ABSTRACT

ANALYSIS OF PLANT POLYPHENOLS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY AND PROTEIN BINDING

by Godfred Ansong

The high molecular weight plant polyphenols known as tannins have both positive and negative effects on human and animal health. Tannin constituents from natural sources have been implicated in astringency of wine, inhibition of carcinogenesis and control of diabetes. The main tannin constituents in white oak, black raspberry and tannic acid were analyzed with HPLC and mass spectrometry. Vescalagin, castalagin, grandinin and roburin E, four ellagitannins purified from the white oak were characterized with a preliminary radiolabeled protein-binding assay. Sanguiin H-10 and its precursors were identified as the main ellagitannins in black raspberry. A hexagalloyl constituent of tannic acid was identified as the active constituent likely to have insulin-mimetic activity. A simple method was devised to assess the protein binding capacities of plant polyphenols by immobilizing unfractionated plant extracts on filter paper disks, and reacting with radiolabeled BSA. Protein binding was proportional to the amount of immobilized extract.

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Abbreviations

APCI	Atmospheric pressure chemical ionization
BSA	Bovine serum albumin
CID	Collision induced dissociation
ESI-MS	Electrospray ionization-mass spectrometry
ESI-QTOF	Electrospray ionization-quadrupole time of flight
FAB-MS	Fast atom bombardment-mass spectrometry
HHDP	Hexahydroxydiphenyl
LC/MS	Liquid chromatography/mass spectrometry
MALDI-TOF	Matrix-assisted laser desorption ionization-time of flight
MS/MS	Double mass analyzers in tandem mass spectrometry
NMR	Nuclear magnetic resonance
PGG	Pentagalloyl glucose
RP-HPLC	Reverse phase high performance liquid chromatography
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TIC	Total ion chromatogram or current
xGG	Galloyl glucose

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First and foremost, I thank God for giving me the strength to carry out these experiments. My gratitude goes to Dr. Ann Hagerman for her time and patience. Her passion for excellence and assertiveness in students has tremendously shaped the way I conduct my experiments and analyze data. I thank the Chemistry & Biochemistry Department of Miami University for the warm reception with which it received me and for offering me the opportunity to use the-state-of-the-art instrumentation to carry out my experiments. I thank Drs. Thomas Asquith of Brown Foreman Distillery and Ian Peat, instrumentation specialist at the Chemistry Department for their assistance in carrying out the LC/MS analysis. Last but not least, I thank my parents for their constant prayers and support.

Chapter One

Plant polyphenols

General characteristics of plant polyphenols

Polyphenols are secondary metabolites that are widespread throughout the plant kingdom. They are a heterogeneous group of compounds that contain two or more phenolic groups and include lower molecular weight compounds such as catechin and ellagic acid, and higher molecular weight compounds such as the polygalloyl glucoses [13]. Polyphenols are known to influence the pharmacological activities of plants. For instance, prevention of lung cancers [30] and the protection of the body tissues against oxidative stress [26] have been attributed to plant polyphenols. The higher molecular weight plant polyphenols (MW $\geq$ 500) are termed tannins and this class of compounds characteristically binds proteins [13].

Classes and biosynthesis of tannin

There are three classes of tannin, the hydrolysable tannins, the proanthocyanidins [13] and the phlorotannins [8]. The hydrolysable tannins have a polyol core (usually D-glucose) in which the hydroxyl groups of the core glucose are esterified by gallic acid (in gallotannins) or hexahydroxydiphenic acid (in ellagitannins). The ester bond can be hydrolyzed by mild acids and bases or by the enzyme tannase to yield phenolic acids and carbohydrates [18]. The proanthocyanidins on the other hand are flavanoid units (both oligomers and polymers) that are linked by carbon-carbon bonds [29]. The carbon-carbon bonds can not be hydrolyzed, and can only be cleaved oxidatively in acidified alcohol to yield anthocyanidin pigments, a characteristic feature of the proanthocyanidins used in their analysis [24]. The third group known as phlorotannin comprises tannins with 1,3,5-trihydroxybenzene (phloroglucinol) monomer units that are linked by carbon-carbon

bonds or aryl ether (C-O-C) bond [8,25]. They are exclusive to the brown algae and the focus of this study will not be on them.

The biosynthesis of hydrolysable tannins starts with gallic acid, whose source is still not clear. Several pathways have been proposed as the source of gallic acid, but the direct dehydration of 3-dehydroshikimic acid (an intermediate of the shikimate pathway) by dehydroshikimate dehydrogenase seems most probable [9,23]. The gallic acid is esterified with an activated D-glucose (UDP-glucose) by the enzyme glucosyltransferase to form monogalloyl glucose (β-glucogallin or 1-O-galloyl-β-D-glucose) [19,20]. Galloyl glucoses from digalloyl to pentagalloyl glucose are then formed by a series of galloylations with the monogalloyl glucose, which serves as a galloyl group donor (Fig. 1.1).

There are two ways in which gallic acids can be esterified with the hydroxyl groups to form higher molecular weight hydrolysable tannins. One type of reaction involves the esterification of galloyl groups to free hydroxyl groups on the core glucose molecule, ultimately yielding the central metabolite β-pentagalloyl glucose (β-PGG). Alternatively, esterification can take place at the phenolic hydroxyl group rather than the aliphatic glucose hydroxyl group, leading to chains of galloyl residues. The bond between two galloyl groups is known as the depside bond (Fig. 1.2). Depside bond formation in the hydrolysable tannins can occur before all the sugar hydroxyls have been esterified. For instance, β-1,2,3,4,6-pentagalloyl-O-D-glucose and 3-O-digalloyl-1,2,4-tri-O-galloyl-β-glucose are isomers in which the latter has one free hydroxyl group on carbon 6 of the core glucose. Two galloyl groups in 3-O-digalloyl-1,2,4-tri-O-galloyl-β-glucose are esterified as a digalloyl residue on the third carbon of the glucose molecule.

The chemistry involved in the esterification of a galloyl residue with a phenolic hydroxyl group is not the same as the esterification with the free hydroxyl group of the glucose molecule. The galloyl transferases involved in the two processes are different. There is a single galloyl transferase involved in the sugar esterification whereas in the esterification at the phenolic residues there are several isoenzymes involved, four of which (galloyl transferases A-D) have been identified [7,9]. Gallotannins with up to twelve esterified galloyl groups have been reported in literature [22].
The ellagitannins are formed by oxidative coupling between sterically adjacent groups.

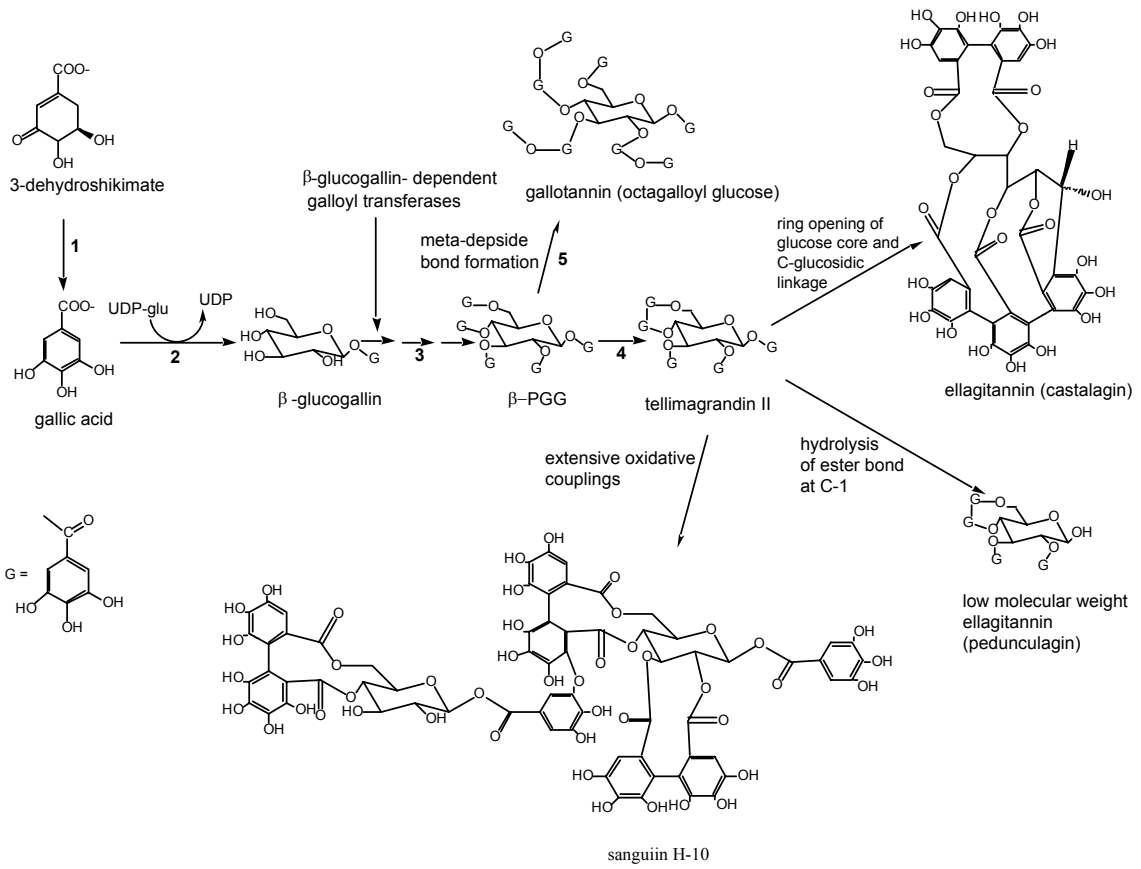

Fig. 1.1: Biosynthesis of hydrolysable tannins. Gallic acid is shown here to be formed from the dehydration of 3-dehydroshikimate by dehydroshikimate dehydrogenase (**1**). A galloyltransferase (**2**) esterifies the gallic acid to an activated glucose (UDP-glucose) to form β-glucogallin (monogalloyl glucose). β-glucogallin dependent galloylation (**3**) of the monogalloyl glucose leads to the formation of β-PGG. Oxidative couplings of galloyl groups on C-4 and C-6 in β-PGG by pentagalloylglucose: O_2 oxidoreductase (**4**) forms tellimagrandin II which serves as a precursor for the formation of high molecular weight ellagitannins. Additional galloyl groups are linked to the β-PGG through depside bonds to form the gallotannins such as octagalloyl glucose. Galloyl transferases (**5**) that link two galloyl groups together are gallotannin-specific.

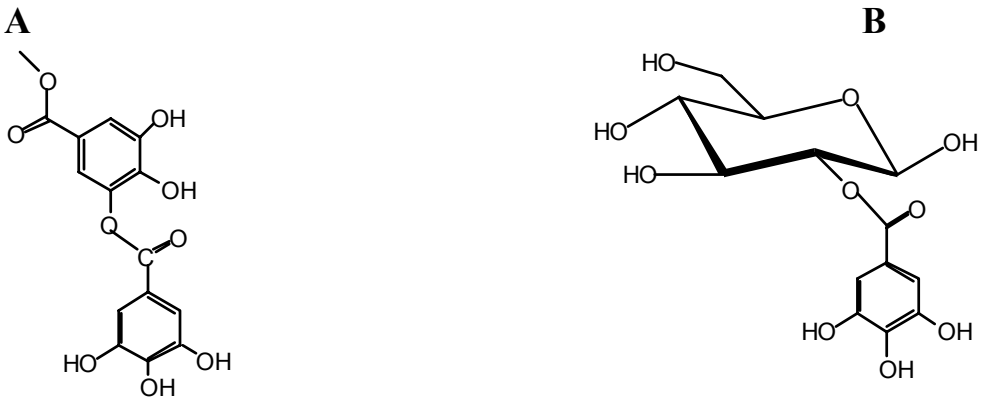

Fig. 1.2: Depside and ester bonds in gallotannins. (**A**) Two galloyl groups linked by a meta-depside bond. (**B**) An aliphatic glucose and a galloyl group linked by an ester bond.

For example, the galloyl groups on C-4 and C-6 in β-PGG are oxidatively coupled by an oxidoreductase to form tellimagrandin II, a simple ellagitannin [21]. Modifications such as extensive oxidative couplings and polymerization occur in tellimagrandin II to form high molecular weight ellagitannins such as the sanguiins (Fig. 1.1). Ring opening of the glucose core and C-glucosidic linkages can also occur in tellimagrandin II to form compounds such as castalagin.

Proanthocyanidin is an end product of the flavanoid pathway. Its biosynthesis starts with the conversion of naringenin chalcone to a variety of flavanones by chalcone isomerase [17]. Flavanone-3-β-hydroxylase then converts the flavanones to dihydroflavonols which are subsequently reduced to leucoanthocyanidins by dihydroflavonol reductase [33]. Anthocyanidin synthase converts the leucoanthocyanidin to an anthocyanidin from which flavan-3-ols are formed with BAN (an anthocyanidin reductase). The final step leading to the formation of the proanthocyanidinin polymer from flavan-3-ols is still not clear. Nevertheless, some mechanisms have been proposed in which a carbocation or a quinone methide formed from flavan-3-ol serves as an initiator monomer unit from which C4-C8 linkages can be formed to generate the proanthocyanidin polymer (Fig. 1.3) [17,33].

Tannin analysis

Gallotannins can be characterized with colorimetric assays such as the rhodanine assay [15] or iodate assay [12]. The rhodanine assay determines the quantity of gallic acid released by hydrolysis through the formation of a colored reaction product between the gallic acid and the rhodanine solution. In the iodate assay, the gallotannin is methanolysed into methyl gallate that reacts with KIO_3 to yield a quinone which is then determined at 525 nm. The ellagitannins are best characterized with the nitrous acid assay [32]. In the nitrous acid assay, ellagic acid released from the hydrolysis of ellagitannin reacts with nitrous acid to form a colored nitrosyl product in pyridine solution. The proanthocyanidins can be characterized by the acid butanol assay. Treating proanthocyanidin under hot acidified butanol in the presence of catalytic trace metal ion

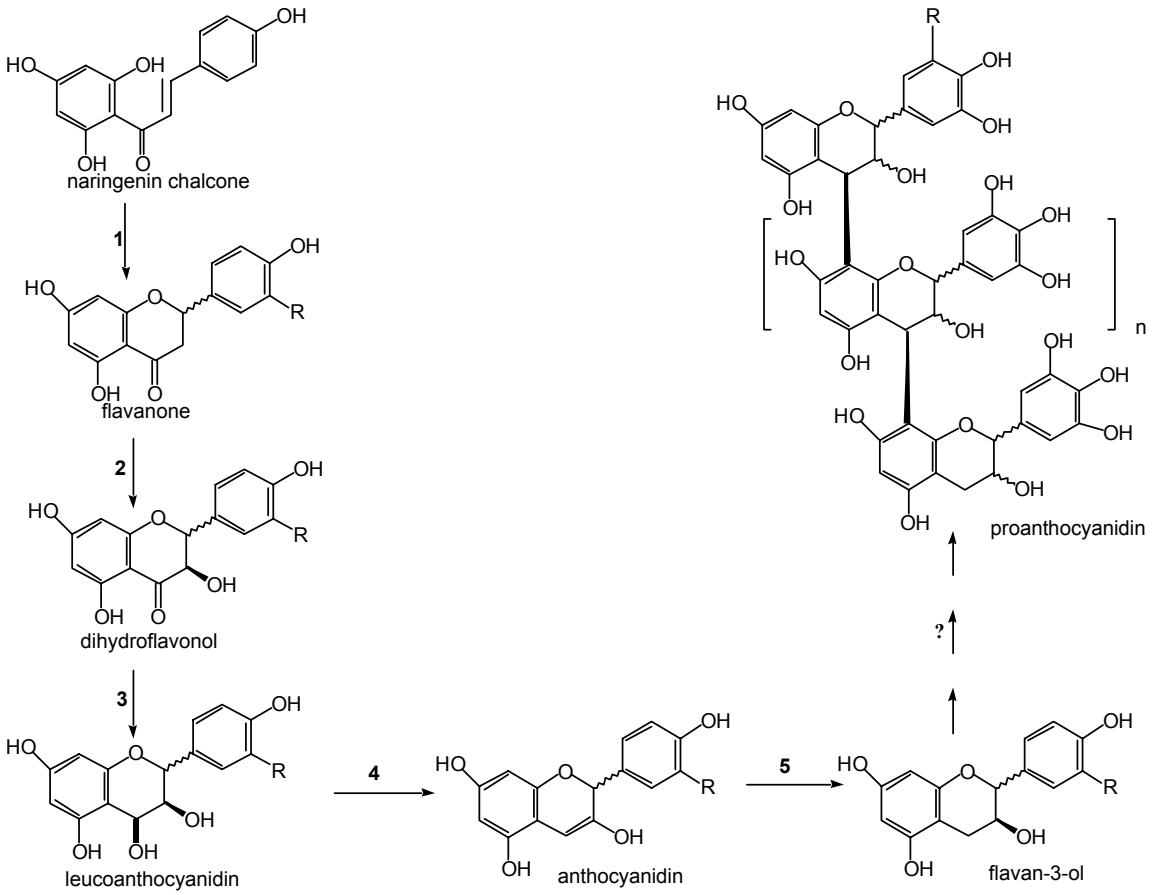

Fig. 1.3: Biosynthesis of proanthocyanidins as a branch of the flavanoid pathway. Naringenin chalcone is converted to the flavanones by chalcone isomerase (**1**). The flavanones are converted to dihydroflavonols by flavanone-3-β-hydroxylase (**2**). Dihydroflavonol reductase (**3**) reduces the dihydroflavonol to leucoanthocyanidin. Leucoanthocyanidins are converted to the anthocyanidins by anthocyanidin synthase (**4**). Anthocyanidin reductase (**5**) reduces the anthocyanidin to flavan-3-ols which are then polymerized into the proanthocyanidins via a carbocation or quinone methide formation in flavan-3-ol.

cleaves the interflavan bond oxidatively giving colored monomeric anthocyanidin pigments [24].

The assays outlined above are chemical degradation assays which only measure released subunits of the parent polyphenol. It is therefore difficult to distinguish between two different tannins that degrade into the same subunits. For instance, beside the hexahydroxydiphenyl group (HHDP), ellagitannins can also have galloyl residues esterified to the glucose molecule making it difficult for the rhodanine assay to distinguish between ellagitannins and gallotannins, both of which can be degraded to yield gallic acid. The assay can also not give an absolute quantification of gallotannins since different sources have different mixtures of gallotannins ranging from mono- to dodecagalloyl glucose.

Using mass spectrometry to determine molecular weights and fragmentation patterns is a widely used method of identifying plant polyphenols [27, 28]. It has the potential to eliminate some of the ambiguities associated with colorimetric assays for tannins by being able to differentiate between tannins that would give similar responses to the colorimetric assay [2, 34]. High performance liquid chromatography coupled to mass spectrometry (LC/MS) is a useful technique that provides selective molecular weight detection of tannin constituents in a plant extract [27]. Molecular ions and characteristic fragment ions obtained from the mass spectrometer are used to identify the constituents in the plant extract. These molecular ions, sometimes with or without reference to authentic standards, have been successfully used to identify tannin constituents. Further fragmentation by collision-induced dissociations (CID) of parent ions, otherwise known as tandem mass spectrometry, offers structural information beside the mass selective identification [4]. Analysis of hydrolysable tannins with the mass spectrometer characteristically shows gallic acid and ellagic acid fragments [27,28]. Mass spectrometric techniques have been developed that are capable of yielding molecular weight distributions in crude tannin mixtures [3,16]. Techniques such as matrix-assisted laser desorption ionization/time of flight (MALDI-TOF) [16], electrospray ionization (ESI) [3] and fast atom bombardment mass spectrometry (FAB MS) [28] have the advantage of having detection limits that are in the nanomolar range for tannins.

Tannins can also be characterized by their ability to bind proteins. The characteristics of tannin-protein interactions have been well studied both in the hydrolysable tannins and the proanthocyanidins. Several techniques such as equilibrium dialysis [5], ESI-MS [31], isothermal titration microcalorimetry [6], size exclusion chromatography [14] and radiolabeled proteins [10] have been employed to study tannin-protein interactions. Tannins with higher molecular weights and larger numbers of hydroxyl or galloyl groups have been found to have high affinity for proteins [1]. However, the interaction may depend on the nature of protein involved. Tannin interactions with random coil proteins may involve cooperative binding while interactions with globular proteins are nonspecific [6]. Protein characteristics favoring strong bonding with tannins include large molecular size, open structures and proline-rich compositions [10]. Tannin-protein interactions can result in either soluble or insoluble complexes in which both hydrophobic and hydrogen bond play key roles in stabilizing the complex [11, 14, 31].

In the first part of this study, molecular ions and fragmentation information obtained from LC/MS analysis of white oak, black raspberry and tannic acid were used to identify the main tannins in these sources. Conventional protein binding assays were used to determine the protein binding capacity of the tannins isolated from white oak (*Quercus alba)*. In the second part, the radiochemical protein binding method [10] was modified to determine the protein binding capacities of five different plant extracts.

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Chapter Two

Purification, identification and characterization of hydrolysable tannins in white oak, black raspberry and tannic acid using LC/MS and protein binding

Introduction

Tannins are implicated in numerous human health issues such as inhibition of carcinogenesis [27], prevention of membrane lipid peroxidation [25] and retardation of microbial growth [12]. Hydrolysable tannins, a subgroup of plant polyphenols, can be found in several plant sources such as berry fruits, galls and heartwood of oak plant.

White oak (*Quercus alba*) is a deciduous plant that grows in the eastern part of the United States. It is a commercial hardwood used in the timber industry and also in cooperage for making barrels for wine aging. The organoleptic qualities (astringency and taste) of beverages are due in part to the polyphenols in grapes used in making the beverages [1, 14], and also believed to be due to the ellagitannin extractives from the oak barrels used in aging the beverages [23]. Castalagin, vescalagin, grandinin and roburin E (Fig. 2.1), have been identified as the main ellagitannins in oak plant [23, 30], suggesting that these four compounds influence the taste of barrel-aged beverages such as wine and whiskey.

Astringency is a tactile sensation in the mouth that arises when salivary proteins are precipitated and their ability to lubricate the mouth is lost [9]. Tannin-salivary protein precipitate formed on the tongue and on the soft palate, causing roughness on the epithelial membrane and resulting in puckering and dryness in the mouth [9]. There is no published correlation between protein binding characteristics of ellagitannins found in oak barrels and wine organoleptic qualities. As part of the study in this chapter, the main ellagitannins in the heartwood of white oak were isolated using preparative normal phase HPLC and identified using analytical RP-HPLC-MS. A preliminary characterization of the ellagitannins was conducted using a protein-binding assay. Our long term goal is to establish a correlation between organoleptic qualities of beverages aged in oak barrels and protein binding capacities of ellagitannins in these barrels.

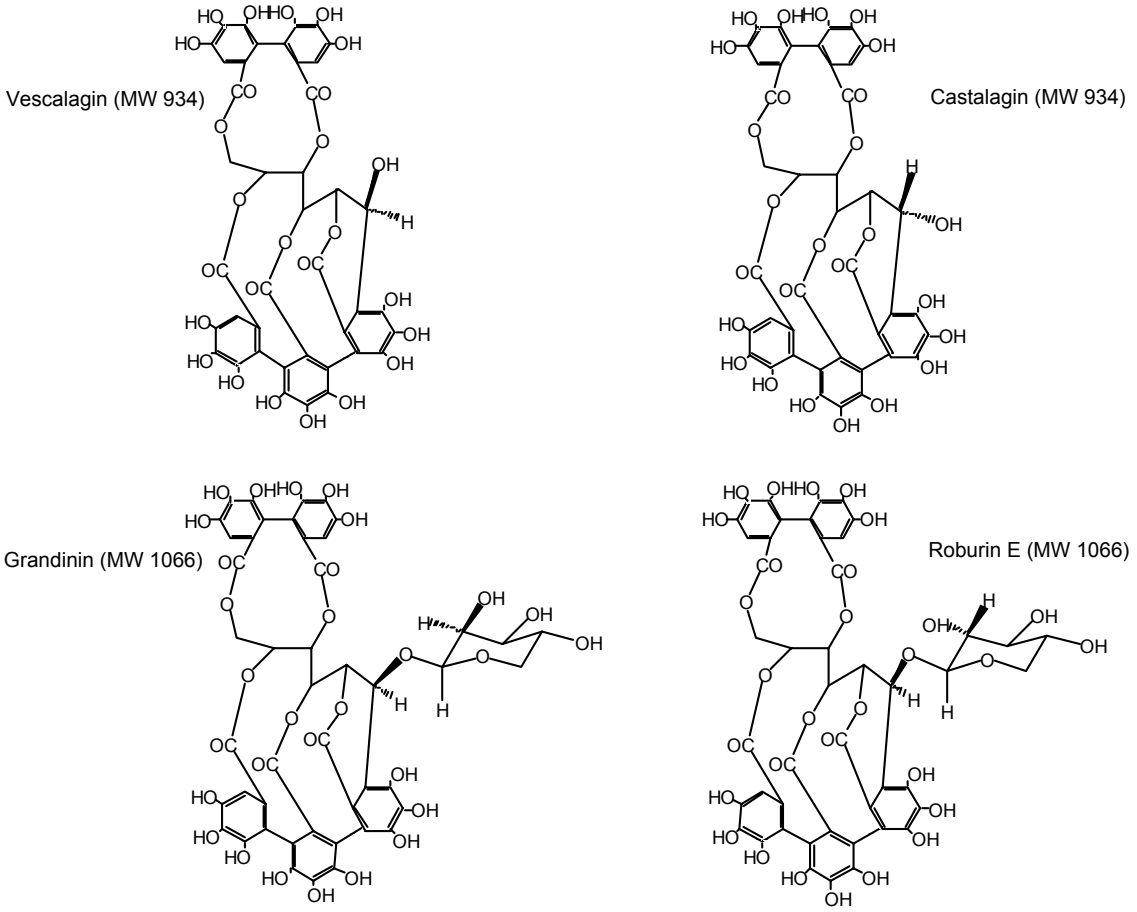


Fig. 2.1: Structures of the main ellagitannins in white oak. These ellagitannins are C-glucosidic compounds in that there is a carbon-carbon linkage between a phenolic group and the C-1 of the ring-opened glucose core. Vescalagin and castalagin differ sterically by the attachment of the hydroxyl group at C-1 of the glucose core. Grandinin and roburin E are glycosides of vescalagin, differing in the pentose sugar substituent attached to C-1 of the glucose core. Grandinin contains lyxose and roburin E contains xylose, which is the 2-epimer of lyxose.

Increasing data link fruit and vegetable consumption to a decreased risk of a number of diseases. Consumption of certain fruits has been reported to provide protection against cancer of the colon [24] and also suppress the oxidation of lipid membranes [15, 25]. Researchers interested in cancer prevention are beginning to focus on black raspberry (*Rubus occidentalis*) as one of the natural sources of cancer chemopreventive drugs. For instance, black raspberry extracts have been found to decrease tumor appearance in rats [6,13]. The active ingredient in black raspberry and the molecular mechanism involved in the inhibition of carcinogenesis are still not clear [8]. The success of commercial ellagic acid at reducing intestinal cancer *in vitro* [31] suggests that ellagic acid, a compound found in raspberries [19], may be involved in the cancer prevention. Since ellagic acid is a degradation product of ellagitannins, it is also possible one or more of the parent compounds might be effective at inhibiting carcinogenesis. In the second part of this study, LC/MS was used to assign the main ellagitannin components to ethanol extracts of black raspberry. The findings from this study may assist investigations into the molecular mechanisms involved in cancer prevention by extracts from black raspberry and possibly lead to discovery of new pharmaceutical agents for cancer prevention.

Tannic acid is a mixture of gallotannins that can be obtained from galls of oak plant. It can inhibit carcinogenesis in human epithelial cells (cholangiocytes) [16]. A constituent of it has also been reported to possess both anti-adipogenesis and glucose transport stimulatory activities in 3T3-L1 cells (Xiao Chen et al., Ohio University, unpublished data). The gallotannin constituent most active in the anti-adipogenesis and glucose transport stimulatory activity assays was tentatively identified as α-pentagalloyl glucose (Xiao Chen et al., Ohio University, unpublished data). α-Pentagalloyl glucose is the anomer of β-pentagalloyl glucose. In α-PGG the galloyl group on carbon-1 is axial rather than equatorial, as it is in the in β- anomer. The enzyme needed for the attachment of an α-substituent to the glucose ring is not known, consistent with the fact that α-pentagalloyl glucose is rare [11]. The third part of the investigation is a follow up on analysis done on tannic acid (Xiao Chen et al., Ohio University, unpublished data). Since the α-anomer rarely exists in nature, LC/MS was used to analyze the main gallotannins in the tannic acid to establish the identity of the active constituent.

Methods

Chemicals and Materials

The dried sawdust of white oak (*Quercus alba*) was from Blue Grass Cooperage (Louisville, KY) and was provided by Dr. Tom Asquith. The radioiodinated BSA was prepared in our laboratory using Chloramine T [2]. Dried extracts from the fruits of black raspberry (*Rubus occidentalis*) were obtained from Dr. Gary Stoner (Ohio State University, Columbus, OH). Tannic acid (Sigma) was provided by Dr. Xiao Chen (Ohio University, Athens, OH). β-PGG was purified from methanolysed tannic acid [5]. Solvents used for LC/MS analysis were HPLC grade and all other solvents were reagent grade.

Instrumentation

An HP 1050 series HPLC (Hewlett-Packard, Germany) coupled to an HP 1050 series UV detector was used for HPLC analysis of tannic acid and purification of oak ellagitannins. Sample injection was done with an HP 1100 series autosampler. An HP 1100 series HPLC (Hewlett Packard, Germany) coupled to an Esquire-LC ion trap mass spectrometer (Bruker Daltonics, Germany) was used for all LC-ESI-MS analysis. Sample injection was done with an HP 1100 series autosampler. All LC analysis and data were obtained using HP Chemstation version 3.1 whereas mass spectrometric analysis was by Esquire Control version 3.0 and data analysis was done using Bruker Daltonics version 4.0.

Extraction of ellagitannins

The air-dried wood dust of white oak was extracted with methanol/tetrahydrofuran solution (75/25, v/v) at room temperature for 2 hours. For every 30 g of the air-dried wood dust, 150 mL of the methanol/THF solution was added. This mixture was then vacuum filtered through Whatman #1 filter paper. The solid residue was discarded and the red-brown filtrate kept for analysis.

Normal-phase HPLC of white oak extract

The redbrown filtrate obtained from the oak wood dust was filtered through a micro-spin filter (0.45 μm pore size), 1mL at a time. It was chromatographed on a Vydac 101TP510 unbonded silica column with the dimensions of 10 x 250 mm, 5 μm. The flow-rate and injection volume were 2 mL/min and 100 μL, respectively. The chromatographic separation was performed using a gradient elution consisting of solvent A: methanol/THF (75/25, v/v) and B: hexane. The elution profile was: 0-10 min, 40-60% B; 10-13 min, 60% B; 13-15 min, 60-80% B; 15-17 min, 80% B; 17-19 min, 80-40% B and 19-25 min, 40% B. UV detection was done at 220 nm. Four main peaks on the normal-phase chromatogram were collected as fractions using an ISCO fraction collector after which the methanol/THF/hexane mobile phase was removed by evaporation under reduced pressure and later with nitrogen gas. Preliminary mass spectrometric analysis was performed at the Campus Chemical Instrument Center, Mass Spectrometric and Proteomics Unit, Ohio State University (Columbus, Ohio) using negative ion electron impact. Peaks that contained compounds with molecular weights 934 and 1066 were collected in large quantity and the mobile phase was then removed by evaporation under reduced pressure. The resulting red-brown suspension was mixed with distilled water and subsequently freeze-dried. The lyophilized extract was brown in color.

LC-ESI-MS of ellagitannins purified from white oak

For the LC/MS analysis of the brown powdery tannin (1 mg/mL in 5% acetonitrile), three different columns were used: an Alltech C-8 column (4.6 x 100 mm, 5 μm), an Alltech C-18 column (4.6 x 100 mm, 5 μm) and an Agilent Zorbax Bonus-RP column (4.6 x 100 mm, 3.5 μm). The mobile phase consisted of A: water and B: acetonitrile both with 0.3% formic acid (v/v). The elution profile for separation on the C-8 column was: 0-15 min, 1-2% B and 15-20 min, 2-5% B. For separation on the C-18 column, the profile was: 0-20 min, 1-5% B; 20-30 min, 5% B; 30-32 min, 5-10% B, 32-42 min, 10% B and 42-43 min, 10-1% B. LC separation on the Agilent Zorbax Bonus RP-column was achieved using a sharp gradient from 5-100% B for 30 min. A second separation was done using a gentle gradient from 5-35% B also for 30 min. The flow rate for all runs was

1 mL/min. The injection volume and UV detection were respectively 20 μL and 220 nm. The mass spectrometric analysis was performed in the negative ion mode with a scan range of 50-2000 m/z. The drying gas temperature was 360^{o}C and gas flow rate was 11 L/min. The nebulizer pressure and capillary voltage were respectively 60 psi and 3.0 kV.

LC-ESI-MS analysis of black raspberry extract

Dr. Gary Stoner's lab (Division of Environmental Health Sciences, School of Public Health and College of Pharmacy, The Ohio State University) supplied four different dry fractions (Ru-F001, Ru-F003, Ru-DH and Ru-ET) of black raspberry extract. Each fraction was dissolved in 0.3% (v/v) aqueous formic acid and 50 μL was chromatographed on an Alltech C-18 column (4.6 x 100 mm, 5 μm) for LC/MS analysis. The elution solvents were A: water and B: acetonitrile both with 0.1% formic acid (v/v). Extract Ru-F001 was separated using the gradient 5-15% B for 35 min. Extracts Ru-F003 and Ru-DH were separated using a gradient profile of 5-100% B for 30 min. Extract Ru-ET was separated with a gentle gradient from 5-30% B for 90 minutes. The UV detection and the flow rate for all runs were 220 nm and 1 mL/min respectively. Mass spectrometric detection for all extracts were performed in the negative ion mode with nitrogen gas as both the drying and the nebulizing gas. The drying temperature was 360^{o}C; the gas flow rate and nebulizer pressure were respectively 10 L/min and 60 psi. The capillary voltage was set at 3.0 kV. The mass scan range for extracts Ru-F003, Ru-DH and Ru-ET was 100-2000 m/z at a skim fragmentation voltage of 47.7 V. Extract Ru-F001 was analyzed at two skim fragmentation voltages, 50.7 and 80.0 V. The mass scan range for Ru-F001 analysis at 80.0 V was 100-2200 m/z while it was 500-3000 m/z at 50.7 V.

Reverse-phase HPLC of tannic acid

The column for the chromatographic separation was an Alltech C-18 (4.6 x 100 mm, 5 μm) fitted with a 25 mm precolumn which was hand-packed with C-18 packing. The mobile phase consisted of A: water and B: acetonitrile both with 0.1% trifloroacetic acid (TFA) (v/v). Separation was performed using an isocratic elution at 20% B with a flow rate of 1 ml/min. The UV detection was at 220 nm. Both unspiked and spiked tannic

acid samples were run for 40 min with an injection volume of 20 μL. Gallic acid and β-PGG were the standards used.

LC-ESI-MS analysis of tannic acid

LC/MS analysis was performed on an Alltech C-18 column (4.6 x 100 mm, 5 μm) with a flow-rate of 1 mL/min using gradient elution consisting of solvents A: water and B: acetonitrile. Both solvents contained 0.3% formic acid (v/v). The elution profile for the separation was: 0-5 min, 20% B; 5-12 min, 20 – 25% B; 12-15 min, 25-20% B and 15-20 min, 20% B. Nitrogen was used as the drying and nebulizing gas in the MS analysis. Nebulizer pressure and capillary voltage were respectively 50 psi and 3.0 kV. The mass spectrometric detection was performed in the negative ion mode with a scan range of 50-2200 m/z. Drying gas temperature was 350^{o}C with a gas flow rate of 10 L/min.

Protein binding assay with ellagitannins purified from white oak

The protein-binding assay with the purified ellagitannin was adapted from [3]. Just before use, an aliquot of the radioiodinated BSA was dialysed for 2 hours at 4^{o}C in buffer A (0.2 M acetate, 0.17 M NaCl, pH adjusted to 4.9) and diluted with 0.68 mg/mL cold BSA to a final concentration of 0.61 mg/mL (specific activity of 0.2 μCi / mg protein). The concentrations of both cold and hot BSA were determined spectrophotometrically at 280 nm using $E^{1\%}_{1cm}$ of 6.6. Aliquots of the cold BSA solution (100 μL) were pipetted into a 0.65 mL microfuge tubes containing 50 μL of the hot BSA solution. Total mass of BSA in assay tube was 98.5 μg. Buffer A was then added to each assay tube to a total volume of 300 μL. The capped tubes were vortexed and then aliquots of 100% methanol (90-40 μL) were added with immediate vortexing. Aliquots of 1.4 mg/mLpurified ellagitannin in methanol (10-60 μL) were also added to the assay tubes with immediate vortexing. Another set of assay tubes was prepared in which aliquots of the cold BSA (0-250 μL) and buffer A were added to 50 μL of hot BSA to a total volume of 300 μL. The purified ellagitannin solution (100 μL of 0.14 mg/mL solution) was added to this set of tubes with immediate vortexing. All the assay samples were incubated at room

temperature for 30 minutes after which they were centrifuged at 13000g for 10 minutes at 4^{o}C. The supernatants were carefully aspirated without disrupting the insoluble tannin-protein precipitate. The precipitate was gently washed with 100 μL buffer A and centrifuged at 13000g for 5 minutes. The supernatant was aspirated and samples counted on a Cobra auto-gamma counter (Packard Instrument, IL, USA). The amount of protein precipitated by tannin in the assay tube that had no cold BSA was taken as 100% bound. Thus, the amount of hot BSA precipitated in the presence of cold BSA was expressed as a percentage of the amount precipitated in the absence of competitor. Background precipitation was subtracted from all values before calculations.

A microfuge tube containing 50 μL of the hot BSA, 250 μL of 2 mg/mL cold BSA and 60 μL of 100% trichloroacetic acid (TCA) was prepared as a protein precipitation check. For background counts, methanol was substituted for the tannin solution. For the total counts, 50 μL of the hot BSA was dispensed into a microfuge tube.

Results

Analysis of ellagitannins in white oak

LC/MS analysis

A preliminary mass spectrometric analysis was done on white oak fractions collected from the preparative normal phase HPLC to scan for the main ellagitannis vescalagin/castalagin (MW 934) and roburin E/grandinin (MW 1066) (Fig. 2.1). A pair of partially resolved peaks that eluted between 12 and 15 min with the normal phase chromatography (B in Fig. 2.2A) contained the four ellagitannins of interest (vescalagin/castalagin, 933.0 m/z and grandinin/roburin E, 1064.9 m/z) (Fig 2.2 B). Peak B was collected in large quantity and lyophilized to yield a brown powdery sample that was analyzed with RP-HPLC-MS.

Three different columns were used in the analytical RP-HPLC-MS, Alltech C-8 and C-18 columns and an Agilent Zorbax Bonus RP column (a C-18 column). Separation of sample constituents on the C-8 column was not successful. All components were washed off the column rapidly in one large peak that appeared between 0.5 and 2.0 min (Fig. 2.3A). A peak similar to this was also observed in the Alltech C-18 column

between 1.0 and 3.0 min (Fig. 2.3B). Beside this peak, there were three other peaks that were observed in chromatograms obtained with the Alltech C-18 column. These peaks were partially resolved. The first peak, which eluted at 9 min, had an m/z value of 1065.1 (Fig. 2.3C) while the second peak, eluting at 11 min, was a mixture of 932.9 m/z and 1065 m/z (Fig. 2.3 D). The third peak eluted at 16 min and had an m/z value of 933.1 m/z (Fig. 2.3E). These values represent the $[M-H]^-$ ions of the main ellagitannins in white oak, castalagin/vescalagin (MW 934) and grandinin/roburin E (MW 1066). Attempts to improve resolution on the C-18 column by changing the mobile phase to methanol/water mixture and acetonitrile/water, both with 40-70 % water were not successful (results not shown). When the gradient was started with a mobile phase that had a large amount of water, the resolution was improved. As a result, an Agilent Zorbax Bonus RP C-18 column, which is known to withstand a mobile phase with high percentage of water [32], was used for the separation with a gradient starting from 95% water (acetonitrile/water, 5/95, v/v). Method development for separation on the Zorbax column was achieved in two steps. The first step used a sharp gradient starting from 5% to 100% of solvent B for 30 min while the second step had a gentle gradient starting from 5% to 35% also for 30 min. Separation with the gentle gradient gave a better resolution of peaks (Fig. 2.4 A). Four sharp peaks (B, C, D and E), well separated from each other, were observed. The first two peaks (B and C) eluted at 9.0 and 12.0 min respectively, and each had an m/z value of 1065 ($[M-H]^-$ ion of roburin E and its isomer grandinin) (Fig. 2.4 B, C). The last two peaks eluted at 14 and 23 min and each had an m/z value of 933 ($[M-H]^-$ corresponding to the ions of vescalagin and its isomer castalagin) (Fig. 2.4 D, E).

Protein binding assay

When protein was held constant, the amount of BSA precipitated increased linearly with increasing amount of the ellagitannin until protein precipitation became independent of added ellagitannin (Fig. 2.5A). In the saturated region, 60.00 μg (60.00 nmol) of the ellagitannin precipitated 31.00 $\pm$ 3.51 μg (0.47 $\pm$ 0.05 nmol) of the BSA. This was 31.5%

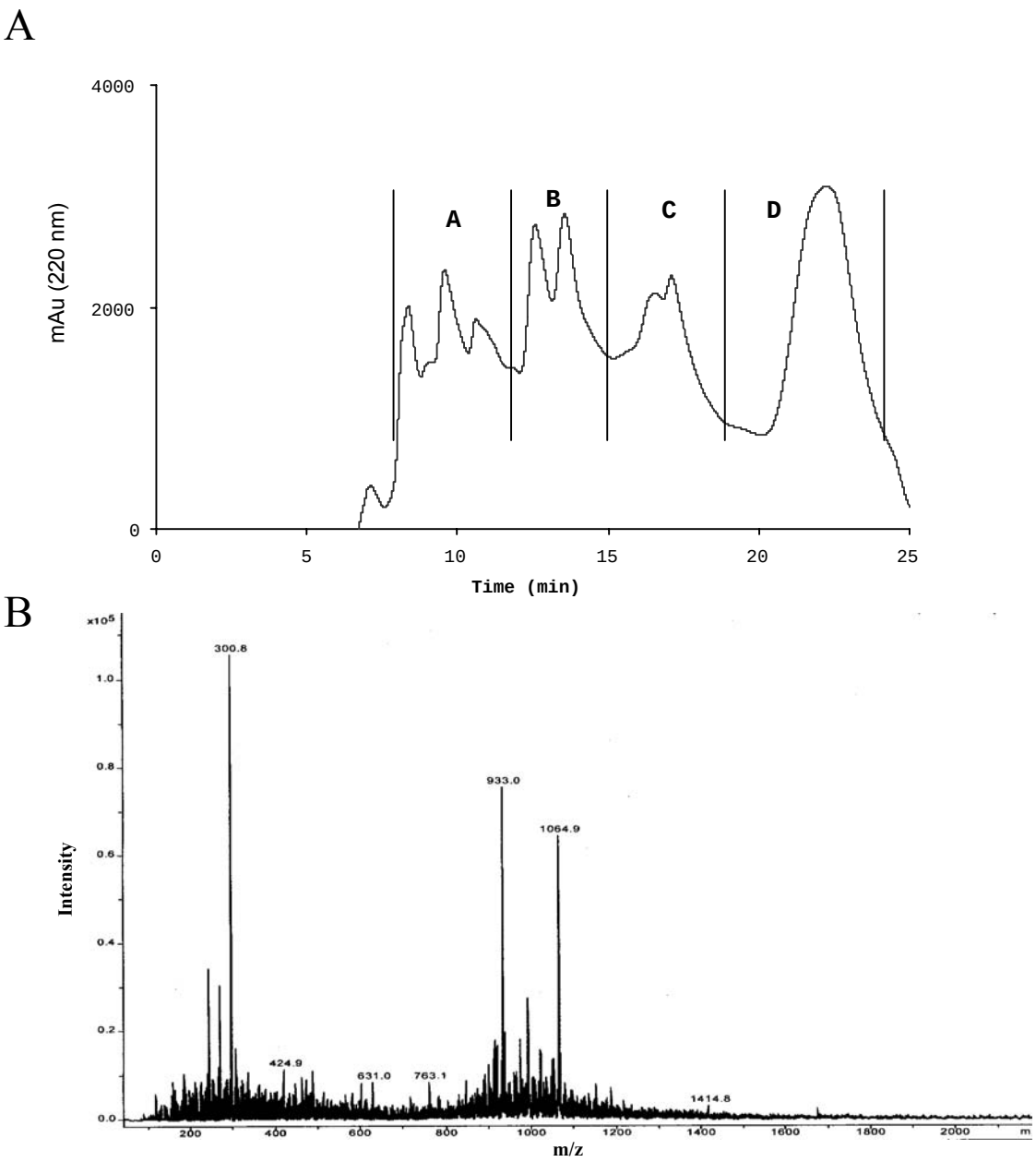


Fig. 2.2: Normal phase-HPLC purification and preliminary mass spectrometric analysis of white oak extract. (**A**) Four fractions were collected using the normal phase chromatography. Mobile phase composition was A: methanol/THF (75/25, v/v) and B: hexane. Flow rate was 2mL/min and UV detection was done at 220 nm. (**B**) Negative ion mass spectrum of peak B indicating the presence of vescalagin/castalagin (933.0 m/z) and grandinin/roburin E (1064.9 m/z). Ellagic acid was observed at 300.8 m/z.

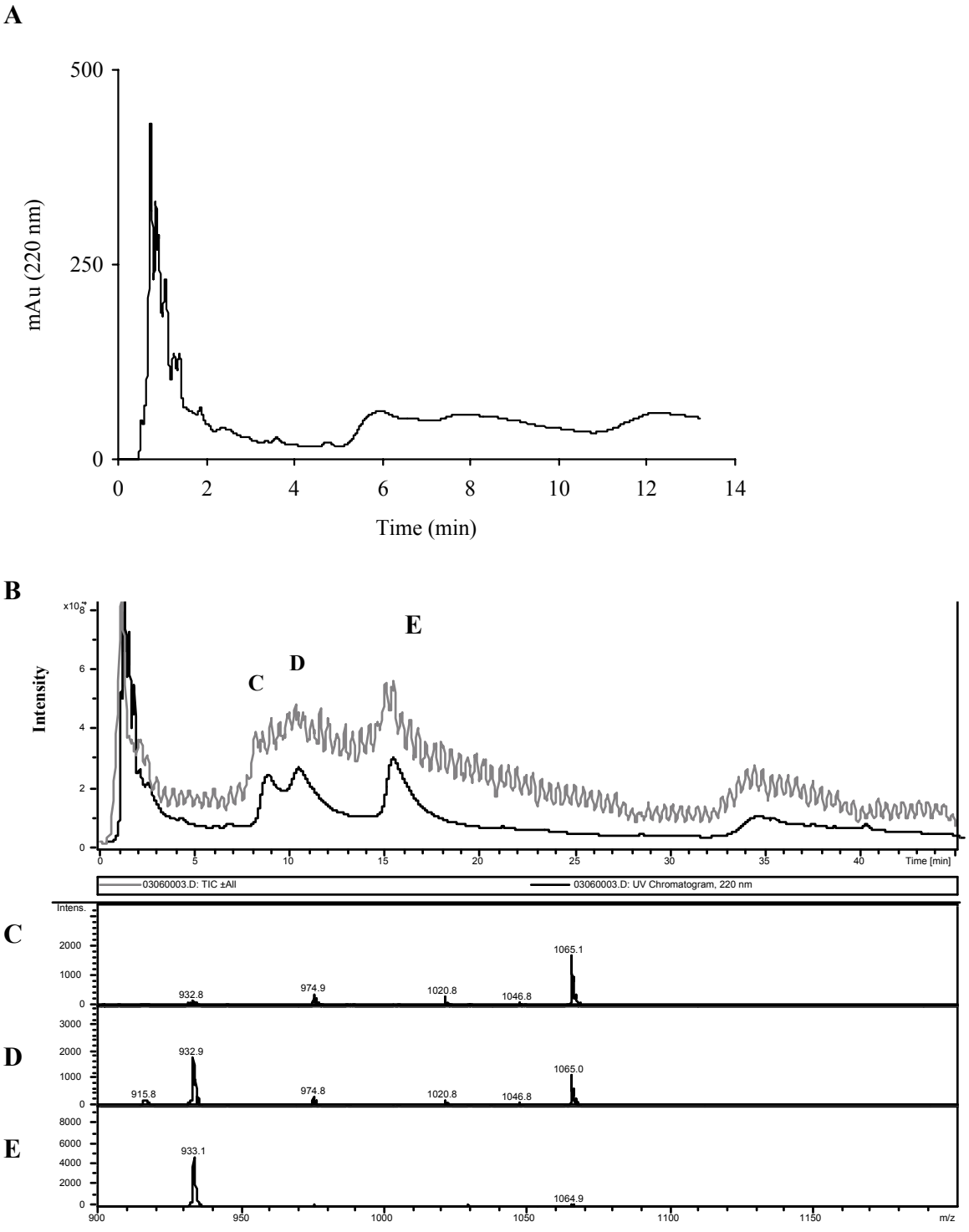


Fig. 2.3: RP-HPLC and LC/MS analysis of ellagitannins purified from white oak using Alltech C-8 and C-18 analytical columns. LC/MS conditions are as described in the text. Gray line shows the TIC while dark line is the UV trace. (**A**) RP-HPLC chromatogram; separation was done on an Alltech C-8 column with a gradient that consisted of A: water and B: acetonitrile both with 0.3 % formic acid (v/v). (**B**) LC/MS analysis on an Alltech C-18 column. The y-axis is only shown for the intensity of the total ion current. (**C**) The negative ion mass spectrum of peak C. (**D**) The negative ion mass spectrum of peak D. (**E**) The negative ion mass spectrum of peak E.

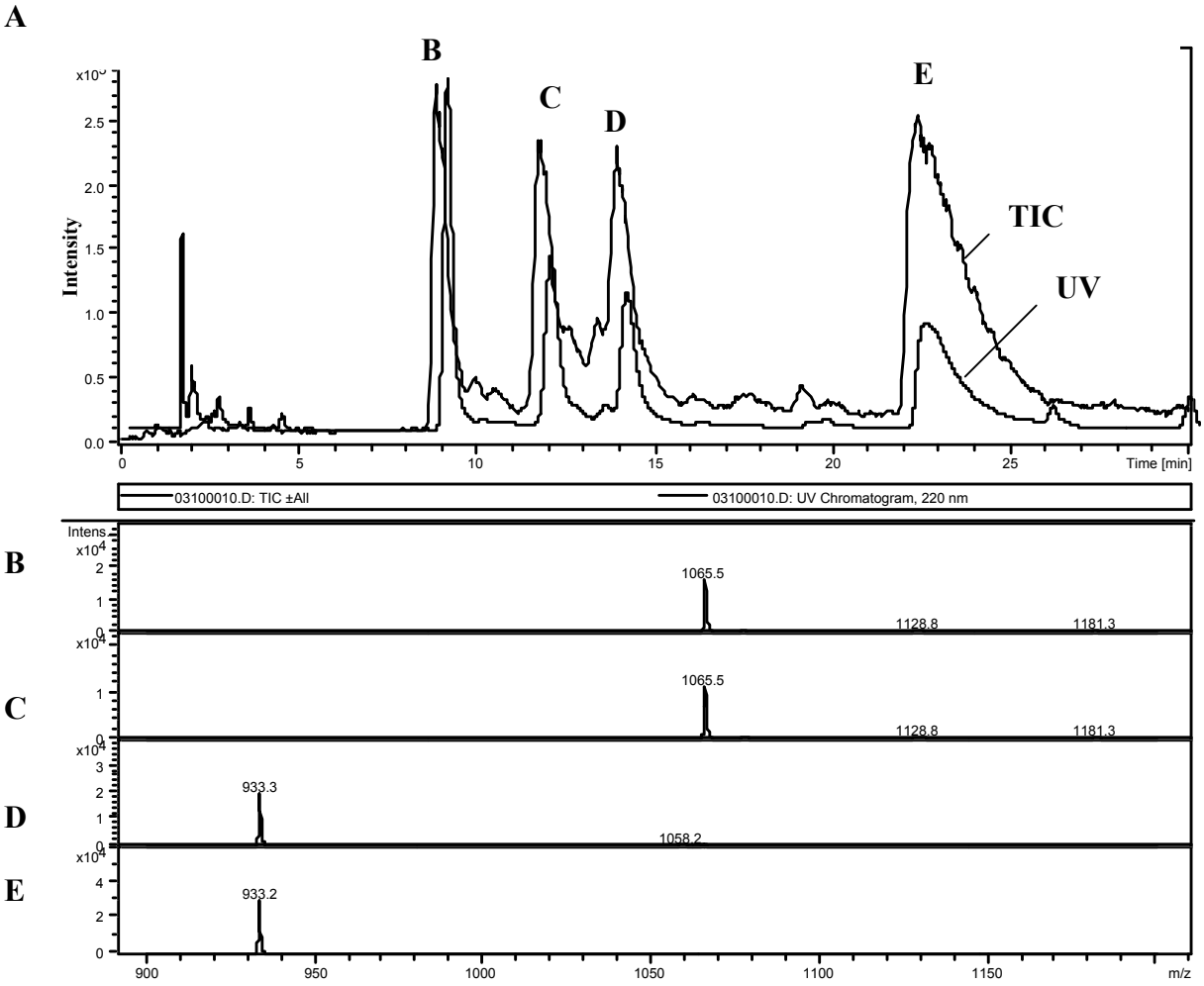
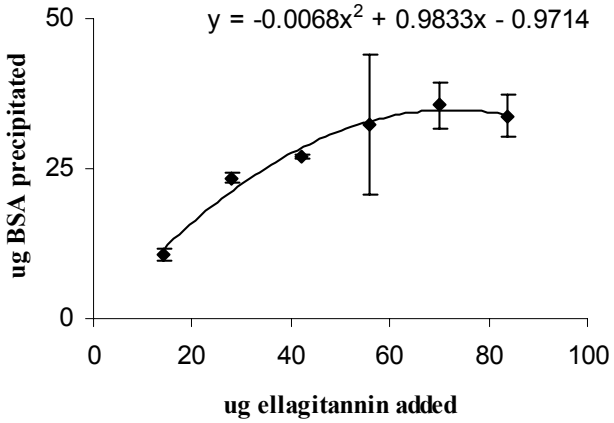


Fig 2.4: LC-MS analysis of ellagitannins purified from white oak using Zorbax C-18 analytical column. Separation was done with a gradient elution that consisted of A: water and B: acetonitrile both with 0.3% formic acid (v/v). (**A**) TIC superimposed on the UV trace. (B) The negative ion mass spectrum of peak B. (**C**) The negative ion mass spectrum of peak C. (**D**) The negative ion mass spectrum of peak D. (**E**) The negative ion mass spectrum of peak E.

A



B

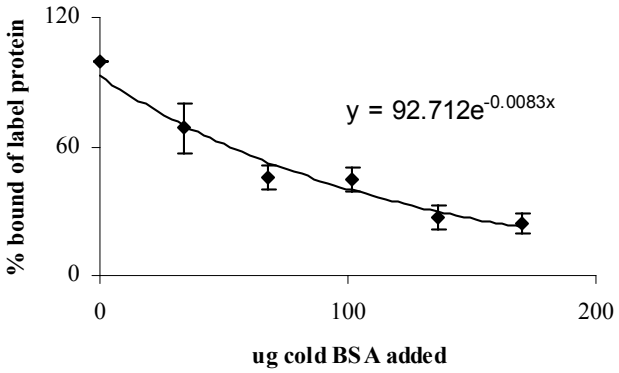


Fig. 2.5: Reactions of purified ellagitannin with BSA. (**A**) The indicated amounts of purified oak ellagitannin were dispensed into assay tubes that contained 98.5 μg labeled BSA and buffer A in a total volume of 400 μL as described in text. The amount of BSA precipitated by the ellagitannin was determined from the linear region of the curve. nmols of ellagitannin and BSA were calculated using the average MW of 1000 gmol and 66,000 gmol, respectively. (**B**) Inhibition of tannin-hot BSA precipitation by various amount of cold BSA. The assay was performed with 30.5 μg hot BSA, various amounts of cold BSA and 14 μg purified ellagitannin in each assay tube as described in the text. The amount of protein precipitated in the absence of the competitor (cold BSA) was set at 100%. The error bars represent standard deviation of three determinations.

of the total BSA present. At the linear region protein precipitation was 0.49 μg BSA per μg ellagitannin (0.007 nmol BSA per nmol ellagitannin).

Tracer BSA precipitation by the ellagitannin in the absence of competitor BSA was set as 100% precipitation; the amount of tracer BSA precipitated in the presence of the competitor was then expressed as a percentage of the amount precipitated in the absence of the competitor [3]. This value was plotted against the amount of competitor added. When the mixture contained 0.46 nmol of the tracer BSA, 1.10 nmol of the competitor BSA inhibited precipitation by 50% (Fig. 2.5B).

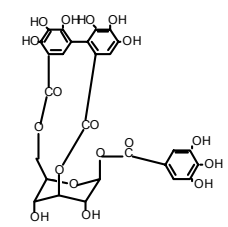
Analysis of ellagitannins in black raspberry

Four different fractions of black raspberry extract (Ru-F001, Ru-F003, Ru-DH and Ru-ET) were analyzed by LC-ESI-MS, and the identities of the main m/z values obtained are shown in Table 2.1. The ion at 783 m/z was found in all four extracts. This ion could represent $[M-H]^-$ of pedunculagin or sanguiin H-10 (Fig. 2.6). The fragments and molecular ions obtained in each extract depended on the magnitude of the fragmentation voltage applied. For instance in extract Ru-F001, increasing the fragmentation voltage to 80 V yielded an ion with m/z value at 301 confirming the presence of ellagic acid, a degradation product of the parent ellagitannins (Fig. 2.7A). The higher voltage was picked as part of the method development process. Since ellagic acid is very characteristic of ellagitannins, we increased the voltage to yield additional known degradation products of the ellagitannins. We also decreased the fragmentation voltage to 47.7 V for fractions Ru-F003, Ru-DH and Ru-ET, and 50.7 V for Ru-F001. At these voltages, m/z values at 1566.9 ($[M-H]^-$ ion of sanguiin H-10), 935 ($[M-H]^-$ ion of galloyl-bis-HHDP-glucopyranose) and 783 ($[M-H]^-$ ion of pedungulangin) were observed in extract Ru-DH. (Table 2.1, Fig. 2.7B). The $[M-H]^-$ ions of corilagin (633 m/z) and galloyl-bis-HHDP-glucopyranose (935 m/z) were also observed in extracts Ru-F003, Ru-DH and RU-ET (Table 2.1, Fig. 2.6).

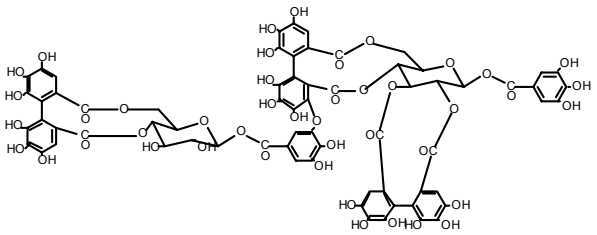
Table 2.1 Identities of ions observed in LC/MS analysis of extracts from black raspberry

	Ellagic acid 301 m/z	Corilagin 633 m/z	Pedun / SanH-10. 783 m/z	GHGP 935 m/z	San H-10 1567 m/z
Ru-F001 (low V)		**x**	**x**		
Ru-F001 (high V)	**x**				
Ru-F003 (low V)		**x**	**x**	**x**	
Ru-DH (low V)		**x**	**x**	**x**	**x**
Ru-ET (low V)		**x**	**x**	**x**	

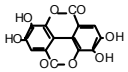
Four fractions (Ru-F001, Ru-F003, Ru-DH and Ru-ET) of black raspberry extract were analyzed with LC/MS using an Alltech C-18 column (4.6 x 100 mm, 5μm). The mobile phase for separation was water/acetonitrile containing 0.1% formic acid (v/v). Separation gradients are described in the text. Fractions Ru-F003, Ru-DH and Ru-ET were analyzed with a fragmentation voltage of 47.7 V while Ru-F001 was analyzed with 50.7 and 80.0 V. Molecular structures of these compounds are shown in Fig. 2.6.
x indicates ellagitannin constituent identified in each extract.
Pedun = Pedunculagin, San H-10 = Sanguiin H-10, GHGP = Galloyl-bis-HHDP-glucopyranose.



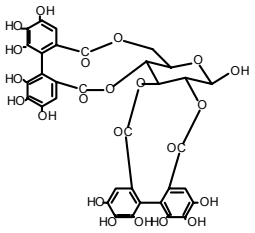
Corilagin (MW 634)



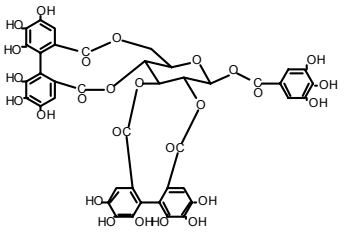
Sanguiin H-10 (MW 1568)



Ellagic acid (MW 302)



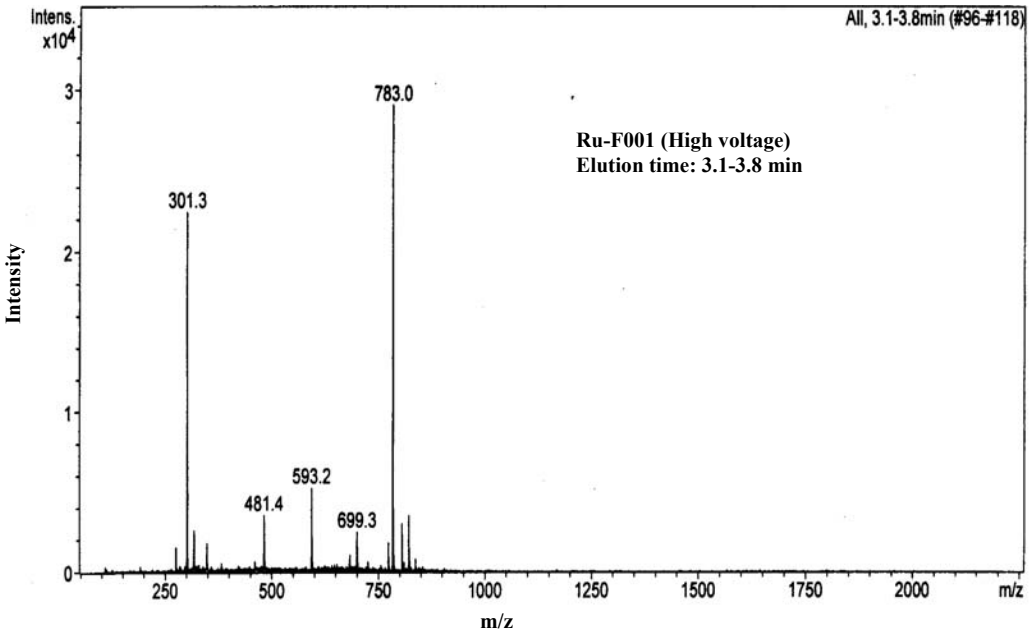
Pedunculagin (MW 784)



Galloyl-bis-HHDP-glucopyranose (MW 936)

Fig. 2.6: Structures of the main ellagitannins in black raspberry. Sanguiin H-10 is a large molecule which when subjected to ESI conditions can fragment to give 633 m/z ($[M-H]^-$ ion of corilagin) , 783 m/z ($[M-H]^-$ ion of pedunculagin and 935 m/z ($[M-H]^-$ ion of galloyl-bis-HHDP-glucopyranose). The m/z value at 783 could also be the doubly charged ion of sanguiin H-10. The mass spectrum of sanguiin H-10 usually shows $[M-H]^-$ ion of ellagic acid at 301 m/z.

A



B

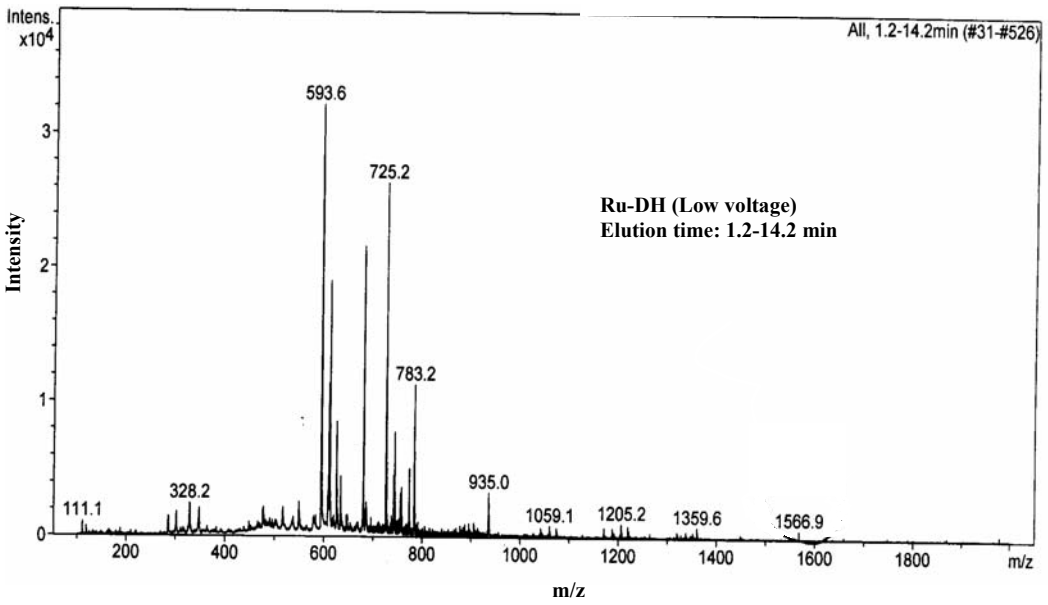


Fig. 2.7: Negative ion mass spectra of extracts Ru-F001 and Ru-DH (**A**) An extracted spectrum of Ru-F001 with high skim voltage (80.0 V) showing the ellagic acid fragment at 301.3 m/z. (**B**) An extracted spectrum of Ru-DH with low skim voltage (47.7 V) showing the $[M-H]^-$ ion of sanguiin H-10 at 1566.9 m/z.

Analysis of tannic acid

Dr. Xiao Chen, our collaborator at Ohio University, proposed that a principal anti-diabetic component of tannic acid (Sigma Chemical Co, St. Louis) was α-pentagalloyl glucose (α-PGG). He based his identification on reversed phase HPLC and mass spectral data. We tested that hypothesis using LC/MS.

In the total ion chromatogram (TIC) of the LC/MS analysis of the tannic acid (Fig. 2.8), the first peak observed was gallic acid (MW 170), which eluted between 1.3 and 1.6 min with an m/z value of 169. Peak W, eluting between 2.3 and 2.7 min had an m/z value of 787.2 and was assigned as tetragalloyl glucose (MW 788). Peak X, which eluted between 2.7 and 3.4 min, yielded both the 787 m/z and 939 m/z ions. β-Pentagalloyl glucose (MW 940) eluted between 3.4 and 4.0 min with an m/z value of 939.1.
The broad peak Y eluting between 5.0 and 6.6 min had an m/z value predominantly occurring at 1091 and was assigned as hexagalloyl glucose (MW 1092). Peak Z, which eluted between 7.2 and 10.3 min in the TIC had an m/z value of 1243 and was identified as heptagalloyl glucose (MW 1244). Beyond peak Z was the much broader portion of the TIC that showed mixtures of $[M-H]^-$ ions of heptagalloyl glucose, octagalloyl glucose and nonagalloyl glucose with m/z values of 1243, 1394.7 and 1546.6, respectively.

In the LC/MS, the fragmentation patterns of the constituents identified in the Sigma tannic acid were also noted since this could provide structural details of individual constituents. In spectra where fragments were observed, they occurred either through the loss of galloyl groups (152 amu) or gallic acid (170 amu) (Fig. 2.9) [26]. The $[M-H]^-$ ion of tetragalloyl glucose (787.2) was characteristic of peaks W and X. The gallate ion (169.2 m/z) and β-PGG (938.9 m/z) were also observed in the spectrum of the tetragalloyl glucose (Fig. 2.10). The abundant ion at 939.1 m/z (Fig. 2.11) was obtained both as an individual peak and as a fragment of all of the higher galloyl-substituted constituents (Figs. 2.12 and 2.13). β-PGG purified from methanolysed tannic acid was also analyzed with LC/MS in order to compare its spectrum with the extracted spectrum of β-PGG constituent in the tannic acid (Fig. 2.11). Like the peak in the tannic acid sample, purified β-PGG eluted between 3.4 and 4.0 min and had a gallate fragment with an m/z value of 168.9. A large number of fragments were observed in the spectra of the

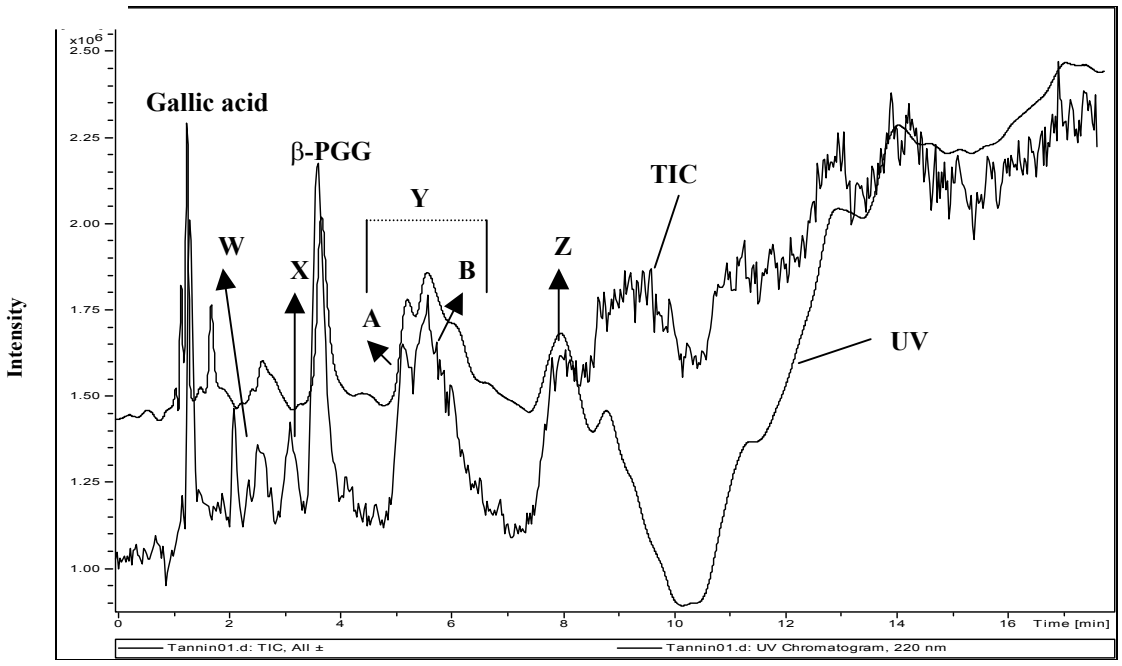


Fig. 2.8: LC/MS analysis of Sigma tannic acid in the negative ion mode. Separation was done on an Alltech C-18 column with a gradient elution. Mobile phase consisted of A: water and B: acetonitrile each modified with 0.3 % formic acid. This shows the TIC of tannic acid superimposed on the UV trace. MS spectra of the various constituents are indicated in Figs. 2.10 – 2.13.

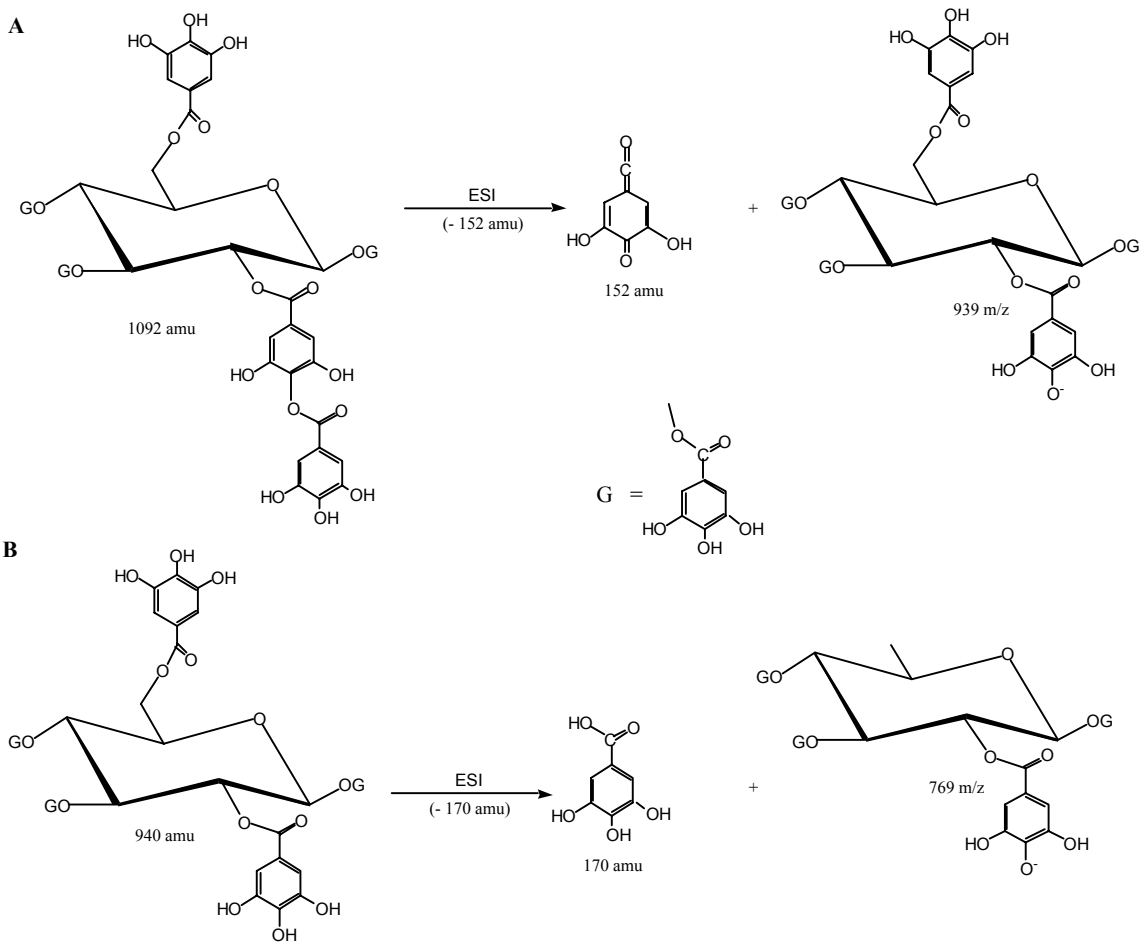


Fig. 2.9: Possible fragmentation routes of hexagalloyl glucose and β-PGG under ESI conditions in the negative ion mode. (**A**) The cleavage of depside bond in hexagalloyl glucose (1092 amu) to generate a galloyl residue (152 amu). (**B**) The cleavage of ester bond in β-PGG to generate gallic acid (170 amu).

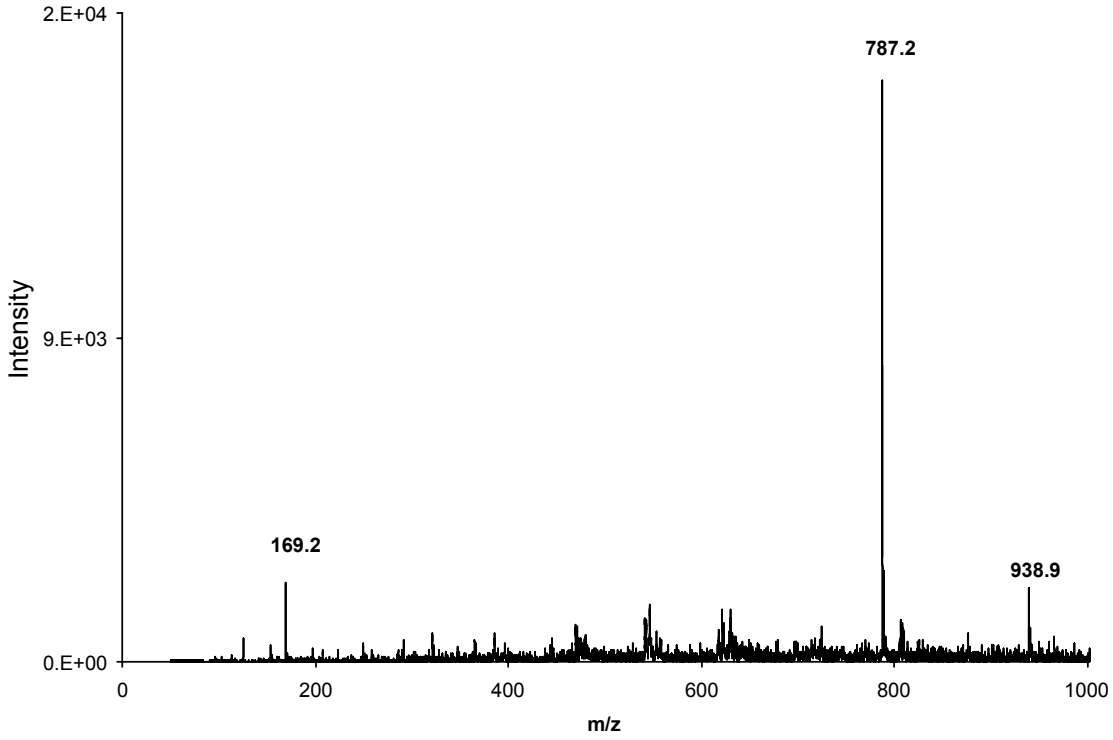


Fig. 2.10: Negative ion mass spectrum of tetragalloyl glucose. Spectrum obtained from the TIC in Fig. 2.8. The tetragalloyl glucose peak eluted between 2.3 and 2.7 min with an m/z value of 787.2.

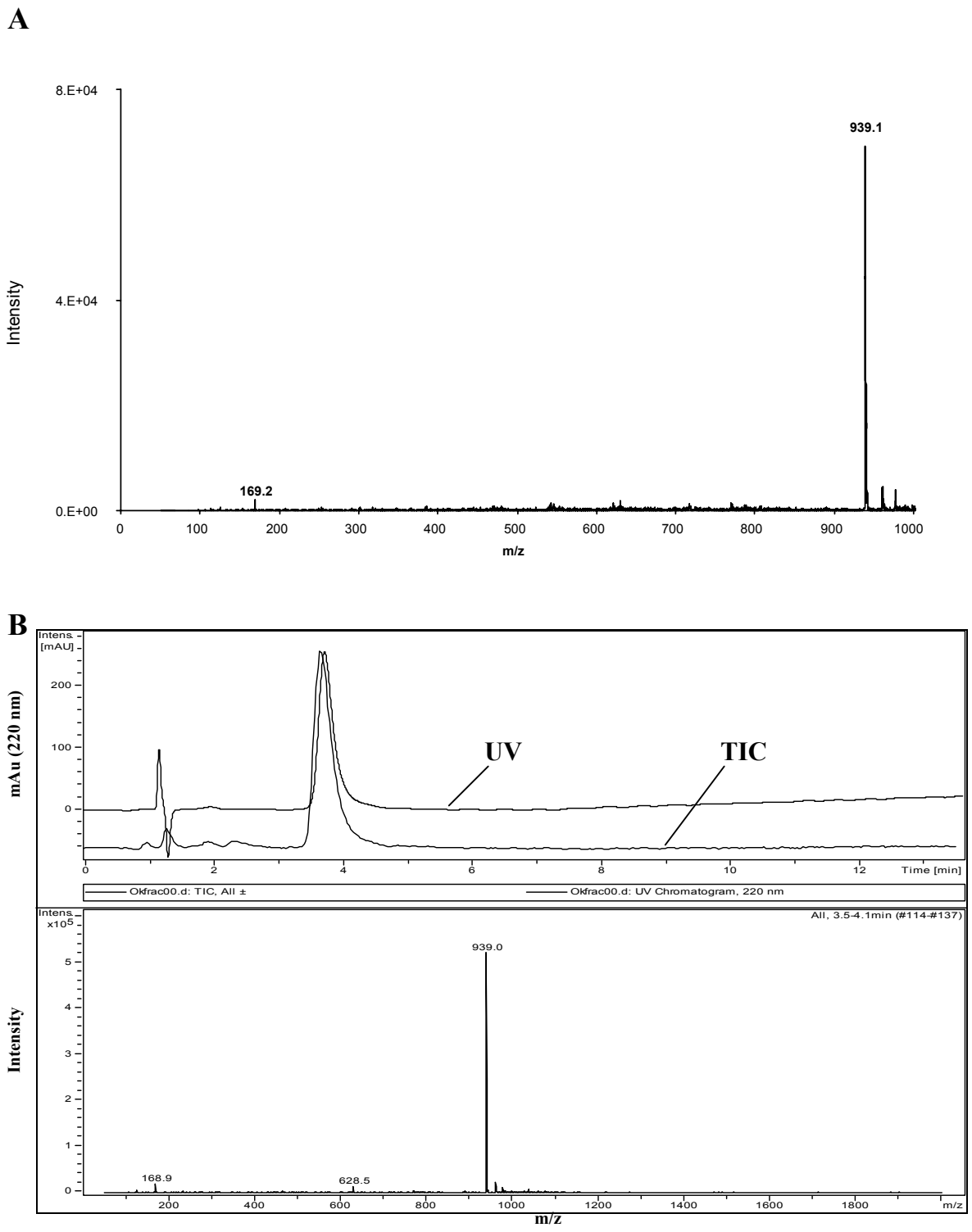


Fig 2.11: Negative ion mass spectra of β-PGG. (**A**) Mass spectrum of β-PGG extracted from the TIC in Fig 2.8. β-PGG eluted between 3.4 and 4.0 min with an m/z value of 939.1 (**B**) LC/MS on β-PGG purified from tannic acid. Top panel shows the TIC superimposed on the UV trace. The β-PGG eluted at 3.8 min. Bottom panel is the mass spectrum showing the m/z value for β-PGG at 939. Mass spectrometric conditions are the same as in Fig. 2.8.

higher substituted constituents (hexa- to octagalloyl glucose) (Fig. 2.12, 2.13); the degree of fragmentation increases with the number of galloyl groups. For example, the region beyond the hepta-substituted constituent showed high degree of fragmentation (Fig. 2.13) in the TIC (Fig. 2.8). Both the $[M-H]^-$ ions of octa- and nonagalloyl glucose were observed in low intensity, probably fragmenting easily to the lower constituents which were observed to be in high intensity.

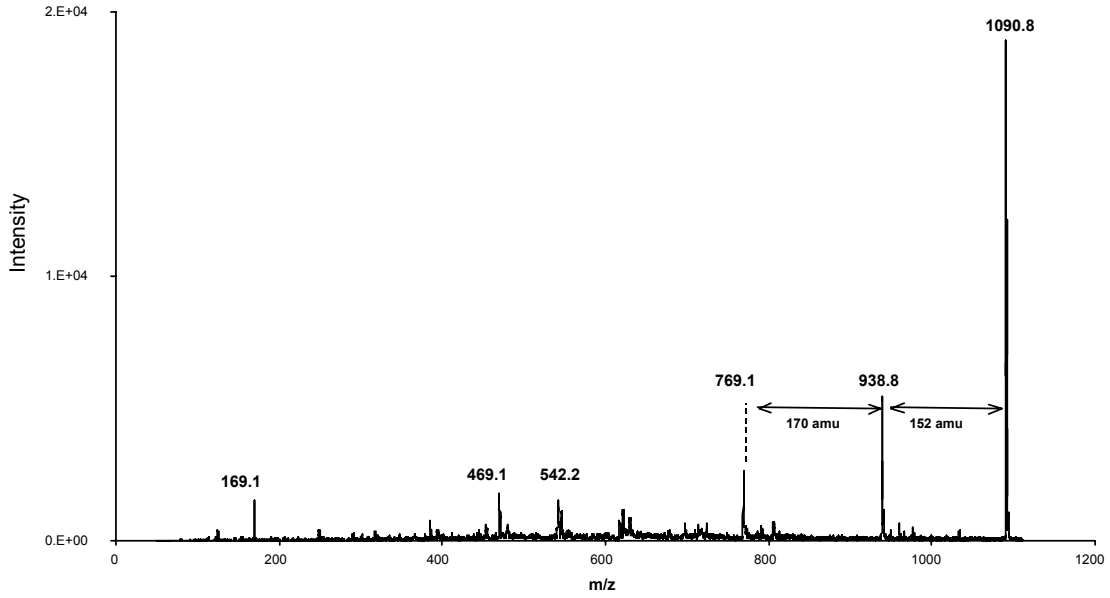
Discussion

Analysis of ellagitannins in white oak

Ellagitannins form up to 2-3% of dry matter in oak wood [23]. Two pairs of diastereomers (vescalagin/castalagin and grandinin/roburin E with molecular weights of 934 and 1066 respectively), constitute about 50% of the total ellagitannins in oak wood [7,23,30]. Vescalagin and its isomer castalagin were first identified and purified from chestnut [17]. Grandinin and roburin E were identified two decades later in pedunculate oak [7]. These four ellagitannins are the first ellagitannins identified that contain a carbon-carbon linkage between an aromatic carbon and C-1 of an open glucose core (Fig. 2.1) [7]. Vescalagin and castalagin differ sterically in the attachment of the hydroxyl group to the C-1 of the glucose core. It has been proposed that the biosynthesis of grandinin and roburin E is based on a condensation reaction between ascorbic acid and vescalagin. The ascorbate substituent attached to C-1 of the glucose core then rearranges into a pentose residue (lyxose in grandinin and xylose in roburin E) on the vescalagin core [22]. In the current study, these four main ellagitannins were identified and purified from white oak using LC/MS. The purified ellagitannins were then characterized using radiolabeled BSA binding assay.

Separation of the white oak extract on the normal phase semi-preparative column followed by mass spectrometric analysis of fractions from the column showed that the two pairs of isomers eluted as two overlapping peaks (Fig. 2.2A). These peaks were collected and analyzed with RP-HPLC and MS. Three different columns were used for the method development. Separation on the Alltech C-8 column failed to retain the

A



B

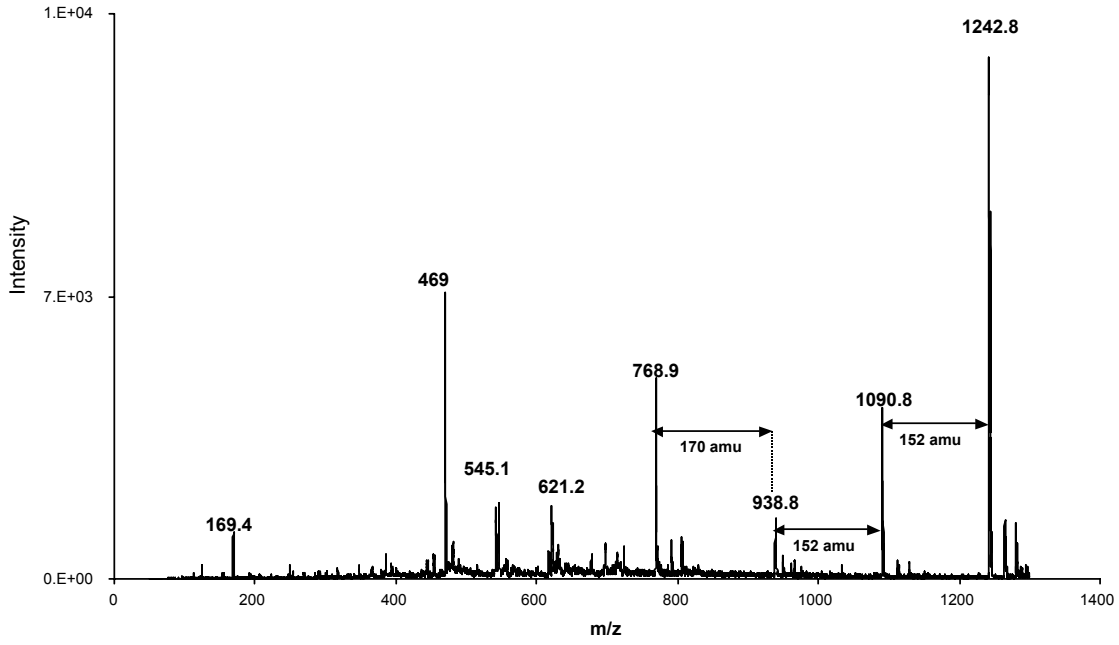


Fig. 2.12: Negative ion mass spectra of hexagalloyl and heptagalloyl glucoses. Mass spectra were extracted from the TIC in Fig. 2.8. (**A**) Hexagalloyl glucose eluted between 5.0 and 6.6 on the TIC with an m/z value of 1090.8. Fragments at 169.1, 469.1, 769.1 and 938.8 m/z represent the loss of gallic acid (170 amu) or the galloyl residue (152 amu). (**B**) Heptagalloyl glucose eluted between 7.2 and 10.3 min on the TIC with an m/z value of 1242.8. Fragments at 169.1, 469.1, 769.1 and 938.8 m/z represent the loss of gallic acid (170 amu) or the galloyl residue (152 amu).

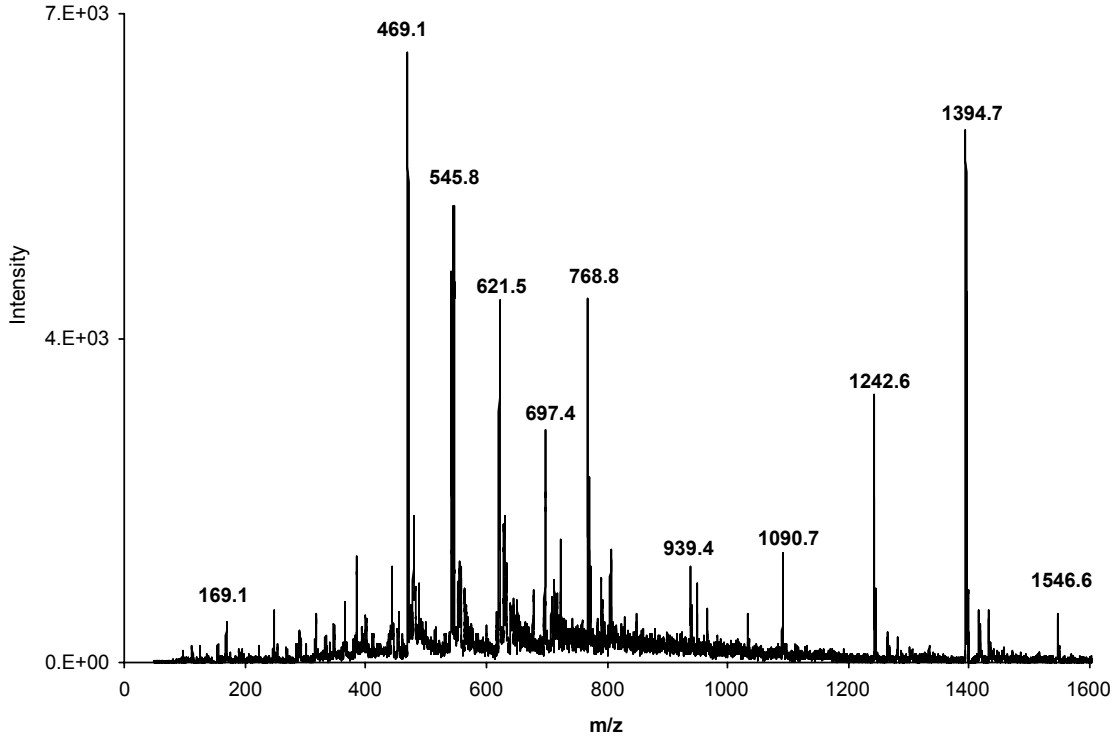


Fig. 2.13: Negative ion mass spectrum of tannic acid showing regions of high fragmentation. This spectrum was extracted from the TIC in Fig. 2.8 (9 – 14 min). The $[M-H]^-$ ions of octagalloyl (1394.7 m/z) and nonagalloyl (1546.6 m/z) were observed in addition to the lower fragments such as β-PGG (939.4 m/z), hexagalloyl glucose (1090.7 m/z) and heptagalloyl glucose (1242.6 m/z).

components. Sample injected on this column was washed off quickly as one large peak. Separation on the Alltech C-18 column partially resolved the four ellagitannins showing some peak overlap and broadening. The difference in retention of components on both columns may be due to the different interactions the components have with the C-8 and C-18 chains; interaction is more hydrophobic in nature on the C-18 column and hence the retention is stronger than on the C-8 column. The aromatic rings in the ellagitannins may have interacted better with the C-18 column than with the C-8 column.

Columns with the same alkyl chains but from different manufacturers perform differently under the same chromatographic conditions. These differences are due to stationary phase modifications employed by these manufacturers to protect the columns from phase collapse and hydrolysis at low pH. Such modifications include end capping and embedding chemical groups [32]. Some C-18 columns cannot tolerate high levels of water since this causes an irreversible aggregation of the flexible C-18 chains thus making it difficult to separate isomers that have the same degree of hydrophobicity [18]. The Alltech C-18 failed to fully resolve the four isomers probably as a result of phase collapse at the high percentage of water used in the mobile phase. Retention was higher on the Agilent Zorbax RP C-18 column; all four ellagitannins were well separated. The Zorbax column is a special reverse phase column designed with rigid C-18 chains that can offer selective retardation of isomers with the same degree of hydrophobicity [32]. The rigid C-18 chains also allow it to withstand mobile phases with high levels of water.

The different retention times of vescalagin and its isomer castalagin may be a consequence of steric differences based on how the hydroxyl group is attached to carbon one of the glucose core. The different retention times between grandinin and its isomer roburin E are probably due to the difference in the pentose sugar substituent on the glucose core. The lyxose (in grandinin) is 2-epimer of the xylose (in roburin E). In RP-HPLC analysis of ellagitannins from oak wood and chestnut, which used different chromatographic conditions from this study, grandinin eluted faster than roburin [30]. In the same study, vescalagin eluted faster than castalagin.

The protein binding ability of the purified ellagitannin was compared to results obtained from another study done using sorghum procyanidin and PGG under the same assay conditions (assay done in 25% methanol and incubated at room temperature) [4].

The procyanidin in that assay precipitated 0.080 nmol BSA per nmol tannin while the PGG precipitated 0.010 nmol BSA per nmol tannin. Comparing the amount of BSA bound by the purified ellagitannin in this study (0.007 nmol BSA per nmol tannin) to the value obtained with the procyanidin, there is a clear indication that the ellagitannin was less effective at precipitating BSA than was procyanidin. However, the amounts of protein precipitated by the purified ellagitannin and the PGG were close in value.

The differences in protein precipitation by the procyanidin (MW 4930) and the two hydrolysable tannins (ellagitannin and the PGG), which have molecular weights between 934 and 1066, suggest that protein precipitation may be a function of molecular weight. Protein precipitation may also be influenced by the presence of organic solvents like methanol, which are noted to disrupt hydrophobic interaction involving proteins [10]. It is possible that the 25 % methanol used in this assay suppressed the precipitation of BSA by the purified ellagitannin. In the findings by Hagerman and Rice [4], BSA precipitation by PGG was suppressed by the presence of methanol while it had no effect on protein precipitation by the procyanidin.

In order to determine whether radiolabeled protein was precipitated as efficiently as unlabeled protein, the amount of unlabeled protein required to cause 50% inhibition of the tracer protein precipitation by the ellagitannin was determined. This is an indirect way of assessing proteins with similar affinity for ellagitannins. We expected to achieve 50% inhibition of tracer precipitation when equal amounts of tracer and unlabeled protein were present. This was not the case; the amount of unlabeled protein required to inhibit labeled protein precipitation was about two times the amount of labeled protein.

Because the amount of BSA precipitated by the purified ellagitannin seemed to have been influenced by the molecular weight or the presence of methanol, a future consideration would be to vary factors such as the assay solution and performing assay with ellagitannins with different molecular weights. Assay temperature has also been found to influence protein precipitation by some tannin, as seen in the work of Hagerman and Rice [4]. The assay with the ellagitannin can be done in a temperature range of 4^{o}C to 40^{o}C to study the influence of temperature. Another consideration is performing the assay separately with each isomer instead of a mixture of all four so that the influence of substituent stereochemistry can be studied.

Analysis of ellagitannins in black raspberry

The phenolic content of raspberry fruit is mostly anthocyanins and ellagitannins [19]. The ellagitannins either have an open glucose core or a glucopyranose core ranging from low molecular weight compounds such as pedunculagin and potentillin to complex or tetrameric ellagitannins such as sanguiin-H 6 and lambertianin C [28].

Dr. Gary Stoner (The Ohio State University) provided four different fractions of black raspberry for us to analyze with LC/MS in order to assign the main ellagitannin components to each of the four fractions. He hoped to relate the measured effect of these fractions on esophageal cancer to their ellagitannin composition. Previous attempts to identify and isolate the active ingredients have been directed at degradation products such as gallic acid and ellagic acid. We used the LC/MS for the screening so that its soft ionization process may allow accurate assignment of intact molecules in these extracts.

Sanguiin H-6 and H-10, and lambertianin C have been identified in red raspberry extracts [19,28]. Sanguiin H-10 is a dimer with a molecular weight of 1568 but analysis with LC/MS is challenging since the compound produces more of the doubly charged di anion at an m/z value of 783 than of the singly charged ion at m/z 1567. Since another common ellagitannin, pedunculagin, produces a molecular ion with m/z 783, it is difficult to establish whether an extract contains pendunculagin or sanguiin H-10. Fortunately, improvements in mass spectrometric analysis such as the use of zoom scans analysis and MS/MS have paved the way for the identification of compounds that produce multiply charged ions. Zoom scanning is a slow scanning technique in a narrow mass range that improves the resolution of an analyte and also allows the charge state of the analyte to be established. The charge state of the analyte is determined from the m/z difference between two high intensity $^{12}C/^{13}C$ isotopes of the analyte thereby helping in the correct determination of the molecular weight [19]. Results obtained from zoom scan analysis can also be confirmed by tandem mass spectrometric data. Sanguiin H-10 for instance, was identified in red raspberry by using both the zoom scan analysis and MS/MS techniques [19]. In MS/MS sanguiin H-10 ionized as a doubly charged molecule at an m/z value of 783 with singly charged daughter ions that had m/z values larger than 783.

In my study, since the MS/MS technique was not employed, the fragmentation voltages were varied in order to facilitate the identification process. The idea of ionizing

at a low fragmentation voltage was to allow the easy identification of molecular ions since constituents in a sample are more easily identified by their molecular ions than by their fragments. Also, by increasing the voltage, we could generate fragments that are very characteristic and unique to the compound of interest. These two fragmentation processes augmented the identification process. By increasing the voltage, the presence of the $[M-H]^-$ ion of ellagic acid was confirmed in extract Ru-F001. This ion also confirmed the presence of oxidatively coupled galloyl groups in the extracts. They are very characteristic of ellagitannins.

The appearance of the corilagin and the galloyl-bis-HHDP-glucopyranose fragments at 633 m/z and 935 m/z respectively in extracts Ru-F003, Ru-DH and Ru-ET confirmed the presence of corilagin, galloyl-bis-HHDP-glucopyranose and sanguiin H-10. Corilagin and galloyl-bis-HHDP-glucopyranose are derivatives of sanguiin H-10 and are related to it either as chemical degradation products or biosynthetic precursors. The molecular ion of corilagin is due to a loss of ellagic acid (302 amu) from 935 m/z. Because the presence of the singly charged molecular ion of sanguiin H-10 at 1567 m/z was only confirmed in extract Ru-DH, it is likely this extract predominantly contains sanguiin H-10 while the other extracts contain the low molecular weight compounds namely, corilagin (633 m/z), pedunculagin (783 m/z) and galloyl-bis-HHDP-glucopyranose (Fig. 2.6, Table 2.1).

Analysis of Tannic Acid

Xiao Chen used both RP-HPLC and ESI-QTOF to analyze the tannic acid. He used a Beckman semi- preparative C-18 column, and an isocratic elution with a mobile phase that consisted of A: water and B: acetonitrile both with 0.1 % TFA (v/v). Flow rate and UV detection were respectively 3 mL/min and 320 nm. His mass spectrometric analysis was done on fractions collected from the HPLC in the positive ion mode with a quadrupole/time-of-flight mass analyzer. In the reverse phase HPLC analysis (Fig. 2.14), he assigned peak TA-3-3 as α-PGG. This peak was found to show some insulin-mimetic activity. α-PGG would be a gallotannin (MW 940) with five galloyl groups esterified to a glucose core. The galloyl group on C-1 of the glucose molecule would be in the axial position, an unusual location for a galloyl substituent [11]. The specific aim of this study

was to establish or confirm the true identity of this peak by LC-ESI-MS analysis in the negative ion mode, and with an ion trap mass analyzer. Chen identified peak TA-3-1 (Fig. 2.14) as β-PGG based on the ESI-QTOF yielding a sodiated β-PGG at 963 m/z ($[M+Na]^+$), and $[M+K]^+$ with an m/z value of 971 (Table 2.2). Chen confirmed the identity of this peak by co-chromatography with an authentic sample of β-PGG in the RP-HPLC. He did not assign peak TA-3-2, but assigned TA-3-3 as α-PGG. Our re-interpretation of his ESI-QTOF data suggested that peaks TA-3-2 and TA-3-3 were sodiated hexagalloyl glucoses ($[6GG + Na]^+$) with an m/z value of 1267 (Table 2.2). These two peaks presumably correspond to the resolved form of peak Y in my LC/MS analysis that had an m/z value of 1091 ($[M-H]^-$ ion of hexagalloyl glucose) (Fig. 2.8). The leading edge of peak Y (A) corresponds to TA-3-2 while the remainder of the peak (B) may be TA-3-3. We identified peak TA-3-4 and TA-3-5 as sodiated heptagalloyl glucose with an m/z value of 1267 (Fig. 2.14, Table 2.2). These two peaks also corresponded to peak Z in the LC/MS analysis (Fig. 2.8). We identified peak TA-3-6 in the RP-HPLC as a mixture of heptagalloyl and octagalloyl glucoses, both sodiated with m/z values of 1267 and 1420 respectively (Fig. 2.14, Table 2.2). We also identified TA-3-7 as sodiated heptagalloyl glucose and assigned the highest galloyl substituted constituent as undecagalloyl glucose (Fig. 2.14, Table 2.2).

In comparing the total ion chromatogram of the LC/MS analysis (Fig. 2.8) to Xiao Chen's RP-HPLC chromatogram (Fig. 2.14), we realized that peaks that eluted as separate peaks in the RP-HPLC co-eluted in the LC/MS as one broad peak. For instance, the two separate peaks TA-3-2 and TA-3-3 in the RP-HPLC were identified as one broad peak in the LC/MS with an m/z value of 1091 (hexagalloyl glucose). The differences in solvent modifier for LC/MS analysis; it decreases sensitivity. Formic acid does not suppress ionization in mass spectrometric analysis but it compromises on peak shape

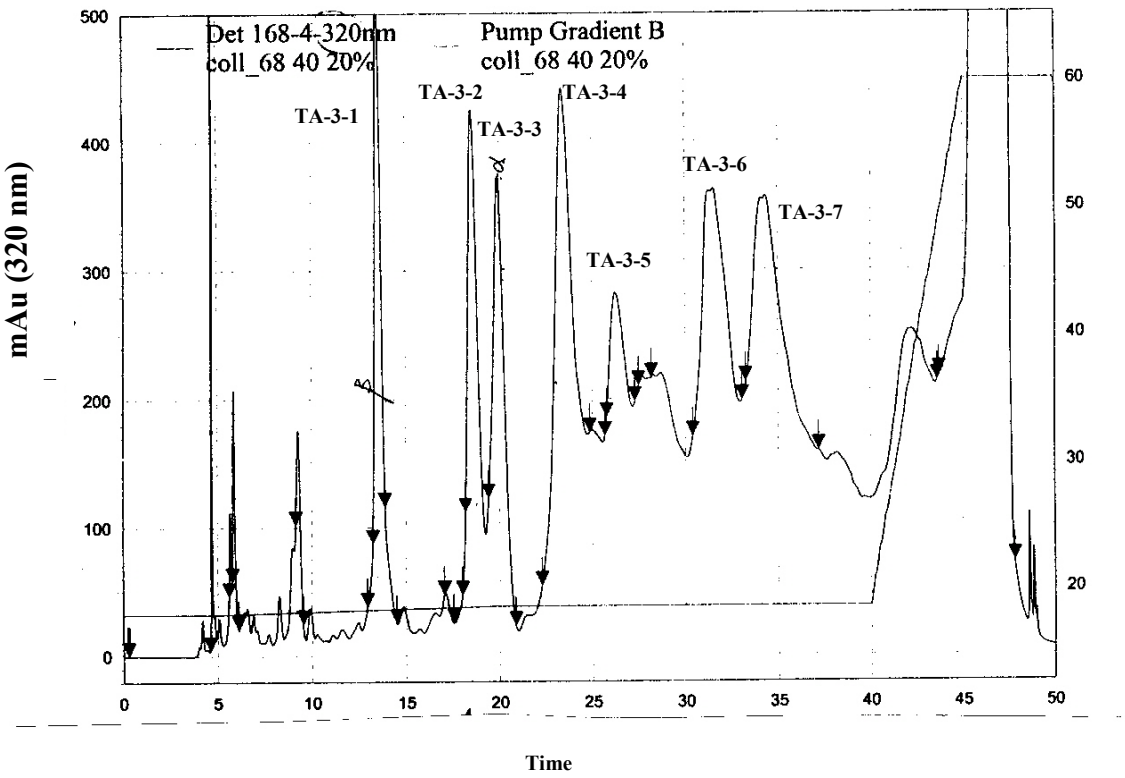


Fig. 2. 14: RP-HPLC of tannic acid using Beckman C-18 semi-preparative column. Separation was done using an isocratic elution with a mobile phase that consisted of water/acetonitrile containing 0.1% TFA (v/v). Flow rate and UV detection were 3 mL/min and 320 nm, respectively. Peaks TA-3-1 and TA-3-3 were tentatively identified as β- and α-PGG (After Xiao Chen, Ohio University, unpublished data).

Table 2.2: Summary of ions observed in LC/MS and ESI-QTOF analysis of tannic acid

Ansong t_R	Ansong m/z, assignment LC/MS (-ve mode)	Chen t_R	Chen m/z, assignment ESI-QTOF (+ve mode)	comments
2.3-2.7	787 tetragalloyl glucose			
3.4-3.8	939 β-PGG	13.4	971 [PGG: K] 963 [PGG: Na] 771 [PGG-170]	TA-3-1 "β"
5.0-6.6	1091 hexagalloyl glucose 939 [6GG-152] 769 [6GG-152-170]	18.1	1115 [6GG:Na] 923 [6GG-170]	TA-3-2 "?"
		19.4	1115 [6GG:Na] 923 [6GG-170] 771 [6GG-170-152]	TA-3-3 "α"
7.2-10.3	1243 heptagalloyl glucose 1091 [7GG-152] 939 [7GG-152-152] 769 [7GG-152-152-170]	22.8	1267 [7GG:Na] 1262 unassigned 1075 [7GG-170] 923 [7GG-170-152] 771 [7GG-170-152-152]	TA-3-4
		27	1267 [7GG:Na] 1075 [7GG-170] 923 [7GG-170-152]	TA-3-5
10.3-15	1395 octagalloyl glucose 1243 [8GG-152] 1091 [8GG-152-152] 939 [8GG-152-152-152] 769 [8GG-152-152-152-170]	30.6	1419 [8GG:Na] 1267 [7GG:Na] 1075 [7GG-170]	TA-3-6
		33.4	1420 [8GG:Na] 1227 [8GG-170] 1262 unassigned 1075 [8GG-170-152] 923 [8GG-170-152-152	TA-3-7

hence the broader peaks observed in the LC/MS.

One other possibility is that, the more conformations or isomers a constituent has, the wider the peak, especially in formic acid-modified solvents. Constituents with larger number of galloyl groups, especially those with more than five galloyl groups, tend to show broader peaks (in some cases, extra peaks) [20,21]. Based on ^{13}C nuclear magnetic resonance spectroscopy (NMR) and methanolysis, these extra peaks have sometimes been shown to be due to positional isomerism within the constituents [20]. Our mass spectrometric data showed that peaks TA-3-2 and TA-3-3 are isomers of hexagalloyl glucose. Peaks TA-3-4 and TA-3-5 were also found to be isomers of heptagalloyl glucose. In another experiment conducted using the same chromatographic conditions as the LC/MS except that formic acid was replaced with trifluoroacetic acid (TFA), we identified five separate peaks (A-E, Fig. 2.15) as possible isomers of hexagalloyl glucose. The mass spectra of the tannic acid showed ions of gallotannins ranging from the gallate ion to undecagalloyl glucose (identified with the ESI-QTOF). The ions at 331 and 483 which are the $[M-H]^-$ ions of monogalloyl and digalloyl glucose respectively were not observed in the LC/MS. They were also not observed in the positive ion mode of the ESI-QTOF. The presence of β-PGG fragment in all the extracted spectra of constituents with more than five galloyl groups suggests that the synthesis of these constituents is based on the β-PGG core.

From my LC/MS and Xiao Chen's RP-HPLC data (Figs. 2.8 and 2.14), constituents with smaller number of galloyl groups (low MW) eluted faster those with larger number (high MW). Retention times were in the order: gallic acid < tetragalloyl glucose < β-PGG < hexagalloyl glucose < heptagalloyl glucose < octagalloyl glucose. Because the separation was done under a reversed phase condition, it may be argued that the retention behavior is linked to a polarity-based separation. Based on the elution trend, constituents with smaller number of galloyl groups may be considered more polar than those with larger number because of a polar mobile phase. However, the literature values of the octanol-water partition coefficient of some of these constituents indicate otherwise. For instance, the octanol-water partition coefficient of β-PGG, hexagalloyl glucose and heptagalloyl glucose are respectively 129, 1.51 and 1.03 [4]. The partition coefficient is correlated to polarity and the lower the value, the more polar

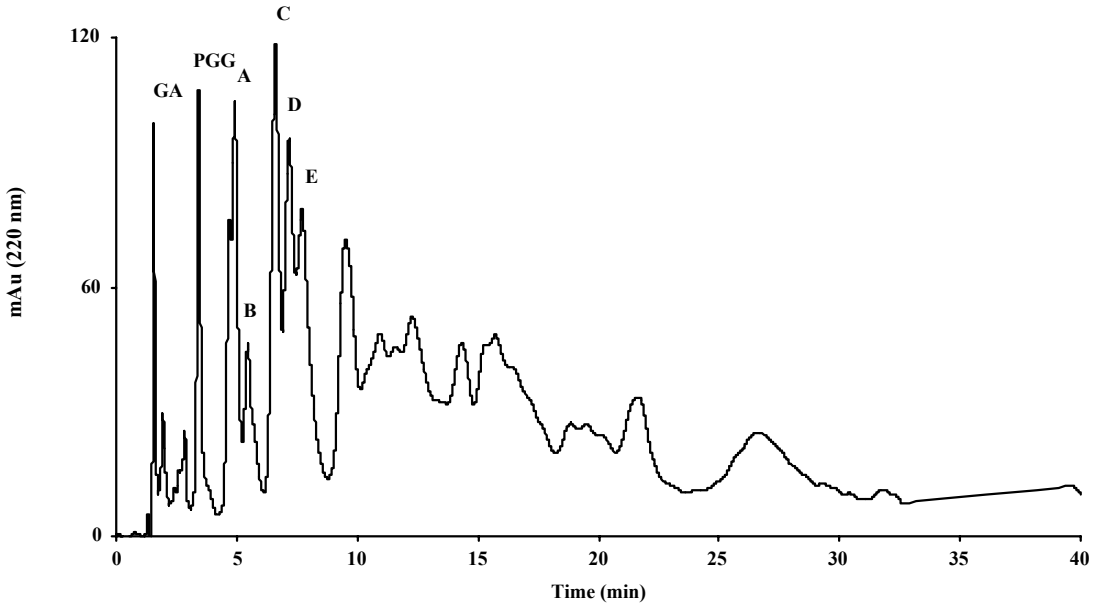


Fig. 2.15: RP-HPLC analysis of tannic acid using Alltech C-18 analytical column. Separation was achieved using an isocratic elution with a mobile phase that consisted of A: water and B: acetonitrile both with o.1 % TFA (v/v). Flow rate and injection volume were respectively 1 mL/min and 20 μL. UV detection at 220 nm. Peak marked as gallic acid and PGG co-eluted with gallic acid and β-PGG standards. Peaks A-E were deduced to be isomers of hexagalloyl glucose based on the LC/MS.

the compound. A retention time dependence on the relative degree of hydrogen bond interactions each constituent has with the column may explain the order in which these constituents elute out of the column. As a result of the large number of galloyl groups, the higher galloyl-substituted constituents had extensive hydrogen bond interactions with the silanoyl groups on the column and were thus retained longer on the column than the lower-substituted constituents.

In Xiao Chen's study, the β-PGG also had anti-diabetic activity, but the TA-3-3 peak, which was confirmed by this study as an isomer of hexagalloyl glucose, was slightly more active. Our experiments in which LC/MS analysis was employed directly demonstrate that Sigma tannic acid does not contain α-PGG but only contains the β-PGG, and that subsequent peaks in the chromatogram are higher galloyl esters, which yield β-PGG (m/z 939) as a fragment upon MS analysis. This suggests that higher galloyl esters could be tested for insulin-mimetic activity.

In conclusion, the combination of the separation power of HPLC with mass spectrometry is a reliable technique for identifying and purifying compounds from natural sources. Additional structural information was obtained from LC-MS through fragmentation voltage variation. In future work with hydrolysable tannins, methods employed by other labs including tandem mass spectrometry (MS^n) and zoom scanning, a peak resolution technique, could be employed. Given our success with hydrolysable tannins, it is reasonable to make our next goal application of LC/MS to analysis of the proanthocyanidins.

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Chapter Three

Protein binding by plant polyphenols: A modified method

Introduction

The biological activities of tannin are related to its interaction with protein. This interaction has some influence on the functional properties of ecological and agricultural systems [1,10,18,23,27]. The importance of tannin-protein interactions in ecological systems is illustrated by the reduced palatability of high tannin forages to herbivores [28]. This process is believed to be due to the astringent sensation experienced when salivary proteins are precipitated by tannins and as a result lose their ability to lubricate the epithelial membranes of the mouth [14]. Tannins can also affect the ecological system through their inhibitory effects on soil microorganisms and enzymes needed for decomposition and nutrient cycling [18].

The effect of tannin-protein interaction in agriculture is seen in animal production in a subsistence setting in which dietary tannins can be either nutritional or anti-nutritional to farm animals [1,23]. Dietary tannins can complex with dietary protein and metabolic proteins such as enzymes thereby preventing protein utilization by herbivorous animal. There is an increased excretion of nitrogenous compound in animals fed on tannin-containing beans [24], ultimately causing growth impairment [2]. It has been shown that young animals that are fed with tannin-containing diets lose weight compared with the normal growth shown by those fed with tannin-free diet [11].

The benefits of tannin have been noted in a few domesticated animals and include the ability to protect ruminants against pasture bloat by interacting with protein and slowing microbial digestion [20]. Tannins also control the level of gastrointestinal parasites in herbivores by binding strongly to endogenous proteins which prevents the intestinal parasites from utilizing them to synthesize their cellular proteins [4,23,29].

In the developing countries where livestock feeding systems are based on browsing, the development of simple methods that can predict the safety of forage fed to farm animals is crucial. One approach will be to study protein-binding capacities of

forage fed to these animals since high tannin levels in the forage can inhibit the functions of essential enzymes needed for metabolic activities. The tannin-protein interaction may be studied by measuring the amount of a radiolabeled protein that the tannin binds with. The radiolabeled BSA precipitation method for tannin quantitation developed by Butler and his group [7], is a sensitive method that requires high-speed (13,000g) centrifugation at 4^{o}C and the handling of small volumes of radiochemical solutions. For some laboratories in less developed countries it is not convenient to conduct all of the steps in this assay. As a result, the radiolabeled BSA precipitation method has been simplified in the current study by eliminating the centrifugation procedure and allowing the radiolabeled BSA bind to tannin that has been immobilized on a paper disk. The insoluble tannin-protein complex formed on the paper disk can be determined by gamma counting. This simplified method was used to measure the protein binding capacity of five different plant extracts.

Methods

Plant materials

Mature leaves of walnut (*Juglans nigra*), tulip poplar (*Liriodendron tulipifera*), and sugar maple (*Acer saccharum*) were collected from Miami University campus (Oxford, OH). White oak (*Quercus alba*) and black oak (*Quecus velutina*) were collected from the campus of University of Missouri (Ellington, MO) and were provided by Dr. Rebecca Forkner.

Sample preparation and immobilization on disk

Mature leaves from tulip poplar, maple, walnut, white oak and black oak were ground in liquid nitrogen and lyophilized. The lyophilized plant tissue, about 80 mg per plant sample, was placed in 2 mL microfuge tubes and extracted with 1 mL of 50% methanol (methanol/water, v/v, 50/50). The extraction mixture was continuously rotated at room temperature for 30 min after which it was centrifuged at 12000g for two min. The supernatant was dispensed onto Whatman #1 filter paper disks (1 cm diameter) in 5 μL increments with brief drying at room temperature after each 5 μL addition.

Analysis of immobilized sample

Acid Butanol Assay

Disks loaded with 5-25 μL of immobilized plant extract (described above) were placed in 15 mL screw-top test tubes with 3 mL of acid butanol solution (conc HCl/n-butanol, 5/95, v/v) and 100 μL of 2% (w/v) ferric ammonium sulfate. The assay tubes were capped loosely for vortexing and heated for 30 min in a boiling water bath. After heating, the solutions were cooled to room temperature and their absorbance determined at 550 nm against an acid butanol blank [25]. This procedure was repeated for extracts that had not been immobilized on the disks.

Another set of disks containing 5-20 μL of 1 mg/mL sorghum procyanidin (procyanidin solution prepared in 50% methanol / 50% water) was used for the acid butanol assay to serve as the standard for the assay. The procyanidin was purified from Sorghum grain according to [6].

Protein Binding Assay

The radioiodinated BSA was prepared using Chloramine T [5]. Just before use, an aliquot of the radioiodinated BSA was dialyzed for 2 hours at 4^{o}C against an acetate buffer (0.2 M acetate, 0.17 M NaCl, pH adjusted to 4.9) and then diluted with a cold BSA solution to a final concentration of 1.5 mg/mL (specific activity of 0.5 μCi/ mg protein). The concentration was determined spectrophotometrically at 280 nm using $E^{1\%}_{1cm}$ of 6.6. Aliquots of this solution (25-250 μL) were pipetted into counting tubes to calibrate the method. Aliquots of 2 mg/mL sorghum procyanidin solution (0-20 μL) prepared in 5% MeOH (MeOH/water, 5/95, v/v) were dispensed onto pencil-labeled Whatman # 1 filter paper disks in Petri dishes (9 cm diameter). The plant extracts were treated in the same way. About 10 mL of the labeled BSA solution was poured over the disks making sure they did not stick together. The petri dish was placed on a shaker at room temperature for 30 min before removing the labeled BSA solution by aspiration. The disks were washed three times with the acetate buffer each time agitating on a shaker for 30 min. They were

then placed in separate counting tubes for gamma counting. The amount of protein bound to each disk was calculated from the standard BSA curve.

Results

Analysis of the immobilized tannin

Acid butanol assay

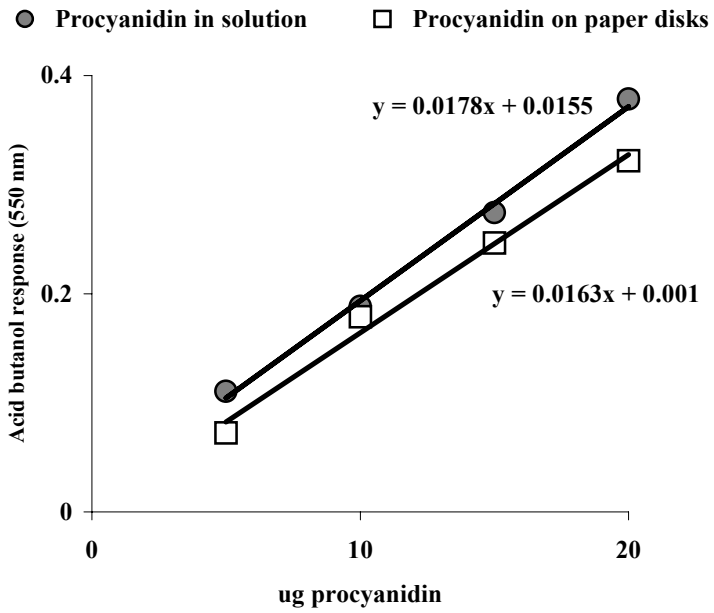
In this study the plant extracts and purified procyanidin standard were immobilized on Whatman # 1 filter paper disks and were reacted with hot acidified butanol. A similar procedure was repeated with the samples in solution. In both cases, the absorbance of anthocyanidin pigments released after the samples reacted with the acidified butanol was recorded. The absorbance response for the assay done on the procyanidin increased linearly with the amount of procyanidin immobilized on the paper disk or in solution (Fig. 3.1). The five different plant extracts showed a similar trend as indicated in Fig. 3.2 for maple extract; the color yield increased with increasing amount of sample.

The acid butanol responses (absorbance values) for the plant extracts were converted to proanthocyanidin levels (procyanidin equivalents) per dry mass of leaf tissue (Table 3.1). Among the extracts, maple was the highest in procyanidin content followed by black oak with tulip poplar and white oak showing intermediate values. The response shown by walnut did not indicate the presence of any procyanidin.

Protein binding

The procyanidin-protein interaction was studied on the Whatman # 1 filter paper disk. The amount of protein bound was dependent on the amount of procyanidin immobilized on the filter paper disk. By increasing the amount of procyanidin on the filter paper disk, the amount of protein bound to the disk was also increased (Fig. 3.1). With a larger amount of procyanidin, protein binding became independent of procyanidin immobilized on the disk. The linear region of the curve is quantitatively useful for calculation of the amount of protein bound per μg procyanidin when protein is in excess.

A



B

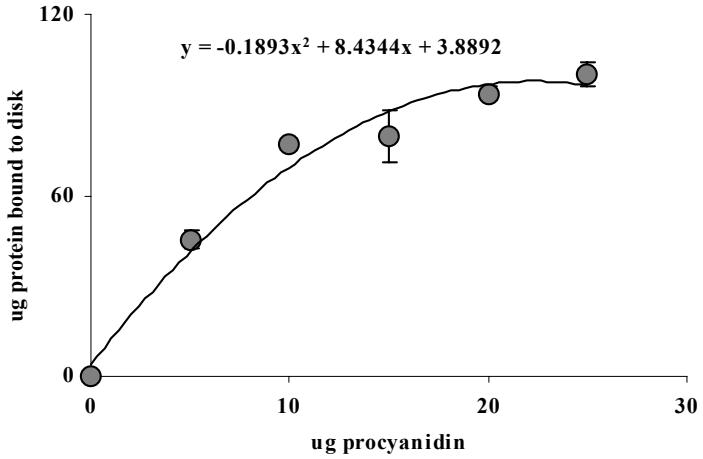
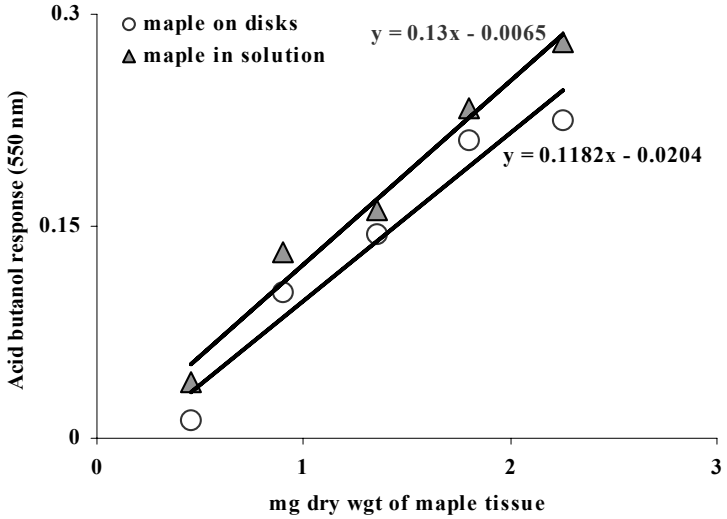


Fig. 3.1: Reactions of procyanidin with acid butanol assay and radiolabeled BSA. (**A**) Aliquots (5-20 μL) of 1 mg/mL sorghum procyanidin were dispensed into screw top test tubes or immobilized on Whatman # 1 filter paper disks (diameter 1 cm). Procyanidin immobilized on the paper disks was placed in separate screw test tubes. Procyanidin standards in solution and on the paper disks were reacted with acid butanol assay solution. Points are the mean of two determinations. (**B**) Procyanidin equivalent to the indicated amounts were immobilized on Whatman # 1 filter paper disks (diameter 1cm) and excess of 1.5 mg/mL radiolabeled BSA solution (specific activity, 0.5 μCi/mg protein) was added to the disks in a petri dish. The petri dish was incubated at room temperature for 30 min and washed. The amount of protein bound was determined by gamma counting. The linear region indicates the binding region. Points are the mean of three determination; error bars are standard deviations.

A



B

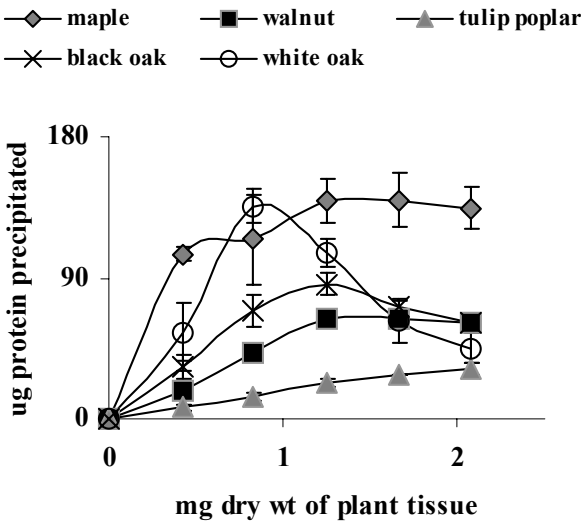


Fig. 3.2: Reactions of plant extracts with acid butanol assay and radiolabeled BSA. (**A**) Aliquots (5-25 μL) of maple extract were dispensed into screw top test tubes or immobilized on Whatman # 1filter paper disks (diameter 1 cm). The maple extract immobilized on the paper disks was placed in separate screw test tubes. The maple extracts in solution and on the paper disks were reacted with acid butanol assay solution. Points are the mean of two determinations. (**B**) Extracts equivalent to the indicated amounts of leaf tissue were immobilized on Whatman # 1 filter paper disks (diameter 1cm) and an excess of 1.5 mg/mL radiolabeled BSA solution (specific activity, 0.5 μCi/mg protein) was added to the disks in a Petri dish and incubated at room temperature. The amount of protein bound was determined by gamma counting. The amount of protein bound by the various plant tissues was obtained from the linear region of the curve as shown in Table 3.1. Points are the mean of three determinations; error bars are standard deviations.

Table 3.1.Tannin composition of the unfractionated plant extracts and the amount of protein bound.

Plant	[1]μg BSA bound	[2] % PC by mass	[3] % MG by mass
Maple	246.0 ± 12.8	4.1	11.9
White oak	161.0 ± 10.0	0.1	10.7
Black oak	80.6 ± 5.4	2.3	8.1
Walnut	47.7 ± 5.0	0.0	9.8
Tulip poplar	15.4 ± 1.8	0.2	5.5

[1]Protein bound, μg BSA bound/mg tissue, is from the slope of the binding curve shown in Fig. 3.2B.
[2]PC, procyanidin equivalents from the acid butanol method standardized with Sorghum procyanidin.
[3]MG, methyl gallate equivalents, using the modified potassium iodate method and the commercial standard methyl gallate (data from [12]).

The amount of protein bound by the immobilized procyanidin was 7.69 $\pm$ 1.92 µg protein/ µg procyanidin.

The protein binding assay with each of the five plant extracts immobilized on the filter paper disk showed a similar response. The binding curves for all five extracts showed that when more plant extract is loaded on the disk, the amount of protein bound by the disk also increased (Fig. 3.2). The steeper slopes of the linear regions of maple and white oak indicated they bound more protein than the other plant extracts (black oak, walnut and tulip poplar) (Table 3.1). The amount of protein bound by maple and white oak was calculated from the linear regions to be 246.0 $\pm$ 12.8 µg protein/ mg dry tissue and 161.0 $\pm$ 10.0 µg protein/ mg dry tissue respectively. The amount of protein bound by black oak, walnut and tulip poplar were between 15 and 90 µg protein/ mg dry tissue.

Discussion

Tannin immobilization on surfaces has been used to recover gold from aqueous solution [22] and uranium in industrial wastewater [26]. It has also been used in trapping enzymes for kinetic studies [13] and for iron sorption [16]. In our study, Whatman # 1 filter paper disks were used to immobilize tannins for protein binding studies, a technique modified from a previous method employed to study tannin-protein interactions [7]. This technique is based on determining radiolabeled protein bound by the immobilized tannin. The ability to immobilize plant tannins on a surface that will not alter their characteristic chemical reactivity offers a convenient method of monitoring tannin-protein interactions. The surface for immobilization should be such that it does not influence the tannin-protein complex.

To ensure that tannin could be immobilized on a surface for protein binding studies, the acid butanol assay was used. The acid butanol assay is a characteristic assay for the proanthocyanidins. In heated acidified butanol, proanthocyanidins are oxidatively cleaved into the brightly colored anthocyanidin monomer units with λ_{max} of 550 nm [25]. The rationale behind the use of this assay is that, if tannins are immobilized on the surface they should be able to respond to the assay.

Cellulose acetate, nylon membranes and Whatman # 1 filter paper disks were tested for tannin immobilization. It turned out that the cellulose acetate could bind proteins even in the absence of tannins (results not shown) making it unsuitable for the protein-binding assay. The nylon membrane did not bind protein in the absence of tannin but did interfere with the acid butanol assay solution. The Whatman # 1 filter paper disk did not interfere with either the acid butanol or protein-binding assay and was confirmed by the acid butanol assay to bind tannin. The acid butanol response obtained from Sorghum procyanidin in solution and on the Whatman #1 filter paper disk showed that the production of anthocyanidin pigments increased linearly with procyanidin amount (Fig. 3.1). The color yield was the same whether the procyanidin was in solution or on disk, an indication that the tannin was immobilized on the disk and did not lose its ability to react with the assay solution.

The acid butanol response obtained from the sorghum procyanidin was used to express procyanidin levels in each plant extract (Table 3.1). The maple extract had the highest level of procyanidin while walnut had none. The differences in procyanidin levels among these extracts may be attributed to factors such as the age of the tissue, time in growing season, environmental parameters and the genetic potential of plant to make different kinds of tannins.

The differences in procyanidin levels may also be due to analytical problems such as the ease with which the proanthocyanidins in these extracts are cleaved by the assay solution, relative amount of proanthocyanidins and hydrolysable tannins in these extracts and the chain length of the proanthocyanidin since short chain proanthocyanidins give low response to the assay [17].

Comparing the amount of protein bound and the procyanidin equivalents in black oak and white oak, white oak bound twice as much protein as black oak but the level of procyanidin in white oak was about 20 times less than that in black oak (Table 3.1). Between tulip poplar and walnut, walnut bound three times as much protein as tulip polar but walnut did not indicate the presence of any proanthocyanidin. This suggests that the proanthocyanidin levels alone did not determine the ability of an extract to bind protein. These plants may contain various hydrolysable tannins that do not react in the acid butanol test but contribute to protein binding. In supplementary data published in

Phytochemical Analysis [12] (Table 3.1) the modified potassium iodate method was used to measure the levels of hydrolysable tannins in these extracts. Both white oak and maple showed higher levels of hydrolysable tannins than the other extracts, which correlated well with their protein binding capacity. Black oak and walnut showed intermediate levels of hydrolysable tannins but the protein binding capacity of black oak was twice as high as that of walnut. The low levels of hydrolysable tannins and proanthocyanidins in tulip poplar correlated well with its protein binding capacity. Relating the amount of protein bound by each extract to the relative amount of hydrolysable tannins and proanthocyanidins, it is obvious protein binding did not correlate with procyanidin levels alone but by both the hydrolysable tannins and the proanthocyanidins in each extract (Table 3.1).

Protein binding capacity depends on the plant species and the specific groups of tannin contained in this plant. For instance, a plant extract that contains a large amount of low molecular weight hydrolysable tannins such as pentagalloyl glucose may bind the same amount of protein as the extract that contains a small amount of high molecular weight hydrolysable tannins provided each has equal amount of proanthocyanidins [3,15]. The high degree of galloylation in the high molecular weight hydrolysable tannins may confer a high capacity for protein [3]. Hydrolysable tannins that have large hydrophobic residues and hence large size may enhance stronger hydrophobic interactions with the protein [8,21]. An extract containing a large amount of procyanidin may bind the same amount of protein as the extract that contains a small amount of prodelphinidin provided each has equal amount of hydrolysable tannins. Prodelphinidins have an extra hydroxyl group on the ring B of the monomer unit and as a result may bind protein more effectively than the procyanidins [19]. The differences in the binding capacities of the five different extracts used in this study might have depended on factors such as molecular weight of tannin in the extract, substituents, structure conformation, and polarity of tannins in the mixtures.

In Fig. 3.1, the linear acid butanol response obtained indicates that the disk binds at least 20 μg of procyanidin. However, the protein binding data obtained from both the procyanidin and the plant extracts showed saturated regions. At the saturated region of the procyanidin-protein binding curve (Fig. 3.1), 18.0 μg of the procyanidin precipitated

85.0 $\pm$ 4.0 μg BSA. The amount of BSA bound by the immobilized tannin may be limited by the geometry of the tannin-protein complex and the surface area of the disk. Two plant extracts namely, black oak and the white oak showed regions on their protein binding curves in which less protein was bound when a larger amount of extract was immobilized on the disks. Tannin-tannin interactions may become more important at higher tannin concentrations and this may affect the protein binding capacity (protein no longer binds to the tannins) [9].

In conclusion, tannin-protein analysis on a surface like the filter paper disk is a simple way of analyzing protein-binding capacities of different sources of tannins. Because tannin-protein interaction influences the nutritional values of plants, the method may find its use in places where browsing is the primary form of feeding for livestock thus helping to predict the safety of tannin-containing forage and ultimately managing the anti-nutritional aspects of it.

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