

THE QUANTIFICATION OF OLIGOMERIC
PROANTHOCYANIDINS IN THE
INNER AND OUTER BARKS
OF SELECTED B.C. CONIFERS

BY

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ABSTRACT

Oligomeric proanthocyanidins form the basis of nutraceutical anti-oxidant products sold under trade names such as Pycnogenol™ and OPC's. These compounds fall in the class of compounds called condensed tannins and are essentially the hot water extracts of certain grape seed or pine species. The oligomeric proanthocyanidin fraction consists mostly of procyanidins, but includes a propelargonidins and prodelphinidins, as well. These compounds may form separate polymers, may be combined in a variety of conformations, and may be gallated or glycosylated.

Although these compounds have been found to have many functions in plants, it is the applications in human medicine that has made them particularly interesting. Oligomeric proanthocyanidins have been shown to be antioxidant, free radical scavenging, anti-inflammatory, and antihistamine. They have been studied for their prevention of cardiovascular diseases, tumors, and virus induced conditions, such as HIV. Traditionally, these compounds have been used by indigenous peoples almost exclusively for gastrointestinal disorders.

Much of the research conducted into the physical properties and biological activity of this class of compounds has excluded reporting the quantity of the compounds extracted from the various tree and grape seed sources. This is largely because standardized quantification is not yet possible. Because proanthocyanidin biosynthetic pathway enzymology is not yet complete, the nomenclature for these compounds has not been definitively established. Compounds require identification before meaningful quantification can be made. However, as research in this field is still ongoing, quantification based on what is already known can be made on the medically active components of proanthocyanidins.

The butanol-HCl (anthocyanidin), vanillic acid, and protein precipitation methods have been devised as methods for the quantification of tannins. However, all of these methods have deficiencies that make them unsuitable for the quantification of proanthocyanidin oligomers with confidence. The butanol-HCL method quantifies coloured proanthocyanidin hydrolysates relative to polymer sorghum or quebracho tannin. Different types of proanthocyanidins yield different colours and different colour intensities, and the colour intensity decreases in strength with the decreasing size of the oligomer. The vanillin acid method, which quantifies the coloured proanthocyanidin - vanillin adduct, actually measures the number of phloroglucinol-like A-rings associated with flavonoids. This reaction is quantified in catechin equivalents, and is sensitive to reaction time, temperature, light, age of the sample and even the water content in the reaction mixture, making this method very undesirable for testing various natural extracts. The protein precipitation method is unable to distinguish between condensed and hydrolyzable tannins.

The method described herein is designed to quantify oligomeric proanthocyanidins by determining the occurrence of 10 signature procyanidins [OPC], identifying seven of the ten signature procyanins, and cumulatively measuring the concentration of the 10 signature peaks relative to a commercial oligomeric proanthocyanidin product, Indena brand LeucoSelect™. Because the quantification is made relative to an already commercialized product, the quantification results predict the amount of salable product in natural product extract. This method is reproducible, rapid, efficient, and can be used to screen for potential new sources of OPC-based products, or to compare the oligomeric proanthocyanidin content in various commercial products. When used to quantify the OPC content in bark extracts, this method was found to provide results similar to those in the literature.

By using this method and screening various bark extracts, it was found that sample freshness played a role in the extractable amounts of proanthocyanidins. In future studies, fresh samples should be used as they most accurately represent the bark composition on live trees. The inner and outer bark quantities of proanthocyanidins were also seen to vary, with the inner bark quantity almost always being greater in concentration than that in the outer bark. Douglas-fir and interior Amabilis fir were found to be the best sources of oligomeric proanthocyanidins at 43.2 and 12.4 mg/g dry inner bark, respectively. The samples of lower mainland Amabilis fir, interior Lodgepole pine, and Caribbean pine from Venezuela, all showed signs of being potential sources of OPC products. Interior and lower mainland Sitka spruce and interior Western hemlock showed little potential as possible sources of OPC's.

By comparing the commercially produced OPC containing products relative to the Indena brand LeucoSelect™ (made from grape seeds) the Capers brand grape seed extract was shown to be 77% similar to the Indena standard. Nu-Greens Prolong, which differed substantially, was shown to be only 7.2% similar to the Indena standard, raising questions about its quantification in units of 'Activity'. The SunForce Pycnogenol™ was found to be only 46.8 % similar, but is sourced from pine bark, which would seem to have a higher concentration of higher molecular weight OPC's, not included in the 10 signature peaks.

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1.0 INTRODUCTION

Oligomeric proanthocyanidins are currently sold as dietary supplements under the summary trade name PYCNOGNOL™. Their current value, based on retail prices, is \$12 million per tonne. In the period between 1980 and 1992, the estimated worldwide consumption surpassed \$800 million dollars (Passwater, 1992). This incredibly high return for what is essentially a hot water extract of pine bark and grape seeds has sparked the creation of several hundreds of small production facilities world wide, several in Vancouver, B.C. alone. Although scientific research on oligomeric proanthocyanidins (OPC's) has declined in the last few years, the lack of information and high profit margin of this product has inspired the development of methodologies directed towards the identification of new sources of these compounds.

Initially, the objective of this thesis was to identify and quantify the condensed tannins in local softwood barks that are suitable for the production of a proanthocyanidin mixture similar to that of commercially produced Pycnogenol™. Pycnogenol™ is a nutraceutical, or herbal medicinal preparation, consisting of monomeric and oligomeric proanthocyanidins, and other phenolic compounds. Before the proanthocyanidins could be quantified, however, several problems were encountered. The complex nomenclature of proanthocyanidins seemed to be poorly defined and in a state of flux, and little quantitative data was available in the literature on the proanthocyanidin content of softwood barks. Papers discussing the occurrence of proanthocyanidins often did not specify the kind or type of proanthocyanidins measured and researchers usually only discussed the effect or structure of proanthocyanidins, rarely their quantities. Most importantly, methods available for screening plant extracts for proanthocyanidins were found to be insufficiently specific or inaccurate to be used for oligomeric proanthocyanidin quantification.

Testing of commercial Pycnogenol™-like products revealed that no standardized composition of these products was evident, and standardization was not possible as no method of directly comparing proanthocyanidins from different sources had yet been developed. Developing such a method became the first problem that had to be solved. The procedure described herein outlines a reliable method for the quantification of oligomeric proanthocyanidins in crude acetone-water bark extracts. This method can also be used to determine the suitability of these extracts to be used as potential sources for the production of Pycnogenol™-like products.

Bark constitutes 5 to 15 % of a tree by weight and contains about 7 % extractives, and approximately 1 % oligomeric condensed tannins. With the oligomeric condensed tannins being sold at \$12 million per tonne (and other extractives being saleable as well: thujaplicins at \$22,000/T and hundreds being currently investigated for potential pharmaceutical use), the time for looking at wood chemicals as readily available, renewable, value added products from wood, has come.

The current paradigm of the local wood industry for tree usage hardly includes wood and forest residues as potential sources of valuable chemicals. Since the breakup of ICC Rayonier and the Western Forest Products Laboratory of Canada over 20 years ago, few companies have invested in chemically based value-added-product development. This mind-set is out of date and needs to be revisited for two major reasons. First, technological advances in analytical instrumentation have created new ways to analyze wood chemicals and find new uses for them. Secondly, the old methods of burning and dumping of wood residues are no longer acceptable, energetically or environmentally.

The annual allowable cut in this province is 70,000 m³ per year. In 1995, Holm estimated that five million tonnes of bark residues were produced per year. Of the entire bark residues produced, Holm (1995) estimated that 42 % was utilized through co-generation, composting and mulch sales. But at an energy value of \$35 per tonne dry bark, McTaggart-Crown (1995), at the Residual Wood Conference, stated that co-generation with bark is the "most expensive way to generate electricity". Also, with the co-generation plant at Williams Lake as an example, the environmental question of what to do with the ash waste must be addressed. Wood floated in the ocean from the harvesting area to the saw mill, as is done where possible in coastal logging practices, becomes loaded with sea salts which produce environmentally hazardous dioxin and dibenzofuran contaminated ash when burned. These sodium and magnesium-rich ashes require special, ground-water sealed dumping facilities.

1.1 OBJECTIVES

The original objective of this thesis was to find and employ an existing oligomeric proanthocyanidin quantification method and apply this method to the inner and outer bark extracts of local softwood species. The selected method of quantification was to allow for a comparison between softwood bark proanthocyanidins, and those found in commercially available proanthocyanidin based products. It also had to be able to ascertain which species would be suitable as potential sources of these compounds.

Two major problems were quickly encountered which required the modification of the original objectives. First, no reliable method of quantification could be found and, after a method was created for the analysis for proanthocyanidins, it was found that there was no clear definition or

standard composition for proanthocyanidins. Even in the commercially available products, large variations in composition were observed. Therefore, a whole new way of thinking about oligomeric proanthocyanidin quantification had to be defined.

At this point, the original objective of surveying the quantities of proanthocyanidins in different bark species was shifted to devising a method capable of more accurately quantifying and analyzing oligomeric proanthocyanidins in bark extracts.

The revised objectives entailed the collection of a number of bark samples from local softwood species. The barks would be separated into inner and outer barks, dried sufficiently to allow for grinding to 40 Mesh and extracting exhaustively with a suitable solvent. The extract would be prepared, fractionated as necessary, and an HPLC testing method would be designed. The HPLC method would be optimized to maximize the resolution of the major oligomeric proanthocyanidins, and be flexible enough to allow for their identification in crude extracts and in the extracts of commercial purified products.

The preliminary results revealed that there was no standard composition for proanthocyanidins in bark (see Section 3.4). Even the commercially available proanthocyanidin based dietary supplements, available in health food stores (made from pine bark and grape seed extracts), contained variable amounts of seemingly the same compounds. In light of this, the second objective became the development of a method of proanthocyanidin identification and quantification, based on the HPLC method, which could be flexible enough to quantify the overall proanthocyanidin content in an extract, even though individual components would be expected to vary by an undetermined amount. This method had to be based on rational

assumptions, and be able to quantify these compounds relative to a pure proanthocyanidin sample.

The third objective was to survey the inner and outer barks of various local softwoods to determine their suitability as potential new sources for the production of Pycnogenol™-like products.

2.0 LITERATURE REVIEW

2.1 HISTORY OF PROANTHOCYANIDIN RESEARCH

The earliest use of tannins was recorded by the Egyptians as far back as 1100 B.C. as a tanning agent for hides. By removing the hair from hides in an alkaline (lime) bath, and placing the clean hides between layers of powdered oak bark, the Egyptians were able to produce an impermeable, non-rotting tanned hide. Though proanthocyanidins are among the active tanning agents in oak bark, it took over 3,000 years for the class of compounds known as proanthocyanidins to gain recognition as a medicinal agent, as well. In 1969, Masquelier first patented flavan-3,4-diols, or leucoanthocyanidins, as a potential cure for various venous and capillary diseases (Masquelier, 1969). He stated that these components reversed the loss of flexibility in the connective tissues of the pulmonary system, which is a contributing cause of some forms of cardiopulmonary diseases. The compounds Masquelier attributed to the cure were initially mis-identified in the patent. Now oligomeric proanthocyanidins are considered as the true active compounds.

Since their original isolation in pine bark and ground nut shells, proanthocyanidins have been identified in a variety of softwood and hardwood barks in addition to apples, grapes, nuts, wines, beer, teas, as well as many of other sources. Although Masquelier chose to mention pulmonary disease in his patent, proanthocyanidins were originally investigated for their anti-oxidant properties. Other researchers have since demonstrated the viability of proanthocyanidins as an alternative medical treatment for venous and capillary diseases. (Blazso *et al.*, 1994; Giess *et al.*, 1995; Passwater, 1992).

Masquelier first began his investigations into proanthocyanidins in the early 1960's after reading

of the account of Jacques Cartier's arrival in Canada (Passwater, 1992). In early winter, 1534, Cartier arrived in the Gulf of St. Lawrence. The story goes that Cartier and his men were landlocked as the gulf began to freeze and, although they could hunt for food, they soon ran out of their store of citrus fruits, which served as a source of vitamin C. Being winter, they could not find a source of vitamin C containing green vegetables or citrus fruits and began to suffer from scurvy. Scurvy is a condition which leads to general body weakness, tender and inflamed gums and joints, loss of teeth. Eventually it causes anemia, hemorrhaging, burst blood vessels, and, finally death. After the death of several crewmembers due to scurvy, an aborigine found the sailors and recognized their plight. He showed Cartier how to make a tea from the bark and needles of a tree recorded in Cartier's diary as the *annis* tree. Very rapidly, the tea restored the health of the remaining crew.

Cartier's account of the *annis* tree did not clearly identify any specific eastern Canadian tree species, and for almost 400 years, no one investigated this seemingly miraculous cure. Upon hearing of this story, Masquelier's curiosity was stirred and he decided to investigate what it was that saved the sailors' lives. During his investigation, he found that Cartier's description of the *annis* tree could have referred to a species of pine tree native to eastern Canada. His investigations concluded that although pine trees contain only trace quantities of vitamin C, not sufficient to cure scurvy as rapidly as noted in Cartier's journal, they contained compounds that seem to multiply its effects. He discovered that these compounds were anti-oxidant in nature and worked in synergy with vitamin C to increase its potency. Masquelier incorrectly suggested in his patent that the anti-oxidant action was due to leucoanthocyanidins (flavan-3,4-diols). It is now thought that the anti-oxidant properties of the bark and needle extract, in conjunction with the vitamin C, cured Cartier's crew.

Masquelier's original research in the 1960's led to the isolation and structural determination of proanthocyanidin dimers, initially, and in more recent literature, trimers and tetramers (Billa *et al.*, 1996). More detailed medical and structural research on proanthocyanidins was carried out in the early 1980's (Tixier *et al.*, 1984), and a stronger resurgence, largely medically oriented, continued through the last seven years, until a few years ago (Giess *et al.*, 1995). Most of the early studies did not clearly characterize the active compounds, and occasionally researchers confused proanthocyanidins and condensed tannins with the hot water phenolic extracts from plants, which are now known to contain far greater variety of compounds than just proanthocyanidins (Hergert, 1989).

As a whole, the quantification of proanthocyanidins has not been well researched, mostly because of the lack of reliable methods. Depending on the source, the literature concentrates on either the structures of proanthocyanidins or their functions, and only rarely on their quantification. Few papers have combined two of these areas, providing useful information on their structure/function relationship (Richardo da Silva *et al.*, 1991; Kaneda *et al.*, 1990). This is likely due to the difficulty and time consuming processes of isolating individual proanthocyanidin monomers, dimers, trimers, and lower oligomers for research.

2.2 NOMENCLATURE OF RELEVANT POLYPHENOLS

Despite Shakespeare,

“What's in a name? that which we call a rose
By any other name would smell as sweet.”

tannin nomenclature, though very complex and occasionally misused, is very important. Part of the difficulty in the identification of plant phenolics is due to their extreme diversity in plants,

and missing information in their biosynthetic enzyme pathways which is essential to accurate nomenclature. In some cases, it is only in the last few years that biosynthetic pathway enzymology has clarified some of the nomenclature. To facilitate better understanding, this section will outline the nomenclature and structure of proanthocyanidins, as far as it pertains to the understanding of the most common polyphenolic occurrences in western Canadian barks.

Proanthocyanidins, OPC's, leucoanthocyanidins, flavan-3,4-diols, flavan-4-ols, flavanoids, flavonoids, cyanidins, procyanidins, catechins and many other names are used by scientists when describing specific structures, or by marketers of proanthocyanidin containing products, when alluding to their potential efficacy.

In the hierarchy of chemical compounds in plants, plant phenolics is the major classification which encompasses a vast number of compounds composed or consisting of phenyl groups. This designation generally encompasses all compounds soluble in aqueous alkaline solutions. These compounds have often been quantified in bark and wood by measuring the mass of their hot water extracts. (Rydolm, 1965). Although this method is simple, it may exclude larger, polymeric, and less soluble polyphenols.

2.2.1 Tannins

Within the broad class of polyphenols is a class of compounds termed tannins. These compounds are phenolic compounds between 500 and 3,000 Daltons, which have the ability to bind with, and precipitate, proteins, alkaloids, and gelatin and form coloured complexes with metals. The name tannin was given to these compounds because they are the active in the tanning process, which keeps leather flexible and resistant to putrefaction. The Celtic word for oak is 'tan', and

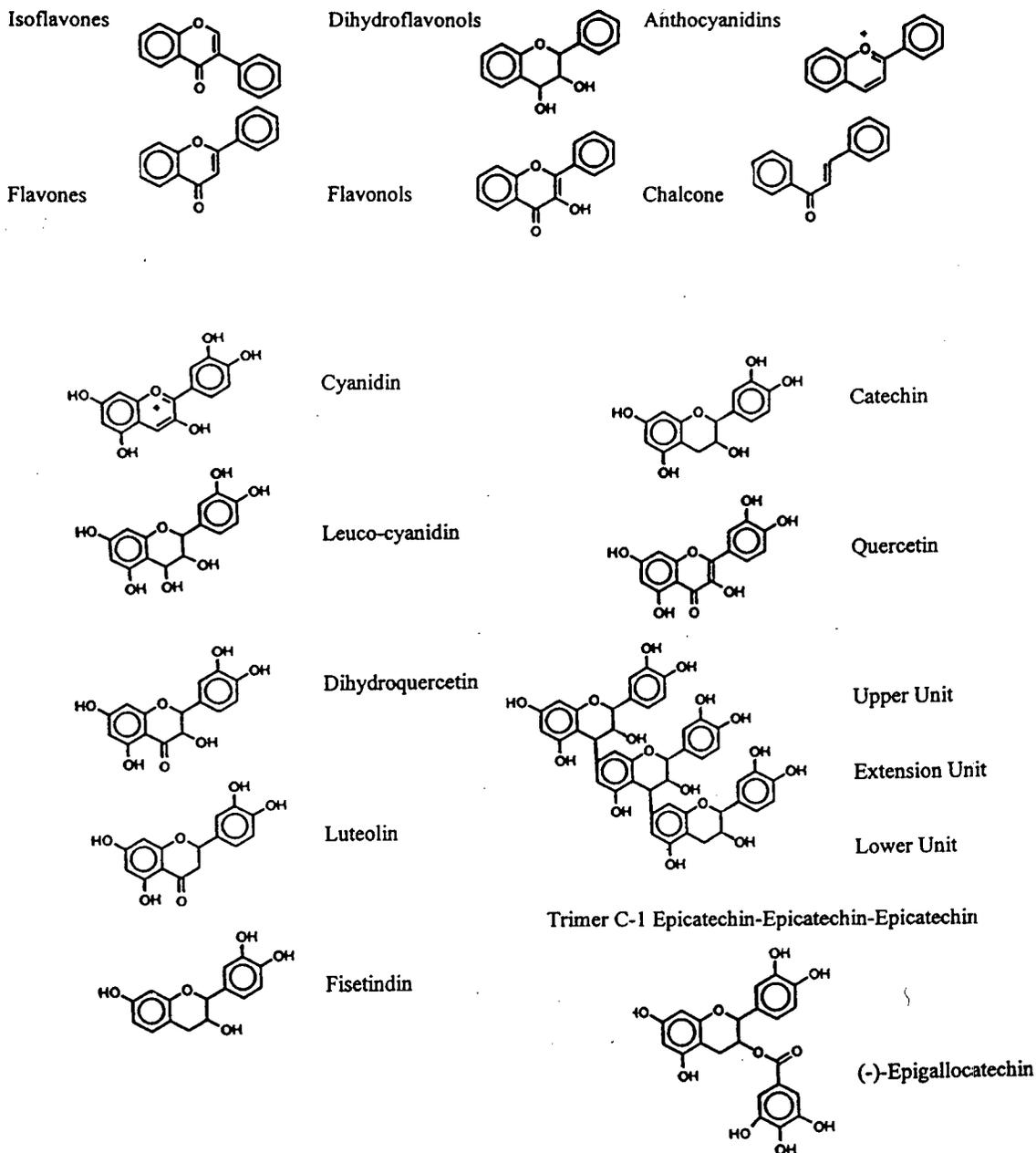
for thousands of years, powdered oak was the tanning agent of choice.

2.2.2 Condensed Tannins

Within the sub-class of tannins are two further distinctions: the first is based on flavonoids and the second is based on esters of sugars and phenolic acids, condensed and hydrolyzable tannins, respectively. In plants, the term 'condensed tannins' has always been synonymous with 'proanthocyanidins', however, this has recently been contested (Stafford, 1993; Stafford 1995) and will be discussed in the next section. Within the class of proanthocyanidins, lies the complex relationship of a number of flavonoid based compounds, bearing the characteristic C₆-C₃-C₆ structure shown in Figure 1. The term flavonoid, coming from the Latin *flavus*, meaning yellow (Geissman and Hinreiner, 1952), was coined in 1952 for the classification of pigments with the C₆-C₃-C₆ structure and initially included coumarins and cinnamic acids as well. Today, due to the advancements in enzymology and analytical techniques, the term proanthocyanidins only refers to oligomeric or polymeric flavan-3-ols (Stafford 1995).

2.2.3 Leucocyanidins

New distinctions in nomenclature specify that the term 'leucocyananidin' should be limited to only flavan-3,4-diol and flavan-4-ol monomers. According to Stafford (1995), flavan-3-ol monomers, anthocyanidins and proanthocyanidins are all enzymatically produced from flavan-3,4-diols. The enzyme responsible for the creation of proanthocyanidin oligomers from the flavan-3,4-diol monomer has not been found, and Ferreira *et al.* (1991) have produced proanthocyanidin oligomers from flavan-3,4-diols spontaneously, without an enzyme. However, because the entire process is enzyme mediated up to this point, it seems probably that the last step in proanthocyanidin synthesis, polymerization, is also enzyme mediated. This is supported



Flavonoid nomenclature by number of hydroxy groups on the B-ring.			
Identification	1	2	3
Flavan-3-ol		Catechin	Gallocatechin
Flavonols	Kaempferol	Quercetin	Myricetin
Anthocyanidin	Pelargonidin	Cyanidin	Delphinidin
Flavan-3,4-diol [†]	Leuco-pelargonidin	Leuco-cyanidin	Leuco-delphinidin
3-OH-Flavanone	Dihydrokaempferol	Dihydroquercetin	Dihydromyricetin
Flavone	Apigenin	Luteolin	
6-Dehydroxy-Flavan-3-ol	Guibourtinidin	Fisetinidin	Robinetinedin

[†]Flavan-3,4-diols and Flavan-4-ols are considered Leucoanthocyanidins.

Figure 1. Structures and nomenclature of common flavonoids.

by analogy by the recent work of Dr. Lewis at the University of Washington, who showed that the last step in lignin and lignan formation, which was previously thought to be spontaneous, is, in fact, enzyme mediated (personal communication).

Flavan-3-ol monomers do not self-condense to form proanthocyanidin oligomers. Flavan-3,4-diols, first discovered by Rosenheim (1920), are very unstable and have never been isolated from extracts (Stafford and Lester, 1986). Rather, Swain (1959) first produced these compounds in 1954 as the reduction product of taxifolin (dihydroquercetin) or dihydromyricetin, forming leuco-cyanidin or leuco-delphinidin, respectively (Stafford and Lester, 1986). These compounds were first obtained in crystal form by Masquelier in 1968 (Ribereau-Gayon, 1972), likely as part of his patent work.

2.2.4 Flavan-3-ols

Procyanidins are defined as flavan-3-ols with a dihydroxylated B-ring. In a hot mineral acid environment in the dimeric form, the upper unit of the procyanidin would be converted to the anthocyanidin: cyanidin. The lower unit would remain a catechin. A prodelphinidin is similar in structure, but with a trihydroxylated B-ring. Under the same hot mineral acid environment, the upper unit is converted to a delphinidin, and the lower remains a gallocatechin. A propelargonidin is again similar to the other structures, but has a mono-hydroxylated B-ring. It is rarely found in nature. Figure 2 shows the biosynthetic pathways of proanthocyanidins and formation of proanthocyanidin polymers. The term dihydroquercetin is synonymous with taxifolin.

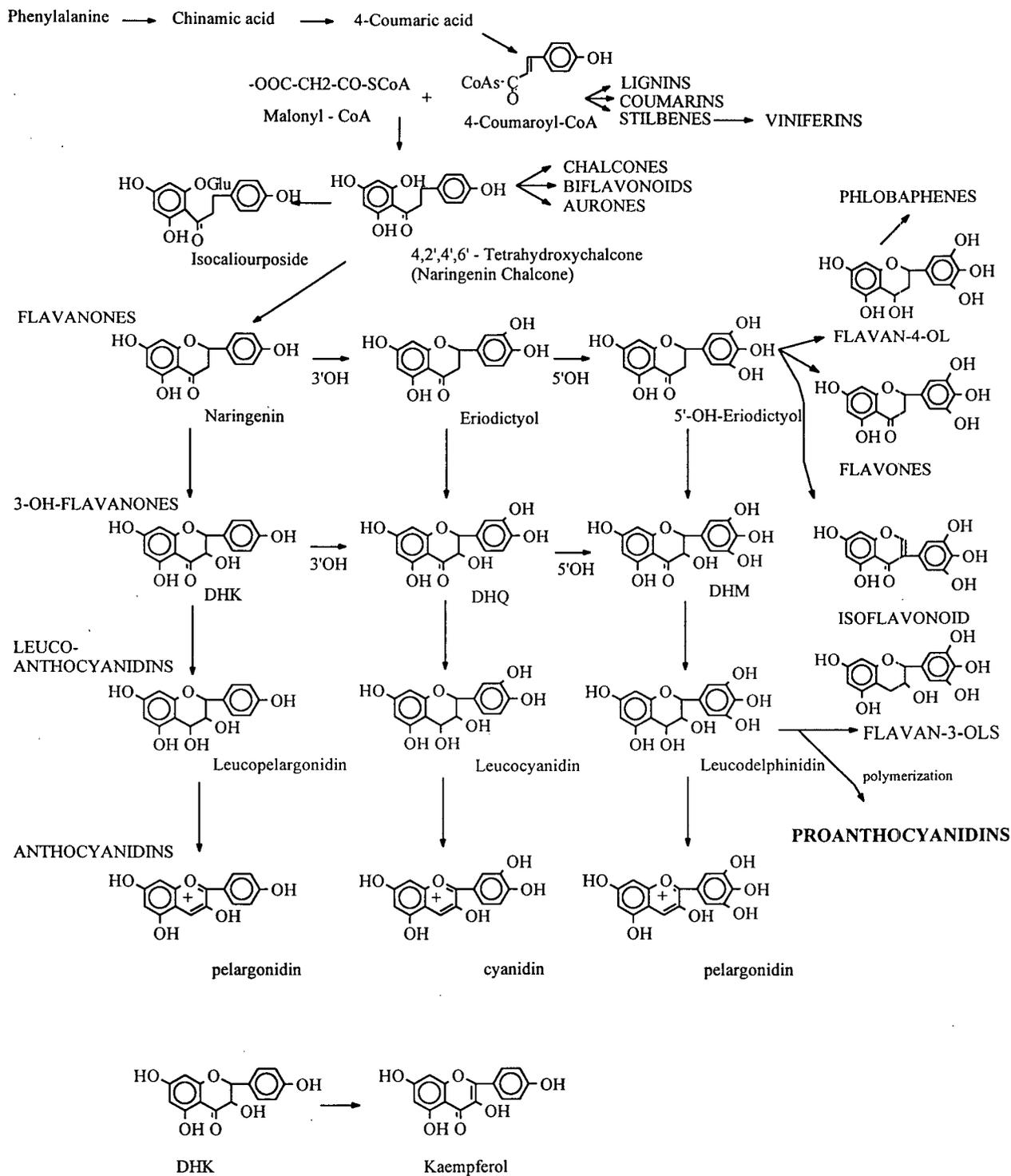


Figure 2. An overview of the C₁₅ pathway to the major subclasses of flavonoids. The grid portion involving the 3' and 3',5', cytochrome p450 hydroxylases and flavanone-3-hydroxylases is emphasized to show possible multiple routes to the dihydroxy and trihydroxy B-rings of dihydroquercetin and dihydromyricetin unless the pathway enzymes are organized into complexes. DHK, dihydrokaempferol; DHQ, dihydroquercetin; DHM, dihydromyricetin. The reaction from dihydrokaempferol to kaempferol is identical for dihydroquercetin and dihydromyricetin (Stafford, 1995).

2.3 STRUCTURE OF PROANTHOCYANIDINS

The most common proanthocyanidin oligomers and polymers are based on catechin and epicatechin monomer unit structures and are known as procyanidins (Figure 2). As in all living systems, however, there is a degree of variability, and pelargonidins and delphinidins and various glycosylated and gallated monomers can also be incorporated into procyanidin chains. Proanthocyanidin chains are not formed by the spontaneous condensation polymerization of flavan-3-ols. They are thought to be formed through enzymatic condensation of leucoanthocyanidins (flavan-3,4-diols) with either a flavan-3-ol unit (eg. catechin, epicatechin, gallic catechin, epigallocatechin) or with an already condensed leucoanthocyanidin, which in the polymerized form takes the form of a flavan-3-ol terminal unit (Stafford and Lester, 1986). Multiple variations in the proanthocyanidin oligomeric structure can occur. Differences in the bonding location (C4→C6 or C4→C8 bonds) and differences in the stereochemistry (α or β orientations) are responsible for the three-dimensional structure of a proanthocyanidin molecule. Differences in the length of the oligomers, and the incorporation of different proanthocyanidin monomeric units, and gallation and glycosylation of the various units, are responsible for many of the physical properties of these compounds.

Chain branching has not been observed, although structures up to only tetramers have been elucidated. This would seem to support the notion of enzymatic linear extension of oligomers in the polymerization of leucoanthocyanidins.

2.4 PROPERTIES OF PROANTHOCYANIDINS

2.4.1 Physical Properties

Proanthocyanidin oligomers are freely soluble in water, acetone, and alcohol. They are less

soluble in ethyl acetate (only monomers and some dimers and trimers) and are insoluble in chloroform and other non-polar solvents. They are light beige tan in colour in their pure form, but darken over time. Proanthocyanidins ionize readily in water with a pKa 8.5 (for procyanidin B-2) (Kennedy and Powell, 1985). They are reversibly precipitated at low pH and have titratable acidic protons, interestingly, at the same pH as carboxylic acid, even though there are no carbonyl groups associated with flavonoids (Powell and Rate, 1987).

Proanthocyanidins are also well known for their reactions with proteins. This property of tannins is responsible for many of their anti-biotic and anti-predatory functions and is also responsible for their taste. The property of astringency, or dryness, is well known to be caused by the tannin-protein interaction, and has been researched for many years because of their effect on the quality of wines.

2.4.2 Astringency

It is difficult to describe the quality of astringency, but Nierenstein (1934) seems to have found a most illustrative description:

“a feeling of constriction, dryness and roughness, along with a sense of stiffening in the movements of the tongue and some loss of taste. These effects are due to the coagulation of the superficial layers of protein both within and without the epithelium which substitutes for the ordinary smooth surface a firmer, less even one over which the tongue can no longer move easily.”

The changes effecting the properties of astringency are related to the molecular size, shape, and diffusivity of proanthocyanidins. Astringency comes from the Latin *ad*, meaning *to*, and *stingere*, meaning *bind*, therefore, referring to a binding reaction. Proanthocyanidins can taste either dry or bitter, and an increase in the size of the molecule, for instance, leads to increased dryness and

decreased bitterness. This change in taste comes from a change in oligomer solubility. Proanthocyanidins have a naturally bitter taste and are known to increase in bitterness with molecular size. However, oligomers at the size of about four monomer units begin to lose their ability to permeate through the tongue's epithelium and their astringency becomes more pronounced.

This hypothesis can be tested by comparing the taste of a procyanidin-rich aqueous solution with a similar alcoholic one. The bitterness of the solution is enhanced in alcoholic solutions as the alcohol is able to dissolve the layer of saliva on the tongue's surface, allowing proanthocyanidins to pass through the tongue's epithelium more easily. However, the astringency of the solution is decreased as alcohol is better able to dissolve the protein-tannin complex, removing the dry sensation attributed to the sensation of astringency. The ease with which an oligomer passes through the tongue's epithelium and enters the blood stream may affect its medical efficacy.

2.5 CHEMISTRY

2.5.1 Acidic Environments

Proanthocyanidins become coloured when exposed to strongly acidic conditions. Bates-Smith (1975) exploited this reaction to identify and roughly quantify proanthocyanidins in various plant extracts. Through exposure to a strong acid, such as hydrochloric or sulfuric acid, a proanthocyanidin is hydrolyzed at the inter-flavan bonds and is converted to an anthocyanidin. The red coloured anthocyanidins absorb UV light at a characteristic wavelength around 450 - 500 nm and can be used as a means of proanthocyanidin quantification. This process only converts the upper or extension units, but not the lower unit which, after hydrolysis, remain as catechin.

Under weakly acidic conditions, a different type of reaction occurs. The weak acid also cleaves the inter-flavan bonds after it is protonated. Figure 3 illustrates this reaction. After cleavage, a catechin and carbocation intermediate is produced. The intermediate can be converted to an anthocyanidin or, in the presence of a suitable nucleophile HX, to a new product. Flavanoids and phenylmethanethiol are both considered good nucleophiles for this reaction (Porter 1992). This method also allows for the re-polymerization of anthocyanidins and is useful for the structural determination of proanthocyanidins.

2.5.2 Basic Environments

Under basic conditions, flavonoids are known to undergo a series of steps known as base-catalyzed rearrangements. Figure 4 shows such a rearrangement as a free-radical process (Porter 1992). During this process, C-ring opening and rearrangement are observed. During the formation of the B-ring quinone methide, the B-ring is opened and rearrangement is allowed to occur. The reversion to the closed ring form produces either catechin or *ent*-epicatechin. Alternately, in the intermediate quinone methide form, the free-radical can redistribute to the A-ring through the delocalization. At the C-8 position, the free-radical can attack the C-2 flavone and form catechinic acid or isocatechinic acid.

Porter (1992) suggested that the aging process of proanthocyanidins is related to the susceptibility of the C2 to singlet oxygen attack. He supports this claim by the observation that exhaustive thiolysis of an epicatechin polymer produced epicatechin and a 4-thiobenzyl ether. The thiolysis of a doubly linked epicatechin polymer results in the formation of epicatechin-(2 β →7;4 β →8)-epicatechin- β -benzylsulfide. The thiolysis of all naturally occurring procyanidin polymers, to some extent, yield the latter C2 product. Also, after aging, condensed tannins

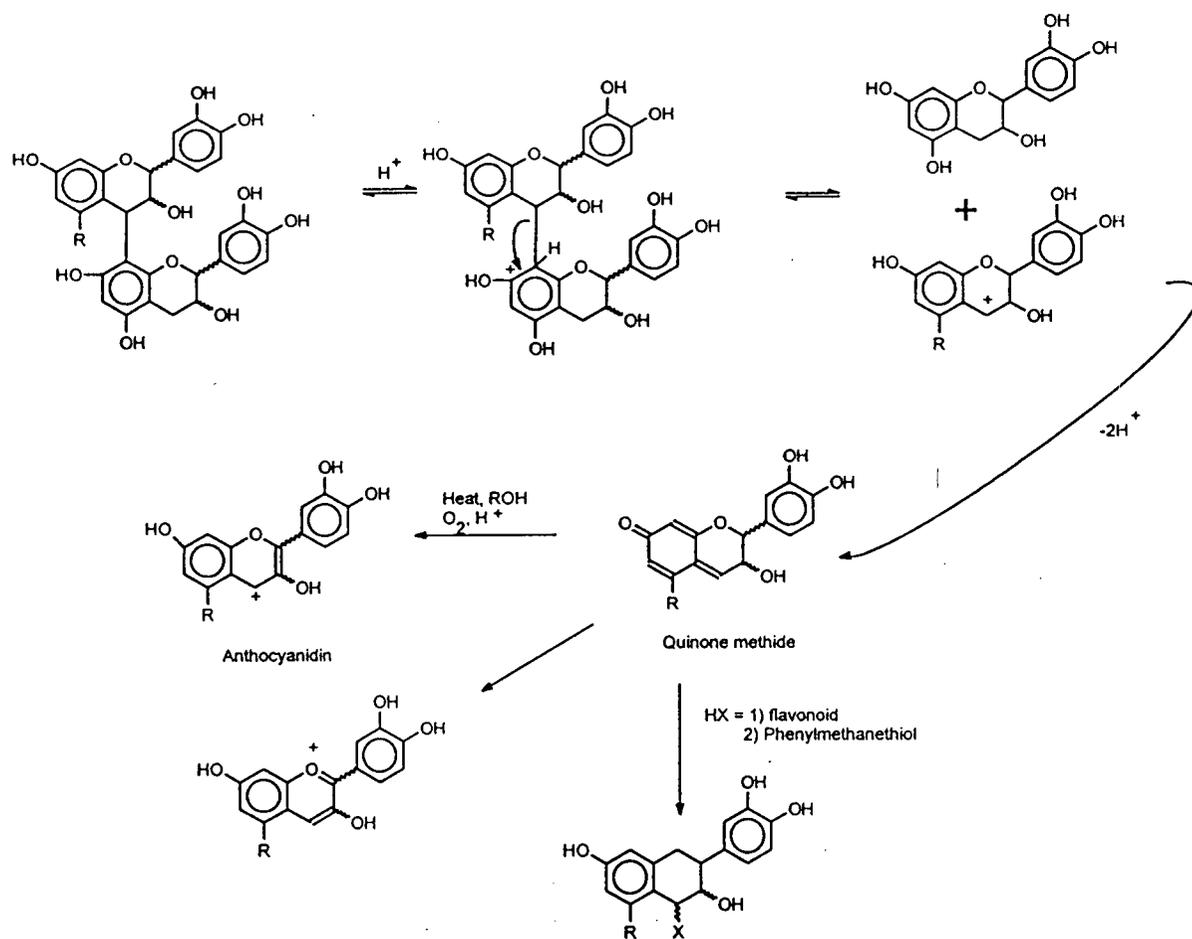


Figure 3. Acid-catalyzed flavonoid reactions. A flavonoid dimer is converted to a quinone methide intermediate. The quinone methide can decompose into an anthocyanidin and a catechin, or can be re-polymerized in the presence of an appropriate nucleophile such as a flavonoid.

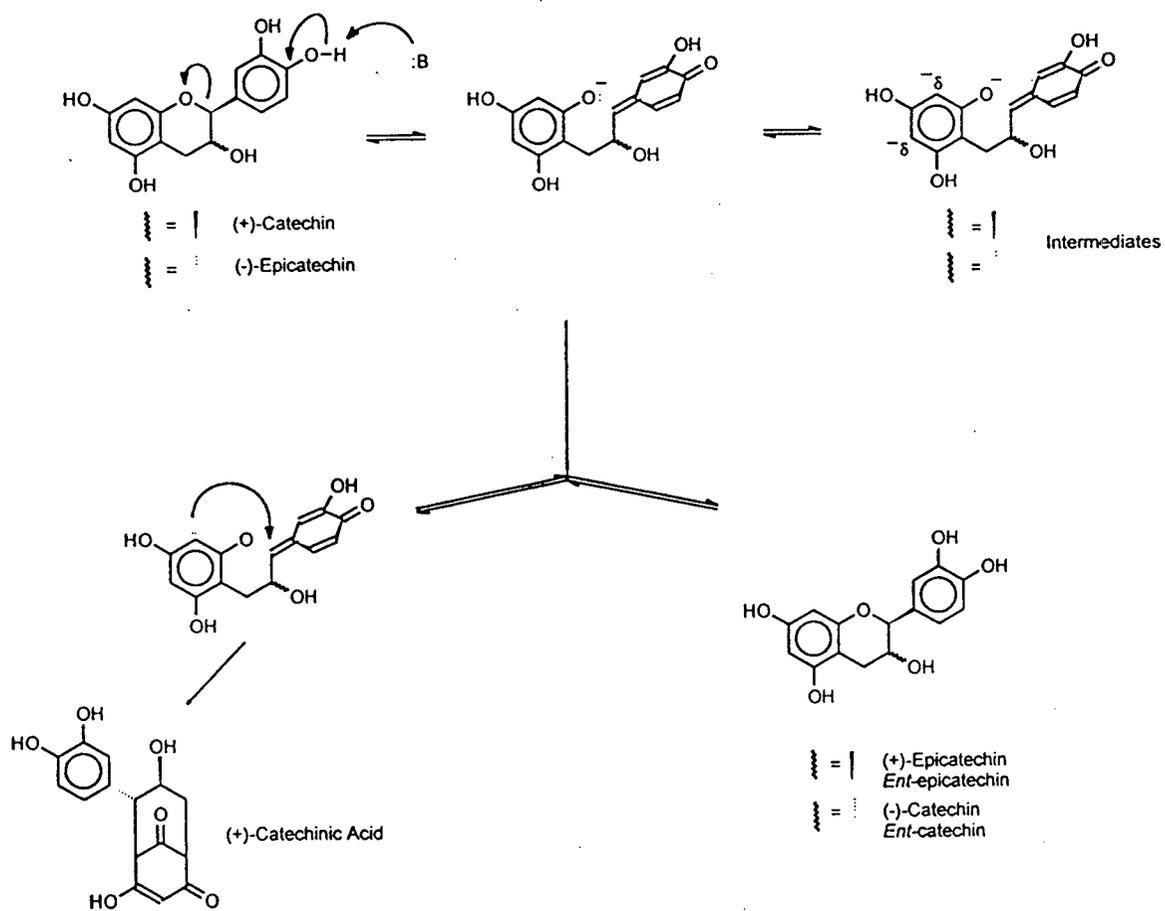
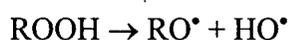


Figure 4. Base-catalyzed rearrangements of monomeric catechin or epicatechin to (+)-epicatechin and (-)-catechin, respectively, or catechinic acid.

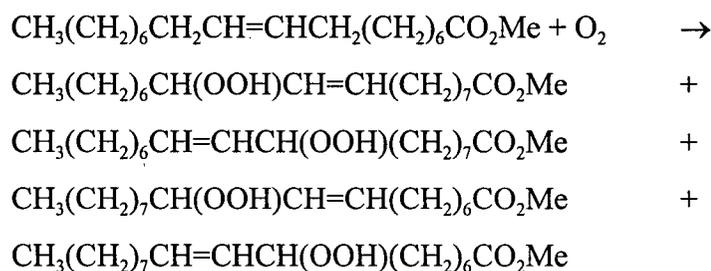
absorb in the 400-600 nm range while fresh condensed tannins absorb in the range of 270-280 nm. Therefore, all naturally occurring proanthocyanidins contain some doubly linked structures, likely formed through aging. It is these structures which contribute to the 400-600 nm absorption. The overall low concentration of the C-2 product in cells, however, would suggest that these products are prevented from building up in cell tissues in nature.

2.5.3 Free-radical and Anti-oxidant Chemistry

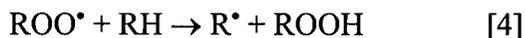
Autoxidation is defined as a radical-chain reaction between molecular oxygen and organic compounds at low or moderate temperatures. Free-radicals are spontaneously formed in alkanes, alcohols, aldehydes, and ethers (Nonhebel *et al.*, 1979). The first step in a free-radical process is initiation. The initiation process can be triggered by thermolysis, photolysis, or by decomposition of hydroperoxides, as shown below. This stage is the most important step in the process of autoxidation, where the rate of autoxidation can be represented by the relationship of the radical initiator concentration being proportional to the square root of the initiator concentration. Equations [1] and [2] illustrate the process of initiation with a peroxide.



Radical propagation often occurs at the reactive allylic or benzylic positions. Some reactions that occur with a methyl oleate are shown below:



The general propagation reaction for this compound is described in equations [3] and [4].



The radical reacts with oxygen and forms a peroxy-radical. The peroxy-radical then reacts with the alkane and forms a peroxide and transfers the radical onto the next molecule. The peroxide is capable of decomposing, as shown in reaction [1] and forming two additional radicals. This reaction can progress endlessly through different susceptible molecules, permanently changing their structure. The rate-limiting step is reaction [3] when only trace amounts of oxygen are available. Normally, reaction [4] is slower, and the stability of the end product is proportional to the reaction rate. The faster the reaction, the more stable the end product (Nonhebel *et al.*, 1979).

Radical termination reactions constitute any reactions that result in the formation of a product in which the radical is eliminated in the form of a covalent bond (Equation [5]). This process dominates under O₂ rich conditions at pressures of oxygen above 10 kPa or 0.1 atm.

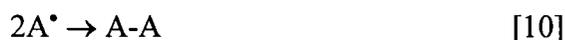


The alkyl radicals shown in Equations [6] and [7] are uncommon, except at very low concentrations of oxygen. This is essentially due to the very high reactivity of radicals with oxygen, as shown in Equation [3]. Hence, the number of alkyl radicals is typically very low.



Anti-oxidants are compounds capable of interrupting the autoxidation chain reaction, preventing the free-radical autoxidation process. To accomplish this, anti-oxidant compounds have

extractable hydrogens. Anti-oxidants, represented as AH below, react with free-radicals, hydrogenating them and becoming themselves radicals. However, instead of propagating the reaction, they react with other radicals to sequester them and form covalent bonds. The kinetics of the anti-oxidant reaction of these compounds is described as:



For a hindered anti-oxidant, such as BHT (butylated hydroxytoluene), reactions [9] and [10] are the predominant reactions. For non-hindered anti-oxidants, all reactions are equally competitive. A mixture of hindered and non-hindered anti-oxidants was found to be more effective because the non-hindered compound quickly abstracts free-radicals and transfers them to the hindered compound where they are stabilized. Non-hindered phenols cannot inhibit autoxidation reactions, only retard them, as the chain reaction is propagated by both peroxy and phenoxy radicals: non-hindered phenols. Once the radical is transferred onto the hindered phenol, it can react with another free-radical, forming a covalent bond and removing the radical from the system (Equations [9] and [10]). Figure 5 illustrates this process.

To determine the anti-oxidant effect of proanthocyanidins, Richardo da Silva *et al.* (1991) used two methods to quantify the anti-oxidant and free-radical scavenging capacity of proanthocyanidins. The first method used a reaction of xanthine and xanthine oxidase, an enzyme found in mammalian cells, which naturally produce superoxides (O_2^\bullet). Using different procyanidins and procyanidin derivatives as O_2^\bullet trapping compounds, they measured the

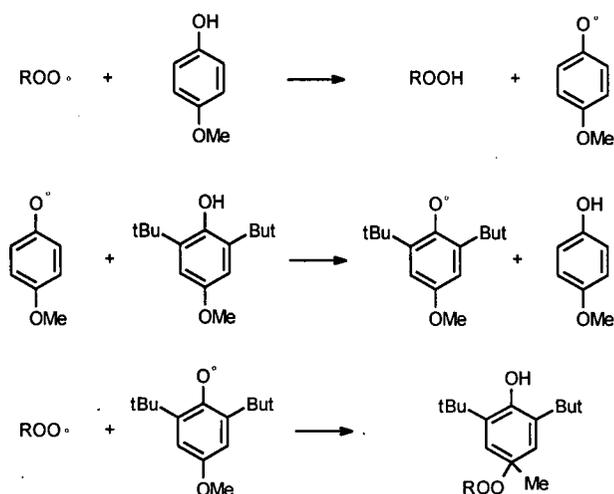


Figure 5. Anti-oxidant reactions of hindered and non-hindered phenols in synergy (Nonhebel, *et al.*, 1979).

concentration of $\text{O}_2^{\cdot-}$ under different pH conditions and concentrations to calculate the initial rate of consumption and the amount of trapped $\text{O}_2^{\cdot-}$. Some of these results are presented in Table 1 and a general comparison of the values show that as oligomeric size increases, so does the ability of the procyanidins to trap $\text{O}_2^{\cdot-}$. Gallated esters, and especially 3-*O*-gallate, were best able to trap the $\text{O}_2^{\cdot-}$, suggesting that gallation, and the site of gallation, bears some importance in the anti-oxidant capacity of proanthocyanidins. These results are compared with Trolox. Trolox-C is a synthetic derivative of α -tocopherol and *d*- α -tocopherol polyethylene glycol 1000 succinate, a synthetic water-soluble form of α -tocopherol. Procyanidins were more effective than α -tocopherol (Ariga *et al.*, 1988). The structure of α -tocopherol, ascorbic acid (vitamin C), and citric acid are shown in Figure 6. Vitamin C and citric acid are naturally occurring anti-oxidants and will be discussed later.

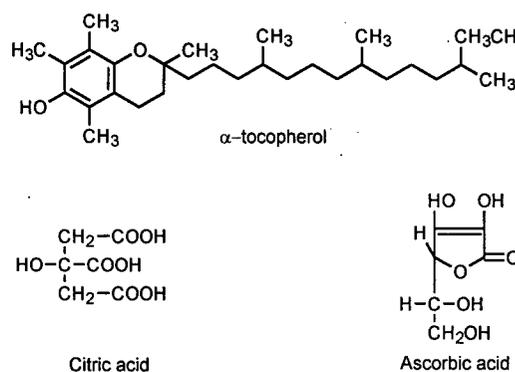


Figure 6. Structures of α -tocopherol, citric acid, and ascorbic acid (vitamin C).

Table 1. Values of superoxide radical trapped at pH 9.0 (nmol/2mL) (Richardo da Silva, 1991).

Compound	at 10E-4 M	By monomer Equivalent
Trolox (industry standard)	37.5	
(+)-catechin	58.5	57.5
(-)-epicatechin	50	50
(-)-epicatechin 3- <i>O</i> -gallate	80	
Procyanidin B2	87.5	65
Procyanidin B5	58	40
Procyanidin B2 3- <i>O</i> -gallate	75	
Procyanidin B2 3'- <i>O</i> -gallate	112.5	
Procyanidin C1	77.5	67.5
Procyanidin trimer 2	67.5	58.5
Procyanidin trimer 3	73.5	57.5

Other research (Matsuzaki and Hara, 1985) showed that the strength of flavonoid anti-oxidants are, in the order of declining strength, epigallocatechin-gallate, epigallocatechin, epicatechin-gallate, epicatechin. All of these flavonoids were found to work in synergy with ascorbic acid, α -tocopherol, citric acid and tartaric acid. Ariga *et al.* (1988) confirmed that procyanidin dimers B1, and B3 were stronger anti-oxidants than α -tocopherol. Taxifolin, quercetin, myricetin, and robinetin were also found to be potent anti-oxidants, as were anthocyanins and malvinidin-3,5-diglycoside, compounds commonly found in grape seeds (Igarashi *et al.*, 1989).

The second test carried out by Richardo da Silva *et al.* (1991), was to quantify the hydroxyl radical (OH^{*}) scavenging ability of proanthocyanidins. This was accomplished by comparing the degradation of 2-deoxy-*D*-ribose by OH^{*} radicals in the presence of various procyanidin monomers, dimers, dimer gallates, and trimers. Again, gallation seemed to increase the efficacy of the OH^{*} scavenging capacity in dimers, however, a general molecular size to efficacy trend was not observed. Hence, the molecular structure played a more important role than molecular weight. Table 2 lists the data in the form of second order rate constants.

Table 2. Second-order rate constants for reactions of scavengers with hydroxyl radical, by the Deoxyribose Assay (Richardo da Silva, 1991).

Compound	rate constant (1/Mol. second)
ethanol	1.2 E-9
mannitol	1.55 E-9
(+)-catechin	2.88 E-9
(-)-epicatechin	3.17 E-9
(-)-epicatechin 3- <i>O</i> -gallate	1.56 E-9
Procyanidin B2	1.41 E-9
Procyanidin B5	1.44 E-9
Procyanidin B2 3- <i>O</i> -gallate	2.4 E-9
Procyanidin B2 3'- <i>O</i> -gallate	3.59 E-9
Procyanidin C1	2.78 E-9
Procyanidin trimer 2	2.26 E-9
Procyanidin trimer 3	2.18 E-9

2.6 FUNCTION

The function of proanthocyanidins can be approached from two sides, their effect in plants as biological agents, as toxins, and their effects in humans, as nutraceuticals. The effects of proanthocyanidins in humans are of greater importance due to their health and economic significance as a dietary supplement, and will be discussed first.

2.6.1 Effects in Humans

2.6.1.1 Free-radicals and superoxides in the human body

As shown in Section 2.5.3, lipids and other biological molecules, are susceptible to autoxidation and free-radical attack. But thermolysis and photolysis are not the only modes of free-radical generation in humans. Free-radicals are generated in an on-going manner as part of the regular cellular function of the body. The plasma and microsomal membrane-associated enzymes, lipoxygenase and cyclooxygenase, are derived from arachidonic acid metabolism. The enzymatic oxidation of arachidonic acid in this reaction leads to the formation of prostaglandins (Figure 7), thromboxanes, leukotrienes, and a number of other compounds that give rise to the production of free-radicals (Freeman, 1984).

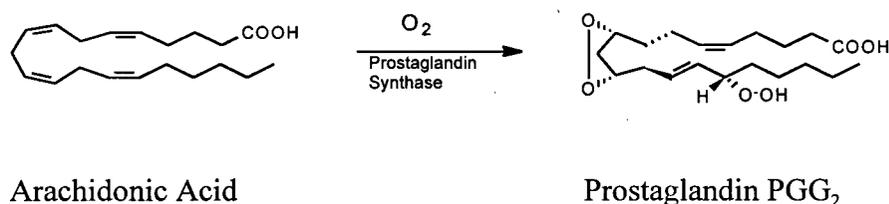


Figure 7. Overall reaction and by-products in the synthesis of a prostaglandin (PGG₂) from arachidonic acid (Nonhebel *et al.*, 1979).

In the cell cytosol, thiols, hydroquinones, catecholamines, flavins, tetrahydropterins, and other soluble components can participate in oxidation-reduction reactions. These reactions contribute to radical production and, as in all cases a peroxide anion, and the resultant H₂O₂, are primary products. Also, enzymes such as xanthine oxidase, aldehyde oxidase, dihydroorotate dehydrogenase, flavoprotein dehydrogenase, and tryptophan dehydrogenase generate free-radicals during their catalytic cycles.

In the mitochondria, superoxide radical generation is influenced by the reduced states of the respiratory chain (electron transport chain) carriers in the inner membrane. As the respiratory chain is intimately associated with free electrons, a potential exists for the accidental generation of radicals in the cell under some conditions.

Peroxisomes are cellular organelles that are comprised of peroxides. Although they are designed to be impermeable to peroxides, to protect the cellular contents, some research has shown that peroxides can diffuse across at least two membrane barriers and through the cytoplasm to cause tissue damage (Jones *et al.*, 1981).

2.6.1.2 Diseases and natural anti-oxidants

Passwater (1992) states that compounds like polyunsaturated fats are easily converted to free-radicals. This leads to an acceleration in the natural aging process by causing damage to the cell membranes and so preventing nutrient uptake and reducing waste disposal from cells. Radicals also can permeate the nucleus and damage or cleave DNA leading to altered proteins, or even to tumors.

The formation of free radicals in the human body occurs naturally, but can also be caused by ingesting foods (or any substances) which are prone to forming free-radicals, or even by sitting in the sun. Regardless how they are formed, free-radicals can have a devastating effect on the human body. Figure 8 illustrates some diseases attributed to free-radicals.

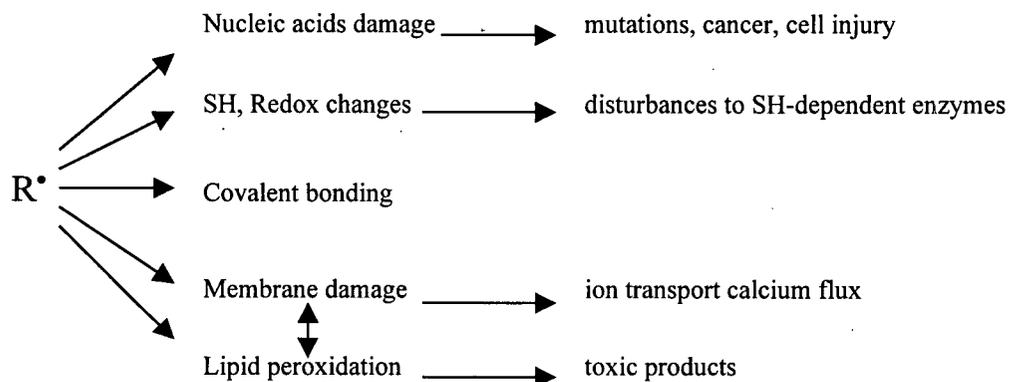


Figure 8. Free-radical and cellular injury. Major routes are shown in which a free-radical can interact with a neighboring component in cells to disturb their metabolic function(s) (Slater, 1984).

Lipid peroxidation is perhaps the most common result of the presence of free-radicals and superoxides in the human body. Lipids make up the bulk constituent in foods and biological (including human) systems, making them particularly susceptible. Simple lipids are made up of triglycerides, steryl and wax esters; compound lipids are predominantly phospholipids, glycolipids, sphingolipids and lipolipids; derived lipids are predominantly fatty acids, soluble vitamins and provitamins, sterols, terpenoids, and esters. Together, these compounds make up a large percentage of the human body's basic building blocks. Plants contain higher unsaturated lipid contents than animals. Although animals have low levels of unsaturated lipids, they contain a certain amount of radical-formation prone, highly unsaturated fatty acids.

The sites of unsaturation in a molecule are susceptible to oxidation (Section 2.5.3), which can lead to complex chemical reactions. In the body, these reactions can lead to coronary heart disease, arteriosclerosis, and cancer and can contribute to the aging process. Some lipid peroxidation-related diseases are listed below in Table 3.

Table 3. Lipid peroxidation - induced diseases and effects. (Jodhav *et al.*, 1995)

<u>Diseases:</u>	<u>Remarks:</u>
1) Hemochromatosis	Organ damage due to iron overload, leading to increased lipid peroxidation
2) Keshan disease	Selenium deficiency causes decrease in glutathione peroxidase activity leading to increased lipid peroxidation
3) Rheumatoid arthritis	Due to iron-induced peroxidation
4) Atherosclerosis	Lipid peroxides and the reaction products of lipid peroxidation such as hydroxyalkanal alter low-density lipoproteins (LDLs), which are important in the development of atherosclerotic lesions
5) Ischaemia	Occurs during reperfusion injury of heart and brain; also results in lipid peroxidation, probably by transformation of xanthine dehydrogenase to xanthine oxidase and by the production of reactive oxygen species.
6) Aging	May be due to lipid peroxidation, but has been confirmed in erythrocytes.
7) Carcinogenesis	Wide speculation about the involvement of lipid peroxidation in carcinogenesis; this is due to genotoxic effects of lipid peroxides.

In studies on the liver, initiation and propagation of lipid oxidation in the liver was catalyzed by iron and microsomal NADPH-Cytochrome-P450-Reductase (Kappus, 1985) This process forms the superoxide radical by the addition of an extra electron to diatomic oxygen molecules, causing the reduction of Fe^{3+} ions. Of all of the radical species ($O_2^{\bullet-}$, HO_2^{\bullet} , H_2O_2 , 1O_2 , HO^{\bullet}) singlet oxygen and hydroxy radicals, 1O_2 and HO^{\bullet} , are the most powerful free-radicals.

2.6.1.3 Natural anti-oxidants

Lipid peroxidation by radicals, and the resultant damage to DNA and other macromolecules, leads to the progressive degradation of the body's ability to protect itself (Madhavi, *et al.*, 1995). The mutation and degradation of DNA by free-radicals, and their imperfect repair, lead to tissue damage and carcinogenesis. Gensler and Bernstein (1981) found that there is a correlation

between the tissue concentration of certain anti-oxidants, such as carotenoids, vitamin E, urate, and enzymes such as superoxide dismutase, and life spans. Vitamin C and E are two natural anti-oxidants found in animals, and although Gensler and Bernstein (1981) did not specifically list vitamin C as a life extending anti-oxidant, it is well known to act in this manner.

Vitamin E is a lipid anti-oxidant and free-radical scavenger. It has been shown to inhibit carcinogenesis and mutagen formation in the repair of DNA and membranes (Gaby and Machlin, 1991). Contested studies indicate that low serum concentrations of vitamin E are associated with higher risks of cancer.

Vitamin C acts differently than vitamin E. Vitamin C is an anti-oxidant that reacts with molecular oxygen to form dehydroascorbic acid. This effectively removes the oxygen, preventing oxidation reactions from occurring. Vitamin C has been shown in studies to reduce the risk of oral, esophageal, gastric, and colorectal cancers. According to Masquelier (Passwater, 1992), proanthocyanidins were found to work in synergy with vitamins C and E to further elicit a number of beneficial effects on the body and will be discussed in the next Section.

2.6.1.4 The effect of proanthocyanidins on the human body

The health benefits of proanthocyanidins have been attributed to their free-radical scavenging and protein binding activities. Passwater (1992) provides a reasonable historical and medical review of one commercial form of oligomeric proanthocyanidins, Pycnogenol™. What is said about Pycnogenol™ will be true in principle of most proanthocyanidins and their derivatives.

Passwater (1992) stated that the actions of proanthocyanidins on the human body are primarily four-fold. The first effect is through their free-radical scavenging capacity. The free-radical chemical functional groups, found naturally in the human body, are usually sequestered by natural anti-oxidants, such as vitamin C and E, to remove them from the body. If they are not removed, they are free to react with various tissues, contributing to a number of ill-defined reactions that contribute to disease and the process of aging.

Proanthocyanidins act against these effects in two ways. Vitamin C and E are both powerful anti-oxidants as explained. Proanthocyanidins have been shown to regenerate vitamin E after it reacts with free radicals in the body. This allows vitamin E to be reused over and over as long as proanthocyanidins are available to regenerate it, making it seem as though the vitamin E is more potent than it really is. Alternately, proanthocyanidins are suspected of protecting vitamin C, sacrificially, by reacting with free-radicals before they react with to destroy vitamin C. This allows more vitamin C to reach its final destination in the cell. The combined synergism of all three anti-oxidants is more effective than only vitamin E or C. But, it is also the effect of the proanthocyanidins alone, being up to 50 times more powerful anti-oxidant than vitamin E and 20 times more powerful than vitamin C, which also plays a beneficial role (Masquelier, 1987).

Vitamin C is also required for the production of collagen. The skin protein, collagen, is required to maintain flexibility and health of skin and tissues. The formation of varicose veins, predominantly in the legs, is a result of the skeletal muscles not sufficiently assisting the conducting of blood through the veins. This occurs most often in legs after remaining motionless in a sitting position for extended periods of time, causing swelling, edema, and pain. After taking

proanthocyanidins, the swelling (edema) was reportedly reduced and the varicose veins subsided. Through the use of proanthocyanidins and their protection of Vitamin C, the benefits of vitamin C can be more fully realized.

The second action of proanthocyanidins is their ability to bind with collagen. During the life of a body, the naturally flexible collagen in skin, veins, arteries, and capillaries, decreases. The flexibility of these tissues is imparted by proteins that form bridging tissues between collagen proteins known as connective tissue. When the skin is stretched, tissues deform. When the skin is released, the connective tissue pulls the skin collagen back into its original shape. Over time, however, these bridges are broken down by free-radicals. Hence, the ability of the tissues to retain its original shape after stretching is diminished. This manifests itself in the form of skin wrinkles, and in some forms of cardiovascular disease. By bonding with the collagen, the proanthocyanidins are able to reinforce or replace the connective tissues.

Briefly, proanthocyanidins are said to prevent heart disease. They are well known in this capacity as they are in high concentrations in red wine, which has a reputation for its cardiovascular health benefits. Anti-oxidants are being researched for their ability to prevent blood clots that form around sites of plaque accumulation in arteries (Masquelier, 1992). Such blood clots are known to cause strokes and heart attacks. Proanthocyanidins also help to give more flexibility to arteries, thus reducing the risk of hardening of the arteries and restores the impermeability of the cell walls in the pulmonary system. This is important as the blood leaking out of the veins at areas other than at capillaries leads to an increase in the hydrostatic pressure outside the pulmonary system, forcing the heart to work harder to generate more pressure to overcome the external

pressure. This is a vicious cycle as the greater pulmonary pressure increases the rate of blood leakage and subsequent external pressure. By creating an impermeable cardiac system, the pulmonary system can work more efficiently while providing the transfer of nutrients and wastes with less effort.

The health benefits of proanthocyanidins have also been seen in cosmetics and are accomplished both directly and indirectly. The skin is composed of the dermis and epidermis. The dermis is the underlying layer of skin where new skin is formed. Collagen, one of the most widely produced proteins, is found in especially high concentrations in the skin and also in other flexible tissues, such as the veins and arteries. In the skin, collagen is held together by elastin, a common protein in connective tissues. The connective tissue serves to maintain the skin's elasticity, strength and smoothness. The epidermis, the top layer of skin, contains no blood vessels and is nourished through the cells of the dermis by diffusion. Degrading enzymes and free-radicals destroy the cells of the dermis and epidermis. Proanthocyanidins were shown to prevent this, making skin appear smooth and wrinkle-free for longer periods (Masquelier *et al.*, 1981; Tixier *et al.*, 1984; Kuttan *et al.*, 1991; Kakegawa, 1985). Research showed that placing collagen fibers in the water containing proanthocyanidins caused them to shorten. In animal testing, this effect was found to shorten collagen-containing tissues up to 70 % in length.

The third and fourth major actions of proanthocyanidins in the human body, according to Passwater (1992), are their inhibition of the inflammatory enzyme and histamine formation, respectively. The free radical scavenging and protein binding properties of proanthocyanidins at the site of inflammation both removes free-radicals and some degrading enzymes which, when present, agitate the wound and increase the degree of inflammation. The Pycnogenol™ mixture

of oligomeric proanthocyanidins, acting in this fashion, was shown to lower the occurrence of stress related stomach and intestinal ulcers by as much as 82 % (Reimann *et al.*, 1977).

The Pycnogenol™ mix of oligomeric proanthocyanidins inhibits the enzyme histidine decarboxylase and thus serves to inhibit the formation of histamines. It also prevents histamine containing mast cells from degranulating and breaking, spilling histamines into the blood, which would cause inflammation and some other characteristic allergic responses (White, 1990; Kakegawa, 1985). The results are that allergic reactions, such as those caused by hay fever, are reduced.

A summary of recent medical research on proanthocyanidins follows. Blazso *et al.* (1994) found that the oligomeric procyanidins from *Pinus pinaster* bark increased pathological low vascular resistance in rats with spontaneous hypertension. Other researchers found that proanthocyanidins were effective against intestinal disorders (Giess *et al.*, 1995), HIV (Eberhardt and Young, 1995), intestinal toxins (Hör *et al.*, 1994), tumors (Gali *et al.*, 1994), and immunodeficiency disorders (Cheshier *et al.*, 1996). Pycnogenol™ has been shown to reduce sports injuries (Tixier *et al.*, 1984), reduce stress ulcers (Reimann *et al.*, 1977), and has been used to treat diabetic retinopathy (blindness) (Passwater, 1992).

Proanthocyanidins have been found to be very safe with an LD₅₀ reported at 3g / kg of body weight in animals (Passwater, 1992). However, other related research focusing on the toxicology of flavonoids measured the denaturation of DNA, caused by flavonoids with Cu(II). The enzyme S₁ nuclease was added to a DNA and Cu(II) containing solution and the denaturation of the DNA

in the presence of the flavonoids quercetin, myricetin or epicatechin was measured. Table 4 shows some of these results. Although proanthocyanidins were found to be good anti-oxidants *in vivo*, they were found to cause DNA mutations *in vitro*. The reason for this is not entirely clear.

Table 4. Comparison of S₁ nuclease hydrolysis following damage to DNA induced by flavonoids (Ahmad *et al.*, 1992).

Flavonoid	DNA hydrolyzed (%) ^a
Denatured DNA control	100
Native DNA control	2.8
Quercetin	49.5
Myricetin	55
Epicatechin	41.3

^aDNA hydrolysis (%) refers to loss of acid-precipitable DNA following treatment with S₁ nuclease.

It should be mentioned that the lack of methodology to properly characterize and standardize proanthocyanidins from different sources had a negative effect on the quality of related medical research. Some medical studies use the crude phenolic/proanthocyanidin plant extract without purification and some use a purified proanthocyanidin fraction. In other studies, an ion exchange column-purified isolate, extracted with hot water or acetone- or methanol-water, is used. In other cases, the commercially available Pycnogenol™ product (Horphag Research Inc., Switzerland) is employed. Few investigators have purified the individual procyanidin oligomers for their studies (Jankun, 1997).

2.6.2 Plants

Proanthocyanidins are found in virtually all plants. Their functions may vary, but generally they are fungi-toxic, anti-predatory, anti-nutritional and have been found in higher concentrations in some plants experiencing environmental stress (Butler, 1989; Walkinshaw, 1989; Chalker-Scott and Kraemer, 1989). As Figure 2 shows, the biosynthetic of proanthocyanidins shares part of the

same pathways associated with the production of stilbenes, isoflavones, and other anti-biotic compounds, as well as lignans and other structural units.

2.6.2.1 Function of Proanthocyanidins in Plants

The chemistry of proanthocyanidins, although not well understood, is far less complex than its bioactivity. Researchers have been able to determine some of the functions these compounds play in plants. Proanthocyanidins' ability to react with proteins, forming tannin-protein complexes, rapidly and reversibly, is the basis of the anti-nutritional character of some plants. When animals chew pasture forage (plants on which pasture animals graze), the mastication or chewing of plant tissues disrupts the cellular membranes, spilling the cell contents into the mouth of the pasture animal. Some forage plants contain slightly elevated amounts of proanthocyanidins, and it has been observed that pasture animals tend to avoid them (Feeny and Bostock, 1968). After the condensed tannins are released into the digestive track of the animal, they react and complex with proteins and other nutritious compounds from the plant, reducing their overall nutrient value. They also complex with the enzymes in the animals digestive track, slowing the rate of digestion and increasing the amount of energy required to digest the remaining components in the plants as new enzymes synthesized. (Waterman *et al.*, 1984). Because forage containing higher concentrations of proanthocyanidins is avoided by pasture animals, they have been given the classification of anti-predatory and anti-nutritional agents. Animals, though have seemingly evolved to overcome at least part of this problem. Tannins bind especially favorably with proline rich proteins. Saliva is especially rich in proline (Beart *et al.*, 1985; Rhaman and Richards, 1988).

The sapwood of trees, as well as many fruits and vegetables, contain phenolic compounds

impregnated in their cell walls. The phenolic rich boundaries act as resistant barriers against wounding and fungal infection (Ostrowsky *et al.*, 1984). Ostrowsky proposed that fungus-resistant beech tree bark has a higher phenolic content than a fungus susceptible tree. A correlation was observed between the tannin content and the natural resistance of trees to various forms of infection and biological attack. Other research showed that biological stresses such as cadmium toxicity, heat-shock, drought, and enhanced UV-irradiation increased the phenolic content in cells. This was thought to be because of the free-radical scavenging properties of the catechin-related oligomers that serve to stabilize cytoplasmic and other cell membranes (Chalker-Scott and Krahmer, 1989). The hypothesis of their function in plants being protectors seems to be supported by their location in plant tissues. However, in large scale replicate testing controlling for only proanthocyanidins must be made to ensure that observed effects are due to the action of proanthocyanidins alone.

2.6.2.2 Localization of proanthocyanidin in plants

Parasites have been observed to feed on the leaves, roots, and within the vacuoles of plants. Proanthocyanidins are anti-parasitic compounds and are present in higher concentrations in the leaves, root, and vacuoles as well. (Chalker-Scott, 1989). They are also found in higher concentration in the outer bark of trees in a non-water-soluble, high molecular weight form. The outer bark of trees is the primary layer of defense and protection against predators, parasites, bacterial attack, and fungi.

Polyphenolic polymers are synthesized and deposited in the bordered pits and parenchyma cells of conifers during the conversion of sapwood to heartwood (Samejima and Yoshimoto, 1982, Hergert, 1971). These polymers are comprised of a number of different compounds such as

polymers of proanthocyanidins, phlobaphenes, Brauns native lignins, and insoluble secondary lignins. In the parenchyma cells, the soluble proanthocyanidins in the sapwood are precipitated through the mixing of the proanthocyanidins with lignin (Hergert, 1989). A similar process occurs in the bark. Substantial amounts of polyphenols and proanthocyanidins are synthesized during the conversion of inner bark (phloem) to outer bark (rhytidome). Up to two to three times more solvent-insoluble proanthocyanidins and lignin-like polymers are found in the rhytidome. The structure of proanthocyanidins changes during the conversion of bark as stilbenes and flavones are incorporated into the polymers, largely at the terminal units. Hergert (1989) found that the periderm (or corky middle bark) has its own distinct set of phenolic polymers that may contain anthocyanidins and fatty acids. In most studies, the inner, outer, and middle barks are not separated because the bark boundaries are not well defined and such separation work is time-consuming. Therefore, experimental data do not always accurately reflect the heterogeneous nature of bark. When attempting to make assumptions regarding the quality of bark for occurrence of a particular chemical, more specific data are required. Figure 9 illustrates the distribution of polyphenols in hemlock wood and bark. In the diagram, proanthocyanidin oligomers would be listed under the designation "water-soluble polyphenols". The exact composition of the water-soluble phenols is not always well defined. Researchers, particularly those who are not phytochemists, have associated the water-soluble phenols with condensed tannins. Hergert (1989) found that although much of the inner hot water-soluble tannins found in hemlock are proanthocyanidin related, the outer bark hot water-soluble tannins are only partially made up of procyanidins. Figure 10 shows the molecular weight distribution of the hot water extracts from the inner and outer bark of red spruce. Much of the outer bark extract has a molecular weight too high to be oligomeric and, as would be expected, it was found (Hergert 1989) that the outer bark proanthocyanidin fraction contains stilbenes and related compounds as

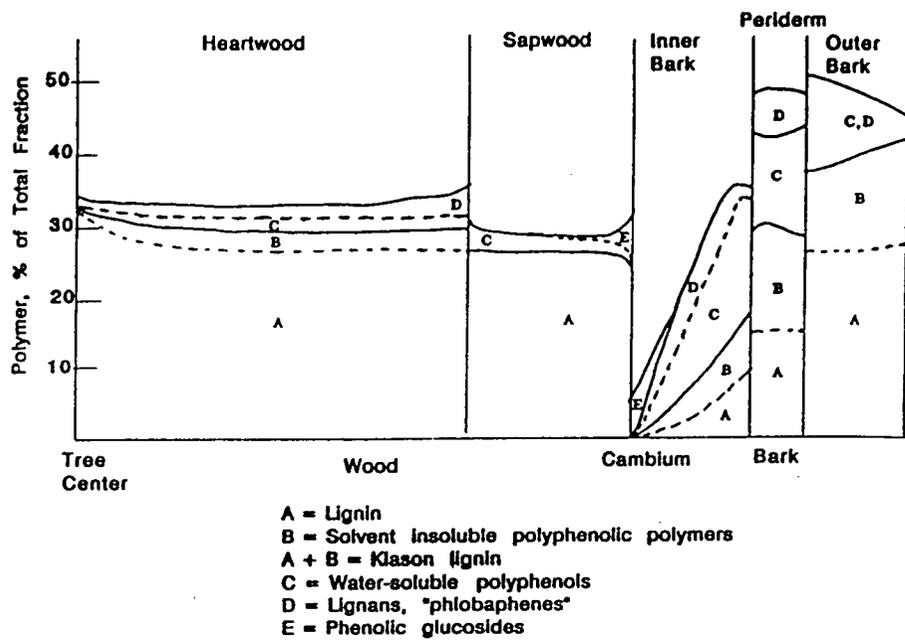


Figure 9. Distribution of phenolic polymers in Hemlock wood and bark (Hergert, 1989).

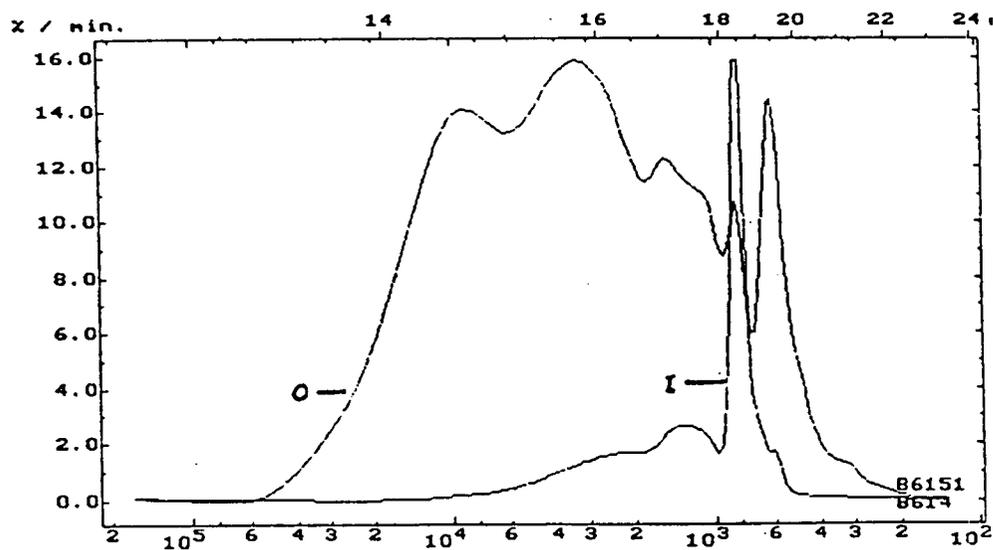


Figure 10. GPC of inner (I) and outer (O) bark 'tannins' of red spruce (Hergert, 1989).

well. Such observations are especially important and need to be taken into consideration when analyzing and quantifying proanthocyanidins from different sources.

2.7 QUANTIFICATION AND ANALYSIS OF PROANTHOCYANIDINS

The most important step in the analysis of proanthocyanidins is the preparation of the sample for extraction. These compounds, as can be seen from their diverse functionality in both plants and animals, are highly susceptible to chemical modification during storage and extraction. Hence, great care must be taken.

2.7.1 Sample Preparation

The greatest factor affecting the extractability of proanthocyanidins is the duration of storage. The longer the materials are stored, the greater the opportunity for degrading reactions to occur, but the effect is unpredictable. In some species, such as sorghum, the proanthocyanidin yield is observed to decrease by 15 % over only 18 days (Broadhurst and Jones, 1978). In freeze-dried oak leaves the proanthocyanidins decreased by 25 % over one year storage (Martin and Martin, 1982). Western hemlock bark proanthocyanidins decreased by one-third when stored in an industrial mill (Herrick, 1980). However, other research showed that room-temperature drying of sorghum grain (Butler, 1982) or Douglas-fir bark (Kurth and Chan, 1952) resulted in a 15 % increase in tannin content of the extract (it was not specified whether the tannin increase was due to a proanthocyanidin increase). High moisture content storage (Herrick, 1980) or high temperature drying (Bates-Smith, 1975) tends to lead to more pronounced losses in proanthocyanidin extractability.

In order to efficiently extract condensed tannins from tissues, they must first be reduced to small

particles to allow for sufficient access by the solvent. The particle size of the extracted tissue also has a large impact on extraction efficiency. In the case of sainfoin leaves, (Bates-Smith, 1973) fine milling to 100 mesh (0.150 mm) doubles tannin extractability. However, in comparing the extractability of dry beans at 20 and 60 mesh, a 25 - 38 % loss of extractability of tannins was associated with the smaller particle size (Deshpande *et al.*, 1986). It is expected that this is due to the high protein content in the beans.

High moisture content of the material makes grinding more difficult, and affects oligomeric proanthocyanidins recoverability. The moisture present during grinding facilitates many reactions, such as autoxidation, which occur more slowly in dry materials. The solvent's interaction with the plant material also becomes important during the extraction procedure itself for the same reasons.

2.7.2 Extraction Procedure

Although the hot-water extraction method is the standard extraction method, and water is a good solvent for the proanthocyanidins, it is not the best extracting solvent. Solvent-water combination is also known to break hydrogen bonds, hence, acetone and methanol-water mixtures were found to be more effective because they are capable of disrupting the fatty acids and protein-complexed condensed tannins (Scalbert 1992). Figure 11 illustrates some of the bonding mechanisms by which some proanthocyanidins are attached in plants. It is only by using a solvent, capable of breaking hydrogen bonds, that most of the extractable proanthocyanidins can be removed.

Many different extraction procedures have been used for the extraction of proanthocyanidins from tree bark and other plant tissues. Scalbert (1992) reviewed several solvents for their tannin extraction strength. Solvents such as acetone, methanol, isopropanol and dioxane, pure or in

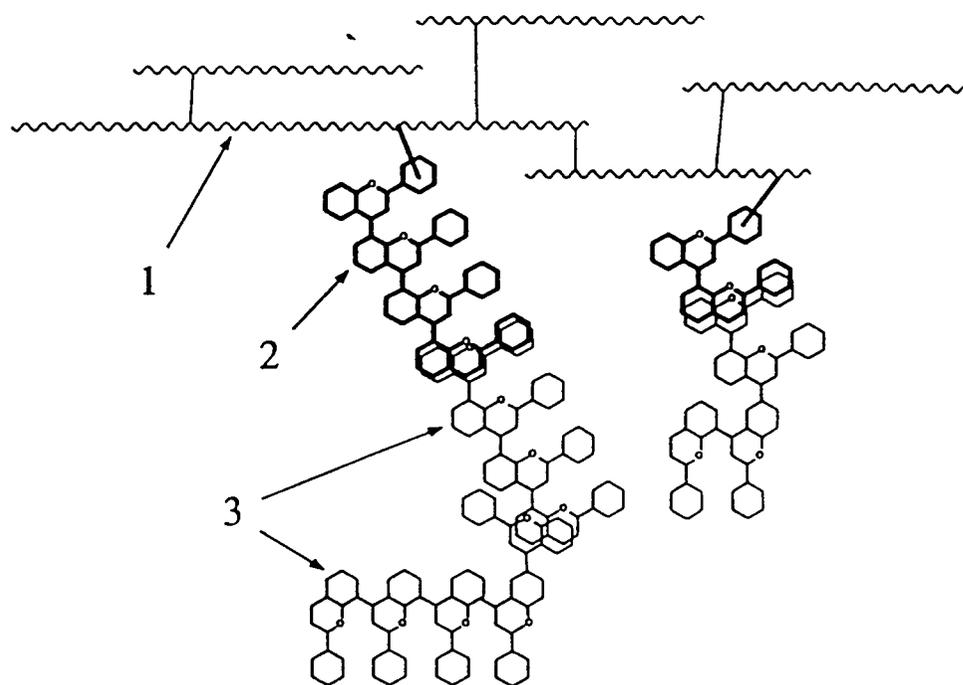


Figure 11. Direct linkage of proanthocyanidins with the cell matrix and proanthocyanidin-proanthocyanidin weak interactions in bark limits leaching by rainwater. (1) cell wall matrix; (2) non-extractable proanthocyanidin; (3) proanthocyanidin extractable with aqueous methanol or acetone, but not with water (Bailey *et al.*, 1994).

aqueous mixtures, were tested. He noted that room temperature extraction with aqueous acetone was the preferential solvent system for oligomeric condensed tannin extraction. Many proanthocyanidins are bound to either polysaccharides or lignins, making them insoluble. Such mixed proanthocyanidin complexes can make up a substantial portion of the bark. Stafford (1989) found that with methanol, only 70% of the tannins in the stem bark of an 80-year-old Douglas-fir were extractable.

In most of the literature, extraction times vary from 15 min to several hours, and the number of extractions varied from one to three. Most often, the extraction procedures are not concerned with quantitative extraction as the results are usually qualitative. Matthews *et al.* (1997) recently conducted a comprehensive quantification of the total proanthocyanidin content in the bark of several softwoods. The extraction procedure involved three separate extractions of 5 g of bark for a total extraction time of 2 h with a 50% aqueous-methanol solvent. Therefore, extraction procedure exceeding the extraction conditions in the literature could reasonably be considered as exhaustive extractions.

Some extraction methods include the use of methanolic - HCl (1% HCl in methanol) as an extraction solvent. This method employs the weak acid to hydrolyze the interflavonoid bonds of the insoluble proanthocyanidin polymers, cleaving them into soluble oligomers. Inter-flavonoid bonds of procyanidins should be labile at pH 3-4 at ambient temperature (Hemingway, 1989). However, in practice, other factors come into play. In sorghum grain varieties, the tannin extract yield was increased with this solvent (Hemingway and McGraw, 1983; Price *et al.*, 1979; Butler, 1982). However, in oak and maple leaves, the dissolved tannin yield was lower with acidified solvents (Hagerman, 1988).

2.7.3 Quantification Methods

The unique reactivity and chemical properties of proanthocyanidins have been exploited to develop three standard quantification methods: the Vanillin-Acid method, the Butanol-HCl (anthocyanidin) method, and the protein precipitation method. However, because these quantification methods are based on the reactivities of the individual oligomers and polymers, they only measure the chemical properties of proanthocyanidins in a cumulative fashion. These three methods will be discussed below.

2.7.3.1 Butanol hydrochloric acid (HCl) method

The Butanol-HCl method is often referred to as the anthocyanidin method, and in essence is an acid-catalyzed cleavage reaction. A small amount of tannin is dissolved in a mixture of 95:5 alcohol:HCl and is heated for 40 minutes at about 90°C. The acid cleaves the inter-flavonoid bond with the subsequent oxidation of the proanthocyanidins to anthocyanidins, as shown in Figure 12. This conversion is not quantitative, but is a good indicator for the presence of proanthocyanidins. The procedure has also been used for the determination of the proportions of propelargonidins, procyanidins, and prodelfinidins in an extract. After hydrolysis, the respective hydrolysis products are separable by paper or cellulose thin-layer chromatography using a Forestal solvent (acetic acid-water-concentrated HCl, 30:10:3, v/v/v). For comparing the relative yields of the hydrolysis products, the absorbency is measured at 550 nm. The spectra may also be recorded with the λ_{\max} in ethanol-HCl at 530 nm for pelargonidin, 545 nm for cyanidin, and 557 nm for delphinidin.

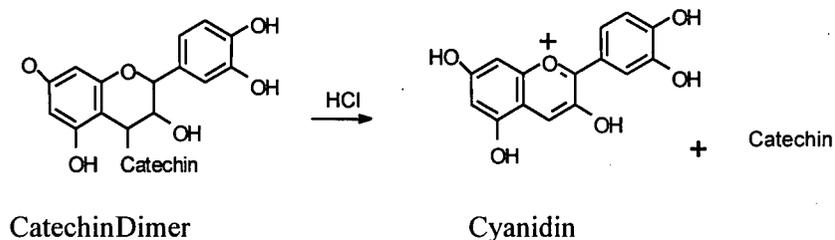


Figure 12. Conversion of a procyanidin to an anthocyanidin.

The conversion of proanthocyanidins to anthocyanidins is not quantitative because it does not go to completion. Porter (1986) used a 2 % ferric ion solution in methanol to catalyze the reaction, which drives the reaction further to completion and increases its reproducibility. In related research, Hemingway (1989) showed that the interflavan bonds between dimers are not equal. In comparing procyanidin dimers, it was found that the dimers with axial flavan units linked at C8 (i.e., 4 β →8) were more easily cleaved under thiolytic hydrolysis conditions than C6 (i.e., 4 β →6) linked flavan units. Therefore, acid hydrolysis of different tannin constituents would be expected to yield different quantitative results.

Although this method is suited for qualitative testing, because of the varied reactivity of proanthocyanidins found in nature, some researchers have used it as a quantification method with some success (Matthews *et al.*, 1997). Using known quantities of tannin, such as quebracho or sorghum tannin, the coloured hydrolyzates produced under identical conditions were used to construct a standard curve. Based on the spectrophotometric response of the tannin, a standard curve is constructed and compared with the unknown sample for quantification.

However, as Hemingway (1989) notes, some interflavonoid bonds are broken more easily than others. As quebracho tannins contain profisetinidin units, the polymers are less easily broken down by hydrolysis. Because sorghum tannins do not contain this unit, they are broken down more easily and give greater colour than quebracho tannins. The hydrolytic products of this reaction (as shown in Figure 12) are catechins for the bottom units, and anthocyanidins for the upper and extension units. Thus, the reaction for dimers has a 50 % maximum theoretical yield, whereas trimers have a maximum of 67 % yield, and decamers give a 90 % yield. Therefore, if the oligomer to polymer ratio of the proanthocyanidins in the standard and sample vary substantially, different calculated quantities would be observed. To further complicate the matter, the actual hydrolytic yield of a dimer is time-dependent. Also, solvent-tannin adducts and phlobaphene by-products, which absorb at 450 nm, have been observed to form and interfere with quantification (Swain and Hillis, 1959).

This method is only capable of roughly estimating the amount of tannin from one sample relative to the other. Without standards, the accuracy and precision of this method varies under different conditions. For these reasons, this method cannot be relied upon for comparative quantification of proanthocyanidins from different sources, which is the primary aim of this thesis.

2.7.3.2 Vanillin - Acid Method

The Vanillin-Acid method was designed specifically for the purpose of quantifying condensed tannins in plant extracts. In this reaction, shown in Figure 13, the proanthocyanidin polymer condenses with vanillin at the C6 or C8 position, changing the UV light absorption maximum from 280 nm, in the UV spectrum, to a deep red at approximately 500 nm. But again, this method measures more the reactivity of the tannin than its quantity, as the colour in this reaction

is “fugitive”. Quantification, by this method, requires that measurements are taken at certain reaction times, or that continuous colour measurement be made to capture the maximum colour intensity.

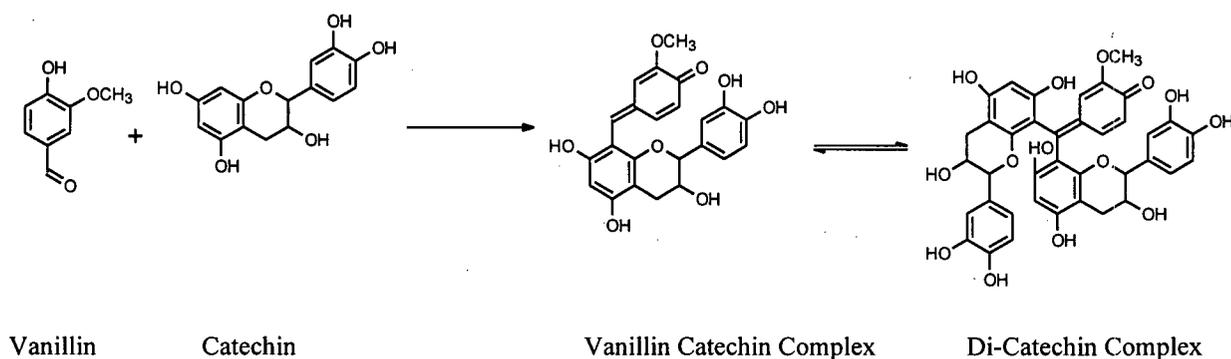


Figure 13. The reaction of vanillin and catechins.

This reaction is carried out at room temperature. A mixture of sulfuric acid and vanillin are added to a condensed tannin containing solution in a small vial. The vial is immediately placed in an ice bath to keep the reaction cool as the reaction rate increases with temperature. The reaction is allowed to continue for a 15 min in the dark, as it is light-sensitive, and is then placed into a spectrophotometer set to 500 nm. The reaction rates for monomers and the various oligomers are different so the reaction is not allowed to go to completion. Rather, the colour intensity is measured after a certain time, while the reaction proceeds.

Vanillin reacts with the phloroglucinol (*meta*-disubstituted) A-ring, and the number of monomer-vanillin adducts is measured by the intensity of the colour. The colour intensity can then be compared with the intensity of standard solutions containing known concentrations of catechin. The concentration of the solution is, therefore, measured in catechin equivalents. However, the

nature of the reaction is such that any compound with the appropriate A-ring configuration (not only proanthocyanidins) can react and contribute to the absorbency at 500 nm. Contrarily, some proanthocyanidins, such as those in quebracho tannins, have been shown to produce only weakly coloured adducts with vanillin as they have resorcinol like A-rings (mono-substituted A-rings). Further, the 'catechin equivalents' quantification units provide no information about the molecular weight of the proanthocyanidin polymers, or the number of molecules.

Although this method was designed specifically as a quantification method, Karchesy *et al.* (1989) note that it cannot be used as such because the reaction mechanisms and effects of different reaction conditions are not completely understood and are prone to cause variance in the results. Variations in fundamental reaction conditions, such as the amount of water in the reaction mixture, the mole fraction of proanthocyanidins in the polymer chains, and the age of the proanthocyanidin preparation all effect the yield of the reaction. Also, different colours of the adduct were noticed for different proanthocyanidins, further complicating this as a standard method of proanthocyanidin analysis.

Brunelle (1998) used a procedure slightly modified from one originally proposed by Broadhurst and Jones (1978) for the quantification of proanthocyanidins by the vanillin-acid method. The standard curve for this procedure, measured by UV absorptivity, increased linearly at low concentration until a maximum. At this concentration, the absorption rapidly dropped off and then increased linearly at lesser slope. Because of this, each sample must be tested at two different concentrations to ensure that the correct part of the standard curve is used. As Figure 13 shows, vanillin must be added in excess to prevent the di-catechin complex from forming.

2.7.3.3 Protein Precipitation

Hagerman (1987) outlines the use of protein precipitation for the quantification of proanthocyanidins. This method exploits the high affinity of tannins for proteins and involves the addition of a small quantity of a tannin containing solution onto a protein-containing agar slab. As the tannins diffuse into the gel, they complex with the proteins in the agar. Because the tannin-protein complexes are coloured, the quantity of complexed proteins can be measured by measuring the area of the diffusion. This method is simple as non-tannin phenolics and non-water-soluble compounds do not interfere with the procedure. Also, it does not require any complex reagents or instruments, and both aqueous and organic solvents may be used. However, the method does not differentiate between hydrolyzable and condensed tannins.

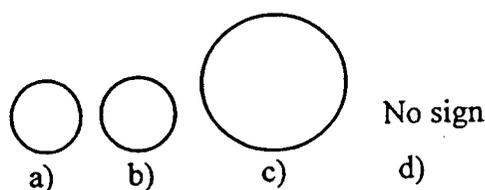


Figure 14. Radial diffusion assay for tannins. An 8- μ l aliquot of the sample dissolved in 70% acetone was placed in each well and photographed after the rings reached equilibrium. (A) 0.60 mg sorghum tannin; (B) 0.60 mg tannic acid; (C) 0.50 mg catechin plus 0.60 mg sorghum tannin; (D) 0.50 mg catechin (Hagerman, 1987).

Figure 14 illustrates how the results would appear and how they can be measured. While 0.60 mg tannin acid and 0.60 mg sorghum tannin result in the same area of complexed proteins (same diameter), a mixture of 0.50 mg catechin and 0.60 mg sorghum tannin complex has a radius of protein twice the diameter of the first two samples. Twice the diameter amounts to four times the area, suggesting that four times the amount of tannins have been complexed. However, a sample of 0.50 mg catechin failed to form a coloured precipitate with any proteins on the agar slab

(Hagerman 1987). This method seems to be too inaccurate and insensitive for the purposes of this study.

2.7.4 Chromatographic Analysis

Although not previously used for proanthocyanidin quantification, a method that has seen some success in the characterization of condensed tannins is high-pressure liquid chromatography (HPLC). Most often, a C-18 reverse phase or a styrene di-vinyl benzene column is used with an eluting solvent gradient of acidified water and either acetone, acetonitrile, or methanol. Most researchers customize their solvent gradient program to their column and solvents (Karchesy 1989; Karchesy *et al.*, 1989). A mostly aqueous eluent is used first with only a small amount of organic solvent. Although all oligomeric proanthocyanidins are water soluble, only the highly water soluble compounds such as gallic acid, catechin, epicatechin, other monomers and, more slowly, some dimers, can be eluted off of the column with pure water. The larger and more hydrophobic molecules adsorb onto the top of the non-polar column. As the eluent changes with the increased concentration of a less polar organic solvent, the medium size molecules are more favorably dissolved in the mobile phase and elute through the column. Factors, such as molecular stereochemical orientation, functional groups, polarity, hydrogen bonding capacity, size, and column temperature also play a part in determining the elution time of a chemical compound in water from a non-polar column.

2.7.4.1 Liquid Chromatography Column Theory

Because the solvent system used in the isolation of proanthocyanidins from plant tissues is capable of stripping a large variety and number of phenolic compounds from bark, many peaks are expected in the chromatogram. Proanthocyanidin oligomers are structurally similar and close

in molecular weight, so, it can be expected that resolution during the investigation will become an issue. Resolution is the separation of two chemically similar compound peaks after passing through a column, and is based on the efficiency and selectivity of the column. The selectivity is primarily a property of the chemical properties of the column and its ability to separate compounds based on their chemistry. Different proanthocyanidin constituents (monomers, dimers, trimers,...) have different polarities. Differences between the monomeric units of the oligomers also effect the chemical structure, polarity, solubility and other properties of these molecules, which change their retention times. The efficiency of the column is the ability of the column to provide well-defined peaks. This can be fine-tuned by adding buffers to the solvent system and, within limits, by adjusting the flow rates and the column temperature during analysis. Many solvent systems for proanthocyanidins include a small quantity of a weak acid, to help keep the peaks well defined and sharp.

There are four primary factors that govern the overall band broadening of a sample as it travels through the column. Plate height theory relates the bandwidth of an eluting sample to the temperature, flow rate, viscosity and physical and chemical properties of the solvent. Equation [12] illustrates the relationship.

$$H = H_p + H_d + H_s + H_m \quad [12]$$

H represents the actual band broadening of a sample travelling though a column. H_p is the broadening which occurs by having multiple and non-equal solvent paths through the packing. This happens most often in old or often used columns. The frequent use and resultant fluctuations of high and low pressure, during use and storage, form channels or pockets in the

column packing. Pockets are areas in which the solvent eddies and delays its conductance through the column. Channeling is caused by the solvent finding a path of lesser resistance to travel through the column more rapidly without interacting fully with the column packing. Over time, small amounts of degradation of the column may facilitate the formation of non-uniform areas in the column that may also lead to the formation of pockets and channels.

H_d represents the longitudinal molecular diffusion along the column by a solute. This form of diffusion is normal as a solute proceeds from high to low concentration. Typically, because of the very low amount of solvent in the column and the relatively short retention time, this factor is not a major contributor to band broadening.

H_s represents a cause of band broadening through interactions between the solute and the stationary phase. It is this interaction of the particles which is attributed to the selectivity of the column. The degree of this interaction determines which compounds elute from the column first. However, in strong interactions the usual interaction between the solute and packing is replaced by a mass transfer effect. Under these circumstances, the solute is taken out of the mobile phase for a short time. The length of time the solute remains bound to the packing, while other solute particles are allowed to proceed, is a measure of the degree of band broadening which is observed.

The single largest factor to contribute to band broadening in the column is the factor H_m . The H_m value represents the broadening caused by the flow of the mobile phase through cracks and fissures in the column packing. Unlike H_p , which is attributed to the three dimensional distribution of the column packing particles within the column, H_m is attributed to the three-

dimensional shape and surface of the individual particles. Imperfect particles can have uneven, rough surfaces, which are disrupted with fissures. In these fissures or around the rough parts on the surface, the solvent flow is slow or can be made to eddy. These delays cause band broadening. The larger the fissure, the longer the solvent will be retained. This factor accounts for 30 - 70 % of the band broadening. To counteract this factor, column manufacturers now produce packing materials as small as 3 μm with uniform and smooth spherical shapes. However, the column used in this research has a 5 μm packing size and uses earlier spherical particle manufacture technology. Although the pore size is 100 \AA , greater pore size variations are common in older packing materials.

2.7.4.2 Detectors

2.7.4.2.1 UV/Vis Spectrophotometers

Two primary methods of detection have been used for proanthocyanidins, UV/Vis spectrophotometry and mass spectrometry. As these compounds absorb UV light at 280 nm, the simplest method of detection has been by monochromatic UV detection, set at this wavelength. Modern methods now employ photodiode array spectrophotometers to measure the UV absorption of the compounds, over a range of wavelengths, from 200 nm to 450 nm. This facilitates the purity of a compound to be checked and allows for better identification to be made. Procyanidins absorb at 280 nm, taxifolin at 289 nm, and gallic acid at 270 nm. If a peak elutes at the retention time of the expected compound, but does not have the same absorption spectrum, the peak is some other compound or is impure.

Bartolomé *et al.*, (1996) have gone one step further in attempting to identify unknown procyanidin peaks in extracts. All procyanidin compounds measured display a peak maximum at

278.9 nm (except the gallated compounds, which vary slightly). By taking the second derivative of the photodiode array (PDA) spectrum, from at least 250 nm to 300 nm, and by measuring the distance in nanometers between the minimum and the maximum absorption, it was found that the monomers, dimers, trimers, and tetramers displayed unique distances. By cross-referencing this to the second derivative maximum (nm) and the convexity interval (the same measurement but on the first derivative of the PDA spectrum), good estimations for the identity of the peak could be made. This method appears to be useful in circumstances where standards are not available, but require these values to be determined beforehand with specialized data acquisition software, and presumes that the sample is free of impurities and overlapping peaks.

2.7.4.2.2 Thermal-Spray Mass Spectrometry

Thermal-spray mass spectrometry detection works on a different premise. Instead of connecting the column end to the UV detector, it is connected to a 30 cm long, electrically heated tube. As the solvent is passed through the tube, it begins to vaporize as shown in Figure 15. During the vaporization stage the solvent and solute are ionized and nebulized. Gas-phase ions are emitted in the form of a supersonic vapor jet of fine droplets or solid particles, into the mass analyzer, which is kept under high vacuum. This type of mass spectrometry is unique as it allows up to 1 mL/min to be injected into the mass analyzer. The sample size is 10 times the capacity allowed in other mass spectrometer methods.

Thermal spray MS requires that the column eluent be at least 30% aqueous buffer. This is necessary as the electronic dissociation of water and the protons and electrons provided by the acid buffer (such as 2.5 % acetic acid in water) serve to ionize the sample during heating in the vaporizer.

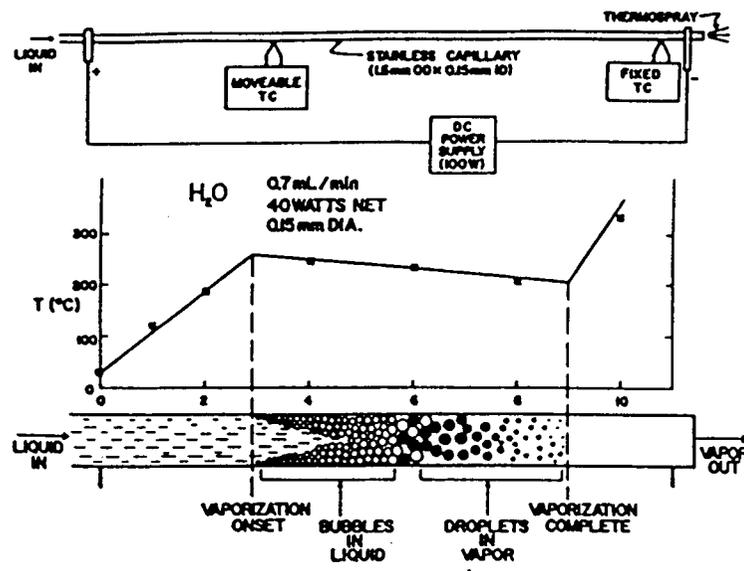


Figure 15. Comparison between the temperature and vaporization stages in the vaporizer (Yergey, 1990).

It is critical that the energy supplied to the vaporizer be controlled accurately. If the energy supplied to the vaporizer probe is too great, the vapor emitted will be prematurely vaporized and superheated. If the energy supplied to the probe is insufficient to vaporize the solvent, the liquid will not be fully vaporized and droplets will be entrained in the vapor jet. Both conditions reduce the sensitivity of the detector. This problem is accentuated when using a non-isocratic solvent program, as required for the analysis of proanthocyanidins. The variation in the solvent composition in a gradient program can have a large effect on the vaporization temperature of the solvent. For a solvent system where the composition is varied from 3% acetonitrile in 97% aqueous buffer (2.5% acetic acid in water), to 80% acetonitrile in 20% aqueous buffer, the vaporization temperature changes from 210°C to 185°C. Most instruments are not capable of changing the temperature of the probe automatically, making the analysis of proanthocyanidins

more difficult, and this limits the efficacy of the procedure.

The mass analyzer works on the premise that by altering the magnetic field in a constant electron potential, or by altering the electric potential under a constant magnetic field, ions with only a specific mass to charge ratio will be focused onto the detector. By varying either the magnetic field or electric potential, as specified, a larger range of ions can be detected and their mass can be determined.

2.8 SUMMARY

In conclusion, the proanthocyanidins that form the major component of commercial oligomeric proanthocyanidins are highly variable in composition. Various amounts of monomeric building units such as catechin and epicatechin, oligomeric building blocks such as dimers, trimers, tetramers and higher oligomers, and polymeric building blocks of high molecular weight condensed units are often referred to simply as condensed tannins (proanthocyanidins). Characterization of proanthocyanidins is constrained through their variability between species and parts of plants (e.g. inner and outer bark). Further, knowledge as to the exact composition and structure of these various forms of proanthocyanidins is scanty and incomplete at best.

Quantification of proanthocyanidins in various plants and health products is further hampered by variable response and reactivity of monomers and polymers in isolated products. Extracts (aqueous or solvent) of plants normally contain a variable range of molecular weight fractions. No pure standards for individual constituents exist, except the monomers, which appear to be only a minor fraction of the extractable proanthocyanidins from the bark. Although variability in the oligomeric composition of proanthocyanidins is well known from HPLC chromatograms

(Appendix A), no attempt has been made in the past to use all the information contained in such chromatograms for summary quantification of Pycnogenol. It must be assumed, therefore, that the proanthocyanidin content of the various oligomeric proanthocyanidin preparations, commercially available today, is based on gravimetric information (weight of the purified extract) or anthocyanidin assay alone rather than on the actual chromatographically identifiable amounts of the oligomeric components. It is therefore, not surprising that nearly all preparations guarantee proanthocyanidin contents as mg OPC / tablet (i.e. 25, 50, or 75 mg per tablet) without characterizing the constituents in the gravimetrically determined extracts. Guaranteed reproducibility in the extract's chemical antioxidant properties can only be ensured through characterization of the contents. Some products are quantified in undefined units of "activity". Presumably, activity may refer to the compound's antioxidant power (potency). However, units of 'activity' may not be sufficient for quantification of the proanthocyanidin content of various bark extracts (the primary objective of this thesis) and should not be sufficiently descriptive for industrial assays. Only observing the occurrence of specific monomers and oligomers can provide accurate quantification data which would ensure chemically reproducible products.

Based on these considerations the objective of the study had to be modified from one of studying the quantification of the extractable proanthocyanidin contents of selected western softwood barks to development of a truly quantitative process capable of encompassing all forms of oligomeric, water- and solvent-soluble proanthocyanidins. Since no suitable proanthocyanidin model compounds are available for the construction of calibration curves, as a compromise, a calibration curve was constructed by using a commercial product (Indena LeucoSelect™) which when dissolved in water, and subjected to UV spectrophotometry, follows Beer's Law. Additionally, this product had to contain all molecular components identifiable on HPLC

chromatograms of natural extracts. The procedure for development of the method, whereby direct comparison of various Pycnogenol sources becomes possible, is described in the following sections.

The reactivity of proanthocyanidins is sufficiently high, to consider them as unstable compounds under even mild, ambient conditions. Hence, these compounds do not store well and are subject to physical and chemical modification during sample storage, preparation, and extraction. It is preferred that samples be extracted as soon as possible after collection. If it is necessary that the samples be stored before use, they should be frozen or, preferably, freeze-dried to preserve their chemical and physical properties.

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Bark

Bark samples were taken from a number of sources, depending on availability (Table 5). Lodgepole pine, Western hemlock, and one sample of Amabilis fir bark samples were removed from freshly felled trees in a log-sort yard near Quesnel, British Columbia. Douglas-fir, and Sitka spruce, and a second sample of Amabilis fir barks were taken from a fresh debarker residue pile from trees harvested in the Mission area. A second sample of Sitka spruce bark was removed from trees scheduled to be felled near Port McNeill, Vancouver Island. One sample of Caribbean pine was imported from Venezuela. The bark samples were either stored in sealed bags in a freezer, or were stored for several months on the bench top until they were prepared for extraction. Frozen samples from the freezer were allowed to thaw and dry for three to four days. Before the bark was completely dried, the inner and outer barks were separated with a knife. Any inner bark that appeared to have black staining due to fungal attack was cut away and discarded. The separated bark samples were dried until hard, and then ground to 40 mesh (0.425 mm) in a large Wiley mill. Table 5 lists the bark samples, their sources, and their storage methods and duration.

Table 5. Bark Sample Locations

Species	Location	Storage Method	Storage Duration
Amabilis Fir (<i>Abies amabilis</i> Dougl.)	Quesnel, B.C.	Bench Top	6 months
Amabilis Fir	Mission, B.C.	Bench Top	2 months
Caribbean Pine (<i>Pinus caribbea</i>)	Venezuela	Bench Top	3 weeks
Douglas Fir (<i>Pseudotsuga menziesii</i> Franco.)	Mission, B.C.	Bench Top	1 week
Lodgepole Pine (<i>Pinus contorta</i> Dougl.)	Quesnel, B.C.	Frozen	6 months
Western Hemlock (<i>Tsuga heterophylla</i> Sarg.)	Quesnel, B.C.	Frozen	6 months
Sitka Spruce (<i>Picea sitchensis</i> Carr.)	Port Hardy, B.C.	Frozen	2 months
Sitka Spruce	Mission, B.C.	Bench Top	6 months

3.1.2 Commercial Pycnogenol Samples

Three oligomeric proanthocyanidin products were purchased from Capers dietary health store in Vancouver.

- A) Capers Naturals, 'Grape Seed Extract'. 60 capsules of at least 50 mg of 95% pure leucoanthocyanins. Encapsulated in a gelatin capsule with rice protein. Recommended dosage: 1-2 capsules daily or as directed by physician. Precautions: Keep out of reach of children. Fillers: unknown (probably micro crystalline cellulose).
- B) Sun Force 'Pycnogenol™', pine bark extract. 60 tablets 25 mg. Recommended dosage: 2-6 tablets daily. Children: 1-2 tablets daily. Precautions: none. Fillers: dicalcium phosphate, microcrystalline cellulose, and magnesium stearate.
- C) Nu-Greens 'Prolong'. 60 capsules of 75 mg grape seed extract, 15 µg resveratrol.
 Proanthocyanidins 95% activity
 polyphenols minimum. 46% activity
 anthocyanidins minimum. 30% activity
 catechins minimum. 18% activity
 Lipoic Acid 12.5 mg
 Beta Carotene (Natural carotenoids) 1500 I.U.
 Recommended Dosage: 1 capsule with breakfast and 1 with dinner.
 Precautions: none. Filler: Rice protein.

A fourth sample of Pycnogenol was obtained from Indena (Italy) under the trade name

LeucoSelect™:

Batch #:	26127/M11
Manufacturing date:	11/1997
Contents:	100.9% LeucoSelect™
Insoluble substances (in water):	1.1%
Water (K. Fischer)	4.0%
Ethanol	8.0 ppm
Ethyl Acetate	<7.4 ppm
Acetone	9.0 ppm
Methylene chloride	< 4.4 ppm
n-Butanol	<15.0 ppm

Authentic samples of catechin, epicatechin, and taxifolin, and the procyanidin samples B-1, B-2, B-3, B-4, and B-7 were provided by Dr. J. Karchesy at Oregon State University.

3.2 METHODS OF SAMPLE PREPARATION AND EXTRACTION

3.2.1 Bark Preparation and Extraction Procedure

A method for the extraction of proanthocyanidins from the bark was derived from the literature. Longer extraction times and greater solvent to bark ratios were used than any method cited in the literature. This was to ensure that the proanthocyanidins would be quantitatively extracted and that the solvent properties, not the solubility product constant or time, would be the limiting factor in the extraction.

Approximately 200 g of bark was used for each extraction to provide for a more uniform sample and prevent small irregularities, such as resin pockets, from overly influencing the sample. The bark samples were added into 3 or 4 L tared Erlenmeyer flasks and cold 70:30 acetone-water solvent was added. Enough solvent was initially added so that the bark samples would be freely suspended in the solvent. This often required the addition of more than 1 L of solvent for the

outer bark samples as they, generally, contain more corky tissue which soaks up more solvent. The solutions were left for 4 h and periodically agitated. The solvent was then decanted and filtered. An additional 400 mL of solvent was added, and the solution was allowed to sit again for 4 h. The solvent was again decanted and filtered. In order to collect at least 500 mL of extract from 200 g of bark, enough solvent was then added to the solution to make a total of 500 mL of collected solvent. The solvent was left in the bark suspension overnight and was then decanted and filtered. A further 100 mL of solvent was added to rinse the bark sample of any remaining proanthocyanidin loaded solvent. This solvent was also collected and filtered.

The collected extracts were rotary evaporated to remove the acetone. Some samples formed soapy bubbles during the rotary evaporation, which were difficult to disrupt. Slow and tedious rotary evaporation with constant reduction of suction, when the bubbles became too large, was the only way of dealing with these bubbles.

The evaporation of acetone caused the precipitation and conglomeration of non-water solubles. This served to separate the fatty acids, lignans, and other non-water soluble extractives from the water soluble phenols. The non-water solubles were scraped from the bottom of the flask and added to the aqueous extract. The residual non-water solubles remaining in the round bottom flask were dissolved in methanol and poured into 30 mL vials. The methanol was evaporated in a low pressure rotary concentrator. This material was also added into the aqueous extract to keep the volumes constant. The total volume was made up to approximately 500 mL.

The aqueous extracts formed precipitates over time. In one case, the precipitate formed up to one fifth of the extract volume. From this extract, 5 mL was filtered through a 0.45 μm filter and

sealed in a vial until testing. After filtering, most samples remained precipitate-free for over two months, and many samples did not form precipitates even in this time.

3.2.2 Pycnogenol Preparation and Extraction Procedure

In order to quantify oligomeric proanthocyanidins, a measurable scientific definition had to be determined. Three different brands of commercially produced Pycnogenols were purchased from Capers, a natural food store located in Vancouver, B.C. The three representative brands were Capers brand (grape extract), SunForce (pine bark extract), and Nu-Greens (grape extract). These tablets or capsules were tested in quantities of approximately 100 mg of Pycnogenol in 10 mL of purified, HPLC grade water. Oligomeric proanthocyanidin material sold in capsule form was removed from the capsules as they may have contained rice protein, to which procyanidins readily bind. Proanthocyanidins were then extracted from the tablets and powders with pure water for at least 8 h on a shaker.

3.3 METHOD OF ANALYSIS

3.3.1 Spiking

In order to confirm the identity of the catechin, epicatechin and taxifolin peaks, all extracts were spiked with 100 μ L of a standardized solution as shown in Table 6.

Table 6. Spiking Solution Concentrations

Compound	Concentration	Ratio
Catechin	50.9 mg / 50.00 mL H ₂ O	1
Epicatechin	48.2 mg / 50.00 mL H ₂ O	1
Taxifolin	48.1 mg / 100.00 mL 7:3 H ₂ O:MeOH	1

3.3.2. Internal Standard

In attempting to find an internal standard for the analysis of Pycnogenols, several compounds were tested to determine their absorbency and to determine whether their retention times interfered with, or matched, those of the proanthocyanidins. Rutin, chalcone, norepinephrine, and catechol were tested as internal standards. Although all were found to have absorption maxima at 280 nm, all except norepinephrine, were sufficiently hydrophobic to be eluted in the range of the proanthocyanidins, and thus interfered with the analyses. Norepinephrine was more water-soluble and eluted well before any proanthocyanidin peaks. One hundred mL of a 1 g/mL aqueous solution of norepinephrine internal standard was added to every sample.

In order to determine if any interaction occurred between the internal standard and the aqueous bark extracts, an aqueous sample of Douglas-fir (*Pseudotsuga menziesii*) inner bark extract was combined with four different concentrations of the internal standard. The proportion of bark extract was kept constant and the Douglas-fir peak profiles were compared at the four different norepinephrine concentrations. Almost no difference in the HPLC profile between the four different internal standard concentrations was found. It was inferred from this that the internal standard did not interfere with or react with the extract, and norepinephrine could be used as the internal standard. These results are shown in Appendix B.

3.3.3 High performance liquid chromatography (HPLC)

3.3.3.1 Instrumentation

The initial HPLC methods development and proanthocyanidin sample analysis was conducted in the laboratory of Dr. J. Karchesy in the Department of Forest Products at Oregon State

University. The HPLC instrument, manufactured by Waters, was computer interfaced and consisted of two gradient pumps, a manual injector and a monochromatic UV detector set at 280nm.

The HPLC system used for later testing was located in the laboratory of Dr. Towers in the Department of Botany at the University of British Columbia. It was also manufactured by Waters, and consisted of a fully computer-interfaced system consisting of two gradient pumps, an auto-injector and a photodiode array spectrophotometer set from 190 nm to 450 nm.

The initial investigations carried out at OSU involved the development and optimization of the extraction procedure and the HPLC analytical method. The bark obtained from various sources in British Columbia or Venezuela were prepared, extracted, and tested by HPLC and small quantities of extract were taken back to UBC for additional analysis. These samples were analyzed under the identical solvent program using a photodiode array spectrophotometer (PDA). The PDA detector helped confirm the identity and purity of the flavonoid peaks.

3.3.3.2 HPLC Column

A PLRP-S column, similar to one used by Bailey *et al.* (1994), was selected for this research. Bailey used this column in the investigation of proanthocyanidins and theafulvins from tea, wine, and cider because of its versatility, and selectivity.

The PLRP-S column (polystyrene divinyl-benzene copolymer) was designed for reversed phase HPLC and has a pore size of 100 Å and an average particle size of 5 µm. It is suitable for use in the pH range from 1 to 13. The non-polar stationary phase is in gel form, and has a recommended

maximum pressure of 2000 psi, limiting the flow rate with an acetone-water-acetic acid solvent system to 1 mL/min.

3.3.3.3 Solvent Program

The solvents used in these investigations are also the same as those in Bailey *et al.* (1994). The solvent program, shown in Table 7, was partially based on literature methods, but had to be optimized at OSU.

Table 7. HPLC solvent system

Time	Flow Rate (mL/min)	%A (acetonitrile)	%B (2.5% acetic acid in water)	Curve
0	1	3	97	
5	1	6	94	5
10	1	10	90	6
40	1	25	75	5
45	1	80	20	6
50	1	80	20	6
55	1	3	97	6

3.3.4 Mass Spectrometry Conditions:

Analytical Software: MSS Data System

HPLC: Waters 510 pump

Mass Spectrometer: Brand: Kratos MS-80 RFA

Conditions: Block source temp 200°C

Scan rate 3 seconds per decade

Probe and Vaporizer Temp: 3% acetonitrile - 210°C
80% acetonitrile - 185°C

3.4 QUANTIFICATION METHOD

HPLC data was acquired from the PDA spectrophotometer at 280 nm. Peaks were identified by retention time, ratio of retention times (by use of a peak retention template), spiking, HPLC/MS, and by UV absorbency spectrum between 190 nm and 450 nm.

Once a peak could be positively identified as one of the 10 signature peaks (discussed in Section 4.1.3) to be used for proanthocyanidin quantification by the above methods, its area was measured and cumulatively added to the other positively identified signature peaks to obtain a composite area value. This value was divided by the area of the internal standard peak to account for the non-reproducibility in the injection volumes. This ratio was recorded and was directly compared with the standard curve. The dilution factors were calculated, and the mass of the oligomeric proanthocyanidins (mg) per gram dry bark was reported. A sample calculation is shown in Appendix C.

4.0 RESULTS AND DISCUSSION

4.1 METHOD DEVELOPMENT

4.1.1 Extraction Methodology

The method for the extraction of proanthocyanidins from bark was designed after comparing a number of literature models. The method used was designed to exceed other designs in extraction time and solvent to bark ratio. This was to ensure that proanthocyanidins would be quantitatively extracted from the bark, and that the solvent properties, not the solubility product constant, nor extraction time, would limit the amount of proanthocyanidins extracted.

Acetone-water (70:30), is commonly cited in the literature (Scalbert, 1992; Souquet *et al.*, 1996; Bae *et al.*, 1994) as the solvent of choice. It is known for its hydrogen bond disrupting properties and its capacity to strip glycosides and protein bound proanthocyanidins from cellulose, lignin, and the extractive matrix found in the bark (Scalbert, 1992). Acetone, being less polar than water, serves to dissolve waxes and the more hydrophobic compounds that may block regular solvent access to the proanthocyanidins.

4.1.2 Peak Identity Confirmation

In the quantification of any complex material by chromatography, peak identity in chromatograms is a primary requirement. Sample spiking is the most convenient method of determining the identity of HPLC chromatogram peaks. For this research, however, only catechin, epicatechin, and taxifolin were available as standards to spike the bark extracts, bulk quantities of oligomeric proanthocyanidin model compounds are not available at the present time. HPLC-mass spectrometry (MS) was selected as an additional means of identifying flavonoid-based peaks. Catechin and epicatechin are present as basic building blocks in all bark

extracts whereas taxifolin is not a basic building block, but a precursor to the leucoanthocyanidin (Figure 2).

The HPLC-MS studies were made to re-confirm the identity of the peaks designated as the Pycnogenol signature peaks in the formation of a template (described in Section 4.1.3). Even though retention times and UV/Vis spectrophotometry could be used to confirm the identities of most peaks, there was some question as to the identity of the peak that eluted at 27 min. UV/Vis spectra showed that this peak in grape-seeds was likely a flavonoid due to its absorption at 280 nm, but was most likely taxifolin in the bark extracts, as it absorbed at 289 nm and co-eluted with taxifolin upon spiking. More information was required to determine if these peaks were pure or if they represented a mixture.

It was thought that mass spectrometry could be used to clarify this ambiguity and confirm the identity of the 27 min peak as well as other unidentified peaks. The investigation was technically limited because the MS-80 HPLC-MS in the UBC Department of Chemistry is equipped with a continuously variable vaporizer probe temperature controller and has only 4 settings. Therefore, as the solvent composition changed during analysis, as programmed by the gradient program, so does the solvent vaporization temperature. For this reason, only the initial major peaks, up to about 19 min, were detectable, but only qualitatively. Information about all the extract components throughout the whole range of analysis could only be roughly estimated. The HPLC/MS data is shown in Appendix D and is explained in the following sections.

4.1.2.1 Indena sample MS-results

The commercial Indena LeucoSelect™ product consistently met the requirements of a model compound. It proved to be usable in constructing a calibration curve for the analysis, in which an increasing amount of LeucoSelect™ was dissolved in a known quantity of water. Further, the HPLC chromatogram of this model compound contained all of the signature peaks that occur in the proanthocyanidin extracts obtained from the various bark species.

One of the major contributions from the commercial Indena LeucoSelect™ product mass spectrometry (MS) investigation was the positive identification of the peak at 4.45 min in the HPLC spectrum. After comparing the MS decomposition pattern to a library of compound decomposition patterns, available at the UBC MS laboratory, the peak was identified as gallic acid.

Catechin was both found to have major decomposition fragments with mass to charge ratios of 291 and 139. By using the data analyzer to show only peaks with these two mass to charge ratios, a chromatogram based on flavonoid peaks was obtained. By overlaying the spectrum of the total extract with the spectra of the peaks with mass charge ratios of 291 and 139, for catechin and its fragment, it could be seen that 8 Pycnogenol signature peaks were represented in this mass spectrum (Appendix D). The two unrepresented peaks were likely in too low concentration to be detected by the MS detector given the limitations of the vaporizer.

4.1.2.2 Douglas-Fir Inner Bark Sample

The data obtained for Douglas-fir showed that the decomposition product of catechin and epicatechin consisted predominantly of fragments with a mass charge ratio of 291 and 139.

Taxifolin has a predominant mass charge ratio of 304. Based on these observations, it was seen that the Douglas-fir extract contained taxifolin and the grape seed extract did not. This meant that the 27 min peak, present in both the grape and Douglas-fir samples, could not be used in the quantification of Pycnogenol because of its ambiguous identification.

4.1.3 Chemical Definition of Pycnogenol™ and Construction of a Pycnogenol HPLC Template

Once the extraction procedure and HPLC method of analysis for proanthocyanidins had been determined and the preliminary data analyzed, several observations were made. The HPLC solvent mixture program was designed to sequentially elute the lower oligomeric proanthocyanidins from the column in less than one hour and was optimized for peak separation. The standards for taxifolin, catechin, epicatechin, procyanidin B-1, B-2, B-3, B-4, and B-7 all eluted with a time frame between 15 and 30 min. Also, two large unidentified peaks were present at approximately 4.5 min and 45 min. These peaks had varying intensities in different samples. The peak at 4.5 min appeared to be a single compound, whereas the peak at 45 min appeared to be a mixture of compounds all eluting concurrently as the solvent was ramped to 80 % acetonitrile at the end of the gradient program. At this time during the run, the acetone content in the solvent was increased from 30 % to 80 % to wash the column clean of high molecular weight compounds.

After the three commercial products were run under the optimized HPLC conditions, a spiking solution containing catechin, epicatechin, and taxifolin was added and the samples were re-run to confirm the presence of these compounds in the extracts. After these peaks were identified on the chromatograms, the three commercial product chromatograms could be overlaid (Appendix E).

Overlaying the spiked peaks corrected for any differences in injection delays that made overlaying chromatograms, based on retention time alone, impossible.

When overlaying the chromatograms of the three commercial products, based on the spiked peaks, eleven recurring peaks of varying intensity were observed in the range from 10 to 40 min. The peak at 27 min could not be unambiguously identified by HPLC/MS (Section 4.1.2.2), and therefore could not be included as a signature peak. Of the remaining ten peaks, seven were identified by corresponding authentic standard retention times, spiking, UV/Vis absorption maxima, and mass spectrometry as: catechin, epicatechin, and the procyanidin dimers B-1, B-2, B-3, B-4, and B-7. The other three peaks are likely proanthocyanidins in nature as the mass spectrometer showed signals at the location of these peaks that corresponded to catechinic fragments showing that they were likely flavonoid based. These peaks were designated as the 10 signature Pycnogenol peaks, and are represented in the retention time template (Figure 16). The signature peaks were identified by UV/Vis spectrophotometry and by the presence of the catechin fragment. This suggests that since all of the compounds are procyanidin in nature, it is justifiable to use the combined peak areas as a reasonable measure of the oligomeric proanthocyanidin content in the various samples of interest.

The gallic acid peak was not included as a Pycnogenol™ signature peak because no evidence in the literature indicates the efficacy of gallic acid, either alone or in synergy with procyanidins. Therefore, variation in the gallic acid content could unduly affect calculated amounts of procyanidins in extracts, thereby seriously affecting the determination of the suitability of an extract as a potential source of a Pycnogenol™-like product.

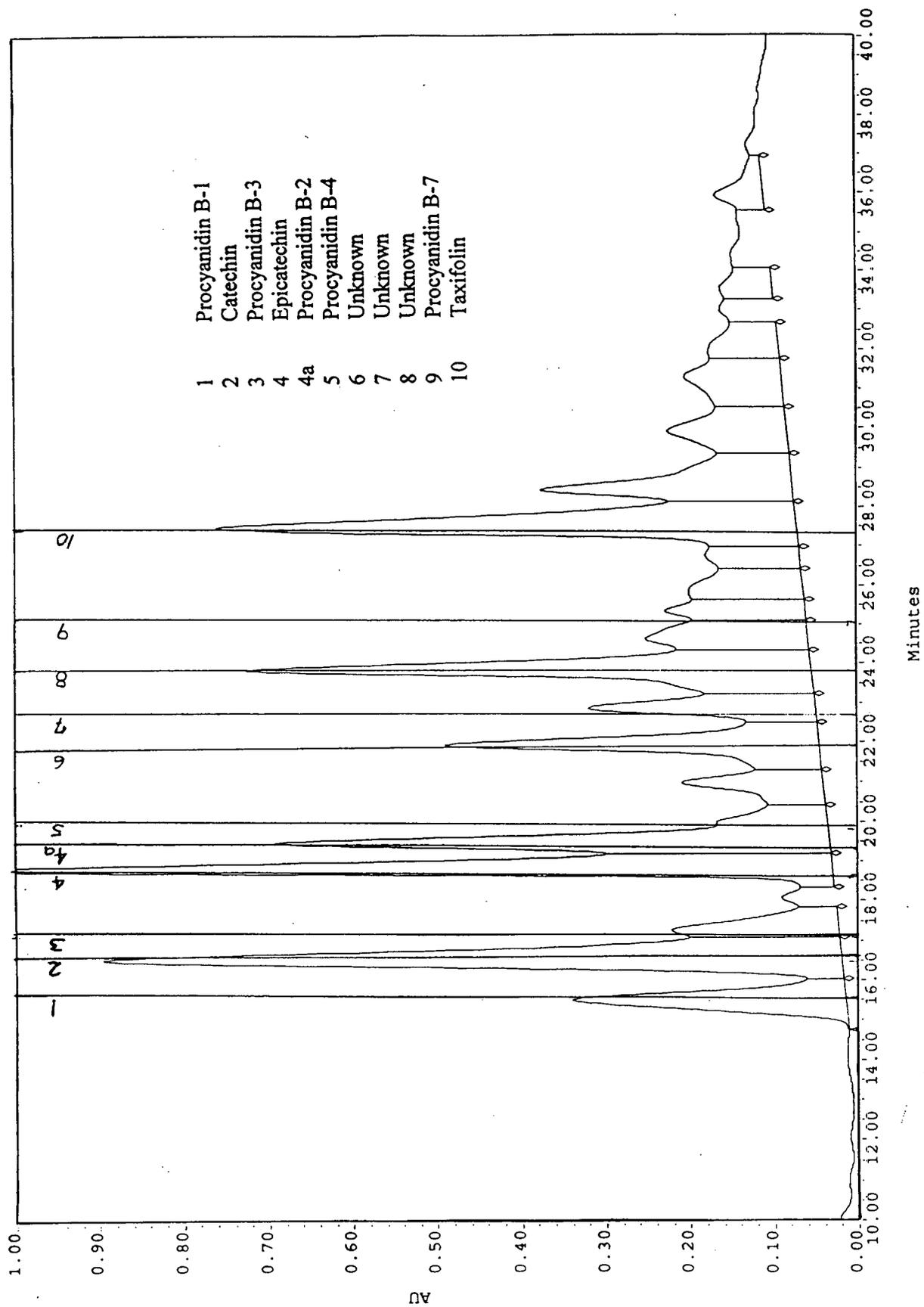


Figure 16. Sample of retention time template overlaid on the Indena LeucoSelect™ HPLC chromatogram. Ratio and distribution of the 10 proanthocyanidin signature peaks.

4.1.3.1 What the HPLC method measures

This method is designed to measure the quantity of Indena LeucoSelect™ in samples as FDA (Food and Drug Administration, USA) approved substrates, based on the components found in commercial products. However, the presence of gallic acid reveals that other non-proanthocyanidin compounds may also be present in approved OPC mixtures (for example, Pycnogenol™), as well. It was acknowledged by Masquelier (1969) that it is the mixture of proanthocyanidins and the small percentage on non-proanthocyanidin polyphenols which make up Pycnogenol™.

The 10 signature peaks only represent a portion of the proanthocyanidins in the extracts. A list of the 10 standard peaks is given in Table 10. The proanthocyanidin and polyphenol composition of the solvent peak at 45 min is unknown, and cannot be accurately determined by this chromatographic method without fractionation since the peaks contain a number of compounds in low concentrations.

The literature did not contain any information regarding the molar extinction coefficients of proanthocyanidins. But, by comparing the individual peak slopes of the different signature peaks, with increasing concentrations, the monomeric catechin, epicatechin, and taxifolin peaks showed a greater increase of slope with respect to the concentration. Concentration response curves of the remaining seven oligomeric components appeared to be quite similar. The molar extinction coefficients (ϵ) of molecules are related to their structures and solubilities. Since the structures of oligomers are similar, and all are freely soluble in water, they are expected to have similar molar extinction coefficients.

4.1.3.2 Validity of Estimations

When observing the final peak at 45 min in the case of the Indena LeucoSelect™, it was found that it accounted for 30 % of the total peak area, whereas the 10 signature peaks accounted for almost 60 %, together making up almost 90 % of the total peak area. Because the solubilities of the oligomers are similar (and hence their similar molar extinction coefficients) the oligomers which make up the 45 min peak are expected to represent about 30 % of the total composition of the extract. However, the majority of the extract material is represented in the 10 signature peaks, thus improving the quality of the estimation. For higher accuracy of identification and quantification, further work would have to be expanded on resolution of the peak at 45 min.

The accuracy of a particular quantification can be estimated by observing the number of signature peaks in the sample extract. The greater the number of signature peaks in the sample extract, the greater the degree of correlation between the standard and the sample. Hence, the better the estimation, the less likely it would be that a massive difference between the composition of the sample extract and the standard would exist.

As is shown in Section 4.2, this method, in spite of the above constraints, produces results similar to those found in the literature and predicts, with reasonable confidence, which tree species would be good potential sources of Pycnogenol™-like compounds. Although this method assumes a ratio between lower detectable and higher unmeasurable proanthocyanidins, this method is more accurate for monomeric and oligomeric proanthocyanidins than any of the other methods, as those methods are based on the composite reactivity response of all proanthocyanidins. Unlike this method, the other methods can neither distinguish between procyanidins, prodelphinidins, propelargonidins, nor distinguish between monomers, oligomers,

and polymers. Therefore, they cannot provide reliable information regarding the potential of an extract as a source of Pycnogenol™-like compounds. It is likely that the antioxidant potency of Pycnogenol varies with the composition of its constituent proanthocyanidin oligomers. However, in this respect, neither the industry nor the scientific community are clear (Richardo de Silva, 1991; Passwater, 1992) and no guidelines are available that would set the composition of the most potent antioxidant product.

4.1.4 Indena Standard Curve

The initial construction of a standard curve was based upon a Capers brand grape seed extract (discussed in Section 4.1.5). The Capers brand product could not be used because it was found to have a non-linear standard curve. One possible explanation for the non-linearity in the Capers standard curve was the aggregation of proanthocyanidins at higher concentrations, as is observed with lignins (Sarkanen, 1979). This would occur more readily at higher concentrations, of high molecular weight proanthocyanidins and would lead to an observed maximum in the standard curve. Lithium chloride is used to prevent the aggregation of lignins when stored in solution. Lithium chloride may interfere with hydrogen bonding between molecules, and thereby remove the lignin's tendency to form high molecular weight aggregates during size exclusion chromatography (Sarkanen, 1979). To test this hypothesis, one standard preparation was made by adding Pycnogenol to water, as usual. A second preparation differed in that 0.05 molar lithium chloride (LiCl) solution was used instead of water.

It was found that the standard curves of both preparations were linear and had very similar slopes (Figure 17). The lithium chloride containing standard curve had a slightly lesser slope than the non-treated standard curve. It was expected that in the case of proanthocyanidin aggregation, the

lithium chloride preparation would have higher concentration values because of reduced aggregation of proanthocyanidins. Lithium chloride is not expected to interfere with UV detection.

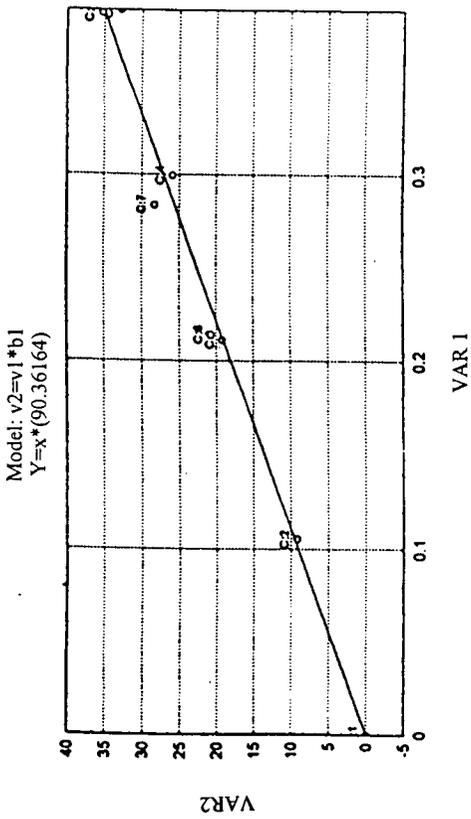
Statistical analysis revealed that these two curves were not significantly different. Hence, both data sets were combined and were subjected to a T-test to determine the p value of the combined curve. The value was found to be 0.001463 (Appendix F). Values under $p = 0.05$ are not considered significantly different, and so the values were combined to calculate the average. The slope of the standard curve comprising both data sets is shown in equation [13]. The statistical analysis of this data is shown in Appendix G.

$$y = 90.36164x \quad [13]$$

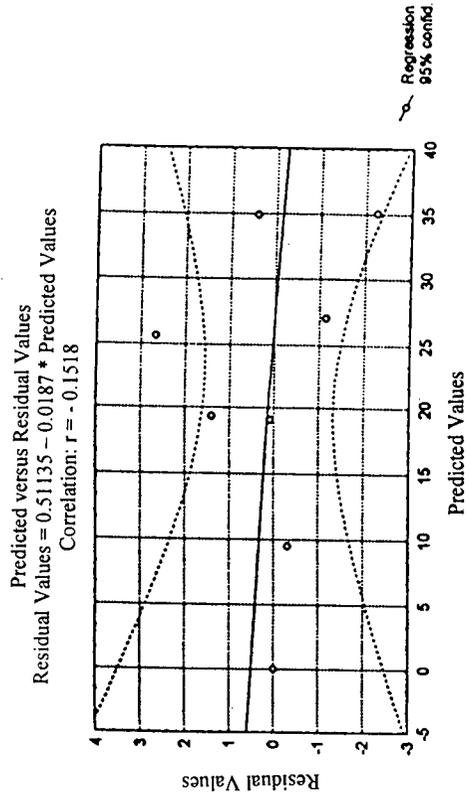
Figure 17a, b, c and d show the plotted statistical data. Figure 17a shows the plotted standard curve. Figure 17b shows the standard curve plotted with the 95% confidence limits. Figure 17c and Figure 17d plot the residual values against the predicted values and the frequency distribution of the residuals, respectively. These plots illustrate that this LeucoSelect™ standard curve has a variance of 98.44 %, and that most observed points fall within the 95% confidence limit. Thus, the few points that fall outside the 95% confidence limit are seen to be due to the small sample size.

4.1.5 Capers Brand Standard Curve

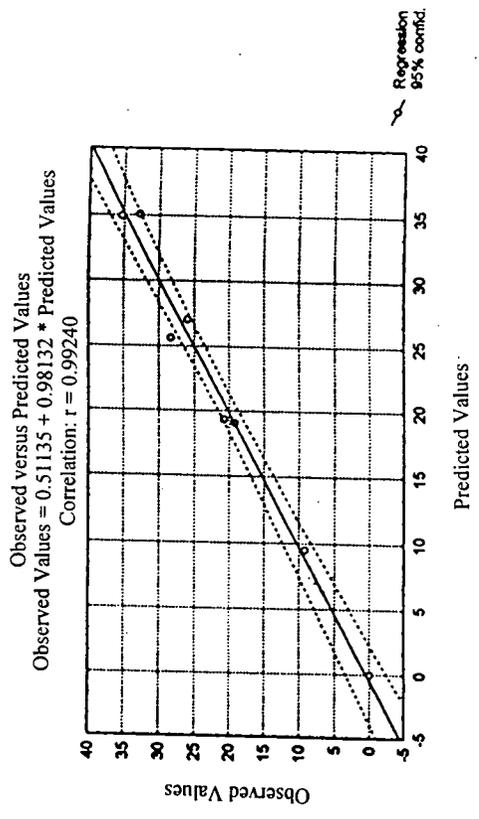
The Capers brand product was initially tested as a potential standard for Pycnogenol quantification, but was rejected after it was determined that its standard curve was non-linear (Figure 18). The standard curve was initially constructed with the Capers brand grape seed extract because it was said to contain 50 mg of 95% pure leucoanthocyanidins, or pycnogenols,



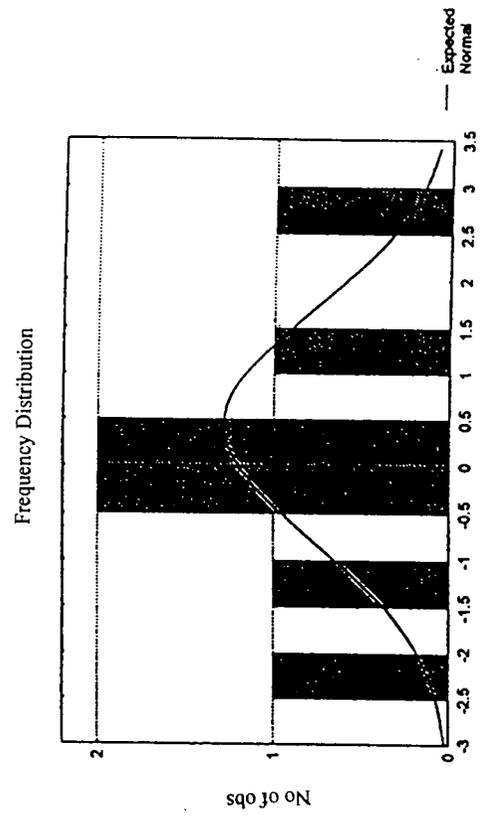
a)



c)



b)



d)

Figure 17. Statistical data for the Indena LeucoSelect™ standard curve: a) plotted values; b) 95% probability (observed versus predicted values); c) predicted versus residual values; d) frequency distribution of residual values.

in each tablet. As such, it appeared to be one of the highest purity products on the market.

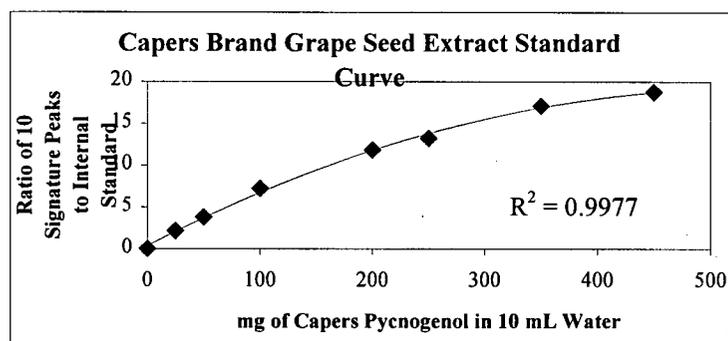


Figure 18. Capers' Brand Pycnogenol Standard Curve for Norepinephrine. Each tablet contains 50 mg Pycnogenol.

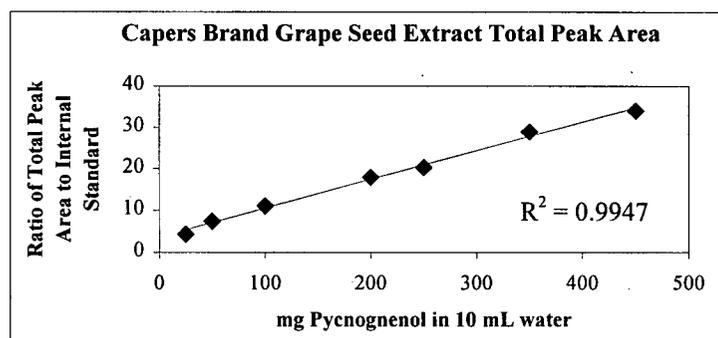


Figure 19. Ratio of the total peak areas to the internal standard.

Figure 19 measures the peak area of all of the peaks in the chromatogram, not only the 10 selected peaks. The y-axis is the ratio of the total peak area to the area of the internal standard. This shows a linear increase in the total chromatogram area for the Capers brand grape extract, with respect to the internal standard.

The ratio of the total graph area to the internal standard increases linearly, and the area of the 10 signature peaks does not. This suggests that an interaction exists between the signature compounds and the other compounds present in the Capers brand tablets, possibly with fillers. Therefore, the Capers brand grape seed extract had to be rejected as a standard for the

quantification of oligomeric proanthocyanidins for the purposes of this thesis.

4.2 QUANTIFICATION OF PYCNOGENOL TAXIFOLIN IN BARK EXTRACTS

4.2.1 Comparison of Quantification with Other Literature Methods

Several methods have been used to quantify condensed tannins, some of which are outlined in Section 1.6.3. The method developed herein differs significantly from other methods as it quantifies only oligomeric proanthocyanidins. However, it is because Pycnogenol™-based products consist of mainly proanthocyanidins that the results derived from this investigation can be compared with the quantitative results obtained by other researcher for condensed tannin. The method herein allows for a good estimation of the monomeric and oligomeric proanthocyanidins to be made by directly measuring the monomeric and lower oligomeric content relative to a standardized product such as Indena LeucoSelect™.

This method is designed to quantify the amount of oligomeric proanthocyanidins in an extract, and not the entire condensed tannin fraction. Therefore, the expected results in this investigation should have lower values than reported by other researchers as other methods measure the total condensed tannin content, including monomers, oligomers, and polymers, in extracts. Also, this method of quantification does not measure other polyphenols; the vanillin-HCl test does. Rather, it directly measures the total procyanidins relative to the LeucoSelect™ standard. In this research, it is necessary to determine the proanthocyanidin content from various sources relative to an already marketed and saleable product

The condensed tannin concentrations in the barks of several tree species were determined by Hathway (1962), and are listed in Table 8. In light of Hergert's (1989) results, it is possible that

these values are high. Hergert found that earlier researchers did not properly purify the condensed tannin fraction of bark extracts, and their samples actually contained substantial quantities of stilbenes, terpenes, other phenolics, in addition to proanthocyanidins .

Table 8. Yields of water-soluble condensed tannins from barks of various trees. (Hathway, 1962)

Species	Condensed tannins content in bark in percent (mg/g)	
<i>Picea abies</i>	5-18	(50-180)
<i>Picea sitchensis</i>	11-37	(110-370)
<i>Pinus densiflora</i>	6	(60)
<i>Pinus ponderosa</i>	5-11	(50-110)
<i>Pinus radiata</i>	17-18	(170-180)
<i>Pinus sylvestris</i>	16	(160)
<i>Pseudotsuga menziesii</i>	5-25	(50-250)
<i>Tsuga canadensis</i>	10-11	(100-110)
<i>Tsuga heterophylla</i>	15-16	(150-160)

The values shown in Tables 10 to 18 represent the oligomeric proanthocyanidin (OPC) content of bark extracts and are not shown with variances. This is for three reasons: this investigation was meant as a survey to test the accuracy of this method on a variety of sources relative to other literature values. More detailed future investigations should be carried out on promising bark species. Because a method of quantification of the extracts had to be developed before the samples could be compared, the duration of time between the harvesting of some of the present bark samples and extracting them was six months. This made it haphazard and prevented accurate quantification of the proanthocyanidin content in some of the experimental bark samples. Following the sample preparation and initial methods development at OSU, only vials of sample extracts were taken to UBC. The numerous sample treatments and re-testing required greater quantities of extract than originally anticipated, hence, insufficient extract remained to make replicate analysis for all samples.

4.2.2 The Vancouver Island and Mission Sitka Spruce (*Picea sitchensis* Carr.)

Few literature sources quantify the condensed tannin or proanthocyanidin content in spruce tree bark. Hergert (1989) studied the condensed tannins from spruce and mentions that Sitka spruce would yield a hot water extract of up to 30% (300 mg/g bark) of the bark weight, which is similar to the 11-37 % reported by Hathway (1962). But Hergert (1989) states that about 20-25 % of this extract consists of substituted stilbene glucosides in both the inner and outer barks. Matthews *et al.* (1997), using the anthocyanidin quantification method, which is sensitive only to proanthocyanidins, found that *Picea sitchensis* had a whole bark procyanidin content of 5.4 % (54 mg/g bark). They also determined Sitka spruce had a M_n (number average molecular weight) of 5.4. The related Norway spruce (*Picea abies*) whole bark contained 3.6 % (36mg/g bark) procyanidins and 0.08 % (0.8mg/g) prodelpinidin; the M_n was reported to be 4.6.

Hergert (1989) reported the occurrence of catechin, gallo catechin, procyanidins B1 and B3, and taxifolin glycosides. The polymeric material was found to be made up of catechin with traces of gallo catechin as terminal units. The chain extenders were found to be procyanidins, prodelpinidins and propelargonidins in a ratio of 20:1:1. The outer bark constituents were found to be copolymerized or mixed with lignin-like polymers and had end units composed of aglycones of stilbenes and taxifolin.

As expected, the inner and outer barks were found to have different extract compositions, but they were also found to differ in composition between different areas within the inner and outer bark. Hergert's (1989) research showed that as the inner bark matures, some of the inner bark solvent-soluble tannins become insolublized. Procyanidins are combined with soluble and

insoluble lignins. As the bark matures and nears the inner-outer bark boundary, the stereochemistry of the proanthocyanidins changes and more taxifolin, phloroglucinol, and stilbenes are incorporated into proanthocyanidin polymers.

The Vancouver Island and Mission area Sitka Spruce (*Picea sitchensis*) inner and outer barks were tested for Pycnogenol relative to the Indena standard curve, as well as the catechin, epicatechin, and taxifolin standard curves with the following results:

Table 9. Quantification data of Pycnogenol in Sitka spruce.

Sample	Pycnogenol Content in the Vancouver Island Sample		Pycnogenol Content in the Mission Area Sample	
	Inner Bark	Outer Bark	Inner Bark	Outer Bark
	mg/g Bark	mg/g Bark	mg/g Bark	mg/g Bark
Indena LeucoSelect™	1.5	0.2	1.0	0.5
Catechin	0.08	0.03	0	0.01
Epicatechin	.05	0.01	0.08	0.01
Taxifolin	0	0	0	0
Cat+Epi as % of Measured Indena Pycnogenol	8.7	20.0	8.0	4.0

Table 10. Occurrence of the signature peaks and taxifolin in Sitka spruce.

Compounds Present	Vancouver Island Sample		Mission Area Sample	
	Inner Bark	Outer Bark	Inner Bark	Outer Bark
Catechin	x	x		x
Epicatechin	x	x	x	x
Taxifolin				x
Dimer B-1		x		x
Dimer B-2			x	x
Dimer B-3				
Dimer B-4				
Dimer B-7				x
Unknown 6 (22.17 minutes)				x
Unknown 7 (23.11 minutes)				
Unknown 9 (24.89 minutes)	x			x

4.2.2.1 Coastal Vancouver Island Spruce

The inner bark was found to contain 8.7 % catechin and epicatechin of the 0.15 % (1.5 mg/g) total Pycnogenol content. The outer bark extract was found to contain 20 % catechin and epicatechin, of the total 0.02 % (0.2 mg/g) Pycnogenol content in the bark. Only three of the 10 signature peaks were present in the inner and outer bark extracts. This bark sample has a low content of proanthocyanidins, and is likely a poor potential source for a Pycnogenol™-like product. Taxifolin, reported by Hergert (1989) was not found in this extract. These results are, on average, one order of magnitude lower than the values reported by other researchers, and in the inner and outer bark extracts only three of the ten signature peaks could be found. However, because this sample was stored frozen for two months, this species should be re-tested.

4.2.3.2 Mission Area Sitka Spruce

The Mission area Sitka spruce, stored for six months on the benchtop, had a slightly lower concentration of Pycnogenol than the Vancouver Island species (1 mg/g). The outer bark samples

showed very low concentrations (0.5mg/g bark) of Pycnogenol. Of the ten signature Pycnogenol peaks, seven were found in the outer bark extract, but these results are also one order of magnitude lower than other reported literature values.

4.2.2.3 Suitability of Stored Bark as a Pycnogenol Source

These results also show that most of the compounds required for Pycnogenol production are absent in all except the Mission area Sitka spruce outer bark sample. The amount of proanthocyanidins suitable for the production of Pycnogenol was overall quite low, between 1.5 and 1.0 mg/g bark in the Mission and Vancouver Island inner bark samples, respectively. From these measurements, it can be seen that the inner bark of this species has a greater concentration of Pycnogenol than the outer bark. This was consistent in both trees.

The Mission area and Vancouver Island Sitka spruce trees were found to contain relatively few Pycnogenol constituents, and in low quantities at that. Therefore, this species is not considered a good source for Pycnogenol™-like products. Though, it is likely that fresher samples would yield higher concentrations of the proanthocyanidins necessary for Pycnogenol production.

4.2.3 Western Hemlock (*Tsuga heterophylla* Sarg.)

Hergert (1989) found that the condensed tannin content in hemlock bark was 12.6 % (126 mg/g bark). Hathway (1962) reported similar values of the water soluble condensed tannin content of hemlock bark between 15 and 16 % (150 and 160 mg/g bark).

Hergert (1989) isolated procyanidins B-1, B-2, B-3, B-4, and monomeric catechin, epicatechin, afzelechin, and epiafzelechin in a ratio of 10:2:1:2, respectively, and calculated the monomeric

units to be procyanidins, propelargonidins, and prodelphinidins in the ratio of 40:2:1. He found epicatechin to be the major terminal unit with lesser amounts of catechin, afzelechin, and epiafzelechin.

Samejima and Yoshimoto (1982) working on *Tsuga sieboldii*, a Japanese species of hemlock, found it to contain 0.2 % (2 mg/g) catechin and epicatechin in a ratio of 35:65, and 0.3% (3 mg/g) proanthocyanidins B1, B2, B3, and B4 in a ratio of 17:53:8:22. This was the only research found in which the amounts of catechin and epicatechin were calculated. The polymeric proanthocyanidins of the Japanese species had a D.P. of 7.8 and a M_n of 2270 and a molecular weight range of 1000-7800. These results are similar to those of Hergert (1989), who estimated, by vapor pressure osmometry, the average molecular weight of the polymer to be 2560 and the average D.P. to be 5. Storage time of the bark before analysis has not been reported for this analysis.

Hemlock bark is known for its high tannin content, and for its high proportion of polymeric tannins. This is reflected in work by Hergert (1989) showing 126 mg/g total condensed tannins in bark, and by Samejima and Yoshimoto (1982) showing that only small amounts (0.5 mg/g) are lower molecular weight monomers or dimers (B-1, B-2, B-3, B-4). But, much of the hemlock bark tannins are known to be hot water soluble.

The quantification results of Western Hemlock oligomeric proanthocyanidins, relative to the Indena LeucoSelect™ standard curve, are listed in the following tables.

Table 11. Quantification data of Pycnogenol in Western hemlock.

Samples	Pycnogenol Content in Quesnel Area Western Hemlock Sample	
	Inner Bark	Outer Bark
	mg/g Bark	mg/g Bark
Indena LeucoSelect™	0	4.5
Catechin	0	0
Epicatechin	0	0
Taxifolin	0	0.33
Cat+Epi as % of Measured Indena Pycnogenol	0	0

Table 12. Occurrence of the signature peaks and taxifolin in Western hemlock.

Compounds present	Inner Bark	Outer Bark
Catechin		
Epicatechin		
Taxifolin		x
Dimer B-1		x
Dimer B-2		
Dimer B-3		
Dimer B-4		x
Dimer B-7		
Unknown 6 (22.17 minutes)		
Unknown 7 (23.11 minutes)		
Unknown 9 (24.89 minutes)		x

4.2.3.1 Western Hemlock Bark

None of the 10 signature peaks could be detected in the inner bark of the Western hemlock sample, which was stored for six months on the bench-top. The outer bark was found to contain only small amounts of Pycnogenol, and catechin and epicatechin were present, but in concentrations too low to detect quantitatively.

It is well known that hemlock contains a high proportion of polymeric condensed tannins, and

only small amounts of oligomers and monomers (Hergert 1989). Samejima and Yoshimoto (1982) found catechin and epicatechin present at 2 mg/g bark and proanthocyanidins B1, B2, B3, and B4 at 3 mg/g bark. The present investigation found the content of proanthocyanidins in the outer bark to be 4.5 mg/g bark, more than the amount found by Samejima and Yoshimoto (1982). This investigation also found the presence of dimers B1, B2, and B4, although B3 was not detected.

4.2.3.2 Suitability of Western Hemlock as a Pycnogenol Source

Although Western hemlock bark is known to contain large amounts of condensed tannins, it has been shown to consist largely of high molecular weight, cold water insoluble polymers. The results of this study support the notion of low quantities of oligomeric, soluble pycnogenols being in the inner and outer bark. Therefore, Western hemlock bark would be a poor choice as a Pycnogenol source, although controlled degradation (by hydrolysis) of the high molecular weight fractions may prove to be profitable.

4.2.4 Douglas-fir (*Pseudotsuga menziesii* Franco.)

More research has been completed on Douglas-fir bark than any other bark species. However, some of the data available on the amount of condensed tannins is ambiguous. Rydholm (1965) reported the taxifolin content in Douglas-fir bark to be 12%, while Hathway (1962) reported that the hot water-soluble condensed tannins, of which taxifolin is one, varies between 5 and 25 %. If the condensed tannin content varies between 5 and 25 %, the extract composition can be expected to vary substantially.

Stafford (1989) showed that only 70 % of tannins are extractable with methanol from the stem bark of an 80 year-old Douglas-fir tree. The degree of extractability is a measure of the efficiency of extraction of proanthocyanidin/lignin polymers (phlobaphenes) which make up a substantial portion of Douglas-fir tree bark.

The extractive and tannin contents are known to vary with the tree's age. The bark nearest to the ground in old trees is lowest in tannin and highest in wax and dihydroquercetin (taxifolin) content. In young trees, the bark contains more tannin and less wax. Also, the bark from the top of the tree, which is younger, contains more tannin than the bark from the base (Kurth and Chan 1953). The periderm, or corky matter in the middle bark was found to contain 20 % taxifolin, where the inner and outer bark contained only 7 %. Therefore, the relative quantity of the type of bark is important and different concentrations of the various bark tissues can drastically change the chemical composition of an extract. Foo and Karchesy (1989) isolated procyanidin B1 and B2, and Malan *et al.* (1992) showed the presence of procyanidin A1 in Douglas-fir.

Matthews *et al.* (1997) found Douglas-fir to contain 2.2 % procyanidins and no pelargonidins based on the whole bark dry weight. The M_n for their sample was 6.2. Bae *et al.* (1994) found a purified sample of Douglas-fir inner bark to have hexamers and heptamers in the greatest concentration, and very low concentrations of dimers, and almost no monomers. This agrees well with Matthews *et al.* (1997), but also suggests that Bae *et al.* (1994) may have used ethyl acetate in their sample purification, which would remove most of the monomers and dimers from the extract.

The quantification results Douglas-fir oligomeric proanthocyanidins, relative to the Indena

LeucoSelect™ standard curve, are listed in the following tables.

Table 13. Quantification data of Pycnogenol in Douglas-fir.

Sample	Pycnogenol Content in Mission Area Douglas-fir Sample	
	Inner Bark	Outer Bark
	Mg/g Bark	mg/g Bark
Indena Pycnogenol	43.2	2.6
Catechin	1.33	0.08
Epicatechin	0.55	0.04
Taxifolin	0.53	0.85
Cat+Epi as % of Measured Indena Pycnogenol	4.4	4.6

Table 14. Occurrence of the signature peaks and taxifolin in Douglas-fir.

Compounds present	Inner Bark	Outer Bark
Catechin	x	x
Epicatechin	x	x
Taxifolin	x	x
Dimer B-1	x	x
Dimer B-2	x	x
Dimer B-3	x	x
Dimer B-4	x	x
Dimer B-7	x	x
Unknown 6 (22.17 minutes)	x	x
Unknown 7 (23.11 minutes)	x	x
Unknown 9 (24.89 minutes)		x

4.2.4.1 Douglas-fir bark yields

The yield of the Douglas-fir inner bark Pycnogenol is 4.37 % (43.7 mg/g), which is very high compared to the inner bark yields of other species. This is likely due the freshness of the bark, the sample having been air dried for only a few days before grinding and testing. Only 0.26 % (2.6 mg/g) Pycnogenol was found in the outer bark, which is similar to the content found in other bark species. The amount of taxifolin detected was far less than reported in the literature.

However, as stated above, the occurrence of taxifolin is known to vary greatly between samples, and could be related to the amount of taxifolin-rich periderm in the bark sample.

This research found both the inner and outer Douglas-fir barks to contain 9 and 10 Pycnogenol peaks, respectively, and both extracts included taxifolin. The yields of catechin and epicatechin for both samples are very similar at 4.4 and 4.6 %, respectively, which is the average amount in most trees tested. These results seem to conform well to the literature values. Matthews *et al.* (1997) found 2.2 % (22 mg/g) procyanidin in the whole bark of Douglas-fir. Because Matthews *et al.* used the whole bark, the total procyanidin value is less than what would be expected in the procyanidin-rich inner bark, and more than what would be expected in the outer bark.

4.2.4.2 Suitability of Douglas-Fir as a Pycnogenol Source

Douglas-fir bark is a good potential source for a Pycnogenols™-like product because of its high yield of lower molecular weight proanthocyanidins. *Pseudotsuga menziesii* bark has been deemed as tea in Austrian nutraceutical specialty stores, consequently, it has been deemed safe for human consumption.

4.2.5 Lodgepole Pine (*Pinus contorta* Dougl.) and Caribbean Pine (*Pinus caribbea*)

Various pine species have been studied for their condensed tannin content, but lodgepole pine and Caribbean pine have not been studied specifically. The water soluble tannin concentration of other pine species was reported to be between 5 and 18% of the total bark weight (Hathway 1962). Although commercial sources indicate large-scale production of OPC's from radiata pine bark, (*P. radiata*) no recent reference was found in the literature as to the soluble proanthocyanidin content of lodgepole pine.

Matthews *et al.* (1997) found *Pinus contorta* whole bark to contain 1.34 % (13.4 mg/g) procyanidins and no prodelphinidins. *Pinus pinaster* whole bark was found to contain 3.1 % (31 mg/g) procyanidin and 0.25% (2.5 mg/g) prodelphinidins; the M_n was found to be 5.1. Blazso *et al.* (1994) showed the presence of procyanidin B-1 in *Pinus pinaster*, which was not found in this study. Blazso *et al.* (1994) also reported the presence of procyanidins B-3, B-6, and B-7. Procyanidins B-3 and B-7 were found in this study, but B-6 was not investigated.

The M_w of the proanthocyanidin polymers in *Pinus longifolia* is 5900. The M_w of catechin is approximately 291. Therefore, the average molecular weight of the polymer in *Pinus longifolia* calculates to be 20 catechin units in size. This makes them large and less easily extractable with an acetone-water solvent.

The quantification results for Lodgepole and Caribbean pine oligomeric proanthocyanidins relative to the Indena LeucoSelect™ standard curve are listed in the following tables.

Table 15. Quantification data of Pycnogenol in Lodgepole and Caribbean pines.

Sample	Pycnogenol Content in Quesnel Area Lodgepole Pine Sample		Pycnogenol Content in Venezuelan Caribbean Pine Sample	
	Inner Bark	Outer Bark	Inner Bark	Outer Bark
	mg/g Bark	mg/g Bark	mg/g Bark	Mg/g Bark
Indena LeucoSelect™	1.9	1.0	0.1	5.2
Catechin	0.06	0.01	0.02	0.05
Epicatechin	0.01	0.01	0.01	0
Taxifolin	0.07	0.05	0.19	0.03
Cat+Epi as % of Measured Indena Pycnogenol	3.7	20	30	9.6

Table 16. Occurrence of the signature peaks and taxifolin in Lodgepole and Caribbean pine.

Compounds Present	Lodgepole Pine Sample		Caribbean Pine Sample	
	Inner Bark	Outer Bark	Inner Bark	Outer Bark
Catechin	x	x	x	x
Epicatechin	x	x	x	x
Taxifolin	x	x	x	x
Dimer B-1				
Dimer B-2	x			x
Dimer B-3				x
Dimer B-4	x			x
Dimer B-7	x			x
Unknown 6 (22.17 minutes)	x			x
Unknown 7 (23.11 minutes)				
Unknown 9 (24.89 minutes)	x			x

4.2.5.1 Lodgepole Pine

The Lodgepole pine inner bark yields 0.19 % (1.9 mg/g) Pycnogenol, which is almost double the 0.1 % (1.0 mg/g) outer bark yield. The inner bark also shows the presence of almost all of the Pycnogenol signature peaks while the outer bark shows only the presence of catechin, epicatechin, and taxifolin. The amount of taxifolin and epicatechin is similar in the inner and outer bark, but catechin is present in much higher concentrations in the inner bark. This sample was stored frozen for six months.

4.2.5.2 Caribbean Pine

The Caribbean pine bark, estimated to be two to four weeks old, was stored moist at temperatures above ambient laboratory conditions. Therefore, this Caribbean pine sample would not be considered a “representative” sample, but may allow for a preliminary survey as to what could be expected from a warm climate pine species. Although no literature could be found on the

proanthocyanidin or condensed tannin content for this species, it is likely that the proanthocyanidins suffered some oxidative degradation and polymerization due to improper sample handling and storage (over-heating) leading to the decreased oligomer solubility.

The amount of Pycnogenol in the outer bark was found to be more abundant than in the inner bark. The reason for this is not clear but likely has to do with the storage method. A greater amount of Pycnogenol in the outer bark is reflected by detection of seven Pycnogenol signature peaks and taxifolin in the bark extract. The presence of these signature peaks allows for the assumption that fresh Caribbean pine bark should yield better results and warrants further investigation.

4.2.5.3 Suitability of Pine as a Pycnogenol Source

Pinus pinaster and *Pinus maritima* are used commercially for the production of Pycnogenol™-like products, and it is likely that Lodgepole pine and Caribbean pine would be good sources, as well. The amounts of Pycnogenol in these samples are quite low, however, there is reason to believe that re-testing on fresh samples would yield better results. Special precautions should be made when testing the Caribbean pine as sample quality control is difficult to ensure due to the hot Venezuelan climate. Samples should be ground and extracted at a Venezuelan laboratory, or should be sent frozen or, preferably, freeze dried for further analysis.

4.2.6 Amabilis Fir (*Abies amabilis* Dougl.)

True firs are the third most common tree species in B.C. forests. Remarkably, no information on the proanthocyanidins or condensed tannins content in fir bark could be found other than a brief mention stating that the extract of the condensed tannin fraction contains reddish-purple

phlobaphenes, which are formed in the secondary periderm (Becker and Kurth, 1958).

The quantification results of fir oligomeric proanthocyanidins relative to the Indena LeucoSelect™ standard curve are listed in the following tables.

Table 17. Quantification data of Pycnogenol in Amabilis fir.

Sample	Pycnogenol Content in the Quesnel Area Sample		Pycnogenol Content in the Mission Area Sample	
	Inner Bark	Outer Bark	Inner Bark	Outer Bark
	mg/g Bark	mg/g Bark	mg/g Bark	mg/g Bark
Indena LeucoSelect™	0.5	0	12.4	0
Catechin	0.02	0	0.35	0
Epicatechin	0.01	0	0.20	0
Taxifolin	0.11	.05	0.13	0.33
Cat+Epi as % of Measured Indena Pycnogenol	2.50	0	4.44	0

Table 18. Occurrence of the signature peaks and taxifolin in Amabilis Fir

Compounds Present	Quesnel Area Sample		Mission Area Sample	
	Inner Bark	Outer Bark	Inner Bark	Outer Bark
Catechin	x		x	
Epicatechin	x	x	x	x
Taxifolin	x	x	x	x
Dimer B-1	x		x	
Dimer B-2	x		x	
Dimer B-3	x		x	
Dimer B-4			x	
Dimer B-7			x	
Unknown 6 (22.17 minutes)			x	
Unknown 7 (23.11 minutes)			x	
Unknown 9 (24.89 minutes)			x	

4.2.6.1 Interior Amabilis Fir

The Amabilis fir sample from Quesnel was left for 6 months on the laboratory bench-top before it was extracted. The yield of Pycnogenol from the Amabilis fir inner bark was very low at 0.05% (0.5 mg/g) while the yield in the outer bark was below the detector threshold, although a small amount of taxifolin was detected. The inner bark showed the presence of catechin, epicatechin, and the dimers B1, B2, B3, and taxifolin. Since no literature references are available, the presence of these compounds in Amabilis fir cannot be compared with other research results.

4.2.6.2 Coastal Amabilis Fir

The coastal fir bark sample was taken two months prior to testing. The Pycnogenol content found in the inner bark was far higher than the amount present in the outer bark. Only epicatechin and taxifolin could be detected in the outer bark sample while the inner bark contained all 10 signature peaks in the extract.

In both the interior and coastal species, the proanthocyanidins were below detector threshold levels in the outer bark, but the inner bark proanthocyanidin concentration varied substantially. The lower proanthocyanidin content in the inner bark is likely due to more than just a difference in sample storage times, as the coastal sample was stored on the bench top for two months and the interior sample was stored on the bench top for 6 months. It is likely that the variance between the respective 0.05 % (0.5 mg/g) and 1.24 % (12.4 mg/g) proanthocyanidin contents in the interior and coastal sample could be due to the difference in harvesting (growing) sites. But again, no quantitative data on the *Abies* genus tannin content could be found.

4.2.6.3 Suitability of Coastal Amabilis Fir as a Pycnogenol Source

Although the coastal and interior Amabilis fir inner bark samples differ in their respective oligomeric proanthocyanidin concentrations, they both contain many of the Pycnogenol signature compounds. Hence, Amabilis may be considered as a potential candidate for the production of a Pycnogenol™-like product, although more research on this species is necessary.

4.3 COMPARISON BETWEEN CAPERS, SUNFORCE, AND NU-GREENS BRAND PYCNOGENOL PRODUCTS

This new method of procyanidin analysis was also tested for suitability in analyzing the procyanidin contents of different commercial Pycnogenol™-like products. As the Indena ‘LeucoSelect™’ sample was the only pure commercially produced oligomeric proanthocyanidin sample available, the Capers, SunForce, and Nu-Greens brand products were tested relative to the Indena brand standard.

Table 19. Quantification data for commercial Pycnogenol products.

The Purity Commercial “Pycnogenol” Products Relative to Indena LeucoSelect™			
Samples	Capers Brand	Sun Force	Nu-Greens
	Estimated Content (%)	Estimated Content (%)	Estimated Content (%)
Indena LeucoSelect™	7.65	4.68	2.62
Catechin	0.384	0.217	0.0
Epicatechin	0.168	0.067	0.028
Taxifolin	0.0	0.054	0.0
Cat+Epi as % of Measured Indena Pycnogenol	5.57	6.07	1.07

4.3.1 Capers tablets

Each Capers 'Grape Seed Extract' tablet weighed approximately 0.5 g and was said to contain 50 mg of 95% pure leucoanthocyanidins, which amounts to 10% Pycnogenol per sample. Relative to the Indena brand LeucoSelect™ standard, the Capers tablets contain 7.65% Pycnogenol (or 76.5% of the Indena LeucoSelect™ content). Both the Capers and Indena brand pycnogenols are prepared from grape seeds.

Differences in grape seed species and minor differences in extraction and purification methods used by different manufactures could lead to changes in the proanthocyanidin composition in grape seed extracts between products. As stated previously, differences in the ratio of the 10 measured signature peaks to the non-measurable oligomeric peaks would result in slight differences in the quantification results.

4.3.2 SunForce Pycnogenol™

The SunForce Pycnogenol™ tablets are said to contain 25 mg pine bark Pycnogenol™ per tablet. SunForce tablets were found to weigh approximately 0.24 grams. Therefore, these tablets should consist of 10 % Pycnogenol. Only 4.68 % was measured. Research on bark proanthocyanidins showed that bark contains a greater amount of high molecular weight oligomers and polymers than grape seeds. Therefore, slight differences in the ratio of signature peaks to non-measurable procyanidin peaks is a likely cause for the discrepancy between the expected content (10 %), and the measured oligomeric proanthocyanidin content (4.68 %). This does not mean that grape seed contains more Pycnogenol than pine bark, only that grape seed may likely contain a greater portion of lower molecular weight proanthocyanidins than pine bark.

4.3.3 Nu-Greens

The composition of the Nu-Greens 'Prolong' extract is listed in Table 20.

Table 20. Composition of Nu-Greens 'Prolong'.

Grape Seed Extract	75 mg
Resveratrol	15 mcg
Proanthocyanidins	95% activity
Polyphenols	min. 46% activity
Anthocyanidins	min. 30% activity
Catechins	min. 18% activity
Lipoic Acid	12.5 mcg
Betacarotene (natural carotinoids)	1500 I.U.

A Nu-Green 'Prolong' tablet weighs 0.4 g and contains 75 mg grape seed extract of 95 % activity of proanthocyanidins or pycnogenols. If the 75 mg of grape seed extract could be considered pure proanthocyanidins, the tablet should contain 18.75 % Pycnogenol. By calculating the Pycnogenol content based on the Indena brand LeucoSelect™, the Prolong tablet contains only 2.62 % Pycnogenol.

The lack of standardized quantification methodology and unregulated units of quantification allows this product to be measured in units of 'Activity'. No clear definition of 'Activity' is provided, nor could it be obtained when the product supplier was contacted. The discrepancy between the expected 18.75 % grape seed Pycnogenol content, and the 2.62 % measured content is too large to be accounted for by a variation in the ratio of the signature peaks to the non-measurable procyanidin peaks, especially as both products are based on grape seed sources. No reasonable explanation can be given for the discrepancy.

One reason for suspecting a suppliers' error is rooted in the dosage instructions on the label. The dosage instructions on the bottle state that one pill should be taken with breakfast and one tablet

with dinner. Proanthocyanidins are very strong protein binders and bind readily to, for instance, milk and egg proteins (for breakfast), or steak and bean proteins (for dinner), effectively removing the proanthocyanidins before they can be absorbed into the blood stream. Such statements indicate a lack of understanding or non-familiarity with the product by the supplier.

4.4 COMPARISON BETWEEN INNER AND OUTER BARKS

Several observations were made between the inner and outer bark content of pycnogenols. Typically, the outer bark would be expected to contain less proanthocyanidins and fewer constituents of Pycnogenol than the inner bark.

As the inner bark is converted into outer bark, it is known that the amount proanthocyanidins are increased and combined with non-proanthocyanidins (perhaps with lignins, lignans, or stilbenes), thereby becoming less soluble. This change seems to impart stability on the oligomeric proanthocyanidins. In most circumstances, when the inner bark pycnogenols are either insolubilized through degradation or polymerization, free monomers and dimers can still be observed in the outer bark. In all bark samples, regardless of the inner bark procyanidin composition, length or method of storage, the outer bark Pycnogenol content remained between 0.2 mg/g and 5.2 mg/g, except in *Amabilis fir* where no pycnogenols were detectable. The pycnogenols in the inner bark are apparently more susceptible to degradation and insolubilization with age, and only remain in the phloem for a few years before being chemically altered by the tree and joining the outer bark where they are insoluble. This insoluble form is more stable as proanthocyanidins can be found here decades after synthesis.

5.0 SUMMARY

Proanthocyanidins are compounds that exhibit large variability in their structure. They are considered to be highly reactive and can undergo physical and chemical changes even under mild ambient conditions. It is expected that the storage duration of some of the samples analyzed in this research affected the quantitative results reported herein. Therefore, samples to be analyzed in the future should optimally be extracted from either fresh or recently freeze-dried samples.

Identification of proanthocyanidins has been hindered by the complex, and often incorrectly used, nomenclature. Also, the testing methods developed for proanthocyanidins are based on their reactivities, which have been found to vary and be insufficiently specific for accurate quantification. These difficulties have greatly limited the amount and quality of research on the quantification of these compounds, and the topic of accurate quantification of both oligomeric and polymeric proanthocyanidins from plants should be revisited due to their importance in both human nutrition and plant protection.

The method described herein utilizes reverse phase high-pressure liquid chromatography to chemically isolate and spectrophotometrically quantify the occurrence of most prevalent procyanidin monomers, dimers, and trimers from plant extracts. No previously existing method of quantification is capable of rapidly, effectively, and accurately determining the amount of high value oligomeric proanthocyanidins suitable for the production of commercial Pycnogenol.

The method of quantifying the amount of usable monomeric and oligomeric proanthocyanidins for the production of Pycnogenol™-like compounds, developed here-in, is usable and provides a good means of estimating the amount of Pycnogenol obtainable from various bark extracts. It can

also measure the suitability of the extract for commercial Pycnogenol production. This method also opens the way to detailed quantitative description of individual (signature) compounds from various proanthocyanidin sources, provided sufficient quantities of the individual compounds, making up the mixture, can be isolated. The individual compounds would have to be isolated by fractionation to construct response (calibration) curves for HPLC analysis of extractive samples. In most samples, the inner bark contains more soluble pycnogenols than the outer bark, but the outer bark Pycnogenol concentration appears to be more consistent. The yield of pycnogenols in the various barks and commercial product extracts is listed in Table 21.

Table 21. Yields of proanthocyanidins in bark extracts and commercial products based on Indena LeucoSelect™.

Species	Location	Inner Bark (mg/g)	Outer Bark (mg/g)
Amabilis fir	Quesnel	12.4	0.0
	Mission	0.5	0.0
Douglas-fir	Mission	43.2	2.6
Pine (Caribbean)	Caribbean	0.1	5.2
Pine (Lodgepole)	Quesnel	1.9	1.0
Sitka spruce	Vancouver Island	1.5	0.2
	Mission B.C.	1.0	0.5
Western hemlock	Quesnel	0.0	4.5

Table 22. Purity of the commercial OPC containing compounds relative to an Indena LeucoSelect™ standard.

Product Brand	Purity of Commercial Products		
	Expected Content (%)	Calculated Content (%)	Purity (%) Relative to Indena LeucoSelect™
Capers Brand OPC's	10	7.65	76.5
SunForce Pycnogenol™	10	4.68	46.8
Nu-Greens Prolong	18.75	2.62	7.2

From the results listed in Table 21, Douglas-fir, Amabilis fir, and Lodgepole and Caribbean pine bark extracts appear to be good potential sources for Pycnogenol™-like products. Sitka spruce

and Western hemlock bark extracts are not expected to be good sources of Pycnogenol.

The quantification of the oligomeric proanthocyanidins, relative to the LeucoSelect™ standard, confirmed that some variances exist in the composition of different commercial products (Table 22). The variations are caused by the lack of standardized isolation, purification, and quantification techniques in the industry. Because of this variation, consumers cannot be guaranteed the quality of the supplements they buy and consume. However, this investigation revealed a major discrepancy between the expected and calculated oligomeric proanthocyanidin content in the Nu-Greens product. Hence, the establishment of this procedure is vital, not only for the determination of new potential product sources, but in the standardized testing and comparison of existing compounds to ensure consumer confidence and safety. The information and methods described herein can be used as a basis for standardized quantification of oligomeric proanthocyanidins from bark and grape extracts, and commercial procyanidin-based products.

6.0 CONCLUSION

The class of compounds termed oligomeric proanthocyanidins shows a highly variable chemical composition. In nature, monomeric and dimeric to oligomeric components are present in widely varying concentrations depending on their source. And, even commercially prepared Pycnogenol products seem to have widely varying proanthocyanidin contents. As of yet, no suitable analytical quantification method is available by which the Pycnogenol content of various botanical extracts can be estimated and compared.

Usually, bark or grape seed extracts have 10 common proanthocyanidin signature components (peaks) whose occurrence can be used for the development of a meaningful HPLC based quantification analytical technique. The method developed in this thesis is based mostly on the occurrence and measured responses of the signature peaks in sample extracts, relative responses obtained from the LeucoSelect™ commercial product.

Among the species tested, Douglas-fir, Amabilis fir, Lodgepole pine and Caribbean pine appear to be good potential sources of Pycnogenol, while Sitka spruce and Western hemlock gave very low yields.

Commercial products tested and quantified as regards to proanthocyanidin content show wide variation not only in composition but also in apparent content of proanthocyanidins termed as Pycnogenols. The method developed herein should allow close control and quantification of the proanthocyanidin content of various extracts, as well as correlation of "potency" with the actual Pycnogenol content of commercial products.

7.0 FUTURE RESEARCH

The method developed in this thesis represents a good procedure for comparative quantification of proanthocyanidin monomers and oligomers in proanthocyanidin extracts. However, this research was conducted on bark samples, most of which were not fresh. The results obtained are consistent with the expected results, but are not representative for fresh bark samples. As a Pycnogenol™-like product would likely be made from a fresh bark sample, this research should be repeated for, at least, fresh Amabilis fir and Lodgepole and Caribbean pine bark samples.

Over \$800 million of Pycnogenol™-like products have been sold between 1980 and 1992 (Passwater, 1992) world-wide. However, in spite of this immense volume (64 T or 2.56 billion tablets), there has been no effort made to standardize the identity of Pycnogenol. Because of this, companies, such as Nu-greens, have produced products such as Prolong™ with uncertain standardization of the Pycnogenol content. This research provides one means of standardizing the quantity of pycnogenols in various samples relative to an authentic standard. Standard curves for measurement of Pycnogenol content have been constructed based on 10 selected peak areas and known benchmark monomeric procyanidins.

Composition of the pycnogenols from different sources of proanthocyanidins are known to vary significantly. Therefore, future research should be directed towards isolation and creation of standard concentration profiles of monomers, dimers and oligomers from various Pycnogenol sources. In this way, different Pycnogenol preparations can be determined more accurately and should be more directly comparable.

Proanthocyanidin extractability is greatly influenced by sample storage. The current method of

sample preparation requires that the bark samples be dried and ground to smaller than 30 mesh for efficient extraction. However, bark samples should be obtained green and dried for no more than a few days, to ensure samples most representative of their chemical composition on the tree. If possible, a wet grinder should be used instead. This would also allow the samples to be ground without drying. A nitrogen purged 70:30 acetone:water solution should be used during the grinding and extraction process. This would ensure removal of the soluble proanthocyanidins and greatly reduce any possibility for the formation of protein-tannin complexes or oxidation during extraction.

Some research has been directed towards the correlation of the proanthocyanidin concentration in extracts and their respective antioxidant and free radical scavenging ability. Such experiments should be conducted in conjunction with this quantification method to develop a positive correlation between the quantification of proanthocyanidins in extracts, based on this method, and the resultant antioxidant and free radical scavenging capacity of such extracts. Such information would improve the estimation of an extract's viability as a potential commercial product, and could be used to compare commercial Pycnogenol products under this criterion.

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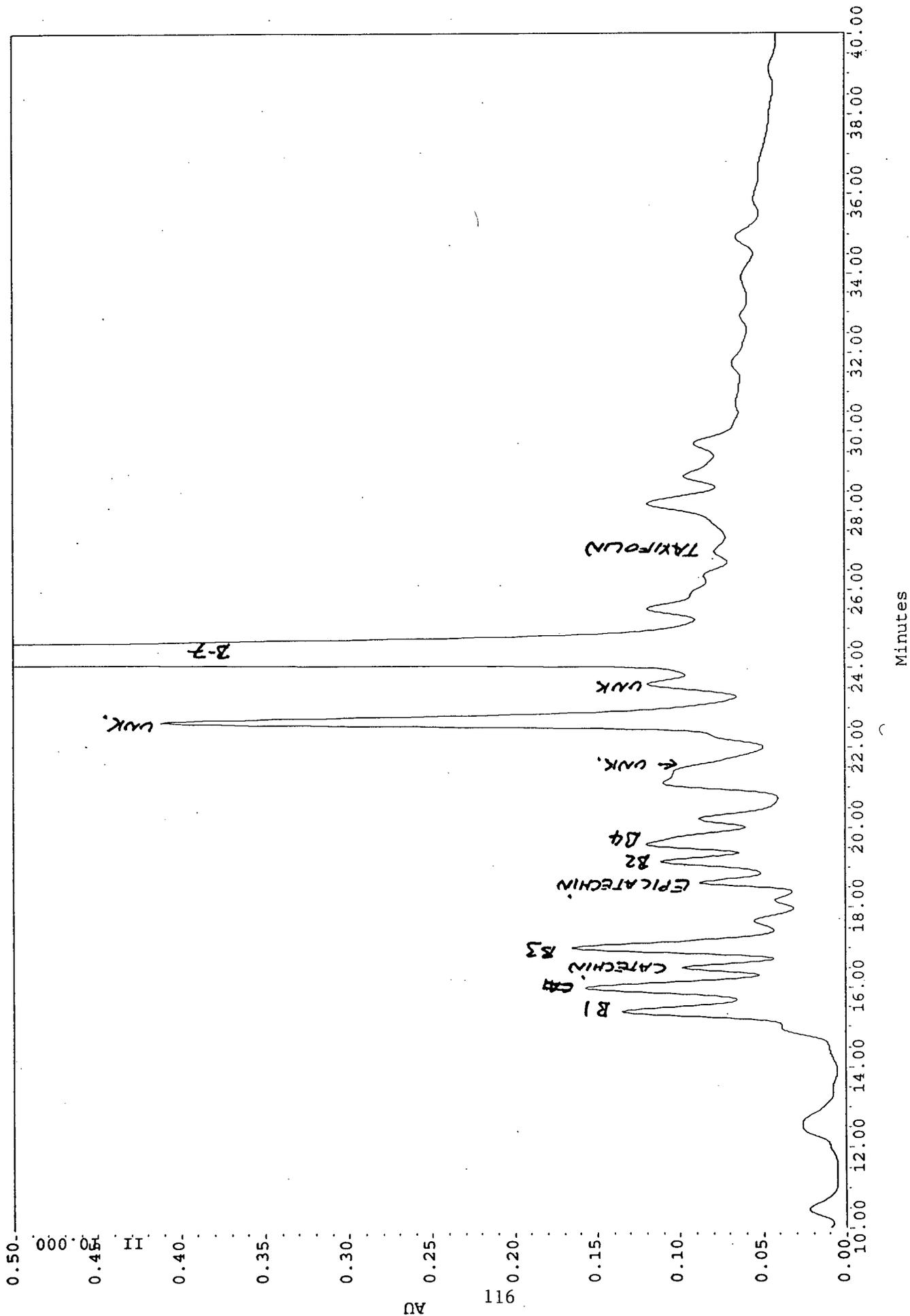
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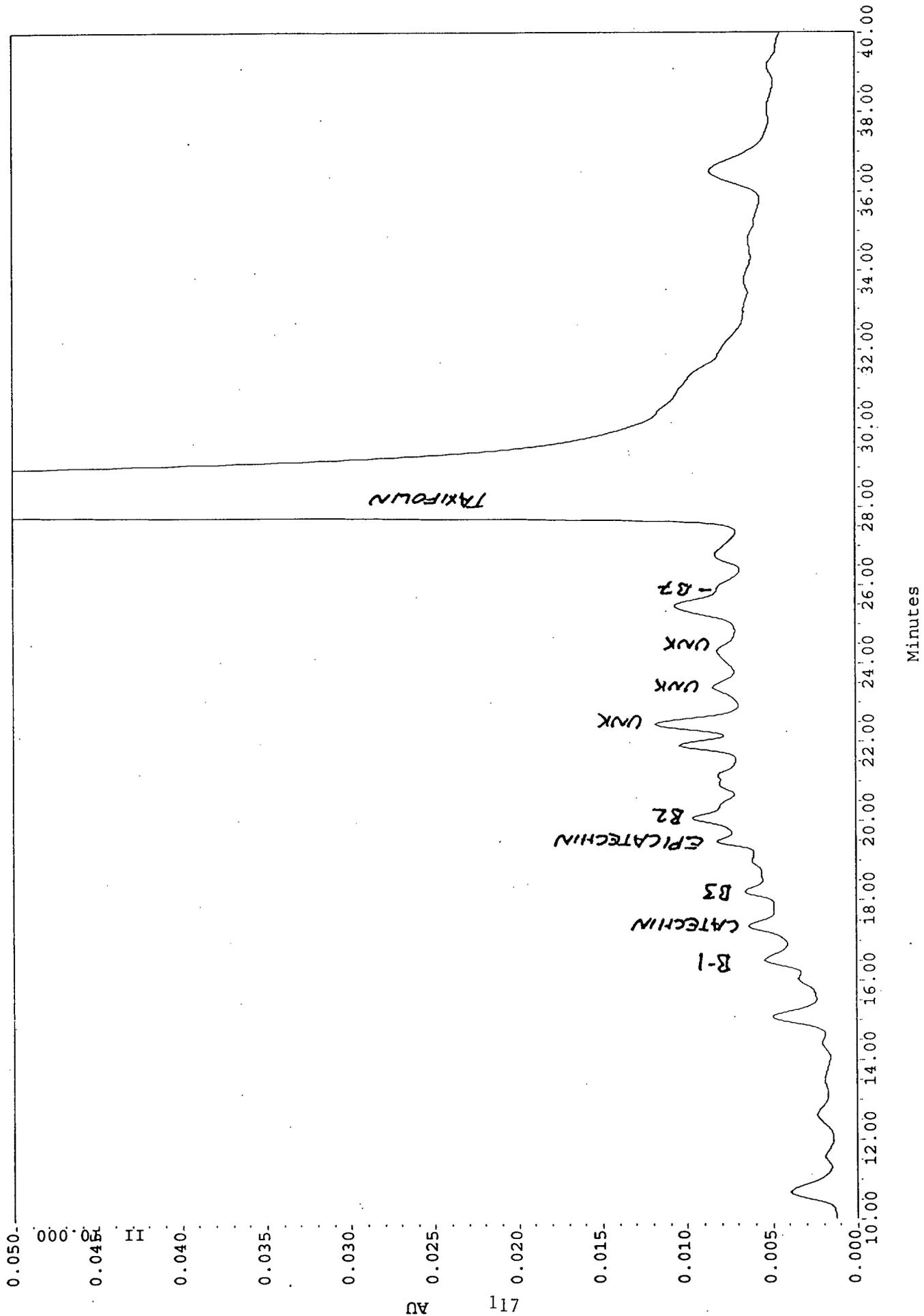
APPENDICES

APPENDIX A
Sample HPLC Chromatograms



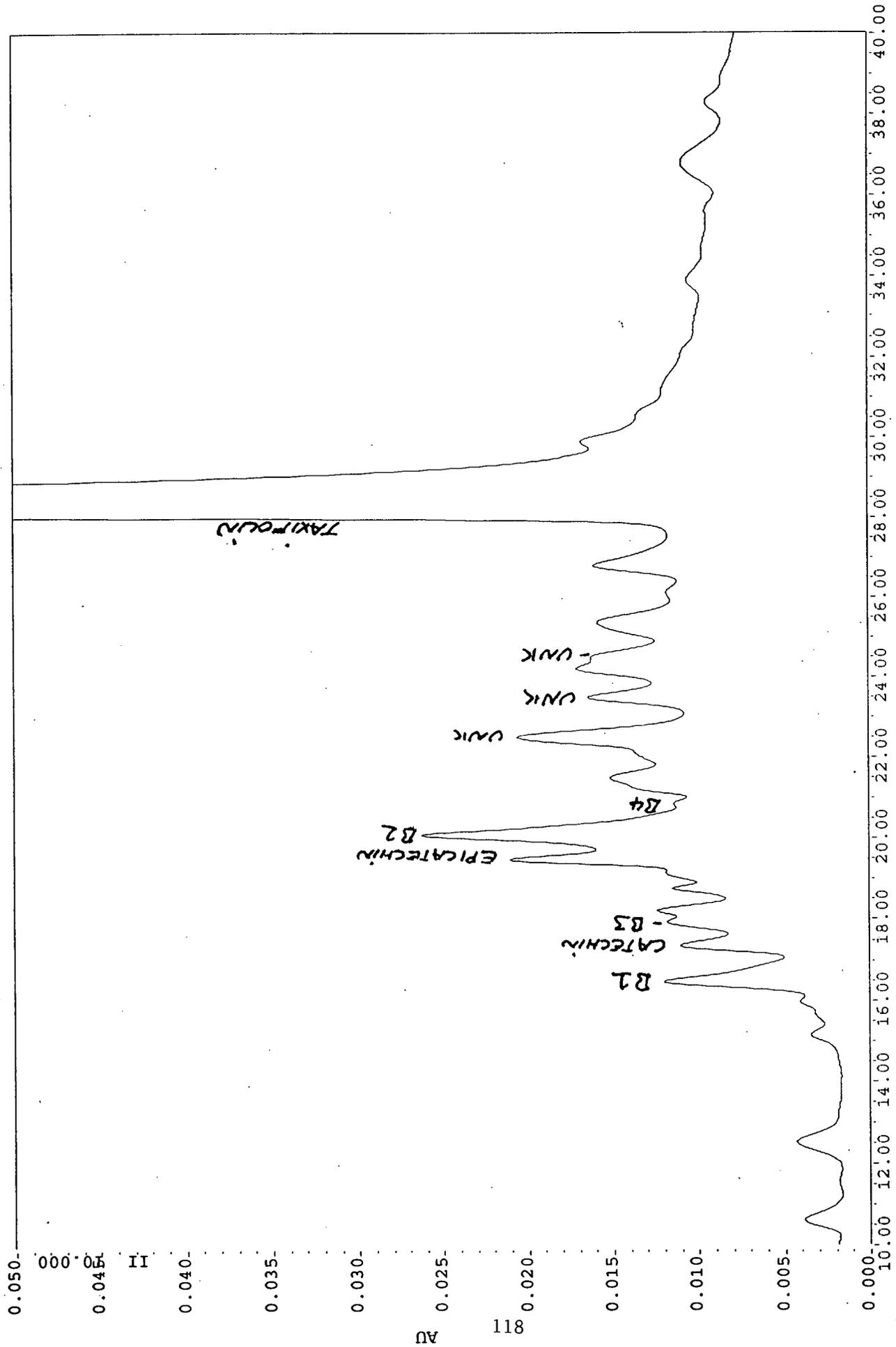
COASTAL SPRUCE INNER BARK.

SampleName: C Vial: 8 Inj: 2 Ch: PDA_280.0nm Type: Unknown



COASTAL FIR OUTER BARK.

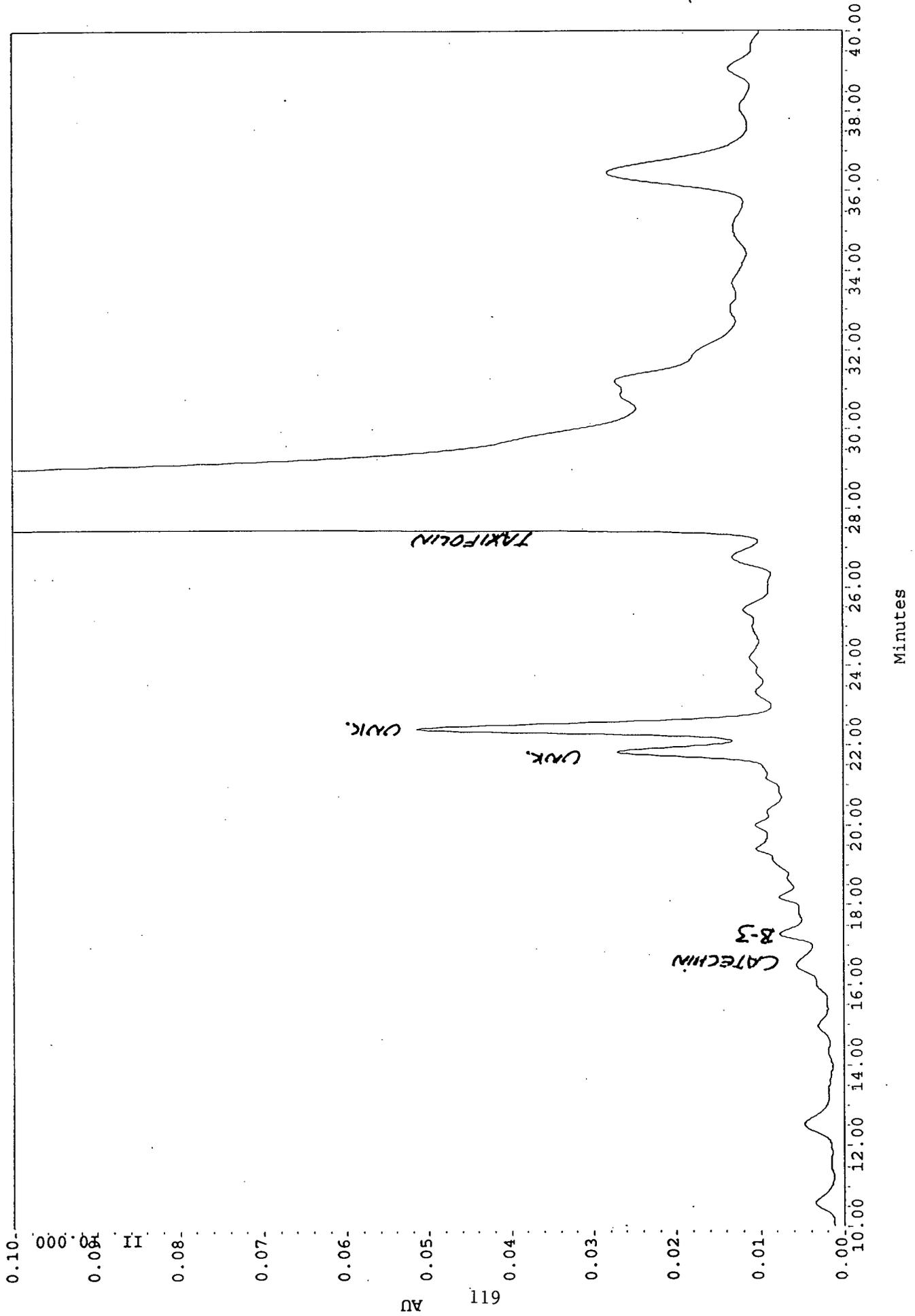
SampleName: H Vial: 13 Inj: 2 Ch: PDA_280.0nm Type: Unknown



Minutes

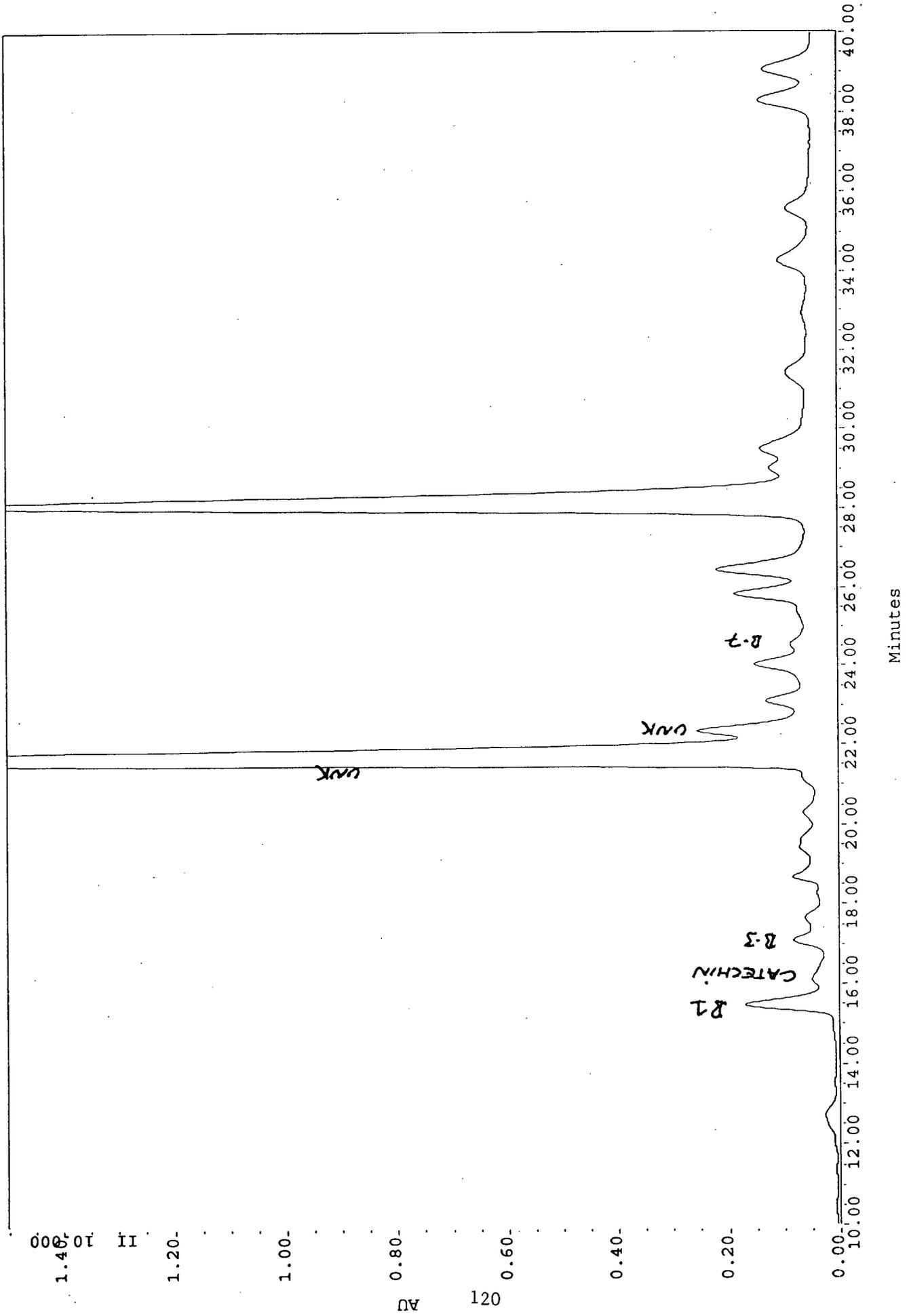
Interior Fir Inner Bark.

SampleName: D Vial: 9 Inj: 2 Ch: PDA_280.0nm Type: Unknown



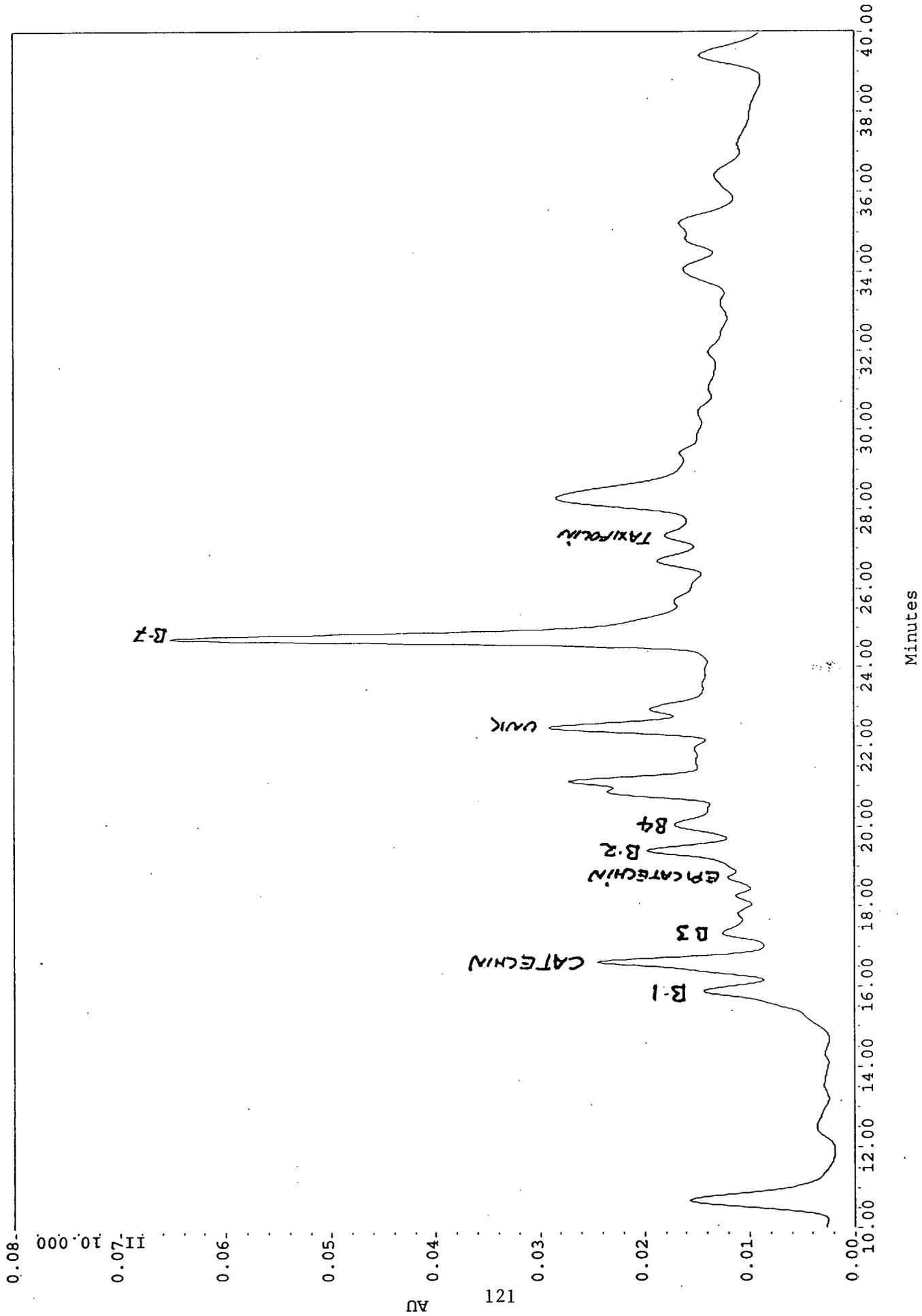
INTERIOR FIR OUTER BARK.

SampleName: I Vial: 14 Inj: 2 Ch: PDA_280.0nm Type: Unknown



SITKA SPRUCE INNER BARK.

SampleName: B Vial: 7 Inj: 2 Ch: PDA_280.0nm Type: Unknown

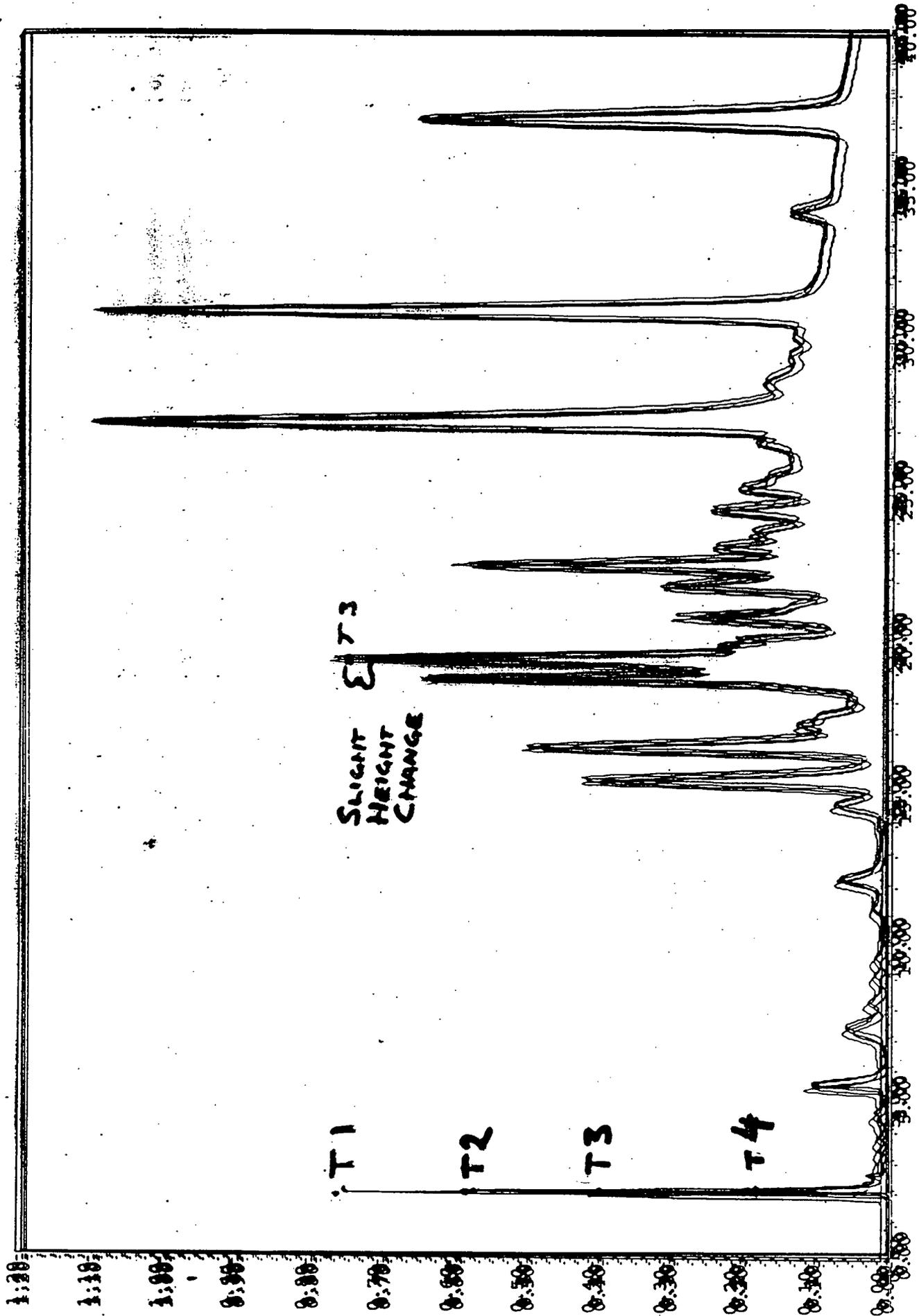


COASTAL SPRUCE OUTER BARK.

SampleName: G Vial: 12 Inj: 2 Ch: PDA_280.0nm Type: Unknown

APPENDIX B

**Internal Standard Interaction With
The Douglas-fir Extract**



SLIGHT
HEIGHT
CHANGE

E
T3

T1

T2

T3

T4

MINUTES

DOUGLAS-FIR INNER BARK IS 4 CONC. OF NOREPINEPHINE CONCENTRATIONS.

SAMPLE NAME: NOE TR VIAL: 10 IRT: 1 CRI: PDA 200.00M TPE: UNKNOWN

APPENDIX C

Sample Calculation

Calculation of Oligomeric Proanthocyanidin Content in Interior Amabilis Fir Inner Bark

Estimated Retention Time	2.04	15.79	16.77	17.46	19.04	19.69	20.17	22.17	23.11	24.13	24.89	27.79
Peak Number	Internal Standard	1	2	3	4	4a	5	6	7	8	9	10
Identification	B1	Catechin	B3	B3	Epicatechin	B2	B4	Unk 1	Unk 2	B7	Unk 3	Taxifolin
Area (units)	5666706	1927505	1878755	365107	2802955	3591186	1014988	810999	1275038	41282	744706	6853339

10 peaks (excluding taxifolin) area / Internal Standard area = 2.62

Mass of Dry Bark 115.65 grams
 Volume of Extract 494 mL
 Indena Standard Curve: y= 90.36164x

Calculation:
 $\frac{2.62}{90.36164} = 0.029$ = 0.029 g x 494 mL extract = 1.24 % Indena LeucoSelect(TM) in Amabilis Fir Inner Bark
 10 mL 115.65 grams bark = 12.4 mg/g LeucoSelect(TM) in Dry Bark

APPENDIX D
HPLC/Mass Spectrometry Data

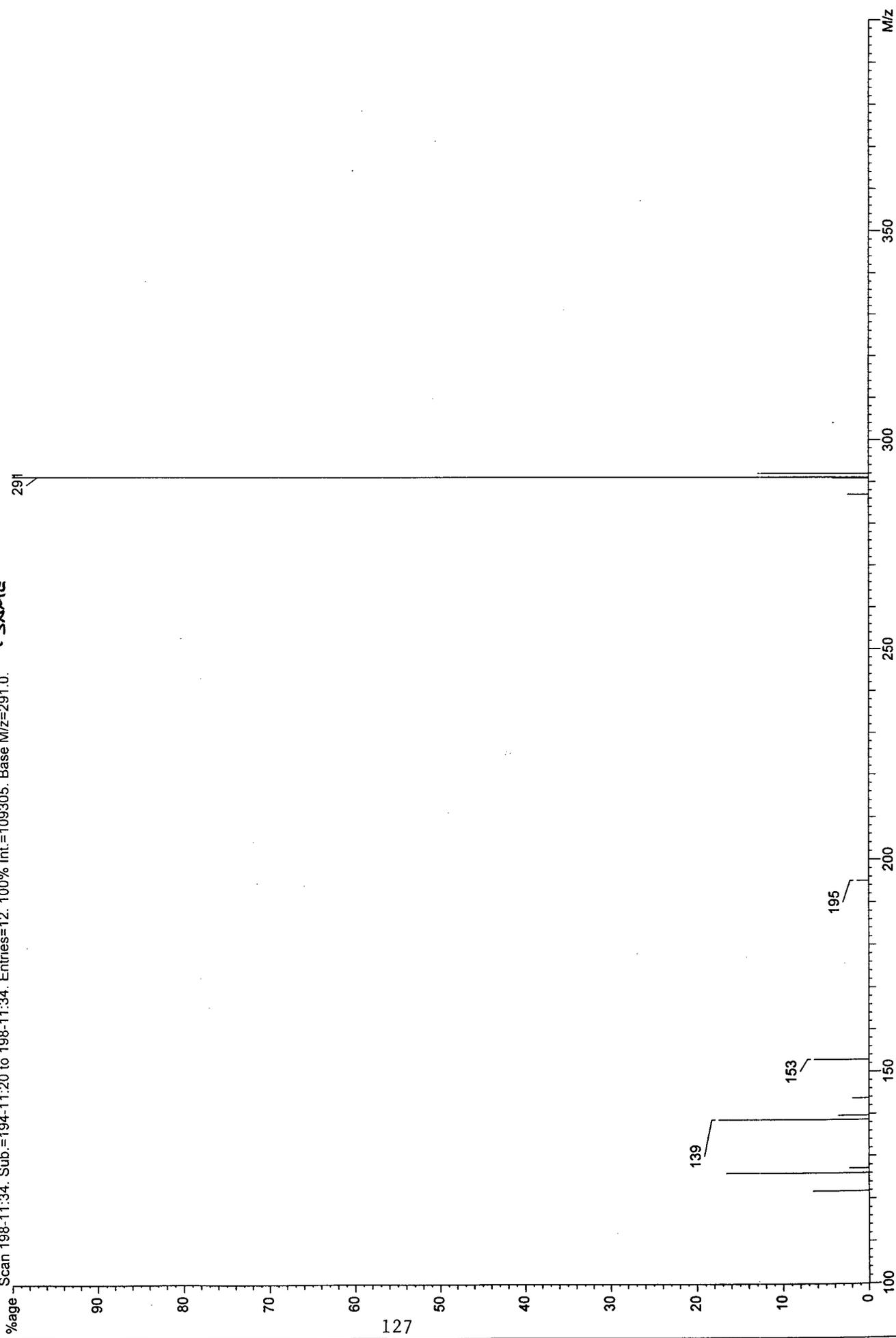
File Name : c:\mswin\data\cmsteric1.mss
Creation Date/Time : 5/7/98 at 9:47:02
File Title : CATECHIN
Instrument : MS80.TSP
Description 1 : 2.5% CH3COOH/CH3CN (97/3); 1ML/MIN
Description 2 : BY-PASS

[EPI] CATECHIN FRAGMENTATION

↳ SAME

SCAN GRAPH:

Scan 198-11:34. Sub. = 194-11:20 to 198-11:34. Entries=12. 100% Int.=109305. Base M/z=291.0.

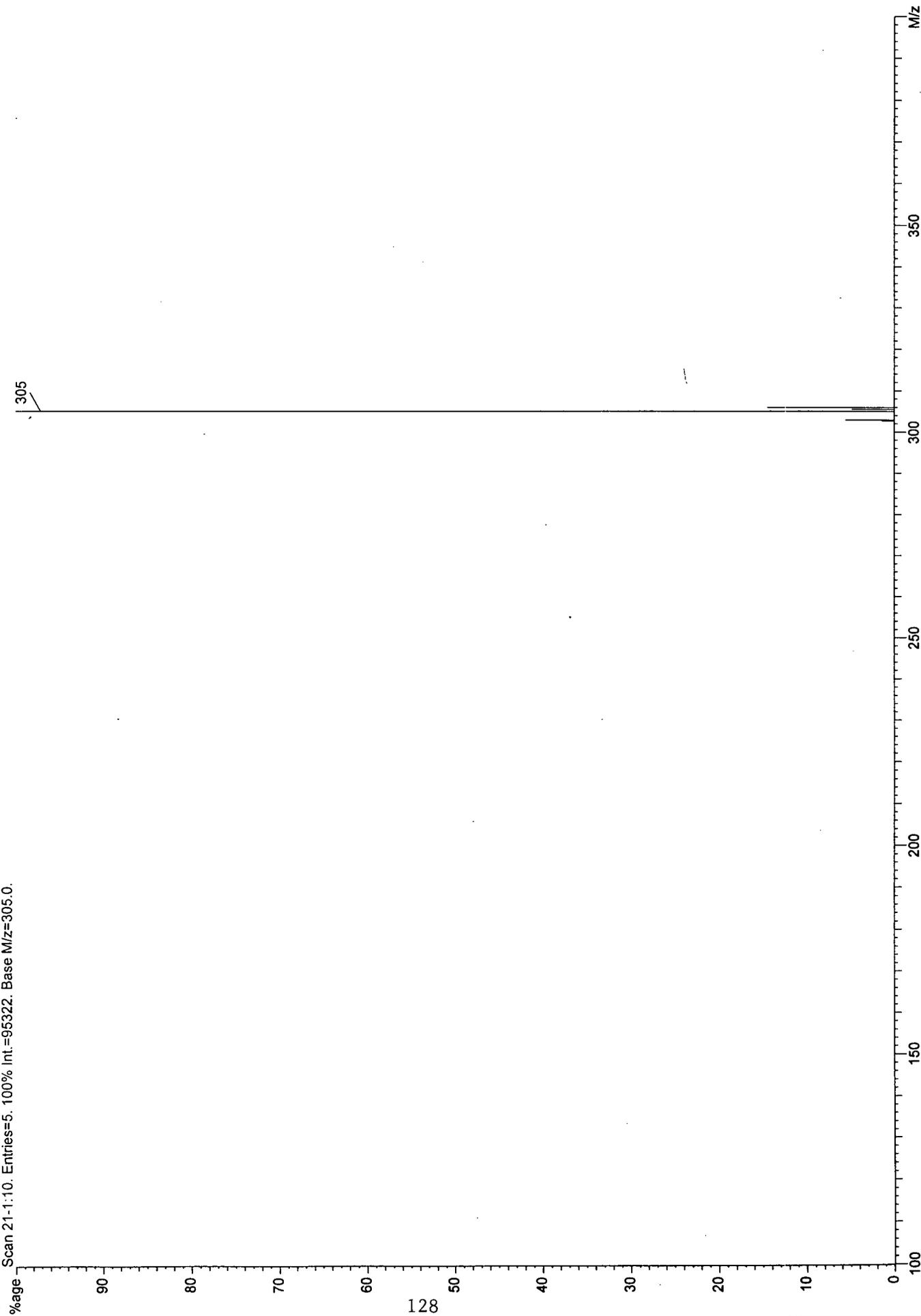


File Name : c:\mswin\data\lcms\eric2.mss
Creation Date/Time : 5/7/98 at 10:25:03
File Title : TAXIFOLIN
Instrument : MS80 TSP
Description 1 : 2.5% CH3COOH/CH3CN (97/3); 1 ML/MIN

TAXIFOLIN FRAGMENTATION

SCAN GRAPH.

%age Scan 21-1:10. Entries=5. 100% Int.=95322. Base M/z=305.0.

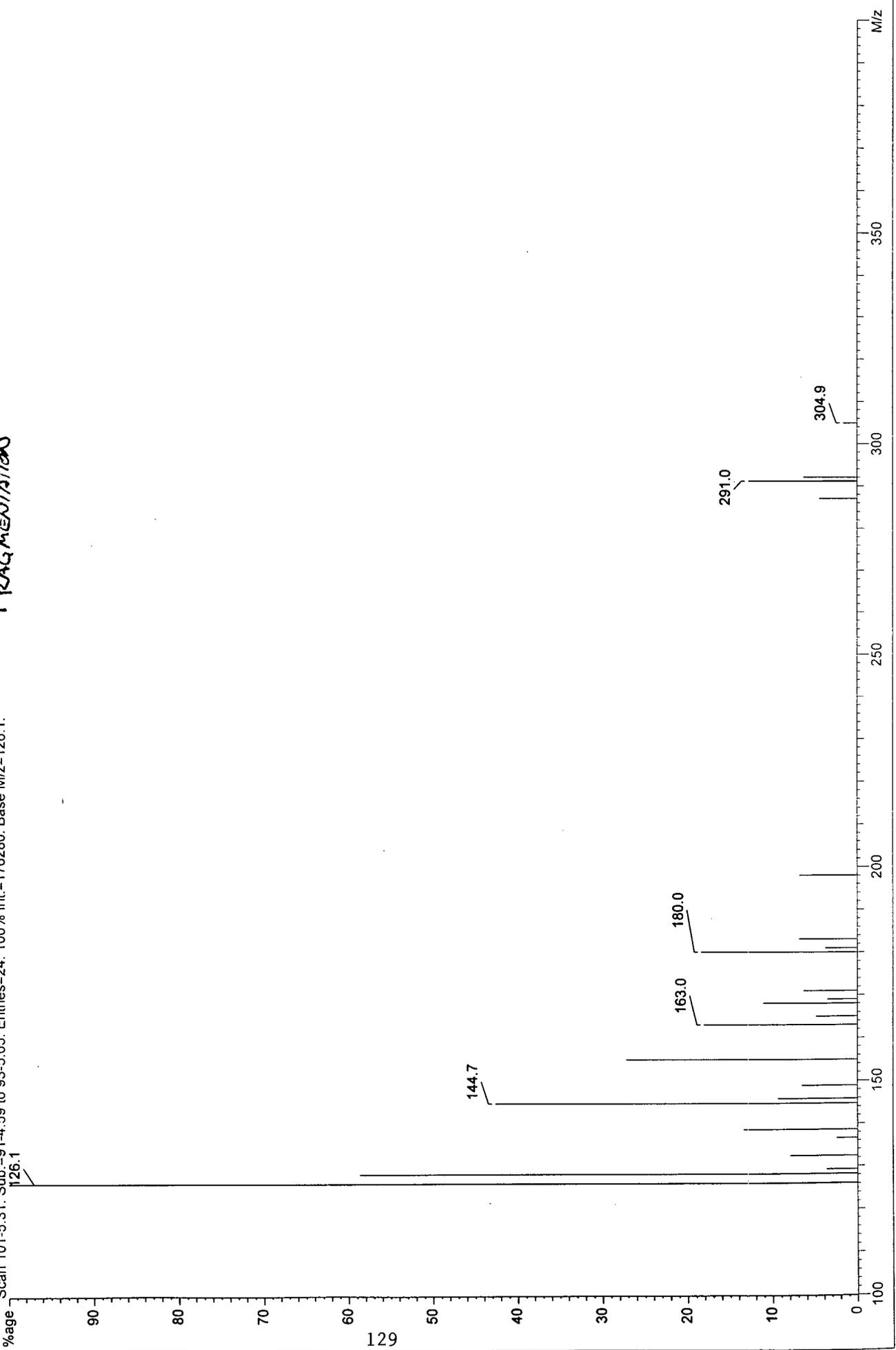


File Name : c:\mswin\data\lcm\slr\c6.mss
Creation Date/Time : 5/7/98 at 11:50:09
File Title : DFIR
Instrument : MS80
Description 1 : DB1-30m, 0.25um; 80C->320C @ 15C/m; He= 27cm/s
Description 2 : 2ul inj (splitless); solvent delay 10 min
Description 3 : S= 250C; D= 300C; I= 300C; Int= 300C

D. FIR INNER BARK AQ EXT.

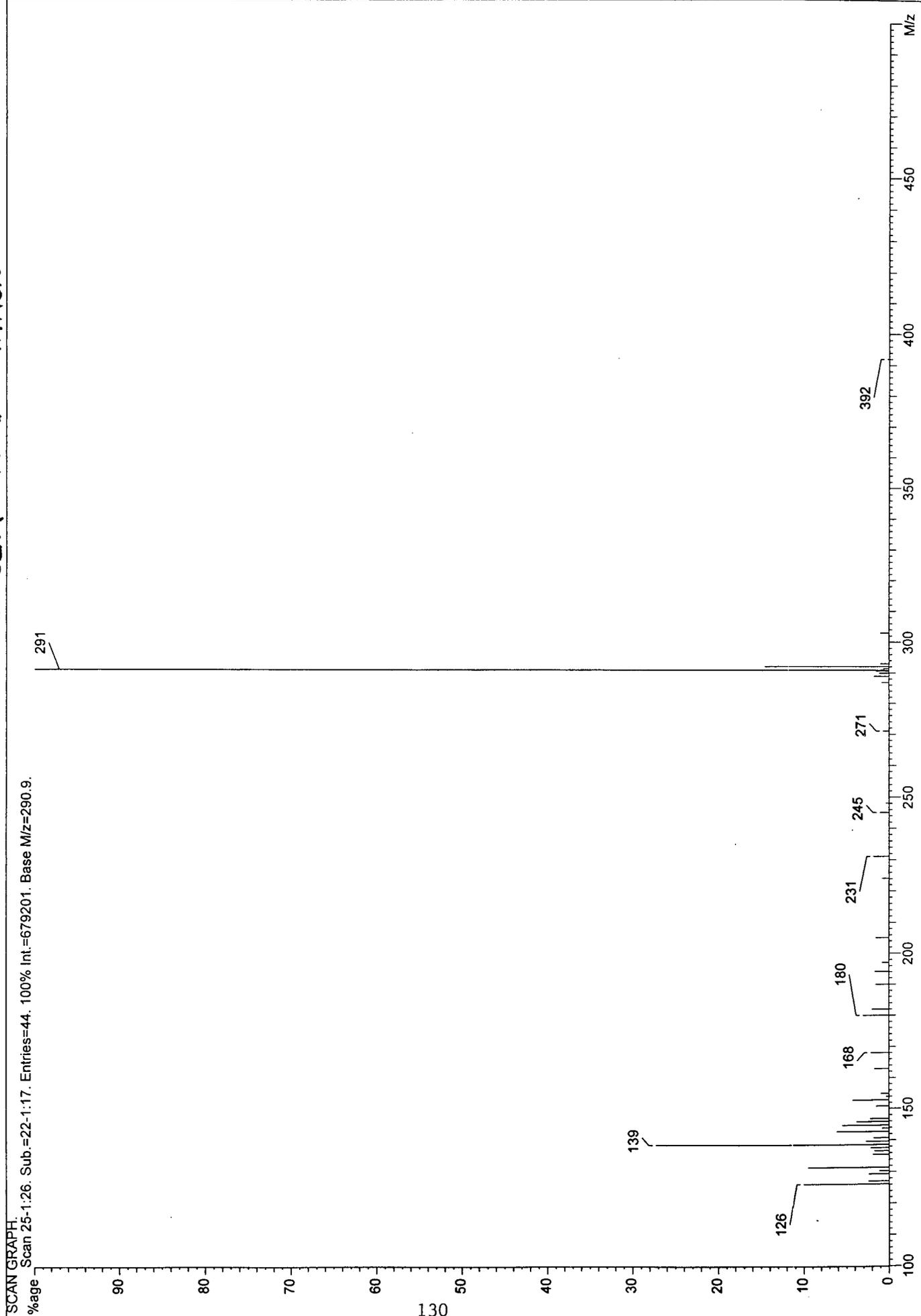
FRAGMENTATION

SCAN GRAPH.
Scan 101-5:31. Sub.=91-4:59 to 93-5:05. Entries=24. 100% Int.=176280. Base M/z=126.1.



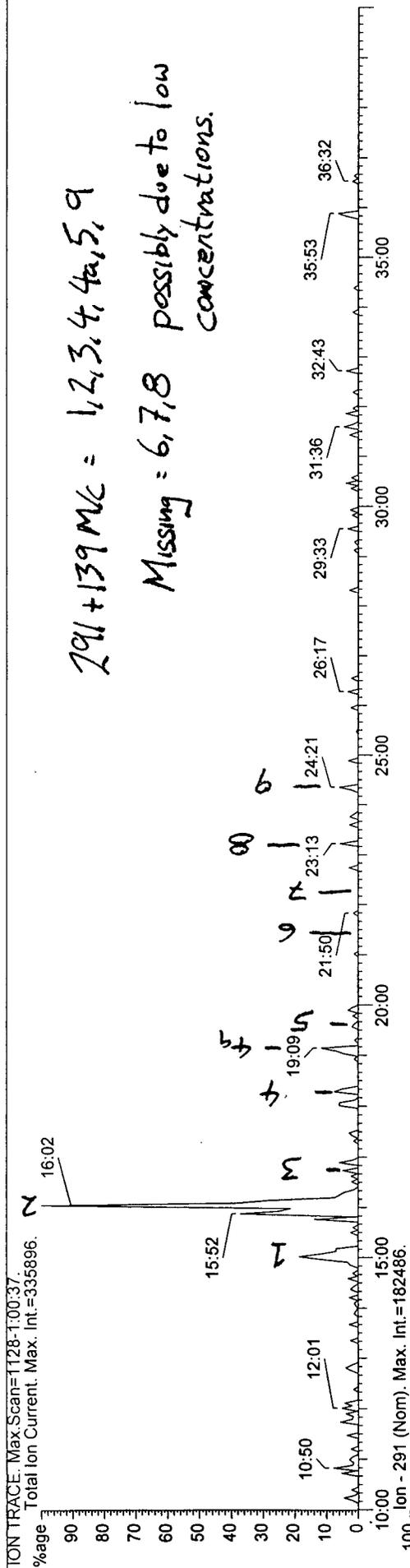
File Name : c:\mswin\data\cms\leric5.mss
Creation Date/Time : 5/1/98 at 11:37:36
File Title : INDENA
Instrument : MS80 TSP
Description 1 : 2.5% CH3COOH/CH3CN (97/3); 1 ML/MIN

INDENA FRAGMENTATION



File Name : c:\mswin\data\lens\lc870.mss
 Creation Date/Time : 5/1/98 at 12:54:37
 File Title : INDENA (THRU COLUMN)
 Instrument : MS80 TSP
 Description 1 : 1 ML/MIN; GRADIENT PROGRAM

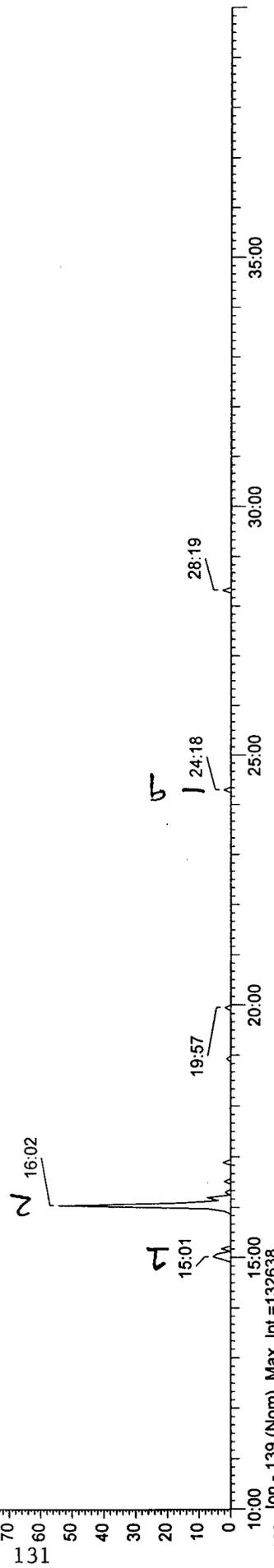
10-40 min



291 + 139 M/C = 1, 2, 3, 4, 4a, 5, 9

Missing = 6, 7, 8 possibly due to low concentrations.

291 M/C = Catechin Peak # 1, 2, 9

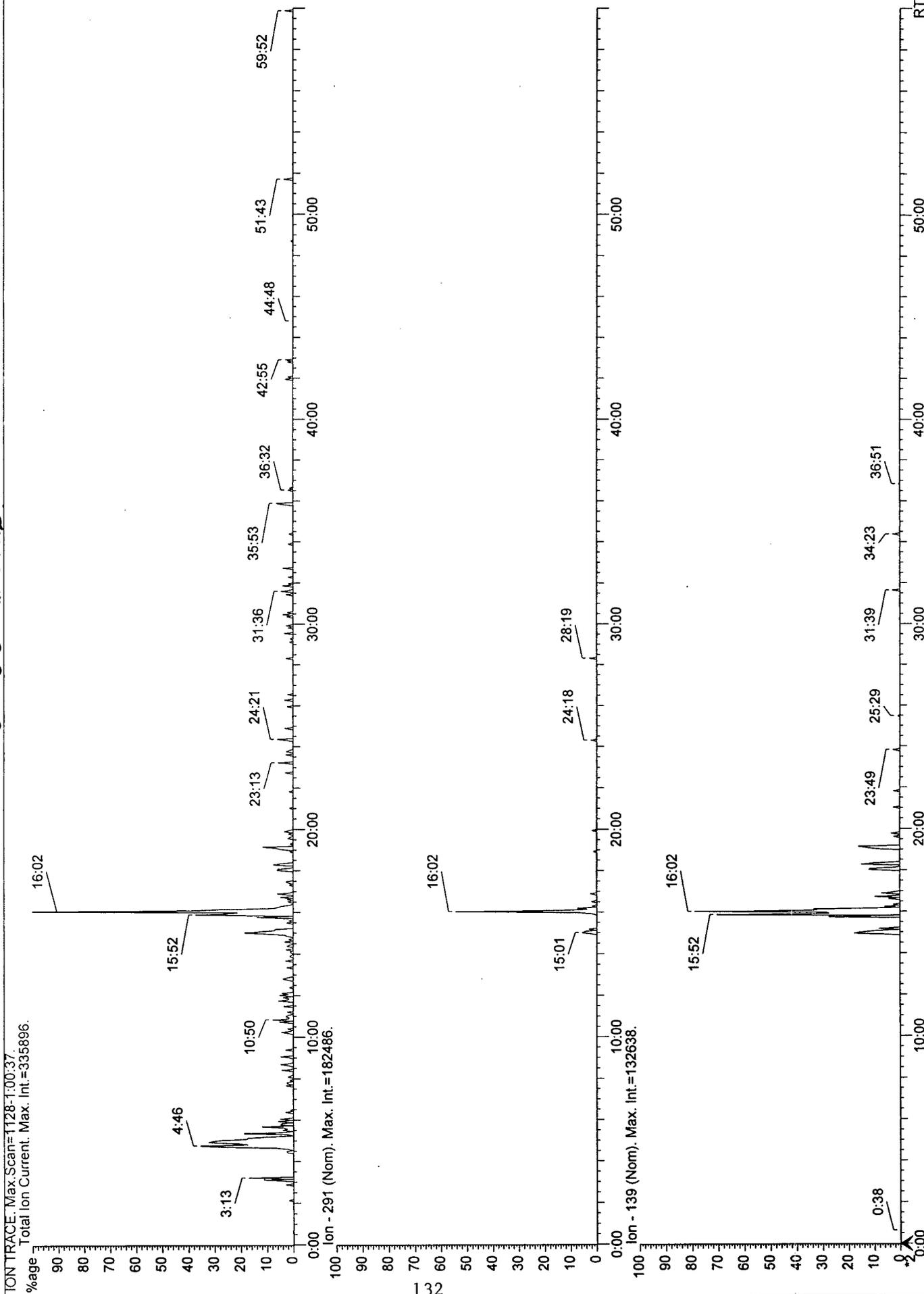


139 M/C = Catechin fragment

Peak # 1, 2, 3, 4, 4a, 5

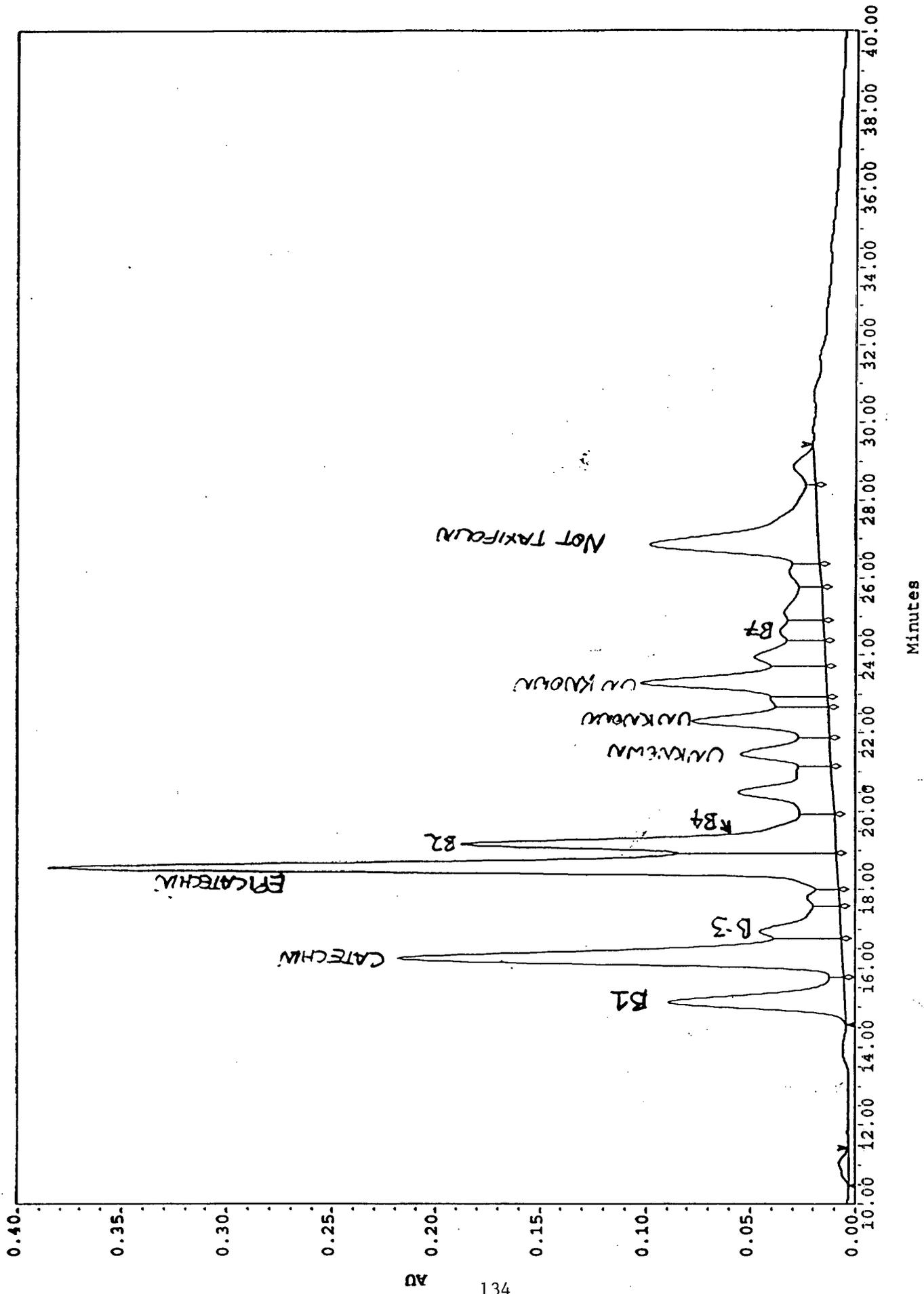
File Name : c:\mswin\data\cms\lc870.mss
 Creation Date/Time : 5/1/98 at 12:54:37
 File Title : INDENA (THRU COLUMN)
 Instrument : MS80 TSP
 Description 1 : 1 ML/MIN; GRADIENT PROGRAM

0 - 60 MINUTES



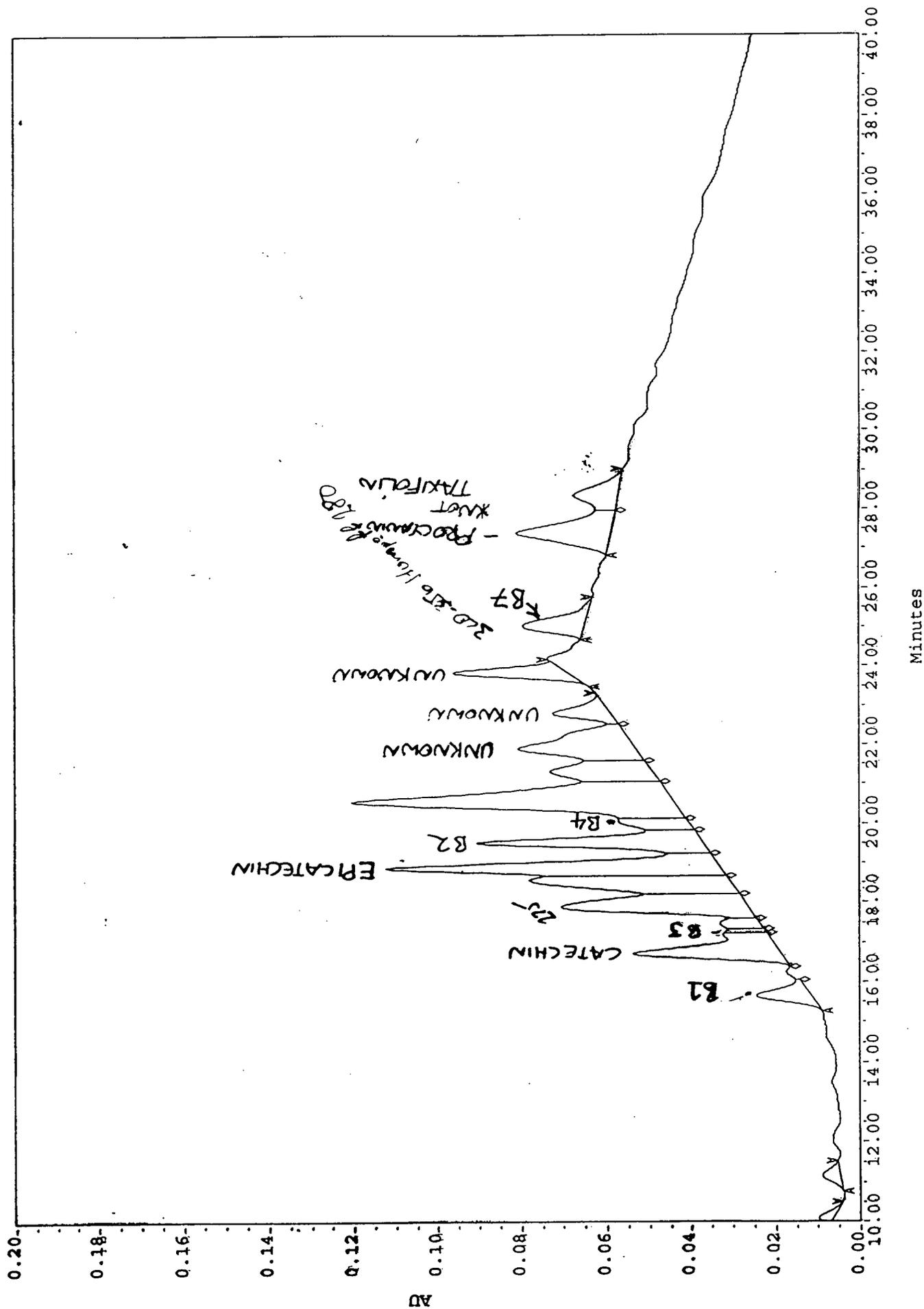
APPENDIX E

Commercial OPC Product Overlay



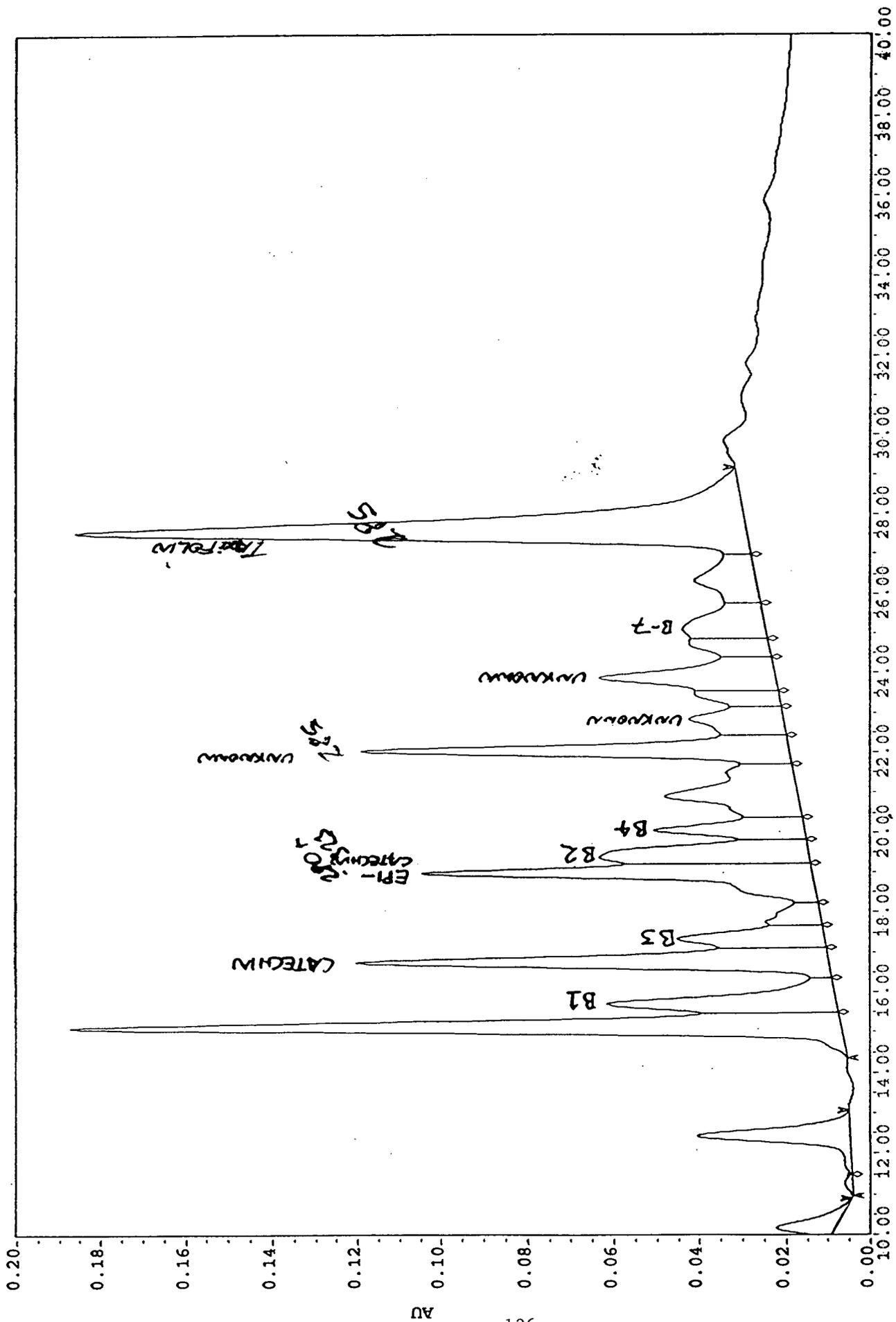
CAPERS - OPLC MIX

SampleName: A5 Spike (RPT) Vial: 9 Inj: 1 Ch: Wvln Chi Type: Unknown



NU-GREENS Prolong™

SampleName: C5 Tablet Vial: 64 Inj: 1 Ch: Wvln Ch1 Type: Unknown



Minutes

SUNFORCE Pycnosamol™

SampleName: B5 Tablet vial: 60 Inj: 1 Ch: Mvln Ch1 Type: Unknown

APPENDIX F

T-Test Data for the Two Indena Standard Curves

T-Test Data for the Two Indena Standard Curves

0.05M LiCl Preparation	
Grams Indena / 10 mL	Response
0.000	0.000
0.105	9.207
0.211	19.194
0.300	25.935
0.388	32.858

Aqueous Preparation	
Grams Indena / 10 mL	Response
0.000	0.000
-	-
0.214	20.746
0.284	28.308
0.387	35.364

Stat. Basic Stats	T-test for Dependent Samples						
	Marked differences are significant at p<.05000						
Variable	Mean	Std.Dv.	N	Diff.	Std.Dv. Diff.	t	df
VAR1	21.45156	11.99088					
VAR2	0.23615	0.13420	8	21.21541	11.85772	5.060522	7

Stat. Basic Stats	T-test for Dependent Samples	
	Marked differences are significant at p<.05000	
Variable	p	
VAR1		
VAR2	0.001463	

APPENDIX G

**Statistical Analysis for the Composite
Indena LeucoSelect™ Standard Curve**

**Statistical Analysis for the Composite Indena LeucoSelect(TM)
Standard Curve**

Stat. Basic Stats	Means and Standard Deviation			
Variable	Mean	st.dev.	minimum	maximum
VAR1	0.23615	0.1342	0.00	0.38840
VAR2	21.45156	11.99088	0.00	35.36415

Stat. Basic Stats	Model: v2;v1*b1 Dep. Var: VAR2 Loss; (OBS-PRED)**2 Final loss: 45.698807870 R=0.99217 Variance explained: 98.440%
N=8	B1
Estimate	90.36164

Slope : $y=90.36164x$
Variance = 98.440%