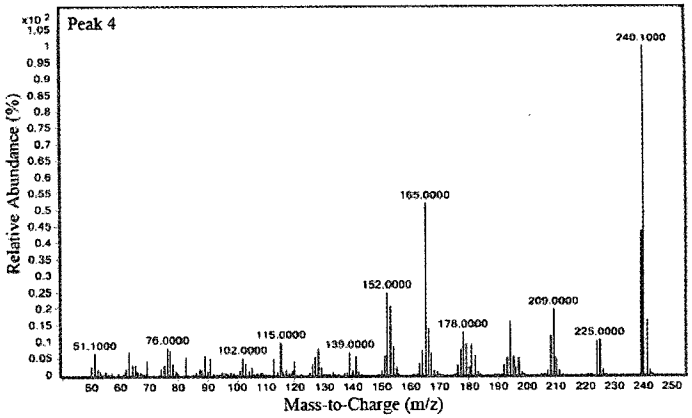

Peak 7
x10 2
Relative Abundance (%)
Mass-to-Charge (m/z)
135.1000
91.0000
148.0000
105.0000
79.0000
55.0998
187.1000
286.2000

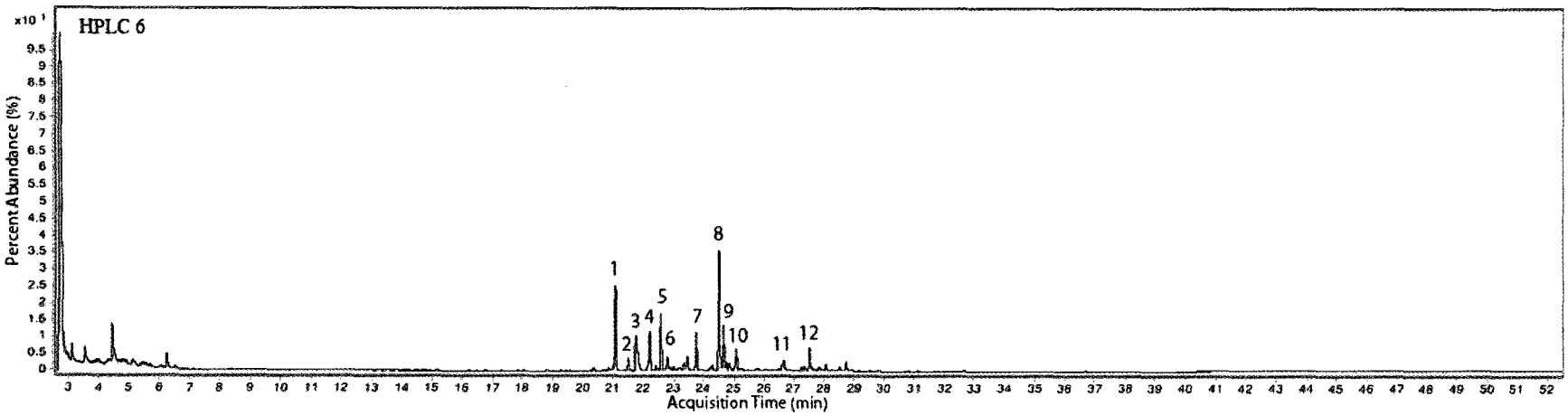

HPLC 5
Relative Abundance (%)
Acquisition Time (min)
1
2
3
4

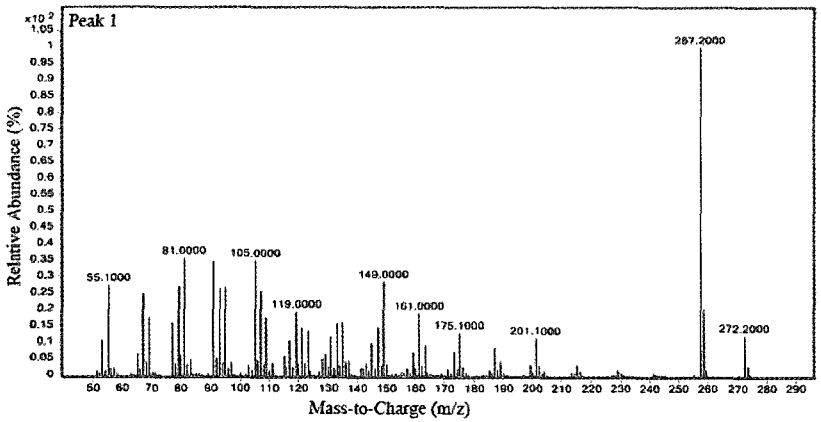

Peak 1
Relative Abundance (%)
Mass-to-Charge (m/z)
240.1000
165.0000
152.0000
178.0000
209.0000
224.0000
115.0000
139.0000
102.0000
76.0000
51.1000

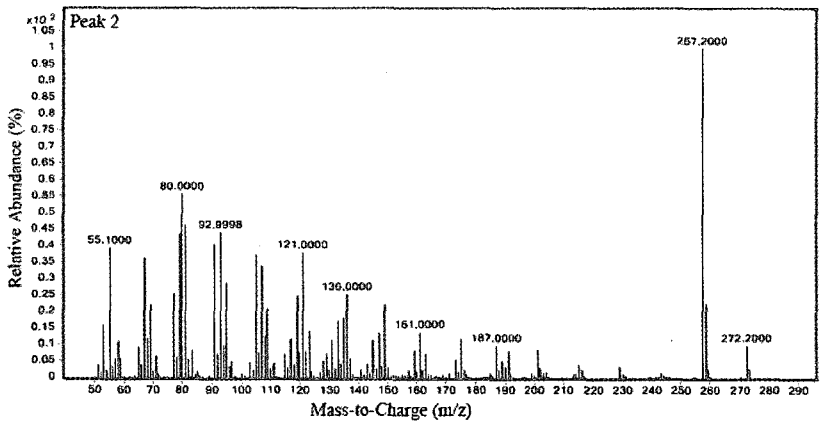

Peak 2
Relative Abundance (%)
Mass-to-Charge (m/z)
81.0000
55.1000
67.1000
95.0000
257.2000
275.2000
109.0000
137.1000
177.1000
192.1000
149.0000
163.0000
245.2000

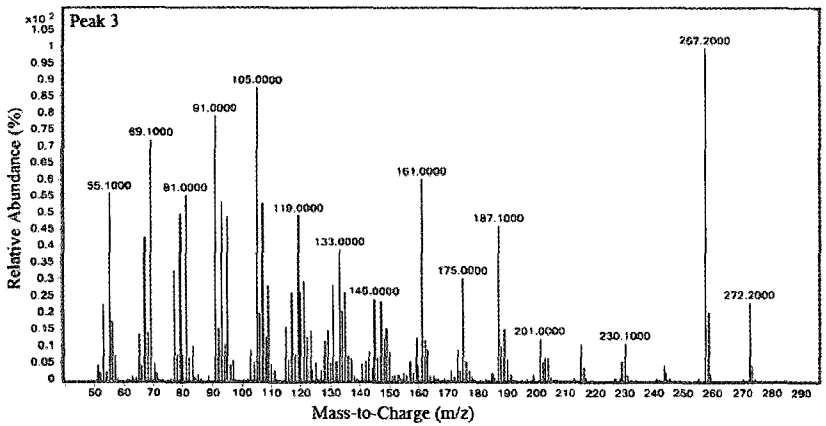

Peak 3
x10 2
Relative Abundance (%)
Mass-to-Charge (m/z)
81.0000
55.1000
67.1000
85.0000
109.0000
123.0000
137.1000
149.0000
163.1000
177.1000
192.1000
245.2000
257.2000
275.2000

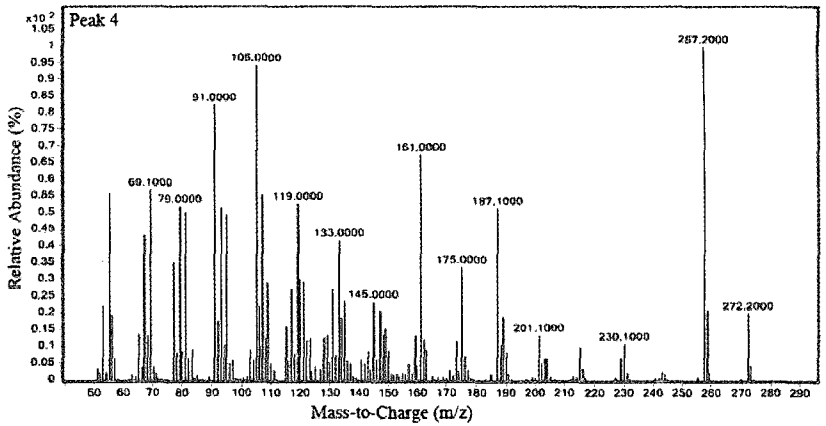

Peak 4
x10 2
Relative Abundance (%)
Mass-to-Charge (m/z)
240.1000
165.0000
152.0000
209.0000
178.0000
225.0000
115.0000
139.0000
76.0000
102.0000
51.1000

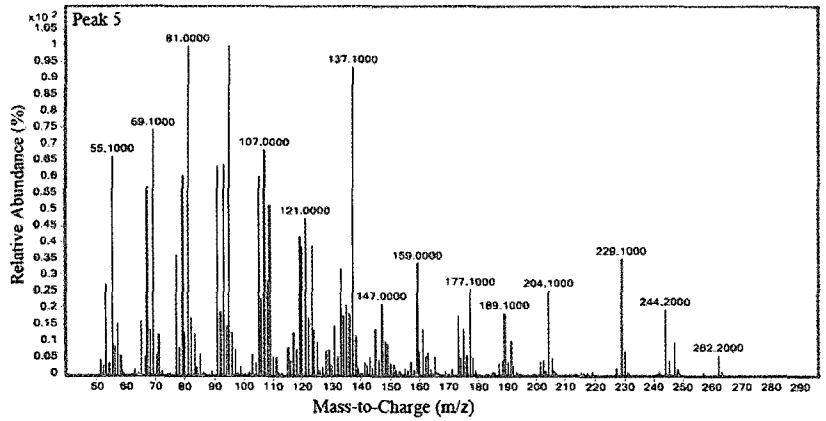

HPLC 6
Percent Abundance (%)
Acquisition Time (min)
1
2
3
4
5
6
7
8
9
10
11
12

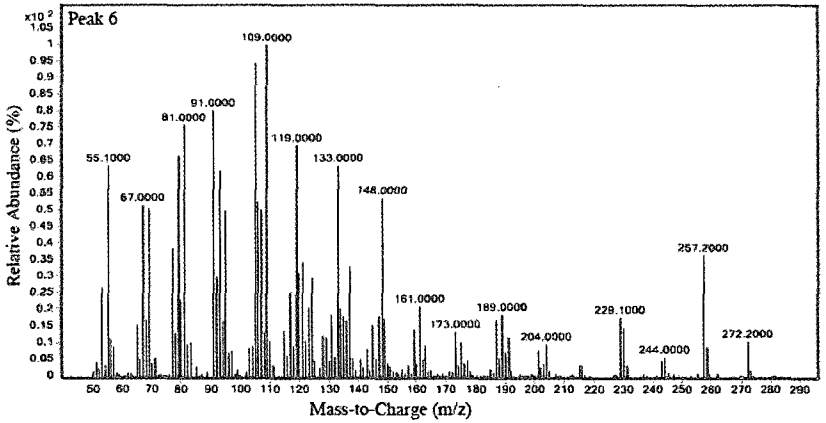

Peak 1
Relative Abundance (%)
Mass-to-Charge (m/z)
257.2000
55.1000
81.0000
105.0000
119.0000
149.0000
161.0000
175.1000
201.1000
272.2000

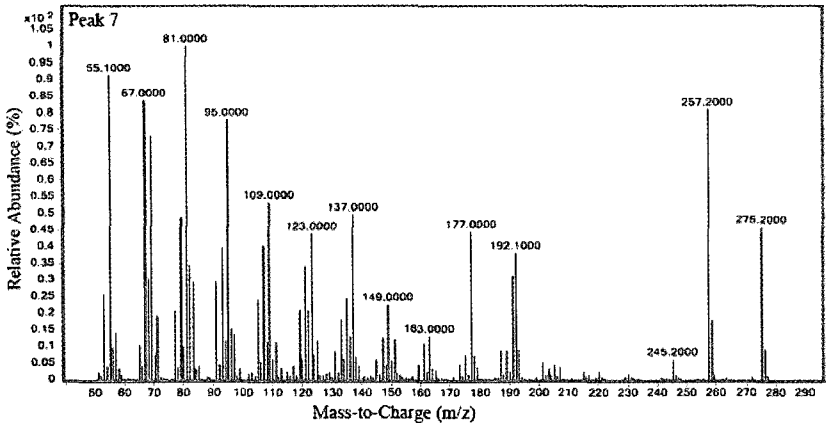

Peak 2
Relative Abundance (%)
Mass-to-Charge (m/z)
257.2000
80.0000
92.9998
55.1000
121.0000
136.0000
161.0000
187.0000
272.2000

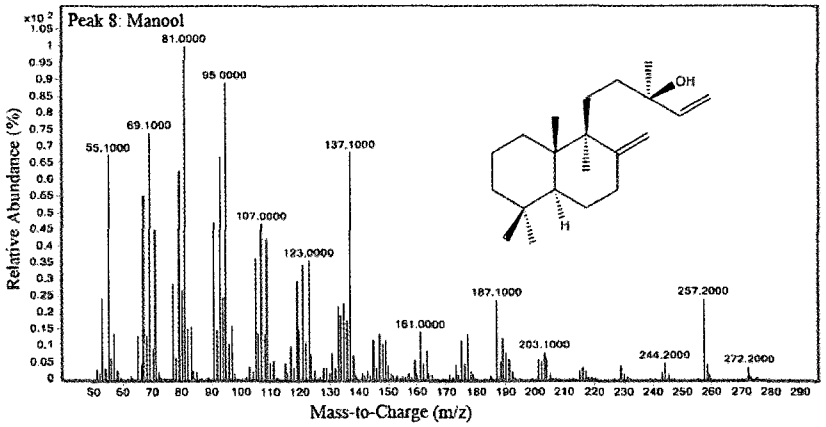

Peak 3
Relative Abundance (%)
Mass-to-Charge (m/z)
267.2000
105.0000
91.0000
69.1000
55.1000
81.0000
119.0000
161.0000
133.0000
187.1000
175.0000
145.0000
272.2000
201.0000
230.1000

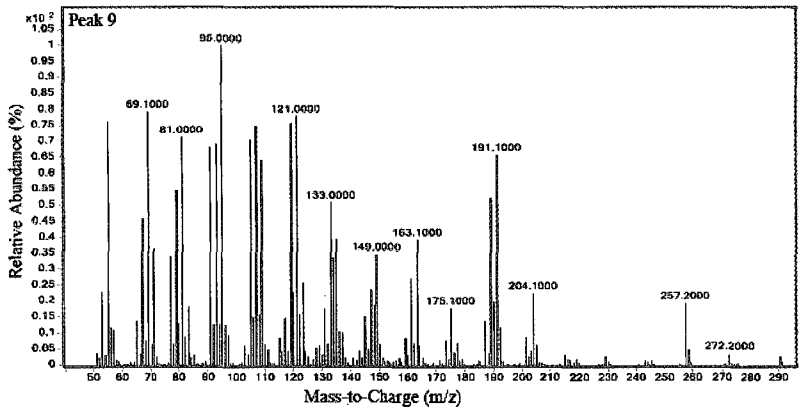

Peak 4
Relative Abundance (%)
Mass-to-Charge (m/z)
267.2000
105.0000
91.0000
161.0000
69.1000
79.0000
119.0000
187.1000
133.0000
175.0000
145.0000
272.2000
201.1000
230.1000

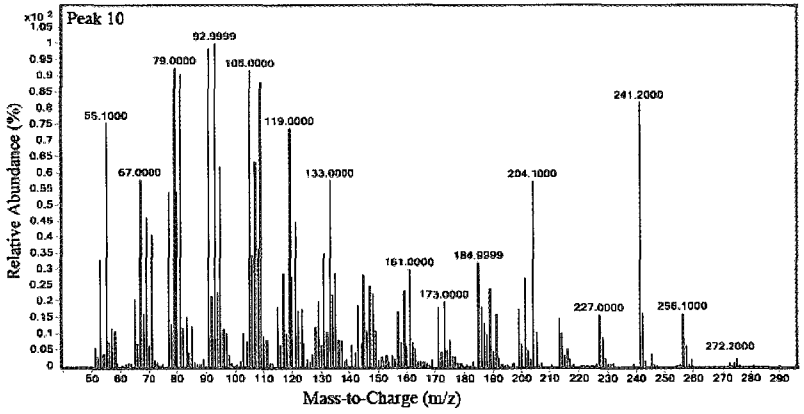

Peak 5
Relative Abundance (%)
Mass-to-Charge (m/z)
81.0000
137.1000
69.1000
55.1000
107.0000
121.0000
159.0000
229.1000
177.1000
204.1000
147.0000
189.1000
244.2000
262.2000

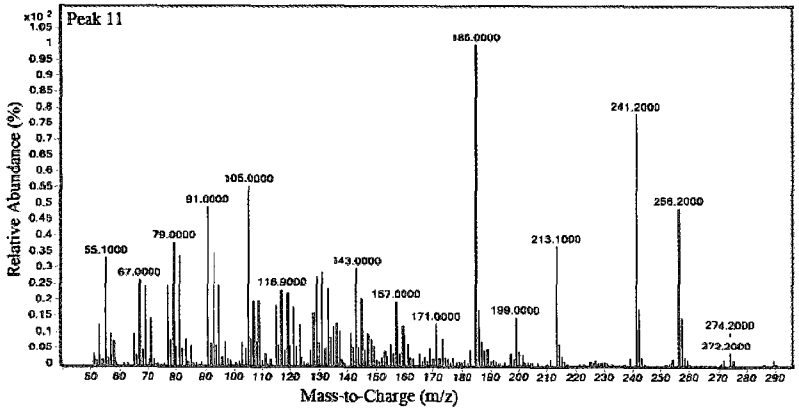

Peak 6
Relative Abundance (%)
Mass-to-Charge (m/z)
109.0000
91.0000
81.0000
119.0000
55.1000
133.0000
67.0000
148.0000
257.2000
161.0000
189.0000
228.1000
173.0000
204.0000
272.2000
244.0000

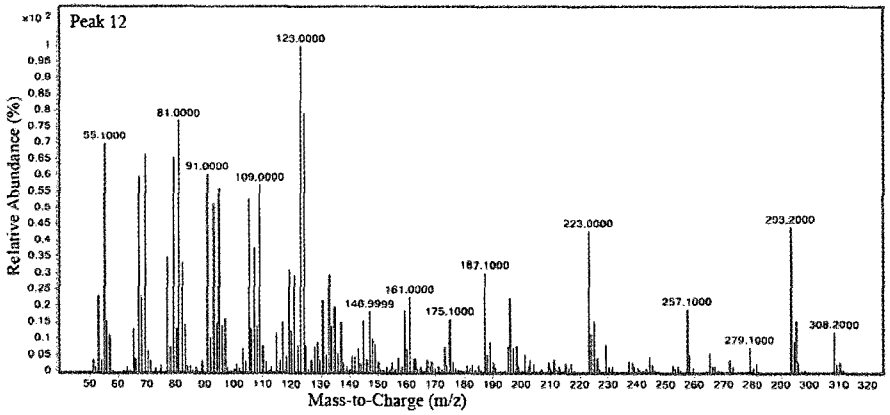

Peak 7
Relative Abundance (%)
Mass-to-Charge (m/z)
55.1000
67.0000
81.0000
95.0000
109.0000
123.0000
137.0000
149.0000
163.0000
177.0000
192.1000
245.2000
257.2000
275.2000

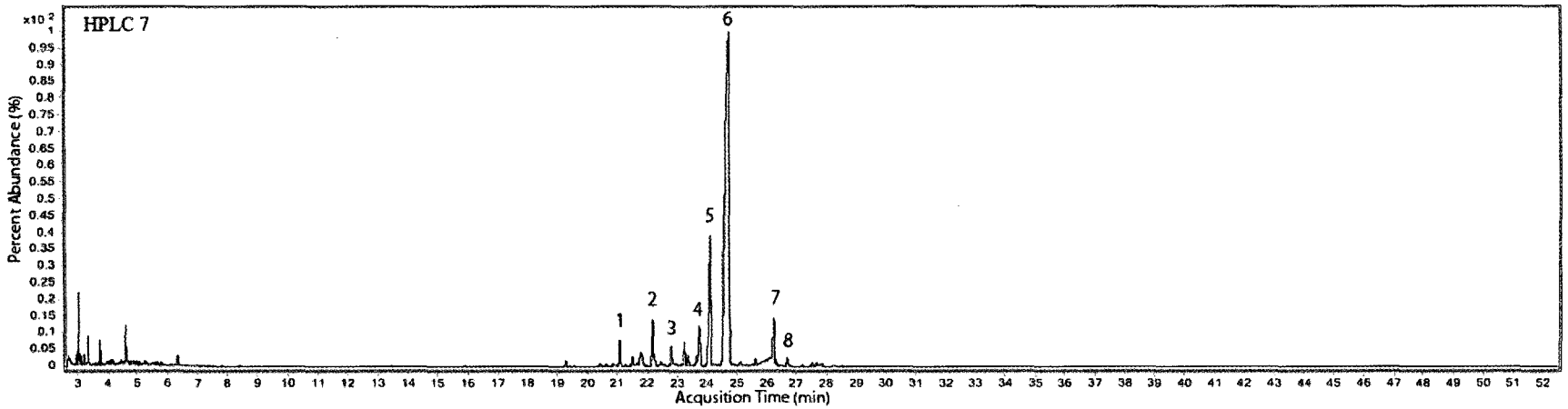

Peak 8: Manool
Relative Abundance (%)
Mass-to-Charge (m/z)
OH
H
55.1000
69.1000
81.0000
95.0000
107.0000
123.0000
137.1000
161.0000
187.1000
203.1000
244.2000
257.2000
272.2000

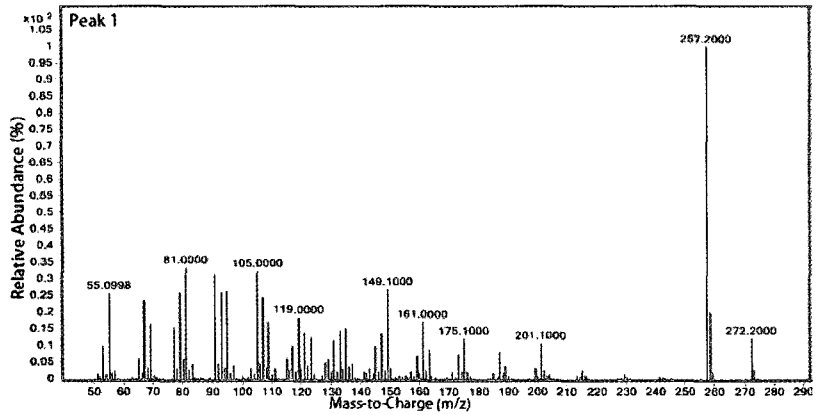

Peak 9
Relative Abundance (%)
Mass-to-Charge (m/z)
69.1000
81.0000
95.0000
121.0000
133.0000
149.0000
163.1000
175.1000
191.1000
204.1000
257.2000
272.2000

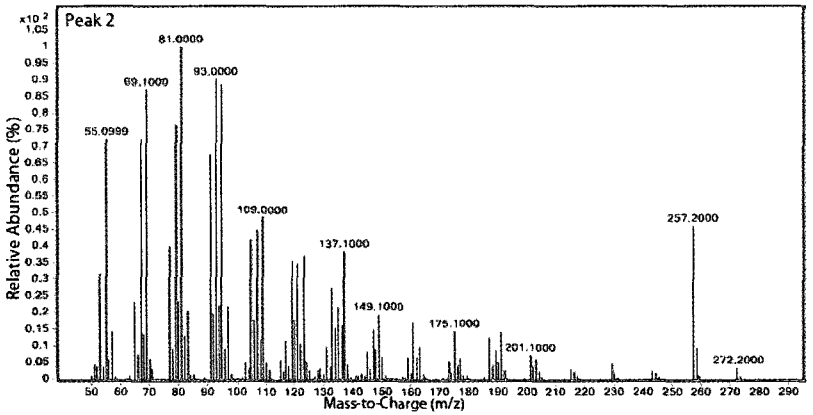

Peak 10
Relative Abundance (%)
Mass-to-Charge (m/z)
55.1000
67.0000
79.0000
92.9999
105.0000
119.0000
133.0000
161.0000
173.0000
184.9999
204.1000
227.0000
241.2000
256.1000
272.2000

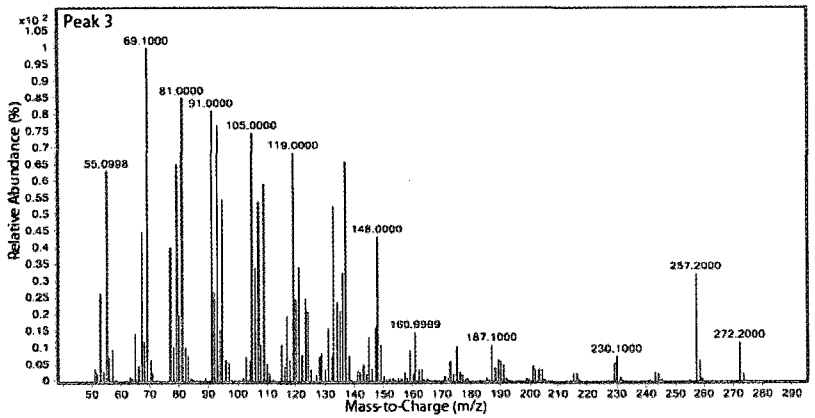

Peak 11
Relative Abundance (%)
Mass-to-Charge (m/z)
55.1000
67.0000
79.0000
91.0000
105.0000
116.9000
143.0000
157.0000
171.0000
185.0000
199.0000
213.1000
241.2000
256.2000
274.2000
272.2000

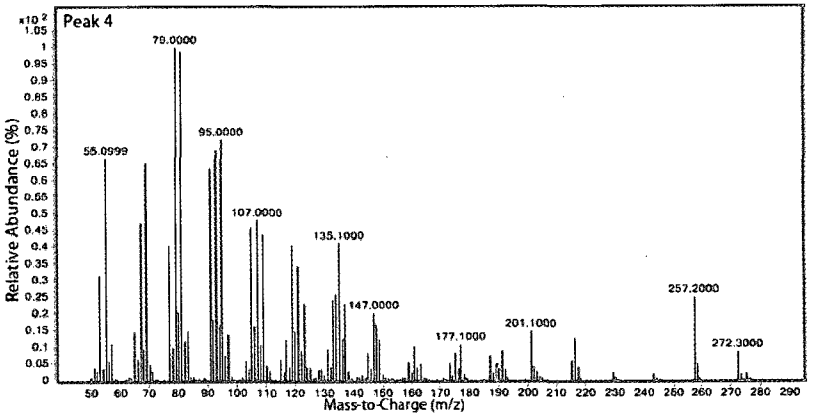

Peak 12
Relative Abundance (%)
Mass-to-Charge (m/z)
55.1000
81.0000
91.0000
109.0000
123.0000
146.9999
161.0000
175.1000
187.1000
223.0000
257.1000
279.1000
293.2000
308.2000

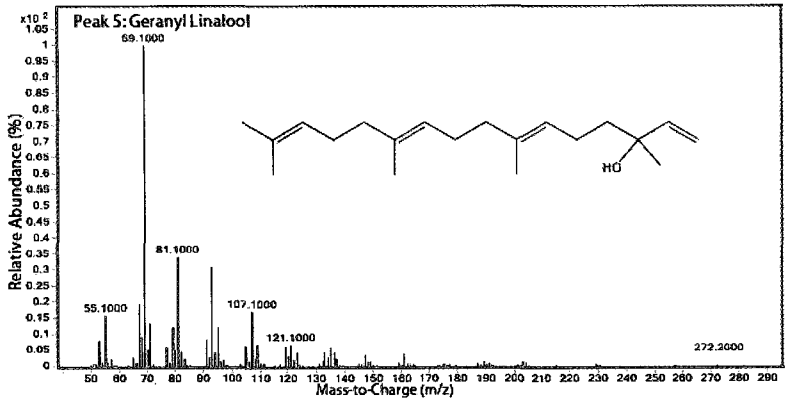

HPLC 7
Percent Abundance (%)
Acquisition Time (min)
1
2
3
4
5
6
7
8

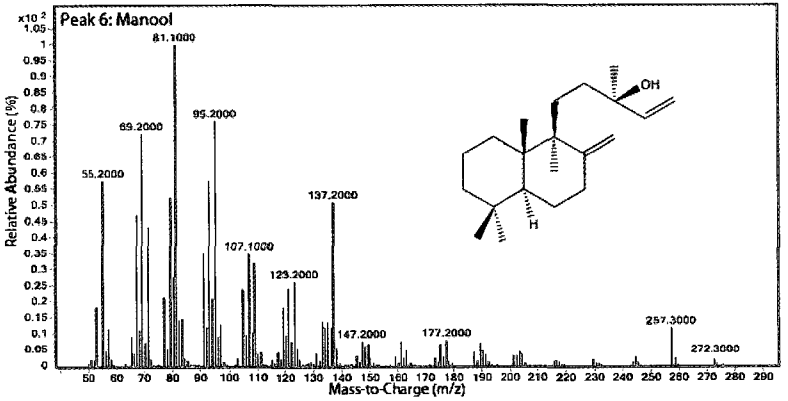

Peak 1
Relative Abundance (%)
Mass-to-Charge (m/z)
257.2000
81.0000
105.0000
55.0998
149.1000
119.0000
161.0000
175.1000
201.1000
272.2000

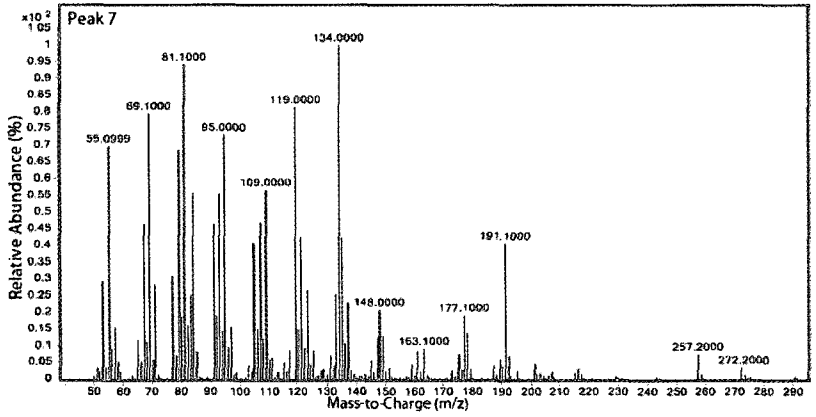

Peak 2
Relative Abundance (%)
Mass-to-Charge (m/z)
81.0000
93.0000
69.1000
55.0999
109.0000
257.2000
137.1000
149.1000
175.1000
201.1000
272.2000

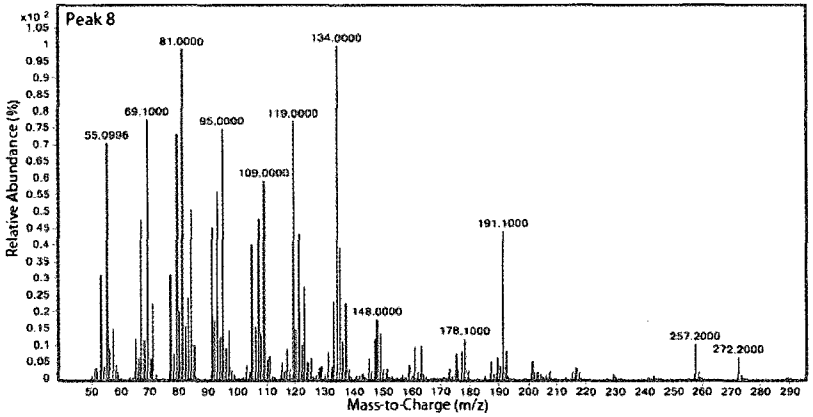

Peak 3
69.1000
81.0000
91.0000
105.0000
119.0000
55.0998
148.0000
257.2000
160.9999
187.1000
272.2000
230.1000
Relative Abundance (%)
Mass-to-Charge (m/z)

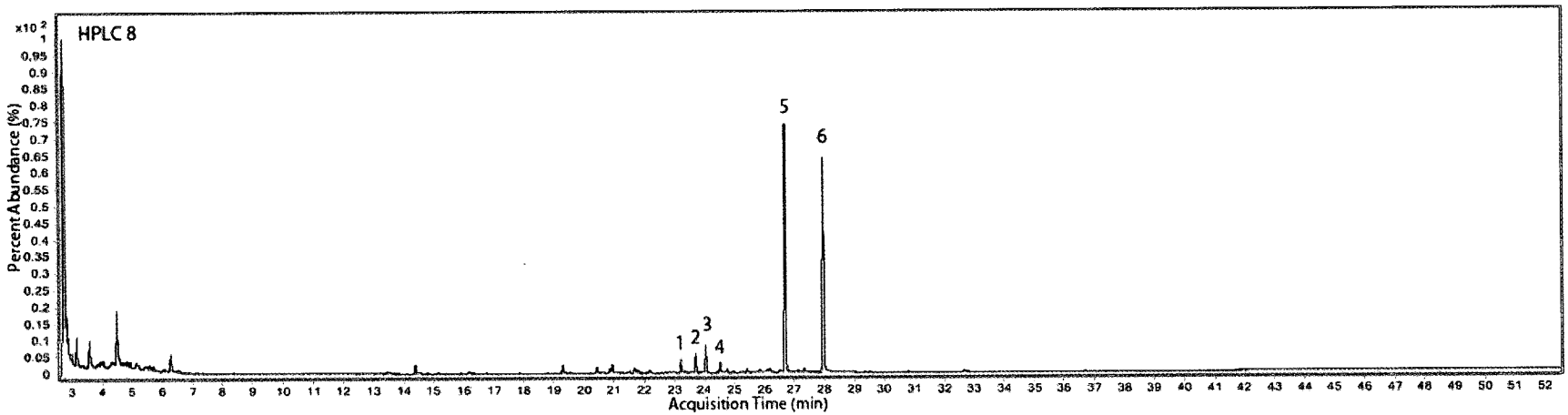

Peak 4
79.0000
95.0000
55.0999
107.0000
135.1000
147.0000
257.2000
177.1000
201.1000
272.3000
Relative Abundance (%)
Mass-to-Charge (m/z)

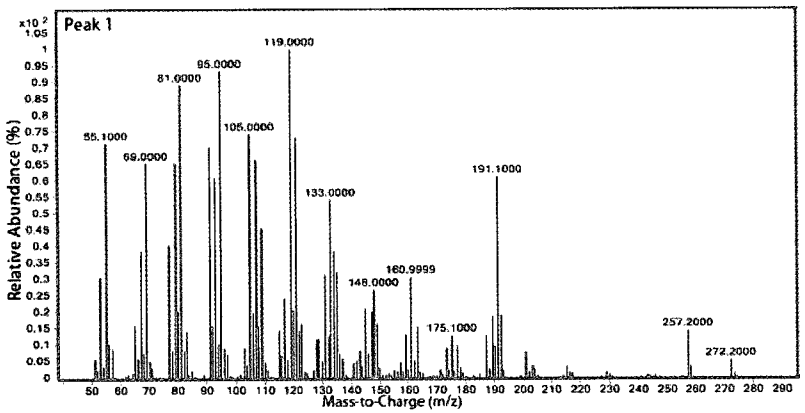

Peak 5: Geranyl Linalool
69.1000
81.1000
55.1000
107.1000
121.1000
272.2000
HO
Relative Abundance (%)
Mass-to-Charge (m/z)

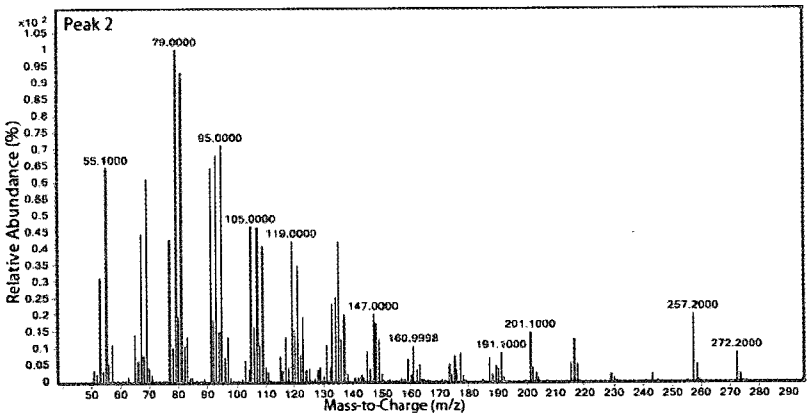

Peak 6: Manool
81.1000
69.2000
95.2000
55.2000
137.2000
107.1000
123.2000
147.2000
177.2000
257.3000
272.3000
OH
H
Relative Abundance (%)
Mass-to-Charge (m/z)

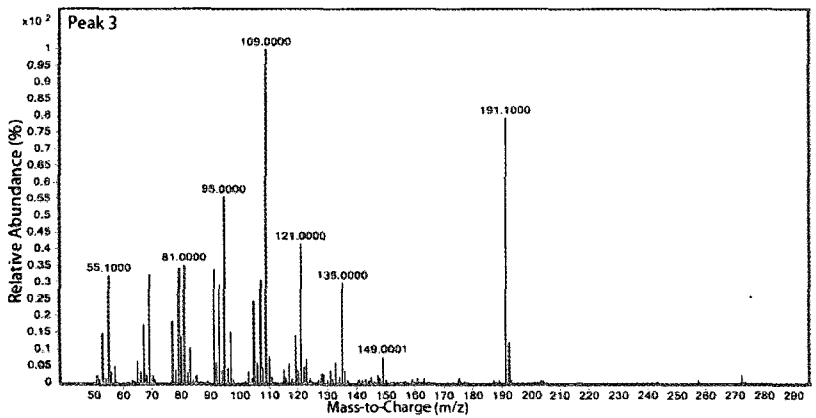

Peak 7
Relative Abundance (%)
Mass-to-Charge (m/z)
55.0999
69.1000
81.1000
95.0000
109.0000
119.0000
134.0000
148.0000
163.1000
177.1000
191.1000
257.2000
272.2000

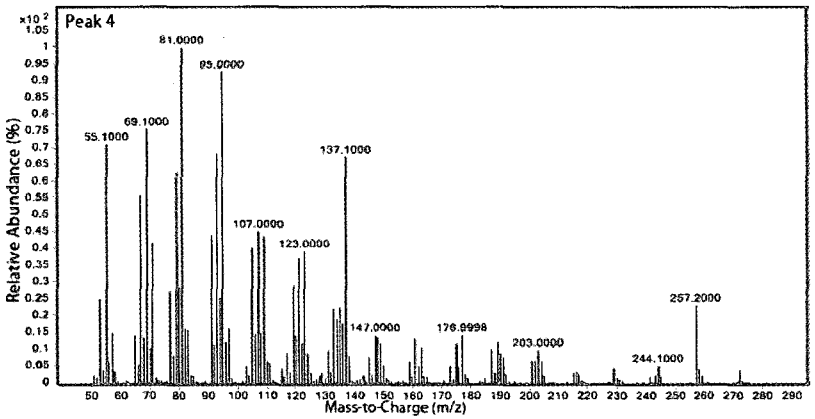

Peak 8
Relative Abundance (%)
Mass-to-Charge (m/z)
55.0996
69.1000
81.0000
95.0000
109.0000
119.0000
134.0000
148.0000
178.1000
191.1000
257.2000
272.2000

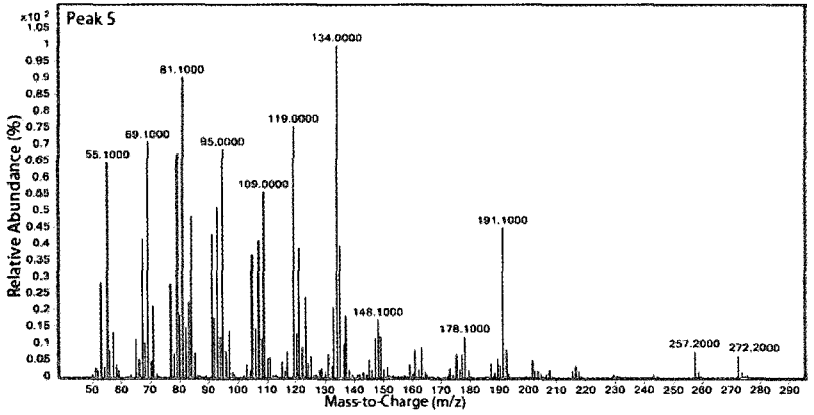

HPLC 8
Percent Abundance (%)
Acquisition Time (min)
1
2
3
4
5
6

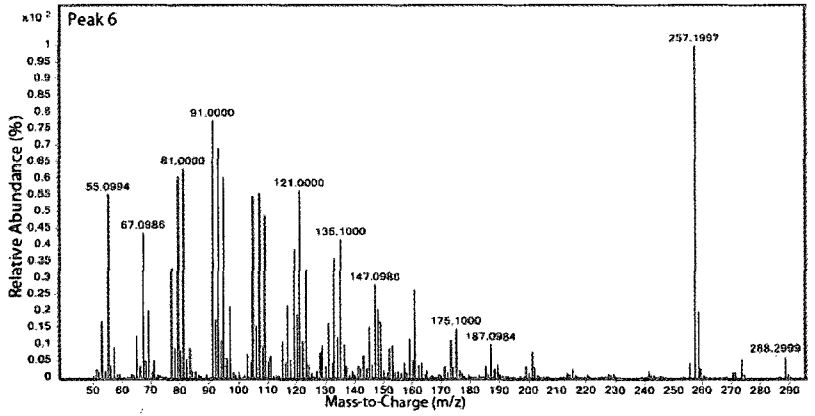

Peak 1
Relative Abundance (%)
Mass-to-Charge (m/z)
55.1000
69.0000
81.0000
95.0000
105.0000
119.0000
133.0000
148.0000
160.9999
175.1000
191.1000
257.2000
272.2000

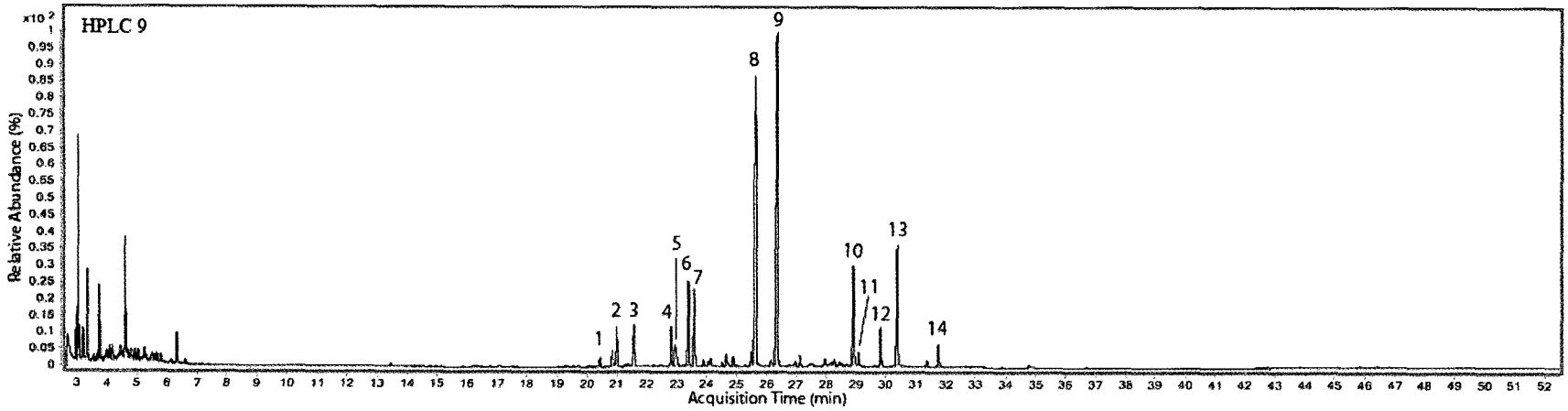

Peak 2
Relative Abundance (%)
Mass-to-Charge (m/z)
55.1000
79.0000
95.0000
105.0000
119.0000
147.0000
160.9998
191.1000
201.1000
257.2000
272.2000

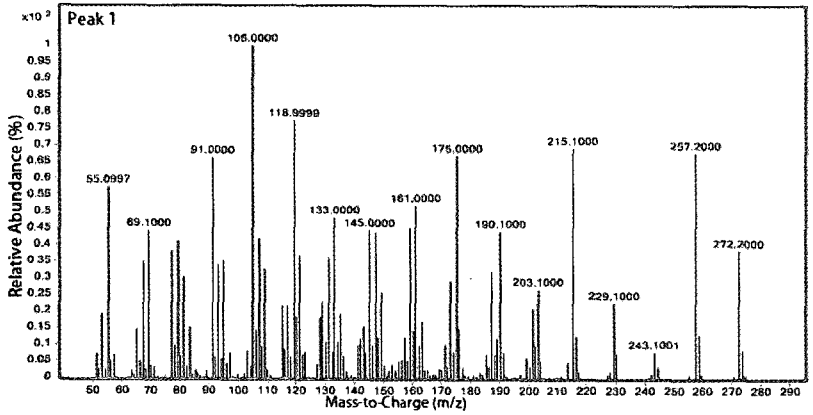

Peak 3
Relative Abundance (%)
Mass-to-Charge (m/z)
109.0000
191.1000
95.0000
121.0000
81.0000
55.1000
135.0000
149.0001

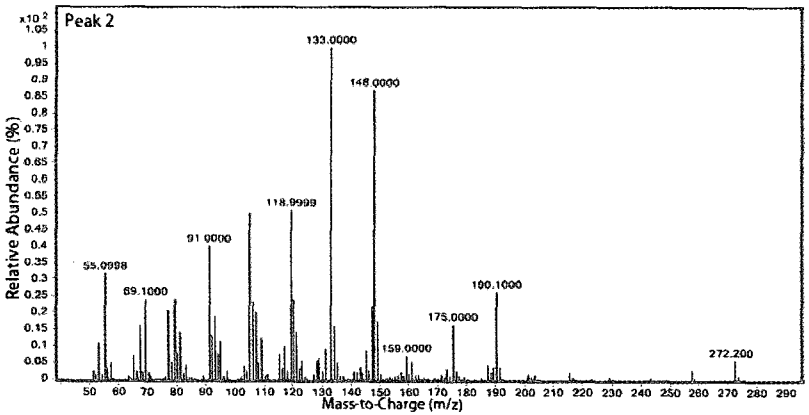

Peak 4
Relative Abundance (%)
Mass-to-Charge (m/z)
81.0000
95.0000
69.1000
55.1000
137.1000
107.0000
123.0000
257.2000
147.0000
176.9998
203.0000
244.1000

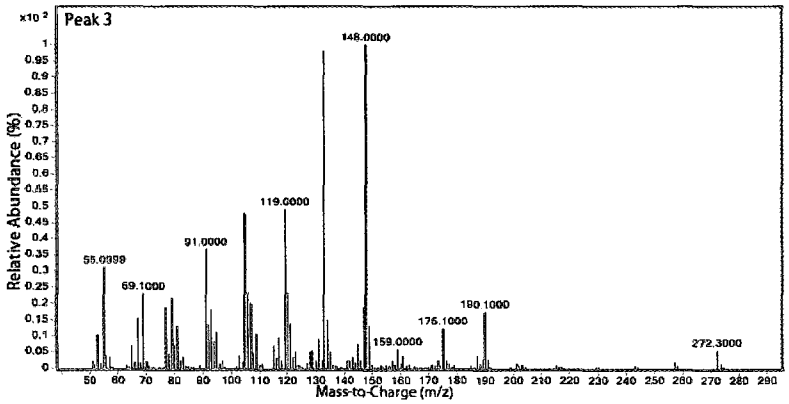

Peak 5
Relative Abundance (%)
Mass-to-Charge (m/z)
134.0000
81.1000
89.1000
119.0000
95.0000
55.1000
109.0000
191.1000
148.1000
178.1000
257.2000
272.2000

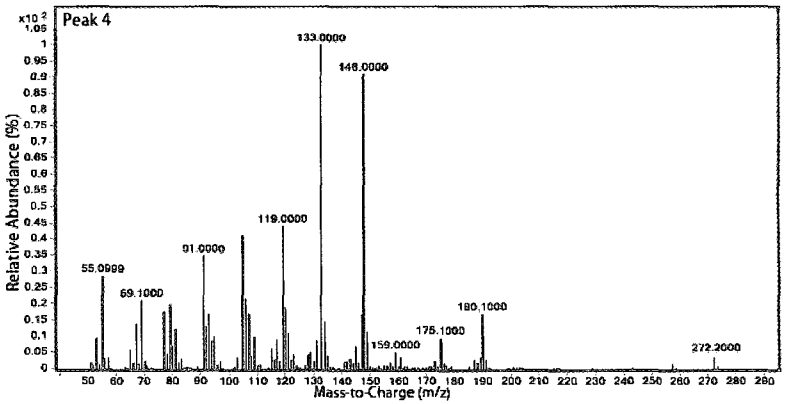

Peak 6
Relative Abundance (%)
Mass-to-Charge (m/z)
257.1997
91.0000
81.0000
55.0994
121.0000
67.0986
135.1000
147.0986
175.1000
187.0984
288.2999

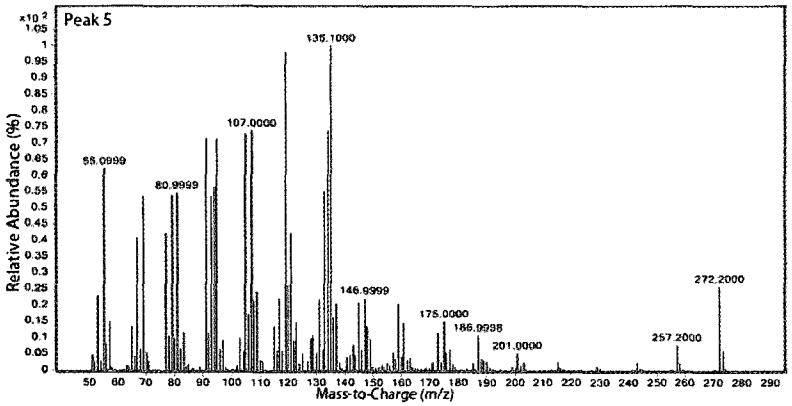

HPLC 9
Relative Abundance (%)
Acquisition Time (min)
1
2
3
4
5
6
7
8
9
10
11
12
13
14

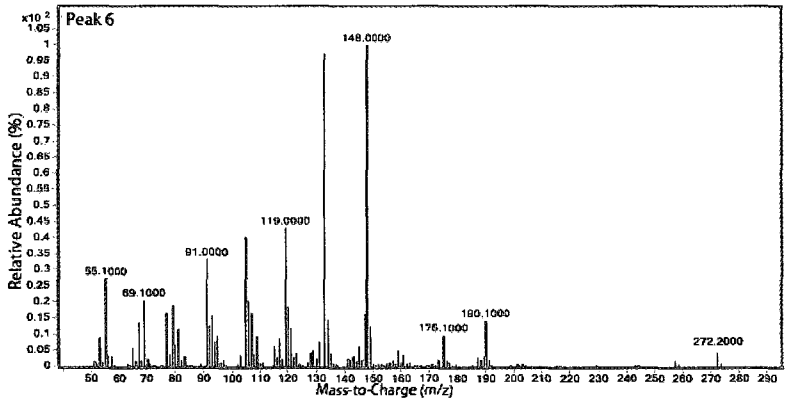

Peak 1
Relative Abundance (%)
Mass-to-Charge (m/z)
106.0000
118.9999
91.0000
55.0997
69.1000
133.0000
145.0000
161.0000
175.0000
190.1000
203.1000
215.1000
229.1000
243.1001
257.2000
272.2000

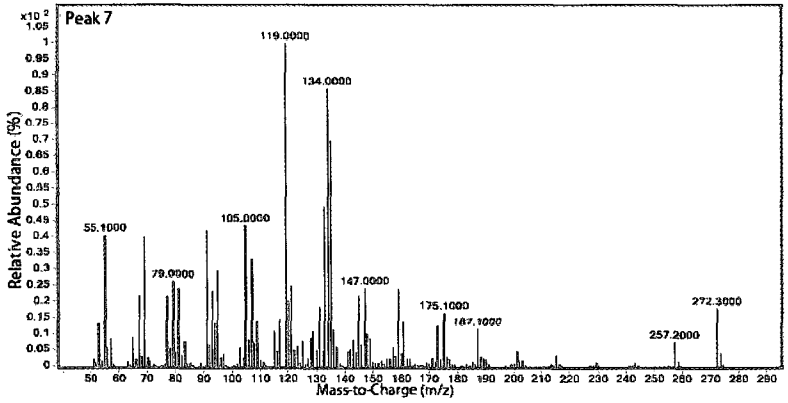

Peak 2
Relative Abundance (%)
Mass-to-Charge (m/z)
133.0000
148.0000
118.9999
91.0000
55.0998
69.1000
190.1000
175.0000
159.0000
272.200

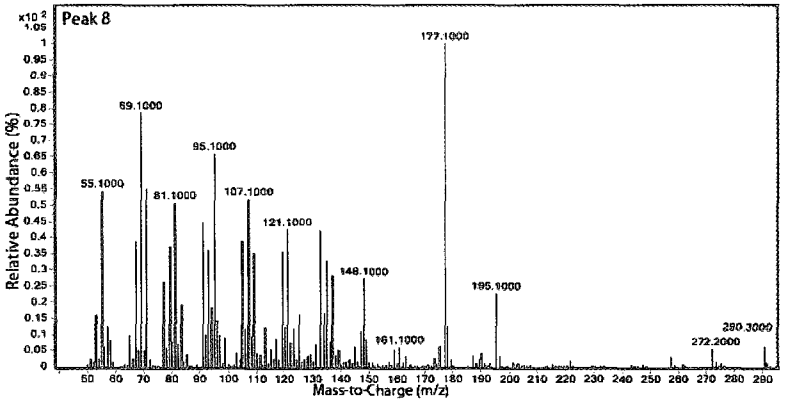

Peak 3
x10 2
Relative Abundance (%)
148.0000
119.0000
91.0000
55.0999
69.1000
180.1000
175.1000
159.0000
272.3000
Mass-to-Charge (m/z)

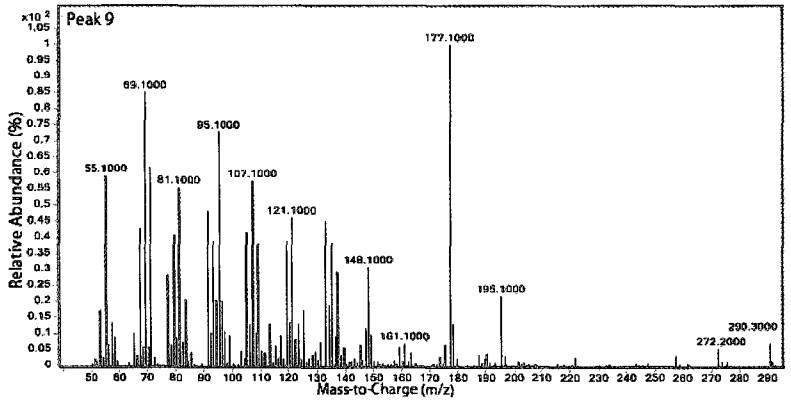

Peak 4
x10 2
Relative Abundance (%)
133.0000
148.0000
119.0000
91.0000
55.0999
69.1000
180.1000
175.1000
159.0000
272.2000
Mass-to-Charge (m/z)

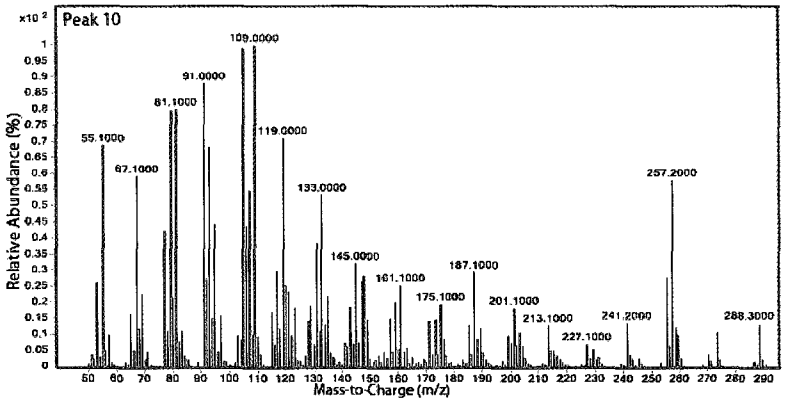

Peak 5
x10 2
Relative Abundance (%)
135.1000
107.0000
55.0999
80.9999
146.9999
272.2000
175.0000
186.9998
201.0000
257.2000
Mass-to-Charge (m/z)

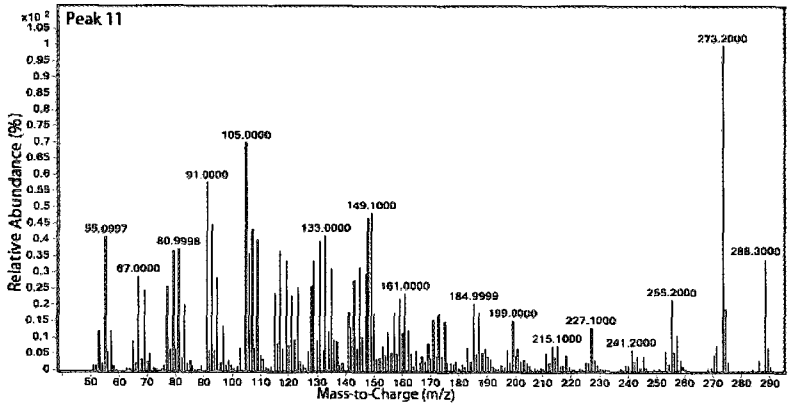

Peak 6
x10 2
Relative Abundance (%)
148.0000
119.0000
91.0000
55.1000
69.1000
180.1000
175.1000
272.2000
Mass-to-Charge (m/z)

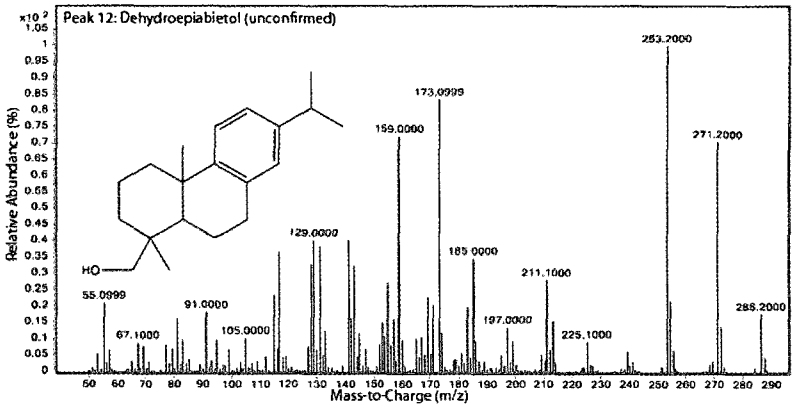

Peak 7
x10 2
Relative Abundance (%)
Mass-to-Charge (m/z)
119.0000
134.0000
55.1000
105.0000
79.0000
147.0000
175.1000
187.1000
257.2000
272.3000

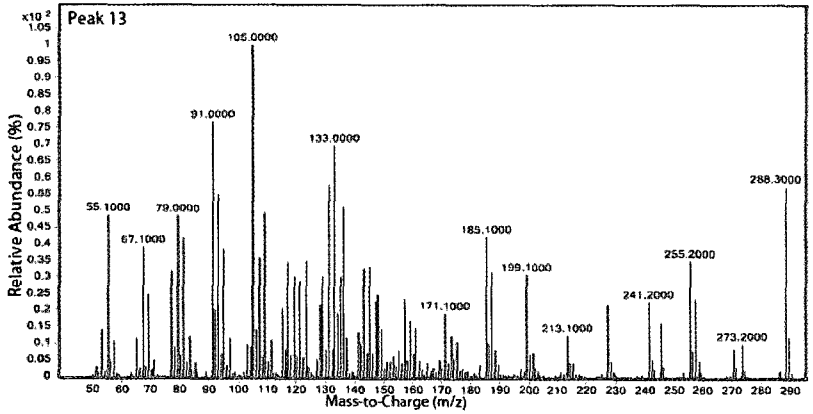

Peak 8
x10 2
Relative Abundance (%)
Mass-to-Charge (m/z)
177.1000
69.1000
95.1000
55.1000
81.1000
107.1000
121.1000
148.1000
195.1000
161.1000
290.3000
272.2000

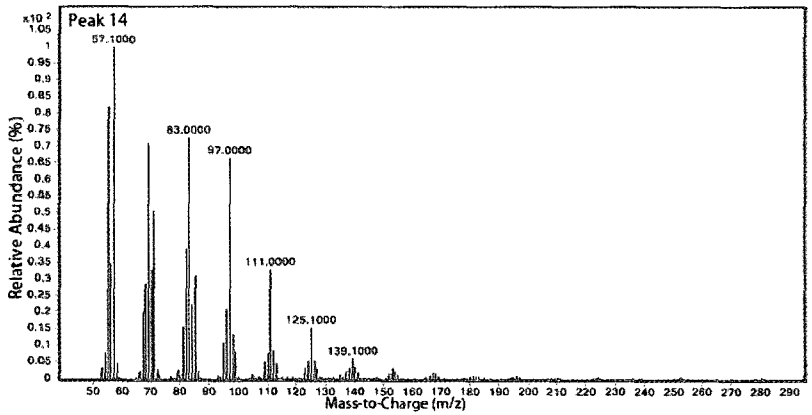

Peak 9
x10 2
Relative Abundance (%)
Mass-to-Charge (m/z)
177.1000
69.1000
95.1000
55.1000
81.1000
107.1000
121.1000
148.1000
195.1000
161.1000
290.3000
272.2000

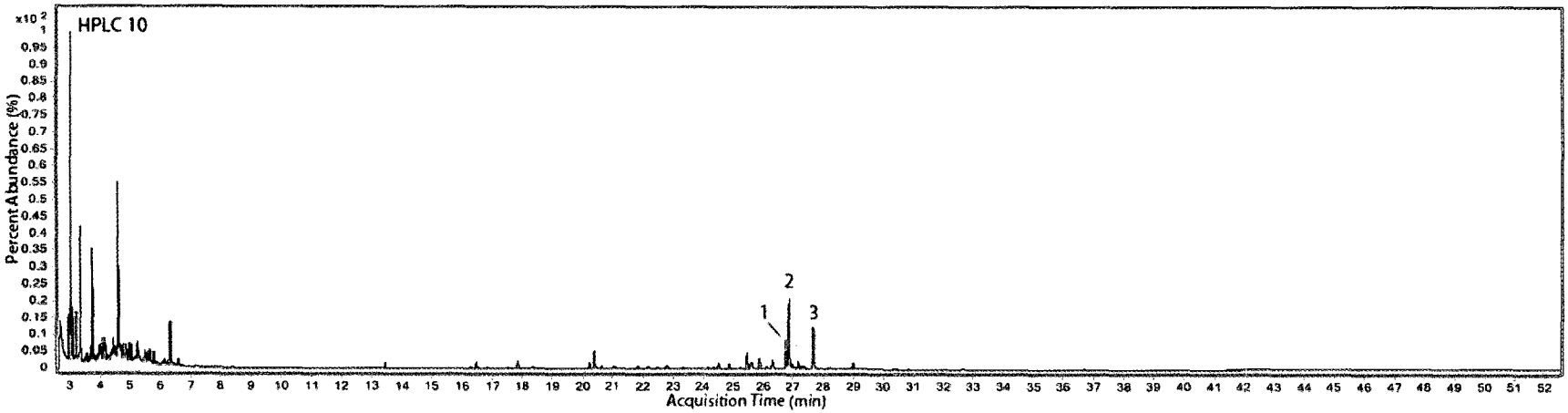

Peak 10
x10 2
Relative Abundance (%)
Mass-to-Charge (m/z)
109.0000
91.0000
81.1000
55.1000
119.0000
67.1000
133.0000
257.2000
145.0000
161.1000
187.1000
175.1000
201.1000
213.1000
241.2000
227.1000
288.3000

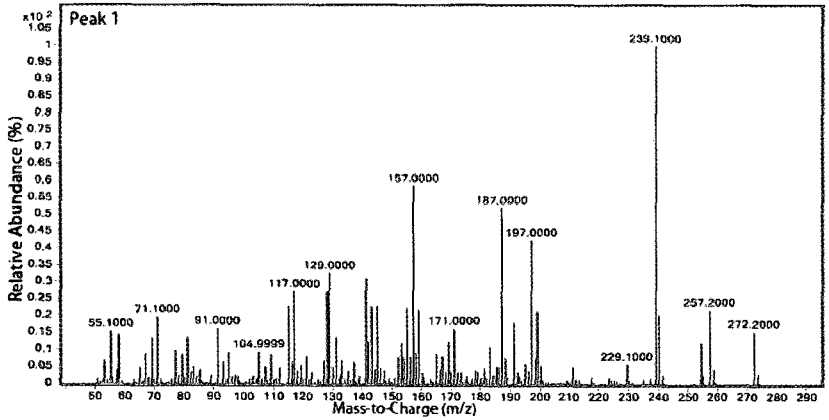

Peak 11
Relative Abundance (%)
Mass-to-Charge (m/z)
273.2000
105.0000
91.0000
149.1000
55.0997
80.9998
133.0000
288.3000
67.0000
161.0000
184.9999
255.2000
199.0000
227.1000
215.1000
241.2000

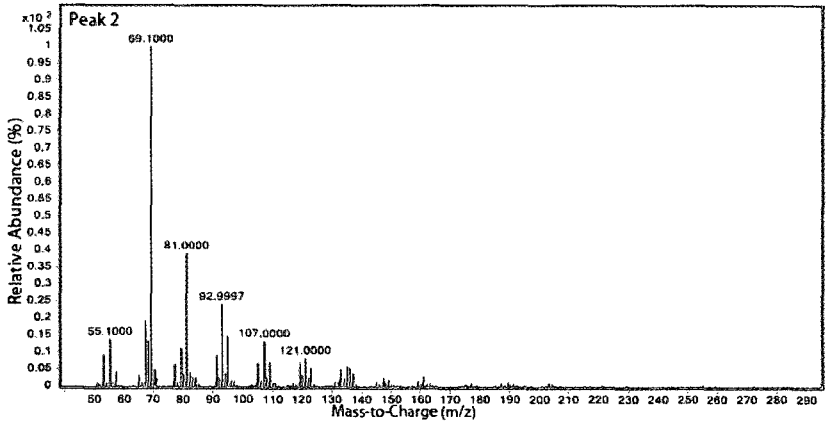

Peak 12: Dehydroepiabietol (unconfirmed)
Relative Abundance (%)
Mass-to-Charge (m/z)
HO
253.2000
173.0999
159.0000
271.2000
129.0000
185.0000
211.1000
55.0999
91.0000
288.2000
197.0000
67.1000
105.0000
225.1000

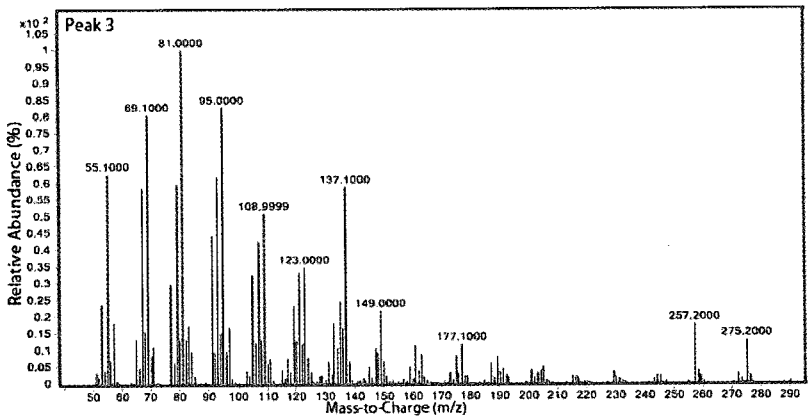

Peak 13
Relative Abundance (%)
Mass-to-Charge (m/z)
105.0000
91.0000
133.0000
288.3000
55.1000
79.0000
67.1000
185.1000
199.1000
255.2000
171.1000
241.2000
213.1000
273.2000

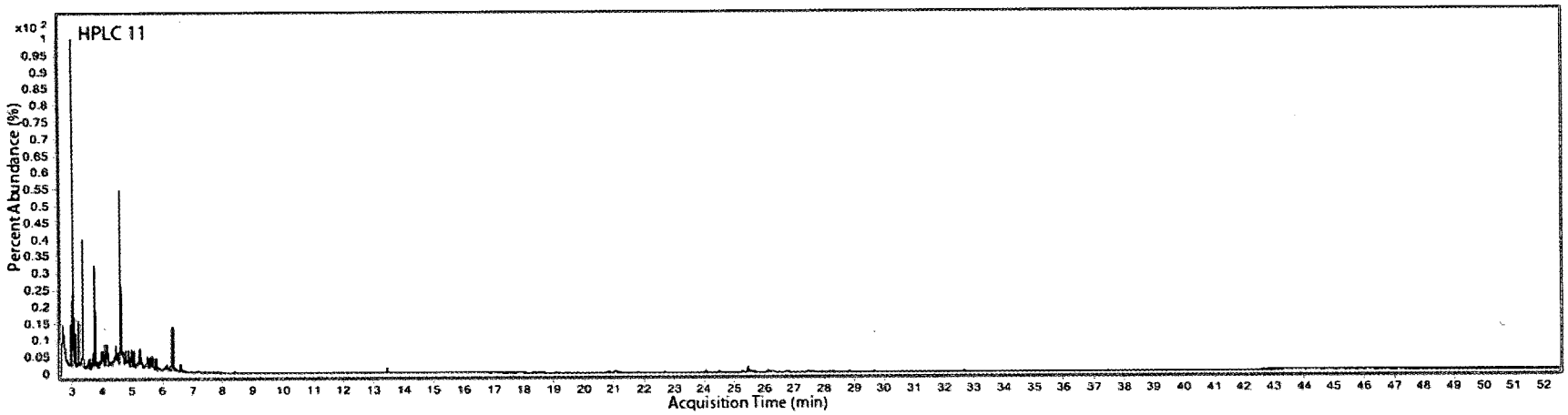

Peak 14
Relative Abundance (%)
Mass-to-Charge (m/z)
57.1000
83.0000
97.0000
111.0000
125.1000
139.1000


HPLC 10
Percent Abundance (%)
Acquisition Time (min)
1
2
3


Peak 1
Relative Abundance (%)
Mass-to-Charge (m/z)
239.1000
157.0000
187.0000
197.0000
129.0000
117.0000
71.1000
55.1000
91.0000
104.9999
171.0000
257.2000
272.2000
229.1000


Peak 2
Relative Abundance (%)
Mass-to-Charge (m/z)
69.1000
81.0000
82.9997
55.1000
107.0000
121.0000


Peak 3
x10 2
Relative Abundance (%)
Mass-to-Charge (m/z)
81.0000
69.1000
95.0000
55.1000
137.1000
108.9999
123.0000
149.0000
177.1000
257.2000
275.2000


HPLC 11
x10 2
Percent Abundance (%)
Acquisition Time (min)

# Chapter 6
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