Application and Characterization of Bioactive Compounds in Peanut Skins, a Waste Product of Virginia Agriculture

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Food Science and Technology

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December 7th, 2010
Blacksburg, VA

Keywords: by-product, peanut, liquid chromatography, mass spectrometry, yeast, optical density

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ABSTRACT

Peanut skins have long been a waste product of the peanut industry. The aim of this project was to find suitable applications for this rich source of natural bioactive compounds. Solvent extracts of peanut skins and a multistep solvent extraction process to yield oligiomeric procyanidin (OPC) extracts were found to be inhibitory towards three types of yeasts (*Saccharomyces cerevisiae*, *Zygosaccharomyces bailli*, and *Zygosaccharomyces bisporus*). All extracts were devoid of solvents that may have interfered with the results. The OPC extract exhibited the highest inhibitory effect, and was chosen for fractionation. Fractionation was conducted by means of a silica or size exclusion high performance liquid chromatography (HPLC) column. Fractions were then subjected to a yeast growth curve assay to determine the active fractions. The fractions were then characterized by liquid chromatography- mass spectrometry (LC-MS). Negative mode electrospray MS determined the fractions to contain mostly procyanidins but also proanthocyanidins. Since it is possible for multiple compounds to display the same molecular ion, multistep MS and retention time differences were utilized to tentatively identify the compounds based upon their fragmentation schemes. However, co-elution was prominent, thus specific compounds responsible for yeast growth inhibition could not be determined. The yeast inhibition assay demonstrated that the procyanidin dimers up to tetramers had the best anti-yeast capabilities.

Dedication

I would like to dedicate this work to those scientists who have worked to advance human culture. Without their hard work and dedication I would not have been able to accomplish the studies completed in this manuscript.

Acknowledgements

I would like to recognize the Department of Food Science and Technology at Virginia Tech as a whole. It truly is a collegial atmosphere. I would especially like to thank my major professor Dr. Sean O'Keefe for always being available to answer my questions. It was his idea for this project and I thank him for always having a forward thinking approach.

I am thankful to Dr. Jodie V. Johnson who conducted the LC-MS^n analysis. He provided me with a wealth of information about mass spectrometry. If it was not for him I would have taken much longer to understand and interpret the data. He is responsible for really speeding up my learning curve as it pertains to electrospray mass spectrometry.

Dr. Renee Boyer was instrumental in developing my understanding of microbiology. Anytime I had a question she was always available to "put the fire out." I am very appreciative of her support. Dr. James Tanko was instrumental in providing advice as it pertains to the chemical structural representation of the MS results. Dr. William Eigel was valuable because of his infinite knowledge of enzyme systems.

I would also like to thank Kevin Wayne Holland for his endless humor and advice as it related to this project along with my Lab Group for their support and collegiality. To Ana and the mouse cat, I thank them for keeping me sane and washing the dishes. Especially to Stella, the mouse cat, my little writing buddy. I will surely miss you, and never forget you. You were always there when I needed a break from writing or interpreting data. To hear your pur-purs always relaxed me and got me through. Lastly, I would like to thank the Biodesign and Bioprocessing Research Center (BBRC) of Virginia Tech, the institution responsible for funding this study.

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CHAPTER 1

INTRODUCTION

Maximizing value of agricultural output is of vital importance for the success of Virginia farmers. Peanuts are an important crop to Virginia farmers. Virginia type peanuts have become increasingly popular since they are larger than the other varieties of peanuts such as Runner and Spanish types. This has made them popular for consumption in whole form. This crop has potentially high value by-products (peanut skins, hulls) that have yet to be adequately investigated for viable use. Another plant by-product, grape seed extract contains polyphenolic compounds responsible for health benefits such as lowering risk of diabetes, coronary heart disease, cancer, and inflammatory disease (Yilmaz & Toledo, 2004). A peanut skin extract could contain beneficial compounds and provide similar effects, ultimately providing a use for a waste product that now has an associated cost.

Peanuts have been extensively studied for their nutritional value and flavor characteristics. Relatively little is known about other components of the peanut plant including the leaf, shell, skin and roots. A few publications have shown these components to also contain polyphenolic compounds such as catechins, phenolic acids, flavonoids, and proanthocyanidins (Dean, Davis, Shofran, & Sanders, 2008; Duh, Yeh, & Yen, 1992; O'Keefe & Wang, 2006).

Research in nutraceuticals has steadily increased over the past decade. Studies indicate that polyphenolic compound extracts may be able to impact chronic illnesses like diabetes. Diabetes is a serious, chronic medical condition that affects an estimated 23.6 million people in the United States (ADA 2008). An increase of oxidative stress is often associated with a diabetic state (Vega-Lopez, Devaraj, & Jialal, 2004). Thus, antioxidants can possibly assist in achieving oxidative balance for diabetics.

Research suggests that polyphenol polymers can play a direct role in the regulation of diabetes. Water soluble polyphenol extracts containing A-type doubly linked proanthocyanidin oligomers from cinnamon have been found to enhance the activity of insulin (Anderson, Broadhurst, Polansky, Schmidt, Khan, Flanagan, et al., 2004). One of the proposed reasons for this occurrence is that proanthocyanidin oligomers function as an insulin mimetic (Jarvill-Taylor, Anderson, & Graves, 2001). A-type proanthocyanidins have been discovered in both peanut skins and grapes (Gu, Kelm, Hammerstone, Beecher, Holden, Haytowitz, et al., 2003; Lou, Yamazaki, Sasaki, Uchida, Tanaka, & Oka, 1999). Another reason for the polyphenolic compounds ability to have a regulating effect on diabetes may be linked to inhibition of α-glucosidase activity. The inhibition of intestinal α-glucosidases delays the digestion and absorption of carbohydrates (specifically oligosaccharides), as a result suppressing hyperglycemia. Similarly, α-amylase catalyzes the hydrolysis of polysaccharides. Polyphenolic compounds have also been shown to inhibit α-amylase activity (McDougall, Shpiro, Dobson, Smith, Blake, & Stewart, 2005). However, much of the research to date is indecisive about which compounds in the extracts are responsible for the inhibition. Synthetic α-glucosidase inhibitors such as acarbose, miglitol, and voglibose are prescribed to Type 2 diabetes mellitus patients but these drugs have certain adverse effects such as hypoglycemia at higher doses, liver problems, lactic acidosis, flatulence and diarrhea (Ani & Naidu, 2008). A natural extract from a waste by-product would be of great interest because it may provide the same benefits as the synthetic drugs discussed previously and accomplish it with a lower associated cost.

Recent outbreaks of food borne illnesses, such as *Salmonella* in peanut products has brought food safety the increased amount of attention. As a result, new technologies have been developed to combat these issues. There have been a number of studies that indicate catechins

and proanthocyanidins may have antibacterial, antiviral, antimycotic, and antiprotozoal effects (Buzzini, Turchetti, Ieri, Goretti, Branda, Mulinacci, et al., 2007). However, finding the combination of specific extract for the appropriate food safety application is difficult. A phenolic extract of Portuguese honey was found to be effective against *Staphylococcus aureus*, however it was found to be ineffective against gram-negative bacteria such as *Escherichia coli* (Estevinho, Pereira, Moreira, Dias, & Pereira, 2008). In a similar study, epigallocatechin gallate (EGCG) was found to be effective against various strains of *Staphylococcus*, but to a lesser extent against gram-negative bacteria (Yoda, Hu, Zhao, & Shimamura, 2004). However, a tea catechin extract containing EGCG was found to be successful at significantly inhibiting the growth of *Escherichia coli* O157:H7 in nematodes (Lee, Kim, Lim, Nam, Youn, Nam, et al., 2009).

The presence of biofilms during food processing applications can lead to numerous food safety issues. Biofilms are difficult to remove and can remain even after sanitary procedures have been completed. In a recent study, grape seed extract significantly decreased the formation of a biofilm (Furiga, Lonvaud-Funel, & Badet, 2009). A peanut skin extract may be able to accomplish the same task. This presents the opportunity of the extract to be incorporated into a sanitizer to limit biofilm formation during food processing.

Phenolic compounds have been known to be responsible for antimycotic activity. Park and others (2006) observed that EGCG has antimycotic activity against 21 clinical *Candida* spp. isolates. Proanthocyanidin compounds seem to show better activity toward the inhibition of yeasts than their originating monomers (Romani, Ieri, Turchetti, Mulinacci, Vincieri, & Buzzini, 2006). Yeasts are a specific type of fungi, so often compounds which show good inhibitory action towards yeasts also show good inhibitory action towards other types of fungi. This would

be of great value given that current antifungal drugs are often prescribed as a last resort, since these drugs are fairly hazardous to the body. A natural antimycotic would be very desirable since its comparative harm to the body would be assumed to be quite less hazardous. Peanut skin extracts show promise based on previous studies for a wide variety of viable applications.

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CHAPTER 2

REVIEW OF LITERATURE

1. Studies Involving Peanut Skins

The first person to bring the beneficial compounds in peanut skins to attention was Dr. Jack Masquelier. He first started working on peanut skins in 1947 and found the skins to contain large amounts of proanthocyanidins (Kilham & Masquelier, 1997). The term proanthocyanidin, procyanidin, oligomeric proanthocyanidin (OPC), and condensed tannins are often used synonymously; however this terminology is not entirely correct. Procyanidins refer to oligomers of epicatechin or catechin only (Gu, Kelm, Hammerstone, Beecher, Holden, Haytowitz, et al., 2004). Proanthocyanidins are a group of flavan-3-ols that include procyanidins, prodelphinidins, and propelargonidins. Prodelphinidins and propelargonidins contain gallo(epi)catechin (3 OHs on the B ring) and (epi)catechin missing an hydroxyl on its B ring, respectively (Figure 1). Catechin gallates can also occur, in which C ring oxygen becomes gallated ($C_7H_5O_5$).

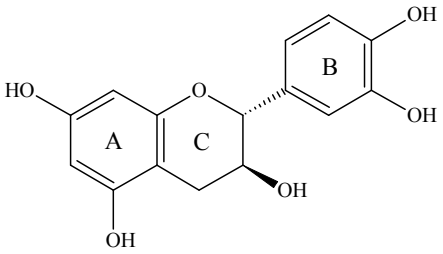

Figure 1. Structure of (+)-catechin. If this structure were to be (-)-epicatechin, the hydroxyl on the C ring would be a down stereo bond.

In 1950, OPCs from peanuts skins emerged as the popular medicine Resvit® which was marketed as a vascular-protective medicine. The peanut commodity market later changed in France in the 1950s. Peanuts had previously been grown in Africa and blanched in France. At this time all peanut processing occured in Africa. Masquelier was then forced to find another source of procyanidins. He found his new source of OPCs from the Landes pine trees. He later patented this procedure in the 1980s (Masquelier, 1987). He also pushed to make OPCs receive

vitamin status as Vitamin P (usually refers to bioflavanoids) for their beneficial action as an anti-inflammatory agent.

It was some time before the next published work strictly on peanut skins (Lou, Yamazaki, Sasaki, Uchida, Tanaka, & Oka, 1999). The study used nuclear magnetic resonance spectroscopy (NMR) to identify A-type procyanidins in peanut skins. The use of NMR was significant because it allowed the elucidation of the linkage and the stereoisomers. A continuation of this work was performed identifying 8 new flavonoids, and two indole alkaloids by NMR and FAB-MS (Lou, Yuan, Yamazaki, Sasaki, & Oka, 2001). Of particular note were the identification of a quecetin 3-O-glycoside, two isorhamnetin 3-O-triglycosides, rutin, isorhamnetin-3-O-rutinoside, and an isoflavone glucoside. Both of these works were based off of the work done by Karchesy and Hemingway in (1986) identifying A-type procyanidins in peanut plants and the identification of luteolin in peanut hulls by NMR (Duh, Yeh, & Yen, 1992). Isorhamnetin-3-O-rutinoside, isorhamnetin-3-O-glucoside, and isorhamnetin extracted from almond skins were found to elute in the listed order using reversed phase chromatography (Mandalari, Tomaino, Arcoraci, Martorana, Turco, Cacciola, et al., 2010).

Utilizing the antioxidant compounds from peanut skins for use in foods has been theorized, but has yet to be implemented. The first published study of this type implemented a methanolic peanut skin extract into sunflower oil (Nepote, Grosso, & Guzman, 2000). The extract was added at the amount of 1.8 mL (1.2 % of extract, 122.4 mg of phenols by Folin-Ciocalteu) to 150 g of oil. Peroxide value of the oil was lower in those samples treated with peanut skin extract (PSE). When the peanut skin extract was compared with butylated hydroxytoluene (BHT) at 0.02 %, PSE was outperformed. Solvent effects of the two antioxidants in solution were not examined. It is therefore plausible this could have contributed

to the results. A follow-up study compared the extraction percentages of methanol, ethanol, acetone, and water at room temperature during 24 hrs by maceration. Methanol, ethanol, and acetone extracts showed the highest phenolic contents in the order listed, but extracts showed no statistical significant difference in extraction percentage by dry weight of matter (Nepote, Grosso, & Guzman, 2002). Water extracted the lowest amount of dry matter, but had similar phenolic content values as acetone. The study was also the first to report proximate compositions for peanuts skins as: 16.60 % oil, 12.32 % protein, 2.83 % ash, and 69.8 % other components. Peanut skins have been estimated to have a 17 % (w/w) proanthocyanidin content, 17 % protein, and 5 % fat (Karchesy & Hemingway, 1986). The authors found that defatted peanut skin extracts using the same solvents led to a lower percentage of dry extract matter, but higher phenolic content by the Folin-Ciocalteu method. However, by the diphenyl picryl hydrazyl (DPPH) radical scavenging assay, solvent extracts obtained from peanut skins and their defatted counterparts were not significantly different ($\alpha.= 0.05$). In 2005 a follow-up study looked at optimizing the extraction process by manipulating the proportion of aqueous ethanol extracts, size of peanut skin particles, time, ratio of solvent/skins, and number of extractions (Nepote, Grosso, & Guzman, 2005). The same assays to assess antioxidant activity as previous studies were used. Extracts containing 30 % - 96 % ethanol-water were not statistically significantly different at extracting dry matter and radical scavenging activity. However, 30 % - 70 % ethanol-water extracts were best at extracting phenolic compounds. A very low percentage of ethanol and very high percentage of ethanol were least efficient at extracting phenolic compounds.

In a similar study Wang and others (2007) analyzed 50 % ethanol defatted peanut skin extracts for total phenolics, total flavonoids, DPPH activity, chelating activity, hydroxyl radical

scavenging, superoxide anion scavenging, hydrogen peroxide scavenging, and the ability to protect erythrocytes against peroxyl radicals. Peanut skin extracts scavenged 97.1 % DPPH, 98.6 % superoxide anion, 89.1 % hydrogen peroxide, 85.3 % hydroxyl radical, chelated 76.6 % of Fe^{2+}, and inhibited 98.6 % of damage to erythrocytes. Results for the DPPH assay were similar to those reported previously at 93 % scavenging activity (Nepote, Grosso, & Guzman, 2002).

Another parameter that could effect the extraction of antioxidants from peanut skins is processing. Yu and others (2005) examined skin removal methods and solvent effects by measuring the total phenolics of peanut skin extracts. Skins from hand-peeled raw peanuts, hand-peeled blanched peanuts, and hand-peeled skins from peanuts heated at 175 °C for 5 minutes were examined. Aqueous methanol and ethanol extracts were explored. They concluded that a combination of roasting the peanuts prior to peeling and ethanol extraction were the most efficient recovery methods(Yu, Ahmedna, & Goktepe, 2005) according to Folin-Ciocalteu and total antioxidant activity (TAA) assays. In a subsequent study they attempted to identify some compounds in the extracts via liquid chromatography-mass spectrometry (LC-MS) and used the DPPH assay to determine the antioxidant activity of the skin extracts (Yu, Ahmedna, Goktepe, & Dai, 2006). In this study 80 % ethanol was used for extraction. Catechins and A-type procyanidins were identified by mass spectrometry. Molecular ions were observed of what they thought to be caffeic acid, chlorogenic acid, resveratrol, and the resveratrol glycoside, piceid. However, with no fragmentation data presented, these results are highly tentative. In addition, their identification of a B-type procyanidin as m/z = 1151 was incorrect, all B-type linkages would have to be m/z = 1153. The correct identification would be an A-type procyanidin tetramer (3 B-type links, 1 A-type linkage). Consistent with other studies,

the peanut skin extracts had high DPPH values. Again, blanching led to the least amount of recoverable phenolics similar to the results of their 2005 study. Microwave-assisted extraction has also been used recently to extract phenolic compounds from peanut skins. The optimum conditions were found to be 90% microwave power, 30 s irradiation time, 1.5 g of skins for total phenolic content, and 90% microwave power, 150 s irradiation time, 1.5 g of skins for ORAC (Ballard, Mallikarjunan, Zhou, & O'Keefe, 2010).

2. Oxidation and Antioxidant Assays

Much of the previous research performed on peanut skins and the compounds it contains has centered on its ability to function as an antioxidant. Oxidation in biological systems is sometimes required for cell signaling purposes, but during most instances is undesirable. Chemically, oxidation occurs when a molecule loses an electron or hydrogen atom. This usually results in replacement by an oxygen atom during a chain reaction. These reactions are initiated by light, heat, metals, or by other free radicals and peroxides. These reactions can proceed in the liquid or vapor phase, are self propagating, and have an induction period. An important class of free radical reactions during which oxidation occurs is autoxidation reactions (Figure 1). The steps of the mechanism are given below (Ingold, 1961).

Initiation	$RH \xrightarrow{I} IH + R\bullet$	(1)
Propagation	$R\bullet + O_2 \rightarrow ROO\bullet$	(2)
	$ROO\bullet + RH \rightarrow ROOH + R\bullet$	(3)
Termination	$R\bullet + ROO\bullet \rightarrow ROOR$	(4)

Figure 2. Steps of Autoxidation. R is any molecule able to undergo oxidation. The initiator is denoted by the letter I.

During initiation the molecule (R) loses a hydrogen radical, in the presence of an initiator (which is usually a metal) (1). During propagation the alkyl radical reacts very rapidly with molecular oxygen to form peroxyl radicals (2). Next a hydrogen atom transfer reaction occurs causing the formation of hydroperoxides (3). At the last stages of oxidation after reaching a maximum, the rate decreases, the peroxyl radicals react with each other and self-destruct to form non-radical products by a termination reaction (4). It was first proposed that an antioxidant functioned by donating a hydrogen atom to the free radical (hydrogen atom transfer), thus neutralizing a free radical and producing a less reactive free radical itself. Recently, it has been suggested that other mechanisms including sequential proton-loss electron transfer (SPLET) and proton coupled electron transfer (PCET) also can occur (Litwinienko & Ingold, 2007). Furthermore polyphenolic antioxidants are capable of trapping up to two radicals because these radicals are too unreactive to continue the chain (Litwinienko & Ingold, 2007). The ability of a molecule to slow the progression of radical chain reactions is the basis of antioxidant assays.

Antioxidant Assays

One of the major problems with antioxidant assays is that there is no standard method to determine antioxidant activity. Each method measures the activity differently, which can lead to a different value for the same antioxidant. There has been a movement to develop a standard method to evaluate antioxidants, but this is yet to become widely accepted. It has been suggested that the oxygen radical absorbance capacity (ORAC) assay, the Folin-Ciocalteu method, and possibly the Trolox equivalent antioxidant capacity (TEAC) assay become standard methods because of their applicability to human biology (Prior, Wu, & Schaich, 2005). These methods will be examined in more depth, along with perhaps the most commonly used assay for antioxidant activity, the DPPH assay.

DPPH

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) method relies on a radical that is also a chromophore to measure antioxidant capacity. DPPH is one of the few stable radicals, and can be evaluated by electron spin resonance (ESR) or by measuring a decrease in absorbance at 515 nm due to reduction of the DPPH· by the antioxidant (Prior, Wu, & Schaich, 2005). Many labs own a spectrophotometer, which has probably added to this method's widespread use. Results can also be compared to standard antioxidants such as quercetin or Trolox and reported as equivalents (Atoui, Mansouri, Boskou, & Kefalas, 2005). The main disadvantages of the DPPH assay are solvent effects and its irrelevance to biological systems since this radical does not occur naturally. The solvent system can effect whether the reaction mechanism is predominately an electron transfer or hydrogen atom transfer. For example, in alcoholic solvents the reaction rate of DPPH is faster, thus leading to an overestimation of antioxidant capacity (Tanko, 2005).

ORAC

Oxygen radical absorbing capacity (ORAC) is a technique that measures the antioxidant capacity of a sample extract against usually the peroxyl radical. However, other radicals such as the hydroxyl radical can also be used (Cao, Sofic, & Prior, 1996). An extract solution expected to contain antioxidants is combined with fluorescein or dichlorofluorescein (are fluorescent) and a radical generator. The extract solution is then compared to a standard solution (Trolox). The solutions are read by a spectrofluorometer. Usually this is accomplished by a plate reader with fluorescence reading capabilities. This allows for many samples to be analyzed in a short amount of time. Once the kinetics of the fluorescence is recorded, the final ORAC values can be calculated using a linear equation between the Trolox standards (reference) and the sample concentrations by calculating the net area under the fluorescein decay curve (Xu & Chang,

2007). The method falls into the category of a hydrogen atom transfer (HAT) mechanism. HAT methods measure the ability of an antioxidant to quench free radicals by hydrogen donation. In most HAT methods, antioxidants and a probe compete for thermally generated peroxyl radicals and the quantitative values are derived from the kinetic curves after monitoring the complete reactive kinetics (Sun & Tanumihardjo, 2007). The obvious disadvantage is that antioxidants and conditions that do not favor HAT will lead to underestimated values. Solvent effects are great with this method. In comparison to DPPH, ABTS, and FRAP, ORAC was affected the most by the solvent used which consisted of aqueous mixtures of methanol and acetone (Perez-Jimenez & Saura-Calixto, 2006). This paper suggested that proteins also interfered with ORAC, having positive ORAC values. However in light of recent developments reporting antioxidant capability of peptides (Sun, He, & Xie, 2004), the implication of proteins as an interferite may have been incorrect. The positive association with a high ORAC value has even led to some manufacturers to label their products with ORAC values (Prior, Wu, & Schaich, 2005) and has even led to the development of a database (Wu, Gu, Holden, Haytowitz, Gebhardt, Beecher, et al., 2004).

Folin-Ciocalteu Assay

This assay is employed to measure the total phenolic content of a substance. This assay has been found to correlate well with the radical scavenging assays DPPH (Katsube, Tabata, Ohta, Yamasaki, Anuurad, Shiwaku, et al., 2004), ORAC and FRAP (Xu & Chang, 2007). This assay is used because it is sometimes difficult to quantitate individual phenols by HPLC or other methods and apply this information to calculate a total phenolic concentration. The premise of this method is rather simple. Phenolics react with the Folin-Ciocalteu reagent (a solution of phosphomolybdate and phosphotungstate). This reagent becomes reduced by the addition of one

or two electrons from the phenolics and the reagent changes color to blue which can be measured spectrophotometrically. The values for this method are reported as known equivalents of an antioxidant. Compounds that have been used as standards for comparison purposes include gallic acid, caffeic acid, vanillic acid, etc. (Prior, Wu, & Schaich, 2005). The drawbacks to this assay mainly are concerned with presence of interfering compounds (Ainsworth & Gillespie, 2007). Tryptophan, quinine, xanthine, ascorbic acid, uric acid, and sulfites also interfere. Sugars at high levels (25 g/L) also interfere. The possibility for interfering substances requires the use of an additional cleanup step, or the results will consist of an overestimation of phenolic content. Flavonoids can also be measured via this method by precipitation with formaldehyde, and comparison of the results with flavonoids included and removed.

TEAC

This assay measures an antioxidant's ability to convert the $ABTS^{\cdot+}$ radical to ABTS. The ability an antioxidant to reduce this radical is the basis of this assay. $ABTS^{\cdot+}$ is colored, so the decrease in absorbance is proportional to the ability of the antioxidant to accomplish the reduction. This can be measured at 658 nm (Perez-Jimenez & Saura-Calixto, 2006). The $ABTS^{\cdot+}$ radical can be reduced by either an electron transfer mechanism or hydrogen atom transfer (Wood, Gibson, & Garg, 2006). Thus this method leads to a more precise measurement of antioxidant activity if solvent affects are likely and the antioxidants in question are able to function by different mechanisms. The value for this assay is reported as Trolox equivalents (Prior, Wu, & Schaich, 2005). The main drawback of this assay is that the $ABTS^{\cdot+}$ radical is not naturally occurring, which makes it of questionable biological significance.

Methods to Measure Oxidation in Foods

Lipid oxidation is the most commonly encountered type of oxidation in foods. It leads to unfavorable changes in food quality and nutritional value. Several methods of determining the extent of oxidation in foods have been developed. One of the oldest and most commonly used measurements to determine the extent of oxidation in oils is the peroxide value. The standard iodometric procedure measures, by titration, colorimetric, or electrometric methods (higher sensitivity methods), the iodine produced by potassium iodide added as a reducing agent to the oxidized sample dissolved in a chloroform-acetic acid mixture. The liberated iodine is titrated with standard sodium thiosulfate to a starch endpoint. The peroxide value is expressed as milliequivalents of iodine per kg of lipid (meq/kg), or as millimole of hydroperoxide per kg of lipid (Frankel, 1998).

The determination of peroxide value is mostly useful for bulk oils that can be analyzed directly. For foods, emulsions or muscle tissues, the lipid is extracted with mixtures of solvents that must be carefully removed by evaporation without decomposition of the hydroperoxides, which can be difficult. Solvents used should not contain antioxidants as stabilizers, which would act as reducing agents and interfere with the peroxide value determination. The method seems to work the best for samples that are oxidized to relatively low levels (peroxide values of less than 50), and that are under sufficiently mild conditions so that the hydroperoxides are not markedly decomposed so to register an accurate peroxide value.

Measurement of carbonyl content is another useful technique to measure the amount of oxidation that occurred. Since the carbonyl value is related to decomposition of carbonyl compounds which contribute to flavor deterioration, it provides information relevant to sensory evaluation. The test serves as a general indicator of the level of oxidation. Total carbonyl

content can be determined in oxidized lipids by the reaction with 2,4-dinitrophenylhydrazine, and the colored hydrazone (2,4-DNPH) derivatives. These are measured spectrophotometrically at 430-460 nm, and expressed as nmol hexanal/kg sample. The anisidine test is another test which measures carbonyl content. This test measures high molecular weight saturated and unsaturated carbonyl compounds in triacylglycerols. This test provides useful information on non-volatile carbonyl compounds formed in oils during processing (Frankel, 1998).

The 2-thiobarbiuric acid (TBA) test is an old and popular colorimetric method used to measure rancidity in some foods and oxidation products in biological systems (Tarladgis, Pearson, & Dugan, 1964). The test is based on the pink color absorbance at 532-535 nm formed primarily due to the formation of malonaldehyde. Polyunsaturated lipids and TBA react to produce a large number of secondary oxidation products (primarily malonaldehyde) which are referred to as TBA-reactive substances or TBARS. The test is standardized by using malonaldehyde generated by acid hydrolysis of 1,1,3,3-tetraethoxypropane, the tetraethoxyacetal of malonaldehyde. Protein and sugar degradation products, amino acids, nitrite, metals, and antioxidants may interfere with the TBA test. Thus, difficulties are often encountered with the TBA test for complex food materials and biological systems containing non-lipid materials contributing to the test.

Lastly, GC methods are capable of determining volatile oxidation products that are either directly responsible for, or serve as markers of flavor development in oxidized lipids. The three commonly used GC methods for this type of analysis are static headspace, dynamic headspace, and direct injection methods (Taylor, 2002). The analytes are low molecular weight secondary oxidation products such as aldehydes and hydrocarbons, and since heat often is used to desorb volatiles off of the GC column, artifacts can be formed in the process. An artifact is a change

from the original chemical analyzed to another product which is not indicative of the starting material. GC analysis has shown some promise in regard to correlation with sensory scores. However, problems can arise with detecting low levels of oxidation products.

3. Proanthocyanidins and Components in Peanut Skins

The most useful technique for qualitatively identifying substances in a complex matrix is undoubtedly liquid chromatography mass spectrometry (LC-MS). Even though mass spectrometry might not give us as much structural information as NMR, it is currently the best technique to interface with chromatography which is needed to separate components before analysis. Since proanthocyanidins are structurally very similar co-elution can be common (Guyot, Doco, Souquet, Moutounet, & Drilleau, 1997). Therefore we need a technique that can separate components even after sample cleanup procedures. Mass spectrometry is similar to an additional chromatographic step because it separates molecules by their mass-to-charge ratio. This allows co-eluting components to be distinguished as long as their mass-to-charge ratios are different or their dissociation pathways are different. Originally mass spectroscopy was limited to gas phase molecules because of the requirement of the molecules to be ionized. This is necessary since the ions are controlled by magnetic or electromagnetic fields. However development of electrospray ionization (ESI) by John B. Fenn in the 1980s allowed liquid soluble samples to be analyzed.

The most common technique for analysis of proanthocyanidins is probably ESI-LC-MS. However one of the first papers to gain structural information from procyanidins in a complex matrix used fast atom bombardment (FAB) ionization. Karchesy and co-workers chose FAB to identify procyanidin oligomers in *Pinus taeda* (Lobolly Pine) bark and *Arachis hypogaea*

(peanut) over NMR since resonance multiplicity and broadening associated with rotational and conformational isomerism often severely complicate the NMR spectra of procyanidin structures (Karchesy, Hemingway, Foo, Barofsky, & Barofsky, 1986). Prior to this study, procyanidins could only be identified by spectroscopy (UV-vis, NMR, IR, etc.) with the aid of pure standards. During this time period, FAB-MS was not interfaced with a chromatographic method, but the authors were still able to identify procyanidin monomers, dimers, and trimers in the pine bark. The results demonstrated that the FAB-MS technique were similar to NMR, and did not require separation of molecules into fractions prior to analysis.

Earlier techniques such as FAB had more limitations because it was a "harder" technique than ESI since it breaks molecules into more pieces upon ionization. For example, this leads to hexamers looking more like trimers in a FAB-MS spectrum. For this reason ESI is a better technique to investigate larger oligomers. However, for lower molecular weight oligomers, fragmentation behavior of FAB is similar to ESI (Cuyckens & Claeys, 2004). Freeze-dried extracts of apple pulp and skin were investigated by ESI-MS finding procyanidins with degrees of polymerization up to 17 (Guyot, Doco, Souquet, Moutounet, & Drilleau, 1997). This paper also confirmed the presence of doubly charged procyanidins in apples which is characteristic of an isotopic distribution differing by 0.5 amu. In a study of the common apple (*Malus pumila*) procyanidins were fractionated by normal phase chromatography and then identified by reversed phase LC-ESI-MS up to a degree of polymerization of 9 (Shoji, Masumoto, Moriichi, Kanda, & Ohtake, 2006). Doubly and triply charged B-type procyanidin species were detected, no prodelphinidin, pelargonidin, or procyanidin A-types were detected. A decamer was tentatively identified as a doubly charged ion, but overlap of isotopic peaks with that of smaller molecular weight species due to inadequate separation led to its tentative identification.

In a study by Yeap Foo and Lu (1999) procyanidins in apple pomace were identified using NMR and MS without chromatography. Pomace was extracted with acetone and separated on an open Sephadex LH-20 column. Two types of B-type dimers were identified (C4→C6) and the more common (C4→C8). Chemical degradation by phloroglucinol confirmed these identifications. Both dimers were found to contain only epicatechin because of the upfield position of the C-2 resonances from NMR data and had $[M-H]^-$ ions of 577. An interesting observation was that the (C4→C6) dimer had a longer retention time on a C18 column than that of the (C4→C8). This proves that these molecules can be differentiated by chromatography even if their MS dissociation pathways are the same. In addition a (C4→C8) B-type trimer and tetramer consisting of only epicatechin units were identified. A B-type pentamer and hexamer were also discovered, but linkage and stereochemistry could not be discerned because the ^{13}C-NMR signals became broad and unresolved with oligomers larger than tetramers. In addition, multiple HPLC peaks were observed either due to various linkages, or inadequate separation. Mass spectrometry signals higher in molecular weight than a pentamer were also very weak, probably due to ionization troubles of higher molecular weight species. Enhancement of ionization of procyanidins can be accomplished by adding an ionization reagent such as ammonium acetate (Prior, Lazarus, Cao, Muccitelli, & Hammerstone, 2001). Yeap Foo and Lu (1999) used straight acetonitrile and water 1:1 without the aid of acid or ionization reagent which may have led to their poor signal intensities.

One of the first studies to use an ion trap mass spectrometer to investigate the structures of proanthocyanidins was conducted by Friedrich and co-workers (2000). Compounds were extracted from malt with aqueous acetone and fractioned with a glass polyamide column. It was possible to determine both the molecular weight and the sequence of the monomeric units

(position of the linkages) with the ion trap instrument because of the ability to perform collision induced dissociation inside the ion trap. However, no differentiation between stereoisomers is possible, and no information about the position and stereochemistry of the interflavanoid linkage (C4→C8 or C4→C8) can be determined from MS alone (Friedrich, Eberhardt, & Galensa, 2000). The authors also provided an argument for why negative mode ESI should be used over positive mode. Polyphenols are weakly acidic compounds, indicating that dissociation is easier than protonation (Friedrich, Eberhardt, & Galensa, 2000). In addition better sensitivity and selectivity have been described for these compounds when the mass spectrometer was operated in negative mode (Poon, 1998). The major contribution of Friedrich and co-workers paper was that it proved that the linkage of the structures of the dimers (epi)catechin-(epi)catechin, gallo(epi)catechin-(epi)catechin, (epi)catechin-gallo(epi)catechin, gallo(epi)catechin-gallo(epi)catechin and trimers of the same type could be determined by MS alone. The fragmentation pathway for $[M-H]^-$ = 593 molecular ion (gallo(epi)catechin-(epi)catechin) was extensively described in this work.

Aqueous methanol extraction of the phenolic constituents in dried plums did not yield proanthocyanidins; neochlorogenic acid and cryptochlorgenic acid were found to be the major constituents (Fang, Yu, & Prior, 2002). Of significance was the identification of quercetin rutionside (also known as rutin) in the extract. Many of the compounds identified in this study were glycosylated. It has been reported that on a C18 column a galactoside will elute before glucoside (Prior, Lazarus, Cao, Muccitelli, & Hammerstone, 2001).

The most extensive study of the dissociation pathway of proanthocyanidins in foods has been conducted by Gu and colleagues (2003). This paper described the major dissociation mechanisms of procyanidins via ESI-MS^n analysis. This study was the first to describe in detail

the dissociation pathway for the breakdown of A-type procyanidins. A-type procyanidins can contain (C4→C8 or C4→C8) and (C2→O→C7) linkages (Figure 3). This was also one of the first papers to use normal phase chromatography to elute proanthocyanidins by degree of polymerization.

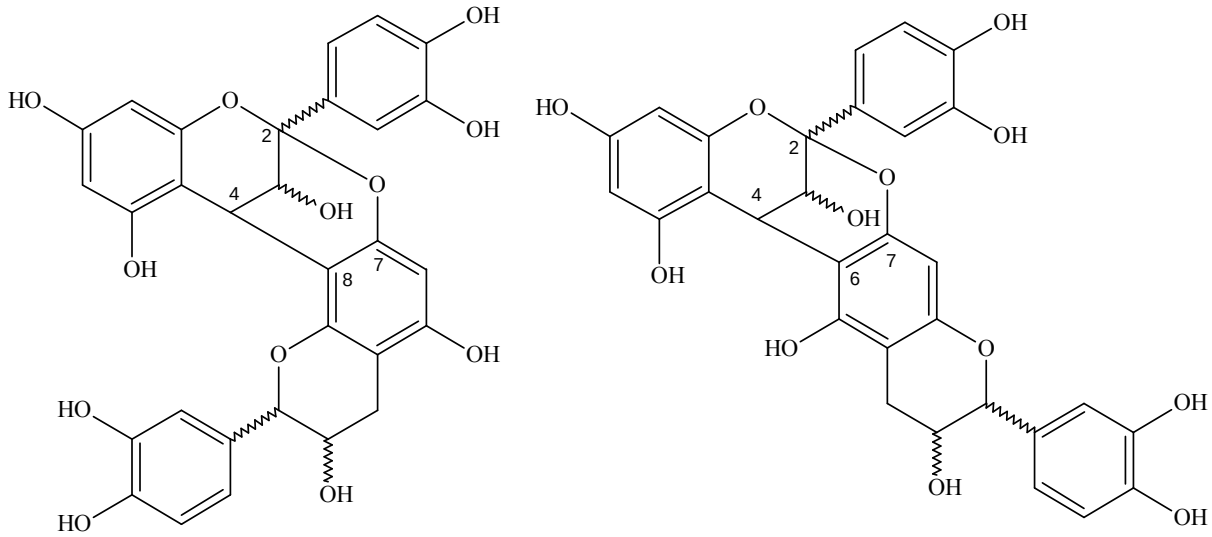

Figure 3. A-type Procyanidin Dimers.

Gu and colleagues were the first to confirm that stereoisomers could give rise to the same [M-H]- ion and dissociation pathway, but separate chromatographically. Four peaks of procyanidin dimers were detected in pinto beans at m/z 575, but they were different stereoisomers that gave rise to the same tandem mass spectra (Gu, Kelm, Hammerstone, Zhang, Beecher, Holden, et al., 2003). Lastly, they provided an alternate theory for why polymeric ions are not frequently seen in ESI-MS^n. Their conclusion was that polymeric procyanidins fragment readily instead of forming multiply charged ions in the negative ESI mode. They did detect some multiply charged species but the constituents were not nearly as numerous as dimers through tetramers. Even today, it is not clearly understood whether ionization efficiency, concentration, or fragmentation of the polymers leads to low amounts being detected. During the same year many of the same authors used their previous knowledge of fragmentation pathways to identify proanthocyanidins

in 88 different kinds of foods (Gu, Kelm, Hammerstone, Beecher, et al., 2003). In addition to using the same normal phase HPLC method to separate proanthocyanidins by degree of polymerization, a reversed phase HPLC method was added as a subsequent step to further purify and identify proanthocyanidins. Thiolytic degradation was performed to identify catechin and epicatechin in B-type linkages. Thiolytic degradation does not break apart A-type linkages, so these linkages could be distinguished, but the stereoisomers could not be determined. Thiolytic degradation was performed, and then the result of the degradation was analyzed by reversed phase LC-MS^n. Since the degradation will result in monomer units, and catechin and epicatechin separate on a reversed phase column, the monomeric units of the proanthocyanidins could be deduced. Of the most significance, was the detection of A-type, B-type, and (epi)catechin glycosides in peanuts. Unlike malt, peanuts were found to contain epicatechin and catechin units according to thiolytic degradation. In this study, peanuts were found to have oligomers no larger than pentamers. It is unclear from the paper whether the nut and skin were part of the material extracted, or only the nut.

B-type procyanidins characterized from grape seeds using ESI-MS were found to contain triply charged species (Hayasaka, Waters, Cheynier, Herderich, & Vidal, 2003). Singly charged species were found up to a degree of polymerization (DP) of 9. Triply charged species (0.3 amu difference between isotopic peaks) represented molecular masses up to a degree of polymerization of 28. Gallo(epi)catechin and gallates of B-types were also found. A study by Passos and co-workers (2007) found grape seeds to contain nongallolyated, monogalloylated, and digalloylated A-type and B-type oligomers using reversed phase LC-ESI-MS-MS run in positive mode. Thiolysis was also performed to validate results. Type-A abundance was 60-80 %, while B-type abundance was 20-40 %. Very little if any A-types have been detected in

grapes. Perhaps grape variety or oxidation played a role in the conversion of B-type to A-type (Kondo, Kurihara, Fukuhara, Tanaka, Suzuki, Miyata, et al., 2000). The study by Passos and co-workers was the first report of A-type galloylated procyanidins.

It has been reported for B-type procyanidins to survive oral administration of grape seed extract to be detected in plasma by LC-MS (Prasain, Peng, Dai, Moore, Arabshahi, Wilson, et al., 2009). In a study of procyanidin dimers metabolized by human microbiota, it was found that the major metabolites were 2-(3,4-dihydroxyphenyl)acetic acid and 5-(3,4-dihydroxyphenyl)-γ-valerolactone (Appeldoorn, Vincken, Aura, Hollman, & Gruppen, 2009). This means that these molecules are stable enough to survive the human stomach, but are degraded in the colon.

Only B-type procyanidins have been found in pine bark (Karonen, Loponen, Ossipov, & Pihlaja, 2004). Proanthocyanidins and prodelphinidins were not detected either. This means that the Resvit® isolated from peanut skins, and that of pine bark likely are different. Doubly charged and a small amount of triply charged ions were found in pine bark. Procyanidins with degree of polymerization up to 10 could be determined. Consistent with studies in grapes, B-type procyanidins, anthocyanidins, vinly-linked anthocyanin flavanols, ethyl-bridged antocyanin-flavanol, and flavanol-anthocyanin compounds were detected in raw red wine using positive mode LC-UV-ESI-MS (Pati, Losito, Gambacorta, Notte, Palmisano, & Zambonin, 2006). Many glycosylated species were also detected. This is not unexpected since the wine did not go through an extraction procedure. A relatively simple distribution of B-type procyanidins were found in Saskatoon Berries by ESI-MS^n, NMR, and thiolytic degradation (Hellström, Sinkkonen, Karonen, & Mattila, 2007). The B-types linkage (C4→C8) and anthocyanidin glycoside were tentatively identified by ^1H and ^{13}C NMR. Fractionation was conducted by normal phase chromatography which led to well resolved peaks. The simple distribution probably aided the

ability of normal phase chromatography to separate the peaks by degree of polymerization before structural analysis.

Almond skins have been identified to contain A and B-type procyanidins and propelargonidins up to heptamers, and A and B-type prodelphinidins up to hexamers (Monagas, Garrido, Lebrón-Aguilar, Bartolome, & Gómez-Cordovés, 2007). Isorhamnetin-3-O-rutinoside, isorhamnetin-3-O-glucoside, and isorhamnetin-3-O-galactoside (elutes before glucoside) were also found in almond skins. Not surprisingly, the compounds found in almond skins were similar to those reported in peanut skins.

Mass Spectrometry Used to Identify Components in Peanut Skins

Lazarus and others (1999) were the first to identify procyanidins in peanut skin using ESI-MS. They analyzed both peanut nutmeat and peanut skins, finding only procyanidins in peanut skins. A-type and B-type monomers through octamers were identified, with doubly and triply charged ions being detected. Oligomers containing more than one A-type linkage were also observed. Their mass spectral data indicated that the A-type procyanidins predominated over B-type procyanidins in contrast to those reported by Karchesy & Hemingway (1986) using FAB-MS. A-type hexamers were ~ 5 times more abundant than ions corresponding to B-type hexamers. Lazarus and others proposed that the differences may be attributed to the isolation methods employed or result from inherent differences in the peanut genotypes used in the respective studies. No galloyated procyanidins were detected in peanut skin samples, but were detected in grape seeds, and tea, but not red wine. Galloylated procyanidins tended to elute late on the normal-phase HPLC trace (Lazarus, Adamson, Hammerstone, & Schmitz, 1999). Almond skins were also analyzed in this study, but were only found to contain B-type procyanidins, in disparity to Monagas and others (2007). In a follow-up study Monagas and

others (2009) studied the flavan-3-ol profile of peanut, hazelnut, and almond skins finding almond skins to contain both A and B-type proanthocyanidins but the B-types were found to predominate. This study also reported propelargonidins. Weak signals corresponding to B-types and A-types with one galloylated unit (up to DP 5) or one (epi)gallocatechin unit (up to DP 8) and containing up to two A-type linkages were also detected (Monagas, et al., 2009).

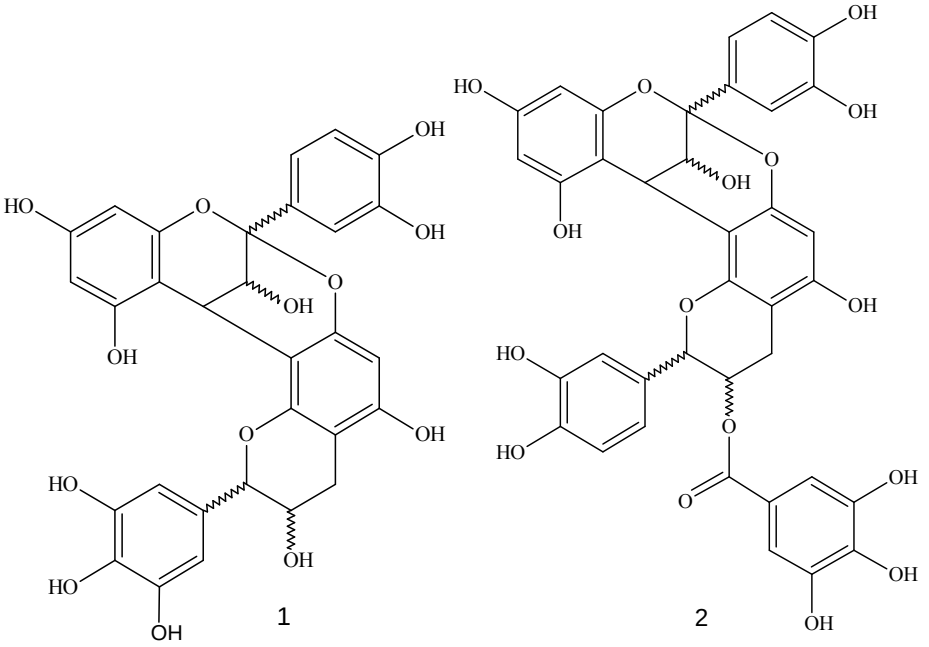

Figure 4. Difference between (epi)gallocatechin (1) and (epi)catechin gallate (2) A-type dimers.

Peanut skins were low in monomeric flavan-3-ols (19 %) in comparison to hazelnut (90 %) and almond (89 %) (Monagas, et al., 2009). Antioxidant capacity determined by ORAC and DPPH was higher for whole extracts from roasted hazelnut and peanut skins than for almond skins; however the antioxidant capacities of the high molecular weight fraction (by Sephadex LH-20) of the three types of nut skins were equivalent despite their different compositions and their degrees of polymerization (Monagas, et al., 2009). In this study, skins were extracted with 80 % acetone in water and separated into a low molecular weight and high molecular weight fraction using Sephadex LH-20 before being subjected to reversed phase LC-ESI-MS. Apparently, CID

was performed, but not reported. The lack of extensive separation before reversed phase LC-ESI-MS enhances the possibility for co-elution and adds to the likelihood that these identifications are likely very tentative.

A recent study isolated procyanidin A-type dimers from peanut skins, and B-type dimers from grape seeds using an efficient isolation procedure (Appeldoorn, Sanders, Vincken, Cheynier, Le Guernevé, Hollman, et al., 2009). The procedure involved defatted peanut skins which were extracted with 20 % methanol in water followed by solvent partitioning with ethyl acetate and concentrated via a rotary evaporator. Fractionation of the extract was first performed via normal phase HPLC and then reversed phase HPLC. Preparative and analytical HPLC were compared. Multiple spectroscopic methods were used: ESI-MS, MALDI-TOF-MS, and NMR. MS was used to determine parent ions, and MS/MS fragements, NMR was used to determine linkage, and stereochemistry. Phloroglucinolysis and then subsequent analysis by reversed phase HPLC-UV was conducted to determine the monomeric units as validation procedure for identification of the B-type procyanidins. Dimers isolated from peanut skins were identified as: epicatechin-(2-O-7, 4-8)-catechin (procyanidin A1), epicatechin-(2-O-7, 4-8)-epicatechin (procyanidin A2), epicatechin-(2-O-7, 4-6)-catechin, epicatechin-(2-O-7, 4-8)-*ent*catechin, isolated from peanut skins for the first time, and epicatechin-(4-6)-catechin (procyanidin B7) (Appeldoorn, et al., 2009). The numbers given above refer to linkages at the carbon numbers of the monomeric units. One peak contained in the reversed phase chromatogram of the peanut skin extract could not be identified due to low purity. Numerous overlaps and poor signal intensities were found in the NMR spectrum. The results were consistent with that of Lou and co-workers (1999) in that all A-type dimers always contained epicatechin as upper units. Procyanidin A1 was the main component of the dimer fraction consisting of ~ 70 % of the

fraction; A2 consisted at ~ 21 % of the fraction (w/w). The other dimers identified were very minor in comparison. This same method was applied to characterize 83 different proanthocyanidin molecular species in a subsequent study involving only peanut skins (Appeldoorn, Vincken, Sanders, Hollman, & Gruppen, 2009). After ethyl acetate partitioning the approximate proanthocyanidin yield was ~ 4 %. Ethyl acetate partitioning was found to remove 82-86 % of dry material, including 34-49 % glucose equivalents. A-type procyanidin oligomers were predominant and represented 95 % of the whole extract. The position of the A-linkages in 16 trimers, and 27 tetramers could be determined by CID dissociation pathway, which in this case appeared to occur at all possible positions (Appeldoorn, Vincken, Sanders, Hollman, & Gruppen, 2009). Normal phase fraction 1 (F1) exclusively contained catechin and epicatechin in a molar ratio of 5:1 (Appeldoorn, Vincken, Sanders, Hollman, & Gruppen, 2009), which is close to the ratio of 9:1 reported earlier (Karchesy & Hemingway, 1986). Normal phase fractionation by degree of polymerization was consistent up to F3 (dimers). After this, F4 contained mostly trimers with smaller amounts of dimers. F5 contained A-type trimers, F6-F7 contained A-type trimers and B-type trimers, consistent with principle that A-type dimers are less polar than the B-type dimers due to the additional bond (Appeldoorn, et al., 2009). Thus B-types will elute later than A-types on normal phase, and the opposite on reversed phase chromatography. F6-F7 is where significant overlap of proanthocyanidins of different degrees of polymerization started to occur, because F6 and F7 also contained tetramers. Fractions eluting further than tetramers are unresolved by normal phase probably due to the large diversity of structures as the degree of polymerization increases (Rigaud, Escribano-Bailon, Prieur, Souquet, & Cheynier, 1993). At this point, identification using normal phase ESI-MS^n alone became difficult and reversed phase ESI-MS^n became essential for identification. Compounds identified

in the fractions after F5 suggest that the molecular diversity is high, thus it seems plausible that this leads to poor peak resolution. Interesting enough, no B-type procyanidins were detected after trimers. The final two fractions F8 and F9 contained pentamers, hexamers, and one heptamer, containing up to three A-linkages.

4. Natural Antimicrobials

The need for inexpensive, natural, antibacterial and antimycotic compounds exists. Consumers are increasingly trying to avoid foods with high levels of chemical preservatives and this is reflected in the food industries' growing interest in finding natural food additives to accomplish the same results (Gould, 1996). The enhanced demand for natural and minimally processed ingredients is can also partly be attributed to stringent regulations governing the use of synthetically derived preservatives (Mandalari, Bisignano, D'Arrigo, Ginestra, Arena, Tomaino, et al., 2010).

Proanthocyanidins as Antimicrobial Agents

A few studies have revealed that proanthocyanidins have antimycotic action. Pine bark extract containing polymeric flavanols (proanthocyanidins) and tannic acid exhibited broad activity towards yeast strains of the genera *Candida, Cryptococcus, Filobasidiella, Issatchenkia, Saccharomyces*: MICs ranged from 200 to 4000 μg/ml of powdered extract (Romani, Ieri, Turchetti, Mulinacci, Vincieri, & Buzzini, 2006). Proanthocyanidins from *Crataegus sinaica* (hawthorn) have even shown antiviral activity (Shahat, Cos, De Bruyne, Apers, Hammouda, Ismail, et al., 2002). O'Keefe and Wang (2006) reported peanut skin extract (PSE) at 0.2 mg/g to have no affect on microbial growth in ground beef or sensory acceptability, but PSE did affectively inhibit lipid oxidation. The PSE was confirmed to have an impact on lipid oxidation,

and also displayed an ability inhibit *Bacillus subtilis, Salmonella typhimurium, Staphylococcus aureus, Streptococcus faecalis, and Escherichia coli* at a level of 0.4 % or higher using a microplate assay to determine the minimum inhibitory concentration (MIC) (Yu, Ahmedna, & Goktepe, 2010). When the PSE was incorporated into raw ground beef and total aerobic bacteria counts were determined, the impact was not as pronounced. This discrepancy likely corresponds with different MIC methods and matrix differences (liquid vs. solid). In addition, different starting concentrations of microorganisms were used, and different levels of PSE extract were applied in each method. Almond skins were also found to be inhibitory towards *S. aureus, Listeria monocytogenes, and S. enterica* in the range of 0.25-0.5 mg/mL using an optical density assay (Mandalari, Bisignano, et al., 2010). No plate count method was performed. Fractionated extracts containing proanthocyanidins and their glycosides from *Ixora coccinea* (Jungle Flame) were found to have antibacterial activity towards the gram positive microorganism *B. subtilis* at 20 mg/mL by means of agar diffusion assays (Idowu, Ogundaini, Salau, Obuotor, Bezabih, & Abegaz, 2010). Volatile oil components and procyanidins of *Cinnamomum burmannii* (Southeast Asia cinnamon stick) were able to inhibit growth of *Bacillus cereus, L. monocytogenes, S. aureus, E. coli,* and *S.* Anatum at MIC levels ranging from 0.39 mg/mL to 2.5 mg/mL (Shan, Cai, Brooks, & Corke, 2007). The scanning electron microscope results were of the most importance. The pictures demonstrate the destructive effects of the crude extract and its fractions on *B. cereus*, *S. aureus* (both gram +) and *S.* Anatum (gram -). The procyanidin fraction had destructive effects on all three organisms, but the most damage was done to *S. aureus*. It was proposed that more destruction was done to the gram positive organisms due to their lack of an extra outer membrane in comparison to gram negative organisms. In addition, phenolic monomers have shown effect on yeast strains. Epigalocatechin gallate (EGCG) was

shown to have inhibitory action towards clinically pathogenic *Candida* species via visual turbidity inspection, although the concentrations of EGCG for antifungal susceptibility were slightly higher than those of commercially available antifungal agents on the whole (Park, Park, Taguchi, Fukushima, Hyon, & Takatori, 2006).

The measurement of optical density has been used to provide accurate measurements of growth of yeasts and antifungal drug test mixtures, the antimicrobial action of synthetic peptides, and the maximum specific growth rates of various organisms (Couto, Barbosa, & Hogg, 2005; Dalgaard, Ross, Kamperman, Neumeyer, & McMeekin, 1994; Enrique, Marcos, Yuste, Martinez, Valles, & Manzanares, 2007; Wesolowski, Hassan, Hodde, Bardroff, & Bilitewski, 2008). This method has also been used to screen a grape seed extract for its applicability as a produce wash (Bisha, Weinsetel, Brehm-Stecher, & Mendonca, 2010) and to model the growth rate of *L. monocytogenes* using absorbance measurements and plate counts (Valero, Pérez-Rodríguez, Carrasco, García-Gimeno, & Zurera, 2006). However, with this kind of relationship it always appears that there is a need for a correction factor to make both sets of data fit (Francois, Devlieghere, Standaert, Geeraerd, Cools, Van Impe, et al., 2005). It often seems that these methods result in an overestimation in the log reductions with respect to plate counts (Lambert, 1998). Lambert (1998) hypothesized that the differences between the methods were due to the level of cell injury, which is masked by the optical density methods, but recognized by the plate counts. Since these methods are often used when a stressor is applied to the microorganisms, another viable explanation may be an observed decrease in colony size and thus optical density, as was observed for *Saccharomyces cerevesiae* when exposed to essential oils (Conner & Beuchat, 1984). However, the deviation seems to be low when two optical density measurements were compared (2-fold dilution, and relative rate to detection methods) (Biesta-

Peters, Reij, Joosten, Gorris, & Zwietering, 2010). Of interest to a Food Scientist is the applicability of optical density methods to food spoilage organisms such as yeasts and molds. Three synthetic peptides showed fungicidal properties towards *Zygosaccharomyces ballii, Zygosaccharomyces bisporus,* and *S. cerevisiae* in a laboratory growth medium and white wine using optical density and a plate count method for growth determination (Enrique, Marcos, Yuste, Martinez, Valles, & Manzanares, 2007). The inhibitory effect was much better in the laboratory growth medium than in wine, suggesting the importance of evaluating antimicrobial activities in the food matrix to be considered for practical application. Results were as expected for the effect of humectants and potassium sorbate on the growth rate of *Z. ballii* in a model aqueous system resembling low sugar products (Gliemmo, Campos, & Gerschenson, 2006). As the a_w level decreased and the level of xylitol in particular increased, yeast growth also increased. These methods seem to work fine to compare treatment to control effects; however it is critical to realize that the method may be overestimating treatment effects due to it measuring a visual property loosely related to cell growth.

Little research exists on the ability of proanthocyanidins to inhibit molds. What research does exists, indicates that proanthocyanidins may not be effective mold inhibitors. When *Aspergillus niger* and *Aspergillus flaveous* was subjected to 10-20 mg/mL of tannic acid or ~ 0.05 mg/mL of tannin containing extract from sorghum, complete growth was observed (Sulieman, Issa, & Elkhalifa, 2007). However, both tannic acid and the tannin extract did show inhibition towards *S. cerevisiae* in this same study. Antifungal activity seems to be better for essential oils than for proanthocyanidins. Essential oils of thyme were found to inhibit all growth of *Aspergillus flavus* at a concentration of 0.35 mg/mL (Omidbeygi, Barzegar, Hamidi, & Naghdibadi, 2007).

Antimicrobial Ability of Essential Oils

Essential oils of Rosemary and Ajowan Caraway (carom seeds) were found to be inhibiting towards the growth of *Aspergillus parasiticus* with the minimum fungicidal concentrations (MFC) occurring in the range of 3000 μg/ml (Rasooli, Fakoor, Yadegarinia, Gachkar, Allameh, & Rezaei, 2008). Extracts from bergamot (*Citrus bergamia* Risso) peel showed no effect on *S. cerevisiae* using the Bioscreen C OD_{600} method (Mandalari, Bennett, Bisignano, Trombetta, Saija, Faulds, et al., 2007). However, eriodictyol (similar in structure to luteolin) was found to be inhibitory to *S. cerevisiae* at a concentration of 800 μg/mL. Much of the antimicrobial activity was found to be synergistic through interpretation of isobolograms. Crude and fractionated essential oils from dill, coriander, and eucalyptus were found to be inhibitory towards *S. cerevisiae* (Delaquis, Stanich, Girard, & Mazza, 2002). In particular cilantro oil was found to be very inhibitory towards Gram positive bacteria and *S. cerevisiae*. Fractions that were rich in carvone and D-limonene seemed to be the most inhibitory. Tea tree oil, garlic oil, and methyl jasmonate have been used to suppress microbial proliferation for fresh cut tomatoes (Ayala-Zavala, Oms-Oliu, Odriozola-Serrano, González-Aguilar, Álvarez-Parrilla, & Martín-Belloso, 2007). Of the three natural antimicrobials, methyl jasmonate performed the best for this particular application.

Modes of Antimicrobial Action

There are abundant numbers of explanations for the reasons why proanthocyanidins display antimicrobial activity. The ability of tannins to complex proteins is well known (Chung, Wong, Wei, Huang, & Lin, 1998; Francisco & Resurreccion, 2008; Hagerman & Butler, 1981). For this reason one of the proposed modes of actions of tannins is to complex with proteins through so-called nonspecific forces such as hydrogen bonding and hydrophobic effects, as well

as by covalent bond formation to cause inhibition of *S. cerevisiae* and more generally yeast inhibition (Cowan, 1999). A more simplistic explanation is the lack of sufficient available amino nitrogen to support growth (Mullins & NeSmith, 1987). However the next year, in a subsequent experiment the same authors found that yeast cell death was due to nitrogen starvation (Mullins & NeSmith, 1988). If the yeast cells were supplemented with a nitrogen source, either organic or inorganic, they would survive as well as control conditions. Another explanation for yeast inhibition due to tannins is iron deprivation (Wauters, Iserentant, & Verachtert, 2001). It was found in this study that growth medium supplementation with more nitrogen or metal ions showed that only iron ions could restore the maximal growth rate of *S. cerevisiae*. Tannins are known transition metal chelators (Leopoldini, Russo, Chiodo, & Toscano, 2006; Malesev & Kuntic, 2007; Rice-Evans, Miller, & Paganga, 1997).

Tiwari and others (2009) list phenolic compounds as affecting enzyme activity, protein denaturation, cell permeability, interference with membrane function, and interaction with membrane proteins as mechanisms of antimicrobial action. Specifically, interference with membrane function can include hindrance with electron transport, nutrient uptake, protein, nucleic acid synthesis, and enzyme activity. Shan and co-workers (2007) observed extensive cell damage in pathogenic bacteria treated with proanthocyanidins. They believed the active components of the extract may bind to the cell surface and then penetrate to the target sites, possibly the phospholipid bilayer of the cytoplasmic membrane and membrane bound enzymes. The effects could lead to inhibition of proton motive force, inhibition of the respiratory chain and electron transfer, and inhibition of substrate oxidation. This could result in the uncoupling of oxidative phosphorylation, inhibition of active transport, loss of pool metabolites, and disruption of synthesis of DNA, RNA, protein, lipid, and polysaccharides leading to cell injury or death.

Through computational analysis it has been ascertained that in procyanidin B-type dimers, the two (epi)catechin monomers can interact with each other, leading to a folded and packed molecule (Mackenzie, Carrasquedo, Delfino, Keen, Fraga, & Oteiza, 2004). In contrast, the rigidity conferred by A-type dimers due to two bonds between monomers results in a more extended conformation. Thus the A-type dimers may be able to penetrate further into a bilayer surface. This property was confirmed when A-type dimers (isolated from peanut skins) and longer procyanidins were determined to have more phospholipid interaction over B-type dimers (Verstraeten, Hammerstone, Keen, Fraga, & Oteiza, 2005). This ability means that these compounds can interact with lipid membranes and thus can modulate membrane fluidity. The alteration of membrane fluidity can affect numerous cellular processes influencing functionality of membrane-associated enzymes, intracellular transport mechanisms, and membrane receptors (Verstraeten, Hammerstone, Keen, Fraga, & Oteiza, 2005). The presence of a rigid cell is a necessity for yeast survival. A strategy was developed to inhibit *S. cerevisiae* and *Z. bailii* by altering cell wall structure with a combination of antimicrobial peptide and β-1,6-glucan oligosaccharides that would lead to an impaired construction of a normal glycoslyphosphatidylinositol dependent cell wall (Bom, Klis, de Nobel, & Brul, 2001). The resulting cell walls were then more susceptible to the action of membrane-active agents, which resulted in total inhibition or slowed growth dependent on concentration and agent. Therefore it is logical that yeasts would be susceptible to decreased growth due to cell wall disruption.

Antimicrobial activity of stilbenoids seems to be related to the existence of an olefinic double bond (Schultz, Boldin, Fisher, Nicholas, McMurtrey, & Pobanz, 1992; Schultz, Cheng, Boldin, Hubbard Jr, Jin, Fisher, et al., 1991). In particular only the silbenoids containing an olefinic double bond (e.g. pinosylvin) have been effective in inhibition of fungi (Kostecki,

Engelmeier, Pacher, Hofer, Vajrodaya, & Greger, 2004). *Z. balii* was inhibited by sorbic acid and anethhol, these chemicals are not stilbenoids, but do contain olefinic double bonds (Fujita & Kubo, 2005). The mechanism is currently not understood, but those stilbenoids containing the olefinic double bond have markedly more activity than those that do not possess the structural property.

5. Aflatoxins in Peanuts

Aflatoxins are natural toxins commonly produced by *Aspergillus flavus, A. parasiticus,* and *A. nomius*. The common toxins produced by these species are B_1, B_2, G_1, and G_2. Their names relate to their fluorescent color (B=blue and G=green). The order of their human toxicity is $B_1>G_1>B_2>G_2$ with B_1 being at least two times more toxic than G_1 (Hussein & Brasel, 2001). In addition these compounds are highly carcinogenic. In the U.S. the level of aflatoxins must be below 15 ng/g (CFR, 2010); in the European Union the level is even stricter, at 2-4 ng/g (Blesa, 2003). Aflatoxin contamination is a problem in a number of foodstuffs including medicinal herbs, peanuts, ginger roots, and corn (D'Ovidio, Trucksess, Weaver, Horn, McIntosh, & Bean, 2006; Fu, Huang, & Min, 2008; Gomez-Catalan, Pique, Falco, Borrego, Rodamilans, & Llobet, 2005; Nakai, Rocha, Goncalez, Fonseca, Ortega, & Correa, 2007). Aflatoxins cost the southeastern U.S. peanut industry approximately $ 25 million annually (Lamb & Sternitzke, 2001). Aflatoxin contamination is most likely to occur at 30 °C due to stress at this temperature, making it more vulnerable to attack by fungi (Scheidegger & Payne, 2005).

Aflatoxin detection can be difficult since the compounds need to be extracted from a complex food matrix. Aflatoxin concentration in the product is not often uniform. This requires that many samples be taken and tested for aflatoxin to ensure the safety of the lot. In one study

the variation due to sampling in powdered ginger was found to be 87 % (Whitaker, Trucksess, Weaver, & Slate, 2009). Aflatoxin are often extracted with chloroform, methanol, ethanol or their aqueous mixtures when applicable (Do & Choi, 2007). Most often a 70 % methanol-water mixture is used. Of concern is that often similar or the same solvents are used to extract proanthocyanidins, therefore it is likely that these extracts could contain aflatoxins. Enzyme-linked immunosorbent assay (ELISA) kits are quite commonly used to detect aflatoxins because of their ease of use (Gilbert & Vargas, 2005). The use of FTIR to detect aflatoxins has been performed, but it has not gained widespread use (Mirghani, Man, Jinap, Baharin, & Bakar, 2001). This instrument may prove to be useful since it is a non-destructive technique, and can be altered into an on-line technique.

Due to the ability of aflatoxins to fluoresce, TLC and HPLC with fluorescence detection are probably the most often employed technique to detect aflatoxins. In addition, LC-MS has been used to detect aflatoxins in the positive mode since it gives increased ionization efficiency over negative mode (Blesa, 2003; Nonaka, Saito, Hanioka, Narimatsu, & Kataoka, 2009). Aflatoxins B_1, B_2, G_1, and G_2 give characteristic $[M+H]^+$ ions at m/z 313, 315, 329, and 331 respectively. The limit of detection LC-ESI-MS for B_1 in peanuts was found to be 0.07 ng/g using a matrix solid-phase dispersion method and 1.35 ng/mL without this aid. Aflatoxins B_1, G_1, and G_2 were detected in peanuts at levels of 0.48, 0.84, and 1.12 ng/g respectively (Nonaka, Saito, Hanioka, Narimatsu, & Kataoka, 2009). This study utilized solid-phase microextraction (SPME) and selected ion monitoring (SIM) to detect aflatoxins at the levels demanded by regulatory agencies for food samples. These studies demonstrate the viability of this method to detect aflatoxins in food samples.

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CHAPTER 3

Separation and Characterization of Proanthocyanidins in Virginia Type Peanut Skins by LC-MS^n

(Manuscript to be submitted to Food Chemistry)

ABSTRACT

Proanthocyanidins were extracted from peanut skins, an agricultural waste product of Virginia. Proanthocyanidin composition of single solvent and multistep extraction procedures of peanut skins were compared by HPLC-UV-vis absorbance. The multistep extraction procedure yielded more procanthocyanidin peaks and larger peak areas. Thus this extraction procedure was chosen for subsequent fractionation. Fractionation was performed by size exclusion (Toyopearl HW-40S) and normal phase (NP) (porous silica) high performance liquid chromatography (HPLC) before reversed phase (RP) electrospray ionization liquid chromatography-mass spectrometry analysis (ESI-LC-MS^n). Proanthocyanidin separation on the NP column was better overall, compared to SEC, and thus was chosen for characterization by MS. Peanut skin procyanidins were found to separate in order of increasing molecular weight on the NP column. The ESI-MS generated positive and negative molecular-type ions and their dissociation product ions were used to identify specific proanthocyanidins and procyanidins. New proanthocyanidin dimeric and trimeric species were identified. These species consisted of one or two (epi)catechin units bound to luteolin or kaempferol. Monomers through nonamers of flavan-3-ols were identified by retention time and their multistep (MS^n) dissociation pathway. Hexamers were identified as both singly and doubly charged negative ions, $[M-zH]^{z-}$, z=1 or 2. Species of greater than pentamers were tentatively identified due to their low ionization efficiency and significant overlap of species both chromatographically and in the MS spectra.

Keywords: Electrospray mass spectrometry, liquid chromatography, proanthocyanidin, peanut skin

1. Introduction

Virginia type peanuts (*Arachis hypogaea*) have become increasingly more popular for consumption in whole form since they are larger than the other prevalent types of peanuts such as Runner and Spanish types. While the nut of the peanut is a prized commodity, the skin is a low-value byproduct. Skins have been used for animal feed or burned for energy. One of their drawbacks for use as an animal feed is their high amount of tannins (also known as proanthocyanidins and procyanidins). Tannins precipitate proteins which can reduce the nutritional value by 30% or more for various animals (Mullins & NeSmith, 1988). Resent research has shown the waste products to be an excellent source of natural phytochemicals such as catechins, procyanidin dimers, and oligomers (Appeldoorn, Sanders, Vincken, Cheynier, Le Guernevé, Hollman, et al., 2009; Gu, Kelm, Hammerstone, Beecher, Holden, Haytowitz, et al., 2003; Monagas, Garrido, Lebron-Aguilar, Gomez-Cordoves, Rybarczyk, Amarowicz, et al., 2009).

Proanthocyanidins found in peanut skins can provide potential benefits by acting as antioxidants, a natural antimicrobial in laboratory media, and vasodilators (Fitzpatrick, Fleming, Bing, Maggi, & O'Malley, 2000; Nepote, Grosso, & Guzman, 2005; Yu, Ahmedna, & Goktepe, 2010). Included under proanthocyanidins is a subclass called procyanidins. Procyanidins consist exclusively of catechin and epicatechin units ranging from dimers to polymers. In this study the catechin or epicatechin (stereoisomers) form could not be distinguished for procyanidin or proanthocyanidin oligomers thus the notation (epi)catechin is given. Proanthocyanidins that contain (epi)afzelechin or (epi)gallocatechin as subunits are called propelargonidins or prodelphinidins, respectively (Fig. 1). In addition there are flavones which consist of a double bonded oxygen (carbonyl) at position 4 of the C ring. Proanthocyanidins, flavones, and

flavanols all fall under the much broader group known as flavonoids. The nomenclature of these molecules presented in Fig. 1 is based on the system presented by Francisco and Resurreccion (2008). In addition, the linkages that make up oligomers through polymers of these subunits are very important. Fig. 2 explains these linkages.

Extraction of the flavonoids in peanut skins is accomplished through the use of solvent extracts. Flavonoids have been extracted from peanut skins by hot water extraction, ethanol, methanol, ethyl acetate, and aqueous mixtures thereof (Lou, Yamazaki, Sasaki, Uchida, Tanaka, & Oka, 1999; Nepote, Grosso, & Guzman, 2000; Nepote, Grosso, & Guzman, 2005; Yu, Ahmedna, & Goktepe, 2005). Nepote and co-workers (2002) compared the extraction percentages of methanol, ethanol, acetone, and water (all at ambient temperature) during 24 hrs by maceration. Methanol, ethanol, and acetone extracts showed the highest phenolic contents in the order listed, but extracts showed no statistical significant difference in extraction percentage by dry weight of matter (Nepote, Grosso, & Guzman, 2002). Water extracted the lowest amount of dry matter, but had similar phenolic content values as acetone. In the follow-up study, Nepote and co-workers (2005) found 70% ethanol, non-crushed peanut skins, ratio of solvent/solid of 20 ml/g, at 10 minutes of shaking and three extractions to be the best conditions. The ethanol extracts of peanut skins had the highest total phenolic contents (TPC), but methanol extracts had the highest oxygen radical absorbance capacity (ORAC) values (Ballard, Mallikarjunan, Zhou, & O'Keefe, 2009). Subsequently, microwave-assisted extraction was found to slightly improve the extraction efficiencies for phenolics from peanut skins (Ballard, Mallikarjunan, Zhou, & O'Keefe, 2010). The extraction of flavonoids has been found to depend on the dissolution of each flavonoid at the cellular level in the plant material matrix, and their diffusion in the external solvent medium (Shi, Nawaz, Pohorly, Mittal, Kakuda, & Jiang, 2005). Another difficulty

complicating the situation of determining phenolic contents of extracts using assay is that the assays are prone to solvent effects (Prior, Wu, & Schaich, 2005).

Chromatography is necessary to determine the flavonoids present in a complex mixture, such as food. The first studies to separate proanthocyanidins in peanut skins used open column techniques. This is evidenced by the use of Sephadex LH-20 columns, and Toyopearl HW-40 (Karchesy & Hemingway, 1986; Lou, Yamazaki, Sasaki, Uchida, Tanaka, & Oka, 1999). After fractionation Karchesey used fast atom bombardment (FAB) MS and nuclear magnetic resonance (NMR) spectroscopy to identify five compounds in peanut skins, mainly monomers and dimers. Lou relied on an additional step, (RP)-HPLC followed by NMR. The extracts were pure enough to structurally identify monomers and dimers, with the carbon linkages and stereochemistry differentiated which is possible via NMR but not MS. Fractionation before (RP)-HPLC analysis is needed to separate structurally similar flavonoids because without this step co-elution becomes common (Pati, Losito, Gambacorta, Notte, Palmisano, & Zambonin, 2006; Yu, Ahmedna, Goktepe, & Dai, 2006). This is especially true when higher degree oligomers are observed (Guyot, Doco, Souquet, Moutounet, & Drilleau, 1997). In this case NMR is ineffective for structural determination.

Separation by degree of polymerization for complex mixtures of flavonoids can be accomplished by normal phase HPLC. Lazarus and co-workers (1999) first used normal phase LC-MS to separate peanut skin procyanidins by degree of polymerization and identify dimers through octamers. Since then it has been combined with RP-LC (or ultra performance (UPLC)) to determine procyanidins and proanthocyanidins up to a degree of polymerization of seven (Appeldoorn, Vincken, Sanders, Hollman, & Gruppen, 2009). However, the separation and

retention mechanisms of oligomeric proanthocyanidins in this mode have not been sufficiently elucidated (Yanagida, Murao, Ohnishi-Kameyama, Yamakawa, Shoji, Tagashira, et al., 2007).

2. Materials and methods

2.1 Samples and Standards

Peanut skins (Virginia variety) were obtained from Tidewater Blanching (Suffolk, VA) and were frozen at -20 °C upon receipt. (+)-Catechin and (-)-epicatechin standards were purchased from Sigma (St. Louis, MO). Acetone, chloroform, dichlormethane, ethanol, and methanol were HPLC-grade quality obtained from Fisher Scientific (Pittsburgh, PA). Acetic acid, ethyl acetate, sodium chloride, and sodium sulfate were also purchased from Fisher Scientific (Pittsburgh, PA). Water was purified using a Millipore Elix® 5 (Molsheim, France).

2.2 Single Solvent Extraction

Peanut skins were extracted by placing 1 g of skins in 20 mL of acetone, ethanol, methanol, or boiling water (99.7 °C) for 15 min using a Fisher Scientific Ultrasonic Model FS20 (Pittsburgh, PA). The solvent extracts were then concentrated to approximately 1/5 the original volume by evaporating the solvent under nitrogen gas. The concentrated extracts were then 0.45 μm filtered using Whatman PTFE Puradisc™ syringe filters (Whatman Inc., Piscataway, NJ) and injected onto the normal phase column (Fig. 3).

2.3 Multistep Peanut Skin Extraction (PSE) Procedure

The method used for extraction of proanthocyanidins was similar to a previously developed process (Masquelier, 1987). Peanut skins (100 g) were extracted with boiling water (99.7 °C) at a ratio of 1 g to 10 mL. Filtration of the liquid was then performed to remove any remaining solid particles. Sodium chloride was added to the point of saturation resulting in the

formation of precipitants. Subsequent filtrations were performed to remove precipitants. Next, the phenolic compounds were extracted in a separatory funnel using ethyl acetate and dried with sodium sulfate. The ethyl acetate extract was then concentrated via a rotary evaporator. Finally, the procyanidins were precipitated with chloroform and filtered. The procyanidin precipitate was powdery, and tan in color. Yields were approximately 1-1.5 g of powder.

2.4 Toyopearl HW-40S Size Exclusion HPLC

Toypearl resin (Tosoh Bioscience) was packed manually into a (30 cm x 8 mm, 20-40 μm) stainless steel column. Separation was run isocratically at a flow rate of 0.5 mL/min using methanol as the elution solvent for 200 min. A low flow rate was needed to not exceed 3 bar of pressure, which could cause deformation of the particles (Tosoh, 2003).

2.5 Normal Phase (NP) HPLC

An Agilent (Palo Alto, CA) 1000 series HPLC separated single solvent extracts or PSE on a Regis Technologies (Morton Grove, IL) GFF II (25 cm x 4.6 mm i.d., 5 μm, 80 Å) porous silica column. This column fits into the category of internal surface reversed phase. The gradient method consisted of solvent A (dichloromethane), solvent B (methanol), and solvent C (water/acetic acid, 1:1, v/v). A flow rate of 1.0 mL/min was applied as follows: 14-28.4% B from 0 to 30 min, 28.4-50% B from 30 to 60 min, 50-96% B from 60 to 65 min, held at 96% B from 65-75 min, followed by column equilibration from 75-100 min. Solvent C was held at 1% C for the entire run. The detection conditions were 210-360 nm for the diode array detector (DAD). The specific wavelengths of 254 nm, 270 nm, 280 nm, 310 nm, and 350 nm were used for detection. The signal at 280 nm responded the best for detection of procyanidins. Fraction collection was performed manually. The fractions were evaporated to dryness and then reconstituted for (RP)-LC-MS analysis.

2.6 Reversed Phase (RP) LC/Electrospray Ionization (ESI) Mass Spectrometry Analysis

The ESI-HPLC-UV-MS system consisted of an Agilent (Palo Alto, CA) 1100 series HPLC coupled to a ThermoFinnigan (San Jose, CA) LCQ quadrupole ion trap mass spectrometer with electrospray ionization (ESI). HPLC separations were accomplished using a Waters Atlantis dC18 (2.1 x 150 mm, 3µ) with guard column. A gradient method consisting of solvent A (5.0 mM ammonium acetate in water to aid ionization) and solvent B (methanol) was applied at a flow rate of 0.15 mL/min as follows: 0-40% from 0 to 20 min, 40-41% from 20 to 25 min, held at 41% B from 25 to 35 min, 41-45% B from 35 to 51 min, 45-95% B from 51 to 68 minutes, and held at 95% B from 68-80 min, followed by column equilibration from 80-110 min. MS and UV (280 nm) detection was performed from 0-80 min. UV detection of procyanidins at 280 nm corresponded well with MS detection (Fig. 4).

HPLC-ESI-MS data were acquired under negative mode using Xcalibur (Thermo Fisher Scientific, Waltham, MA) and analyzed with the same software. The mass spectrometer was operated in the normal mass range (NRMS m/z 50-2000) and the high mass range (HRMS m/z 50-4000) for samples that were found to contain multiply charged species. However, mass accuracy and resolution are poorer for the HRMS compared to the NRMS. The isolation window was increased for the higher m/z ions (NRMS) due to wider isotope distribution and the thought that the high MW oligomers might be more susceptible to CID during the isolation event. Tandem mass spectrometry (MS^n) collision-induced dissociation (CID) product spectra were obtained for data-dependent MS^n scans with 37.5% maximum CID energy, q-CID of 0.25 and CID time of 30 ms. For m/z-specific product scans, the isolation of the precursor ion was performed with a variety of isolation widths depending upon the ion of interest and potential interferants, typically from 1.5 to 3.0 u but otherwise with the same CID parameters above. For

ions resistant to CID at the lower energies above, 42.5% maximum CID energy and q-CID of 0.3 were used. Zoom MS (zMS) for mass ranges of interest was done to determine multiply-charged species, and isotope patterns. The optimized electrospray/ion optics parameters were as follows: spray voltage, 3.2 kV; sheath gas (nitrogen), capillary voltage, -12 V; capillary temperature, 250 °C; tube lens offset voltage, 0 V. At least one blank run consisting of 1:1 water:methanol was run between different samples to monitor sample carryover.

3. Results and discussion

3.1 Peanut Skin Extraction

Two different extraction methods were evaluated. The first method involved production of single solvent extracts from peanut skins. The extracts were then analyzed via normal phase HPLC/UV. The chromatograms were evaluated for the number of resolved peaks and their peak areas according to UV 280 nm absorbance. The NP peak areas of the unknowns and catechin were compared to determine the relative amount of the compound present. Since catechin and epicatechin have similar molar extinction coefficients (280 nm), and the extracts contained oligomers and polymers of these basic units, it was assumed that the same molar extinction coefficients at 280 nm could be used to estimate proanthocyanidin concentrations. A similar procedure was also employed recently (Appeldoorn, Vincken, Sanders, Hollman, & Gruppen, 2009). In addition Luthria and others (2006) found UV area data to correlate well with determination of total phenolics by the Folin-Ciocalteu assay. Mixtures of water and organic solvents were also investigated, but this did not significantly increase extraction efficiency (data not shown). Sonication was performed because it has been found to aid in the extraction by resulting in greater penetration of solvent into cellular materials; improving mass transfer to and

from interfaces; and facilitating the release of contents by disrupting the biological cell wall on the surface and within raw materials (Mason, 1998).

The type of solvent used to extract the procyanidins from the peanut skins did not seem to have much of an effect on the type of compounds extracted, and their concentration (Fig. 3). This is not uncommon, for example, one solvent or mixture may yield a high amount of dry matter, but have relatively low activity, while a solvent yielding a low amount of dry matter may have high activity (Ballard, Mallikarjunan, Zhou, & O'Keefe, 2009; Yu, Ahmedna, Goktepe, & Dai, 2006). In light of these discrepancies and the reality that the solvents tested showed relatively little variation; water was chosen as the major extraction solvent. Water is an inexpensive and non-hazardous solvent. The use of water as the major extraction solvent for the PSE procedure allowed for a major reduction in the amount of chemical waste produced in comparison to a procedure using organic solvents.

In order to effectively concentrate and purify the proanthocyanidins, a multistep solvent extraction procedure was performed. The boiling water extract was subjected to a salting out procedure to remove protein and to a lesser extent fat and sugars. Proanthocyanidins are readily soluble in ethyl acetate. In addition ethyl acetate has a low boiling point which makes it ideal for concentration. The proanthocyanidins were extracted from the concentrated salt solution with ethyl acetate and concentrated by evaporation of the solvent. Chloroform was added to the concentrated ethyl acetate mixture to precipitate proanthocyanidins and remove any remaining fat. Proanthocyanidins precipitate because of their low solubility in chloroform. Solvent partitioning with ethyl acetate has been used previously to purify and concentrate proanthocyanidins (Appeldoorn, Vincken, Sanders, Hollman, & Gruppen, 2009; Yu, Ahmedna,

& Goktepe, 2005). Hexane or chloroform was also in these procedures to remove lipid, however this was the first step in the extraction process instead of the last.

3.2 Normal Phase vs. Toyopearl Separation

Size exclusion chromatography has been used to separate procyanidins by molecular weight (Fitzpatrick, Fleming, Bing, Maggi, & O'Malley, 2000). One would expect the later eluting fractions to contain smaller molecular weight compounds according to the usual SEC mechanism. However, the reverse of the expected was observed. It has been hypothesized that for proanthocyanidins Toyopearl chromatography functions as an adsorption technique instead of SEC (Andersen & Markham, 2006). This would explain why low DP molecules eluted early in the SEC run.

Separation of the extracts by NP had the advantage of being faster, and separating the extract overall better than SEC (Fig. 5). For these reasons, it was chosen as the preparatory step prior to (RP)-LC-ESI-MS. NP chromatography has been used to separate procyanidins in saskatoon berries, blueberries, apples, and almond skins (Hellström, Sinkkonen, Karonen, & Mattila, 2007; Khanal, Howard, Brownmiller, & Prior, 2009; Monagas, Garrido, Lebrón-Aguilar, Bartolome, & Gómez-Cordovés, 2007; Shoji, Masumoto, Moriichi, Akiyama, Kanda, Ohtake, et al., 2006). The mechanism or mode of separation for proanthocyanidins on a normal phase column is not totally understood. By examining the mechanism of separation of proanthocyanidins on a hydrophilic interaction chromatography (HILIC) it was found that hydrogen bonding and solute hydrophobicity, cooperatively contribute to the DP-based separation, but the hydrogen bonding effect predominates in HILIC mode using an aqueous acetonitrile mobile phase (Yanagida, et al., 2007). The authors also discovered that hydrogen bond formation between two different compounds is generally enhanced under hydrophobic

conditions more than aqueous conditions. Thus in this study, the NP separation likely occurs by the very strong hydrogen bonding between the silanol group on silica-beads and the hydroxyl group of solute functioning as the primary retention mechanism. Therefore adsorption is likely the chief mechanism of separation, not hydrophobicity.

The mechanism by which procyanidins separate on a normal phase column is probably not only an adsorption mechanism. Procyanidins are probably displaying a combination of hydrophobicity and size exclusion behavior on a NP column to a lesser extent. The normal phase pore size is probably better equipped to separate procyanidins than larger SEC pore sizes in this situation. The column used in these experiments was a GFF II. This column has some liquid reversed stationary phase inside the pores. The exact type of this stationary phase is not disclosed by Regis Technologies. In this specific case, there might have been some partitioning also occurring and influencing the separation.

3.3 Identification of Compounds Using LC-MS^n

Compounds **1** & **2** have been found to occur in numerous agricultural products, and in peanut skins (Appeldoorn, Vincken, Sanders, Hollman, & Gruppen, 2009; Karchesy & Hemingway, 1986; Monagas, et al., 2009). Identification of (+)-catechin and (-)-epicatechin were performed by comparison with known data on their dissociation pathway and retention time match (Fig. 6). Their identification is highly certain. The rest of the compounds identified are of a tentative nature because their identifications are based upon MS alone. The uncertainty of these identifications mostly refers to stereochemical differences. Thus if the identifications are incorrect, it would most likely be due to the stereochemical position of one or two bonds.

A 316 amu flavonoid **3** with one sugar group attached was also found in fraction A. The $[M-H]^-$ ion was m/z 477. This ion dissociated in CID to m/z 315 (loss of a 162 amu sugar) and

produced characteristic fragments for isorhamnetin. Likely candidates for this identification are rhamnetin, isorhamnetin, tamarixetin, nepetin with one hexose attached. Since these molecules are very similar structurally, the exact identification could not be determined from MS alone. Isohamnetin-3-O-glucoside has previously been identified in almond skins (Monagas, Garrido, Lebrón-Aguilar, Bartolome, & Gómez-Cordovés, 2007). It could not be determined from MS analysis alone whether the sugar group was glucose or galactose since both molecules have the same molecular ion and fragmentation patterns. Had there been both hexose versions in the extract, the identification could have been elucidated since the galactoside has been known to elute before the glucoside on a C18 column (Prior, Lazarus, Cao, Muccitelli, & Hammerstone, 2001). Compound **4** is likely the same 316 amu flavanoid with two sugar units (rutinose) attached. Isorhamnetin-3-O-rutinoside is shown in Fig. 7 as an example of one of the probable candidates.

Fraction B contained dimeric species. Compound **5** has been found in peanut skins previously, and showed characteristic fragments that agree with these studies (Appeldoorn, et al., 2009; Gu, Kelm, Hammerstone, Beecher, et al., 2003). Compound **6** is a novel compound not identified elsewhere, to the best of our knowledge. The fragmentation scheme is given for this molecule in Fig. 8. The major fragmentation routes of proanthocyanidins are: quinone methide fission (QM), retro-Diels-Alder fission (RDA), and heterocyclic ring fission (HRF) as previously explained (Gu, Kelm, Hammerstone, Zhang, Beecher, Holden, et al., 2003). This compound fragments according to RDA and HRF. The MS2 base peak at m/z 447 shows that HRF was the primary dissociation pathway for this molecule. Fragments indicative of a water loss were also seen at m/z 555 and 403, from the m/z 573 $[M-H]^-$ ion and the RDA fragment m/z 421. Instead of fragmenting according to QM, this molecule fragments according to direct cleavage of the

interflavan bond (Friedrich, Eberhardt, & Galensa, 2000). The presence of the carbonyl group on the C-ring hinders QM fission in this instance. The MS3 spectra suggested the compound is likely [(epi)catechin→luteolin] due to fragments that are mostly characteristic of luteolin. However, these fragments were of low intensity and no other data are currently available to prove its existence over kaempferol. With present data it can only be concluded that both are possible.

Compounds **7**-**11** are likely procyanidin A-type dimers. For example, A-type linkages can consist of (C4 →C8, C2→O7) as shown in Fig. 2 or (C4 →C6, C2→O7) linkages. Both linkages show the same fragmentation patterns by mass spectrometry, therefore the best spectroscopic method to determine the linkage is via NMR. These compounds will chromatographically separate due to structural differences (i.e. linkage, or stereochemistry). Similarly, Appeldoorn and co-workers (2009) observed 4 (RP)-LC peaks corresponding to A-type procyanidins, 1 B-type (eluting first), and 1 peak eluting last that could not be identified due to low purity. Based upon their RP-LC/MS and NMR data on peanut skin dimers data in Table 1 suggests: **7** is [epicatechin-(C4 →C8),(C2 →O7)-catechin], **8** is likely [epicatechin-(C4 →C8),(C2 →O7)-catechin enantiomer], **9** is [epicatechin-(C4 →C8),(C2 →O7)-epicatechin], **10** is [epicatechin-(C4 →C6),(C2 →O7)-catechin]. Compound **11** could probably not be determined in the Appeldoorn (2009) study because of low concentration and is most likely another A-type displaying different stereochemistry than those already discussed. The last compound identified in fraction B was **12** which is likely a biflavanone called manniflavanone (Fig. 9). The (-)ESI-MS/MS of the m/z 589 $[M-H]^-$ ion resulted in the m/z 463 product ion due to loss of 126 amu. The m/z 303 product ion corresponded to a quinone methide process. These

fragments agree with the data presented on manniflvanone previously (Ferrari, Terreaux, Kurtán, Szikszai-Kiss, Antus, Msonthi, et al., 2003).

Fractions B and C exclusively contained dimers exclusively, with procyanidin A-types being the main species. More B-types were seen in fraction C vs. B due to their better retention on a normal phase column. Eluting just before the B-types was **13**. The formation of the m/z 439 product ion via loss of 152 amu from the m/z 591 $[M-H]^-$ ion indicated RDA as the primary dissociation mechanism. The m/z 465 product ion from m/z 591 shows HRF was occurring to a lesser extent. Quinone methide ions at m/z 303 and m/z 285 indicate (epi)gallocatechin and (epi)catechin subunits, respectively, through an A-type linkage.

B-type dimers in sum made up approximately 44% of fraction C. The product spectra of the $[M-H]^-$ ions of **14, 15, 17** displayed similar product ions as for **5**. The mechanism of B-type fragmentation has been extensively described elsewhere (Andersen & Markham, 2006). Again, their differences in retention time were likely due to linkage or stereochemistry. A trace of a B-type dimer with a (epi)gallocatechin subunit was also detected **16**. The spectra matched somewhat with that reported previously, but not all of the fragments match (Gu, Kelm, Hammerstone, Beecher, et al., 2003). This was probably due to its low abundance in the fraction and possible co-elution with other substances. Compounds **18, 19, 22-24** eluted after the B-types, constituted about 49% of fraction C, and showed similar MS2 dissociation spectra to the compounds in fraction B. This elution order on RP is expected since A-type dimers are less polar than B-types because of their additional bond (Appeldoorn, et al., 2009).

As further proof for compound **6**, **20** and **21** showed similar MS dissociation spectra. These compounds eluted in NP later than **6** suggesting it may be the epicatechin derivative of **6**. Other explanations for delayed elution include linkage site, or kaempferol as a subunit. MS3

spectra for both retention times were more convoluted than in fraction B, therefore no definitive identification of flavone subunit could be discerned.

Fraction D contained mostly trimers with one A-type linkage. Again, multiple peaks were seen for the same m/z due to differences in monomeric units and the position of the linkages (Table 1 and Fig. 2). Compound **25** is likely a procyanidin trimer with 3 A-type linkages. Another possibility would be luteolin or kaempferol joined to 2 B-type linkages. Either luteolin or kaempferol would need to be the bottom unit, since the molecule is only able to have a C4 linkage. However this molecule would show more QM products, which **25** does not show. Compound **35** displays a less convoluted spectrum, probably due to less co-elution, but is likely the same molecule as **25** displaying linkage or stereochemical differences. The main dissociation pathway is through HRF (Fig. 10). RDA also occurs to a lesser extent forming an m/z 709 fragment. Similar dissociation for HRF and RDA for m/z 861 $[M-H]^-$ has been observed, however the dissociation pathway is not exactly the same probably due to a lower collision energy and differences in the instrumental conditions applied (Appeldoorn, Vincken, Sanders, Hollman, & Gruppen, 2009; Gu, Kelm, Hammerstone, Zhang, et al., 2003). Gu and co-workers (2003) were able to observe QM fragments when they used a collision energy of 120% for this molecular ion. The collision energy used in the experiment for this molecule was 37.5%, therefore it is expected that QM fragments may not be observed for this data set due to the inability to break the A-type linkages at the collision energy used for this experiment. Compound **26** matches well with previously reported data (Monagas, Garrido, Lebrón-Aguilar, Bartolome, & Gómez-Cordovés, 2007), and follows the dissociation pathways of B-type dimers discussed previously confirming its identity as a procyanidin dimer with 3 B-type linkages.

The major species by far in this fraction was a procyanidin trimer with 1 A-type linkage (~ 89 %). Compounds **27, 28, 30, 31,** and **34** fell into this category. All of these compounds contained characteristic fragments that placed the A-type linkage on the bottom unit of the trimer (Appeldoorn, Vincken, Sanders, Hollman, & Gruppen, 2009; Gu, Kelm, Hammerstone, Zhang, et al., 2003). Appeldoorn and co-workers using ultra performance liquid chromatography (UPLC) were able to determine 2 compounds that put the A-type linkage on the top unit of the trimer (minor species detected). Perhaps, this version does exist in peanut skins, but our column was not able to distinguish the other linkage, to allow for detection.

Compound **29** almost perfectly matches the dissociation spectra previously published (Gu, Kelm, Hammerstone, Zhang, et al., 2003), thus confirming its identification as [(epi)catechin→A→(epi)afzelechin→B→(epi)catechin]. Compound **32** is likely a trimer with 2 A-type linkages with one (epi)gallocatechin subunit. Some highly tentative fragmentation data suggests the (epi)gallocatechin resides on the top unit of the trimer. However, **32** was in low abundance and did not fragment well because of its multiple A-type linkages. Therefore a higher concentration and higher collision energy will need to be employed for future experiments. Compound **33** is likely an B-type dimer linked to luteolin or kaempferol through an A-type linkage. The major fragment was m/z 569, this would indicate direct cleavage of the B-type interflavan linkage (Fig. 11). It is not currently understood why this pathway would be favored over direct cleavage of the interflavan linkage of the flavone. Low intensity fragments were also seen at m/z 289, 287, and 283 (most intense) indicating cleavage was possible at multiple sites. The major fragment m/z 733 corresponded to HRF indicating a 126 amu loss. The major fragment m/z 707 corresponded to RDA representing a 152 amu loss.

Fraction E contained mostly tetramers, but also contained an intermediate amount of trimers, and a small amount of dimers. Compound **36** was in such a low abundance that enough ions could not be trapped to obtain good fragmentation data. The fragmentation of compounds **37, 41, 45, 48**, **49** and their derivatives have already been discussed previously in this study. It is currently unknown why they elute later than expected.

Compound **38** was the first tetrameric species detected in Fraction E. The fragmentation data matched almost perfectly with Appledoorn (2009) data on m/z 1151 in peanut skins. The characteristic fragments identified the molecule as a tetramer with one A-type linkage at the bottom (last linkage) unit. Compound **46** exhibited slight differences in fragmentation data, but the data corresponded best with the same identification as **38**. Again, stereochemistry probably led to the difference in retention time. Compound **47** exhibited similarities with **38** and **46**. However **47** had two different fragments (m/z 861 and 573) that correspond to the A-linkage being at the top (first linkage) unit.

The m/z 863 trimers in fraction E showed similarity with those discussed previously. However, the fragments at m/z 573 and 451 indicated that the A-type linkage was at the top unit for **39,** and **40** rather than the bottom unit as in **43**. The data describing the site of the linkages is in agreement with that of previous reports (Appeldoorn, Vincken, Sanders, Hollman, & Gruppen, 2009; Gu, Kelm, Hammerstone, Zhang, et al., 2003). Compounds **42** and **44** are tentatively identified as [(epi)catechin→A→(epi)catechin→A→(epi)catechin→B→(epi)catechin] only because the m/z 859 fragment was slightly more prominent than m/z 861. Either the conditions used in this experiment or the extract composition do not allow us to discern the site of the linkages for these molecules, unlike the previous reports cited above. The most likely reasons for multiple molecular species present in fraction E are either lack of separation or degradation

of these species in the instrument. Gu and others (2003) reported that with an increase in DP the stability of the ions markedly decreased. This anomaly may be starting to occur in fraction E.

Fractions F-H are characterized in Table 2. These fractions displayed singly and doubly charged ions, i.e. $[M-zH]^{z-}$ (z =1, z =2 respectively). Since mass spectrometers measure the mass-to-charge ratio of ions, doubly charged species will show up in the mass spectrum at (M-2H)/2, M = neutral molecular weight. The normal mass range (NMR), high mass range (HMR), daughter ion fragments of NMR ions, and zoom MS were all used to determine the charge state of molecules within these fractions. Mass accuracy and resolution of ions in the HMR are poorer than in the NMR, therefore masses and their isotopes may not be as exact. It is currently unknown whether B-type oligomers exist at a lower concentration in these fractions. The 2 amu difference of pseudomolecular ion of B and A-types leads to a complicated isotopic distribution pattern for multiply charged species that cannot be distinguished even using zoom MS. This can theoretically occur with A-types as well. This distribution pattern can be distinguished with high resolution MS, but not with the instrument that was used for this analysis. Therefore, the diversity of linkages might actually be underestimated in this study.

Consequently, poor chromatographic resolution of the species in these fractions could possibly overestimate the diversity. However, this is less likely, since the same $[M-H]^-$ ions for lower DP substances have been found to elute at multiple retention times in a study that investigated the same agricultural product and were confirmed to be different by other techniques than MS (Appeldoorn, et al., 2009). In the present study, it was common, to observed multiple compounds eluting at different retention times which produced the same $[M-H]^-$ ions (Fractions A-E). Therefore it is likely the diversity is underestimated due to the lack of

sensitivity and selectivity to detect species that were present in fractions A-E, but not fractions F-H (B-types, propelargonidins or prodelphinidins).

Fraction F contained tetramers and pentamers. Co-elution of multiple molecular species became common starting with this fraction. For this reason the relative percentage of molecular species could not be determined for this fraction, or subsequent fractions. However, through fragmentation data the site of interflavanic linkage could be determined (Table 3) upon comparison with previously published data for fraction F (Gu, Kelm, Hammerstone, Beecher, et al., 2003; Gu, Kelm, Hammerstone, Zhang, et al., 2003).

Most of the data matched well with that of previous reports referenced in this manuscript. The linkage could not be determined for compound **60** because it contained fragments at m/z 861, 863, and 575. This molecule is probably [(epicatechin)catechin→B→(epicatechin)catechin →B→(epicatechin) catechin→A→(epicatechin)catechin] due to the presence of m/z 863 and 575 fragments. However, m/z 861 was the BP. It was likely possible that its presence occurred because of co-elution with compound **59**. In fact most of the identified molecules in this fraction contained product ions likely related to other species that co-elute. This is likely due to a small percentage of co-elutant ions being trapped along with the ions of interest. This makes identification of molecular species in co-eluting peaks possible, but of a more tentative nature than if the peaks were completely separated.

Fragmentation data was extremely complicated for fractions G and H due to isotopic distribution, diversity of the number of linkages and types, along with co-elution issues. For this reason fragmentation data is not provided in Table 2. However, the number of linkages and degree of polymerization should be fairly accurate with pseudomolecular ion information.

Fraction G was the first fraction to show doubly charged molecules (z = -2) i.e. $[M-2H]^{2-}$ ions. Compound **69**, an A-type hexamer was confirmed to be doubly charged from zoom MS scans, which showed the isotopes of m/z 862 (the most prominent ion) to differ by 0.5 atomic mass units which is characteristic of doubly charged molecules. The A-type hexamer **71** NRMS m/z 1725 $[M-H]^-$ ion at the same retention time was also seen at m/z 1727 in the HRMS. Thus it is unknown if **69** and **71** (and similar molecules in G and H) are the same molecule showing a singly and doubly charged state or different. It is possible that the hexamer with one A-type linkage could have had a propensity to lose two protons, while the hexamer with two A-type linkages did not. Nuclear magnetic resonance spectroscopy could distinguish between the two molecules. However, obtaining a pure compound to analyze even with modern chromatographic techniques (UPLC or HILIC) would be very difficult. A study using matrix-assisted laser desorption/ionization (MALDI) time of flight (MS) found similar issues analyzing high molecular weight proanthocyanidins in peanut, hazelnuts, and almond skins (Monagas, et al., 2009). The $[M-H]^-$ ions of compounds isolated from the skins showed good agreement (within 0.2 amu) with their theoretical values ending up to pentamers. For DP 6-12 the actual $[M-H]^-$ ion m/z values diverged 0.2-1.4 amu from their theoretical values. However, HRMS data showed good agreement with NRMS data in this study for compounds **71**, **73**, **77**, **79**, **81**-**84**, **88**-**90**, **94**, **95**, **99**.

Fraction H showed heptamers, octomers, and nonamers. The spectra were the most complex in this fraction due to overlap of isotopic distribution patterns of species, which made it difficult to determine the number of linkages, and charge state. For this reason the most intense isotope was determined to be the $[M-H]^-$ ion (NRMS). However this could have led to a correct or incorrect identification based upon the theoretical isotopic distribution (Fig. 12). For this

reason the identification of species in this fraction is the most tentative. It is fairly certain that the degree of polymerization is correct, but the number of linkages may not be.

4. Conclusions

Peanut skins contain many types of proanthocyanidins, but overall contain more A-type proanthocyanidins. Peanut skins contain mostly dimeric, trimeric, and tetrameric species of proanthocyanidins. The multistep extraction procedure is an effective means of concentrating procyanidins from peanut skins when compared to single solvent extracts. This data corresponded well with previous studies in the area, in particular, with previous studies identifying compounds in peanut skins. However, four novel compounds were identified in this study. Higher molecular weight proanthocyanidins which produced $[M-2H]^{2-}$ ions were found to exist in peanut skins unlike in previous reports. Molecular species that display a DP of greater than five were shown to display highly complex mass spectra. For this reason their identification in this study is tentative.

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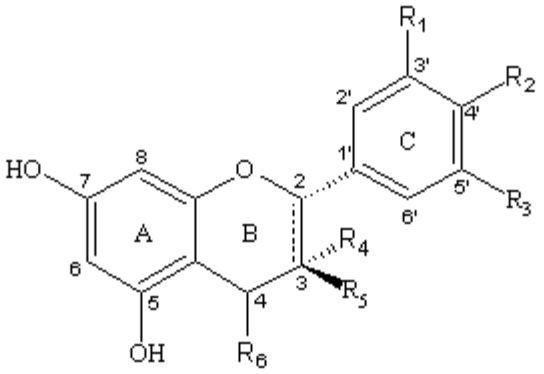

Name	R_1	R_2	R_3	R_4	R_5	R_6
Catechin	H	OH	OH	H	OH	H
Epicatechin	H	OH	OH	OH	H	H
Afzelechin	H	OH	H	H	OH	H
Epiafzelechin	H	OH	H	OH	H	H
Gallocatechin	OH	OH	OH	H	OH	H
Epigallocatechin	OH	OH	OH	OH	H	H
Epicatechin-3-gallate	H	OH	OH	OH	gallate	H
Luteolin	OH	OH	H	-	H	O
Kaempferol	H	OH	H	-	OH	O

Fig. 1. Structures of some common monomeric flavonoids. The dashed line between positions 2 and 3 on the C ring indicates the presence of a double bond for luteolin and kaempferol.

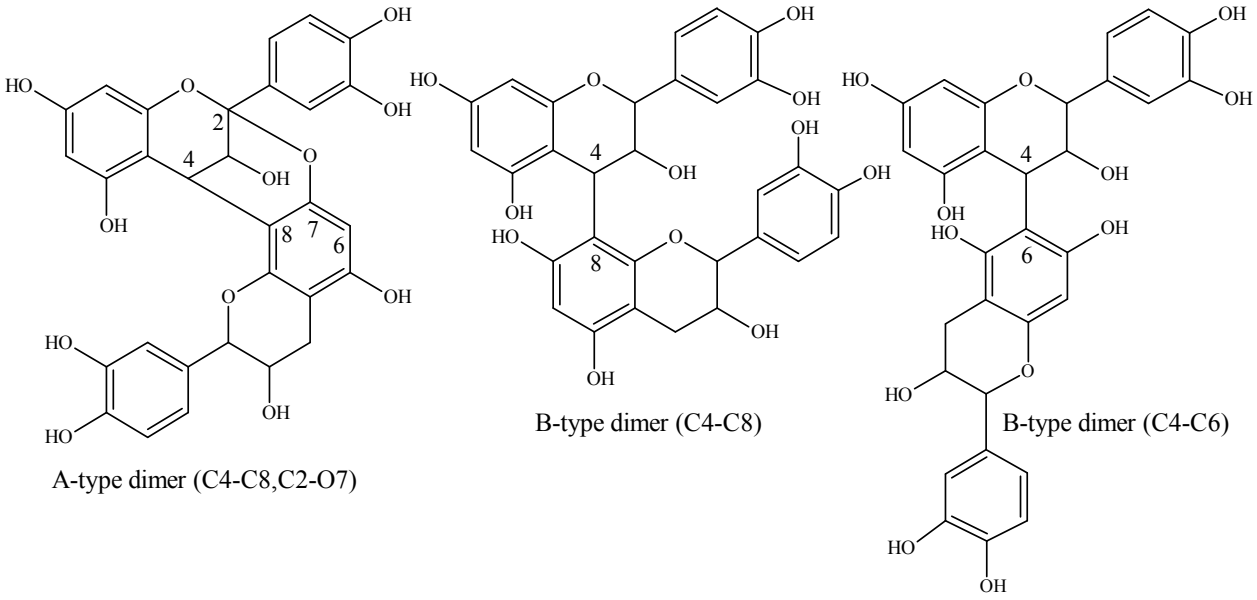

Fig. 2. Linkage types possible for proanthocyanidins. In addition there is a fourth linkage type where the A-type linkage is (C4-C6,C2-O7). In that case the bottom unit would be flipped 180°.

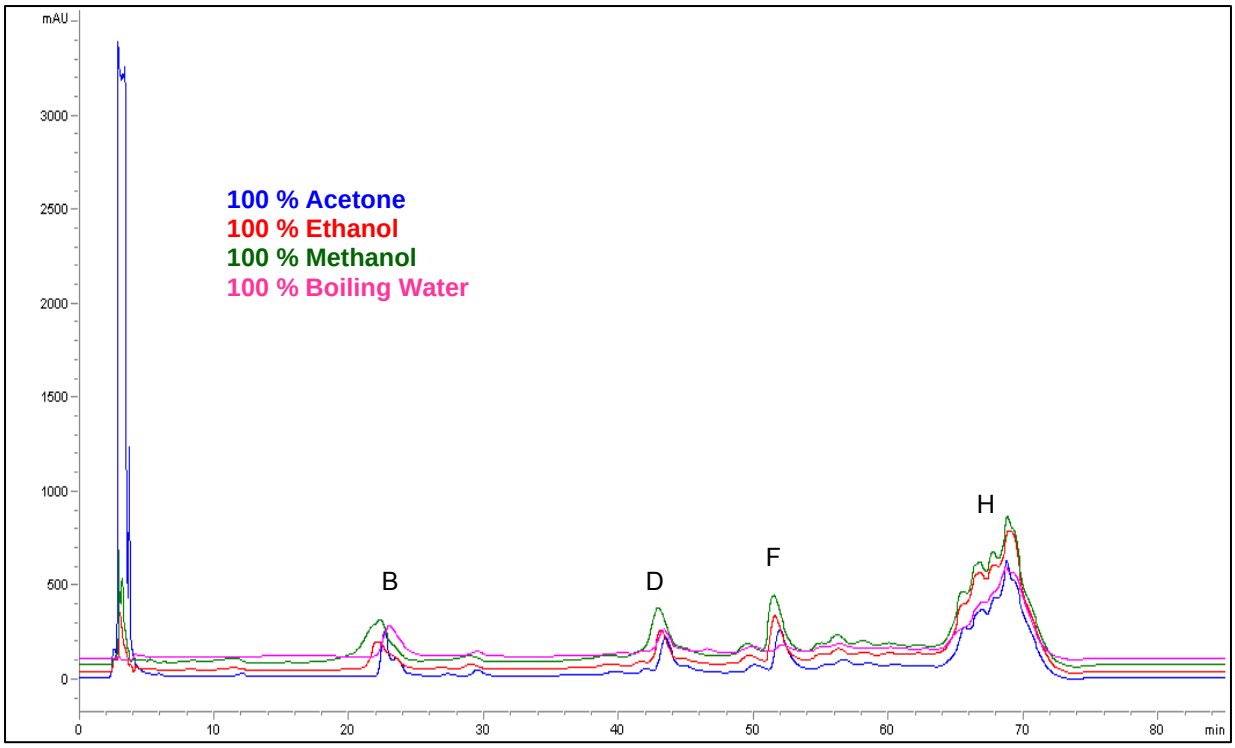

Fig. 3. Extraction efficiency of different solvents. Concentration was compared by UV 280 nm absorbance along with the ability to separate procyanidins by degree of polymerization via normal phase chromatography.

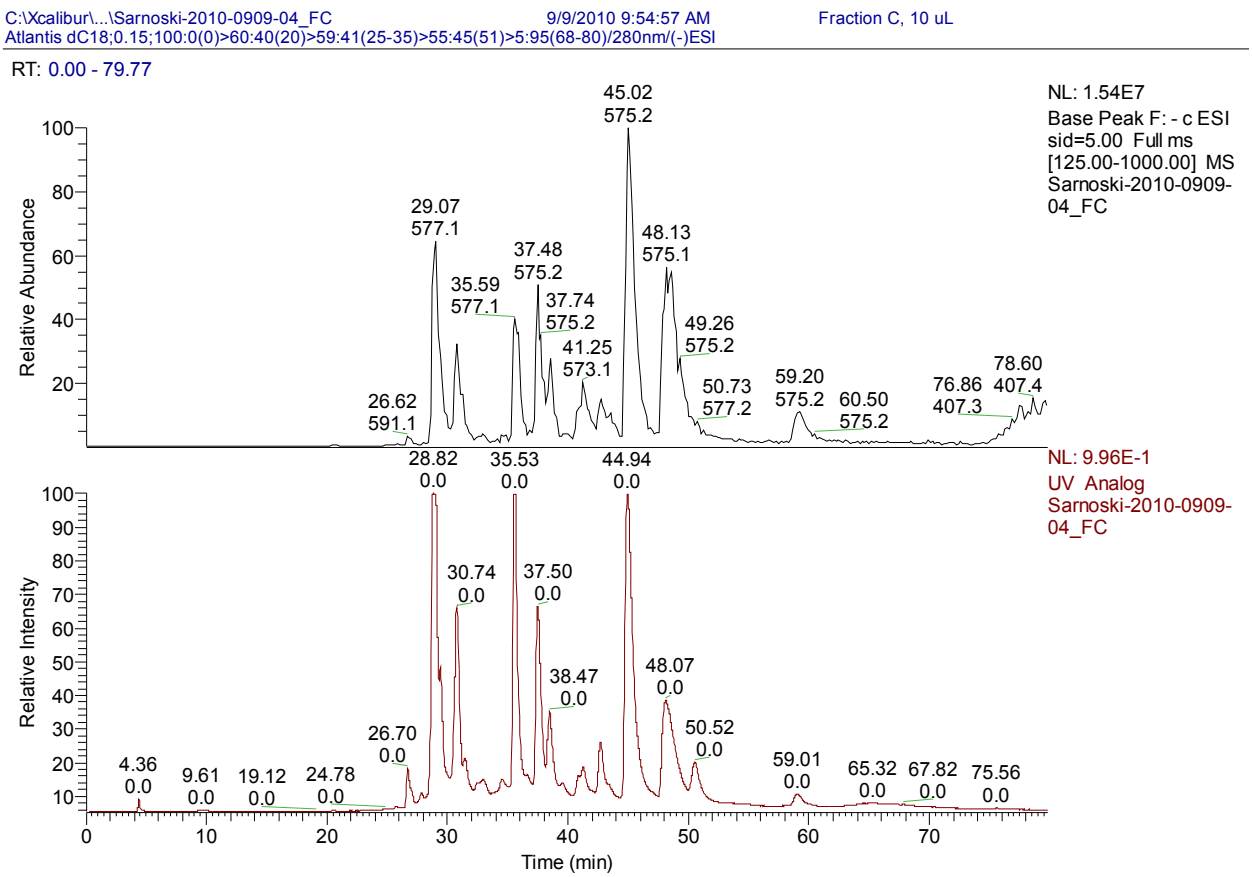

Fig. 4. Fraction C (NP). UV 280 nm data corresponded well with negative mode ion data.

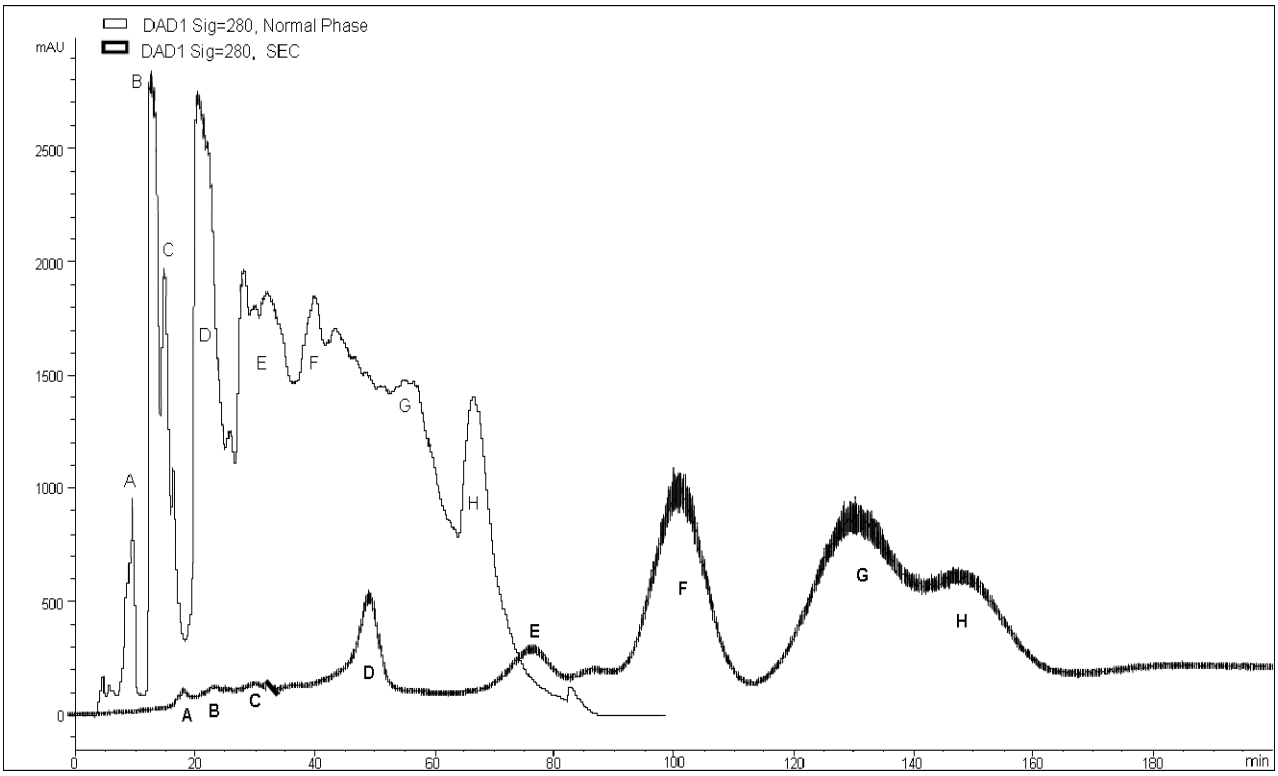

Fig. 5. Overlay of normal phase and SEC (bold) separation of the PSE extract.

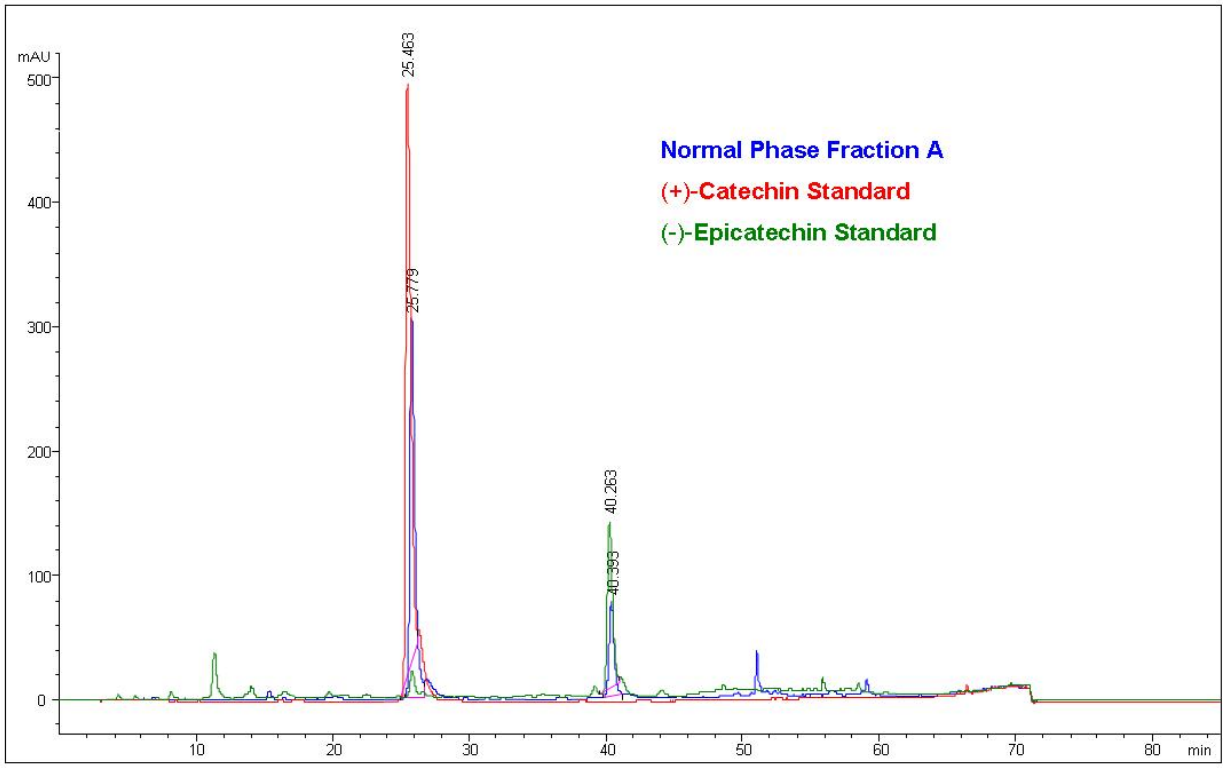

Fig. 6. Retention time matching of components in Fraction A with standards. UV 280 nm spectra and retention times were compared using reversed phase chromatography.

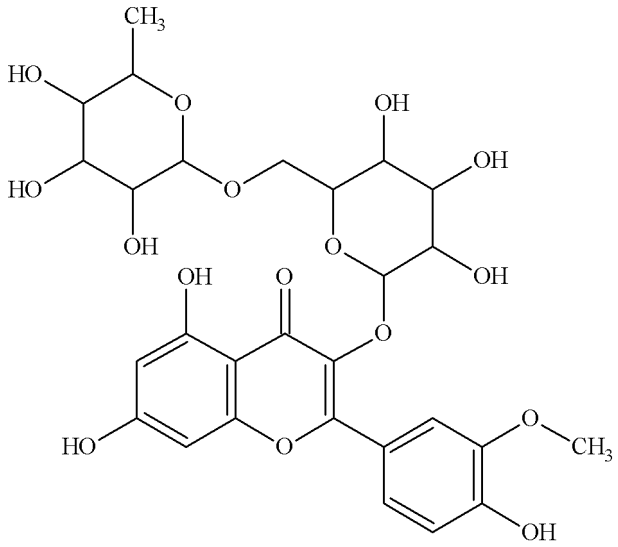

Fig. 7. Isorhamnetin-3-O-rutinoside.

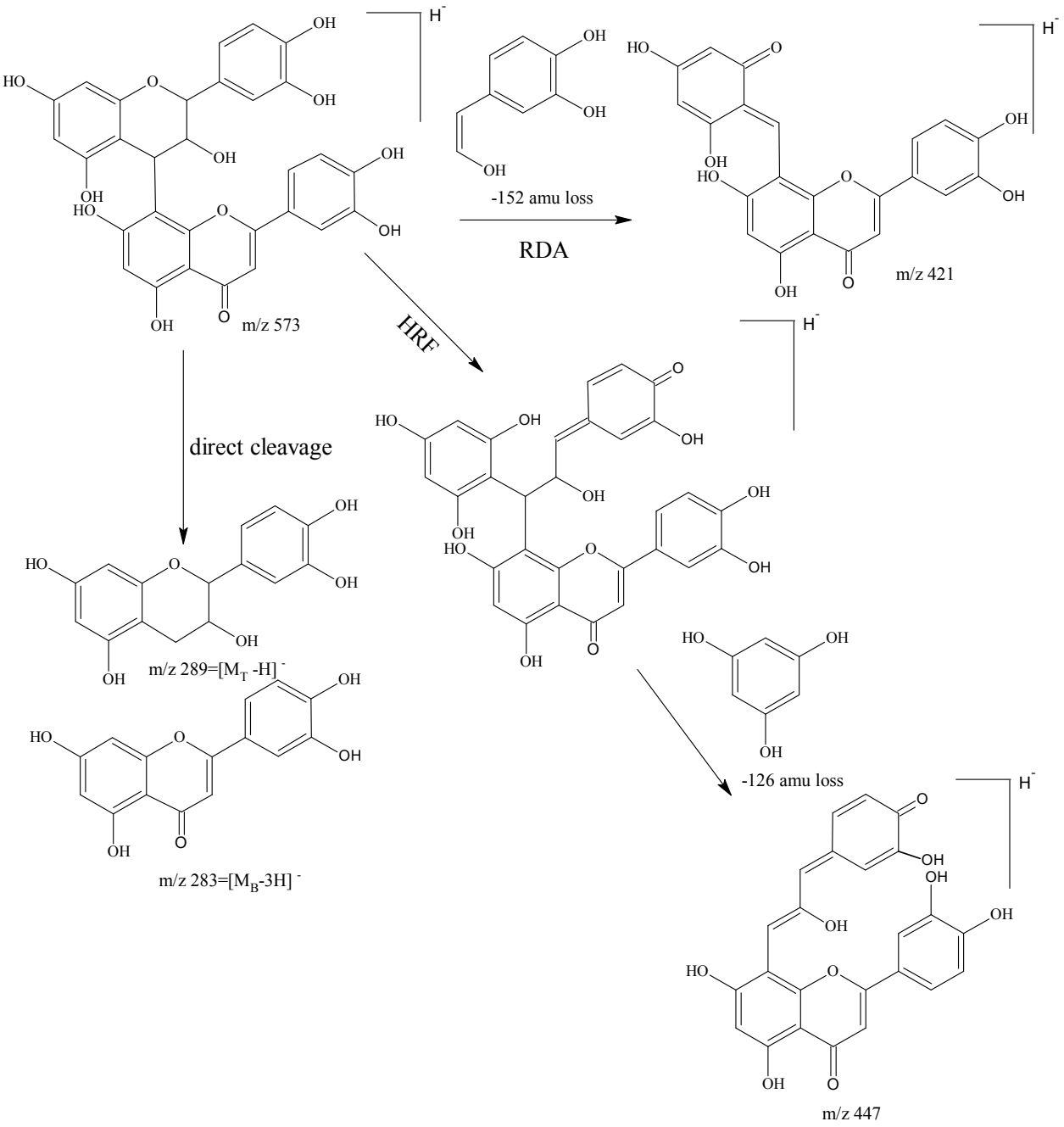

Fig. 8. Fragmentation scheme for luteolin or kampferol proanthocyanidin dimer. Mechanism shown with luteolin as the flavone. The mechanism for direct cleavage is not shown in full detail. It is likely that two hydrogen atoms are taken from the A ring of the bottom (B) unit (luteolin) with one coming from the hydroxyl and another from near the site of the interflavan linkage.

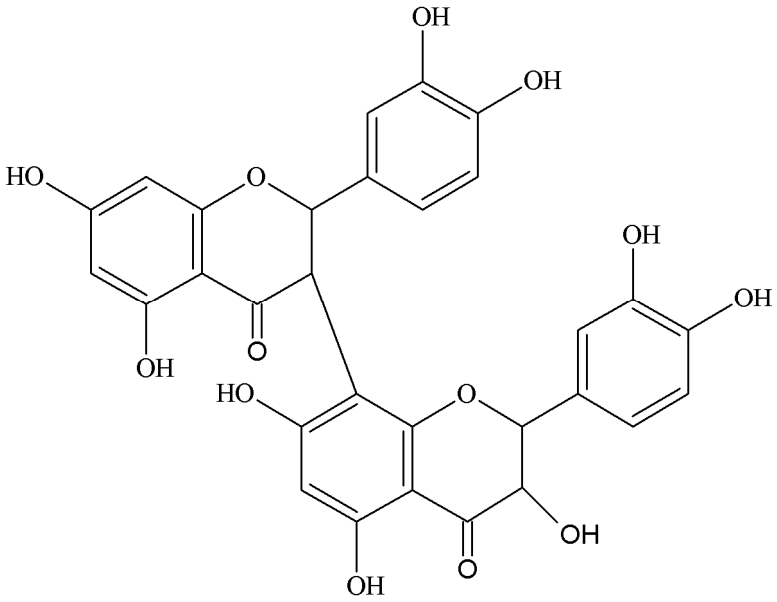

Fig. 9. Manniflavanone

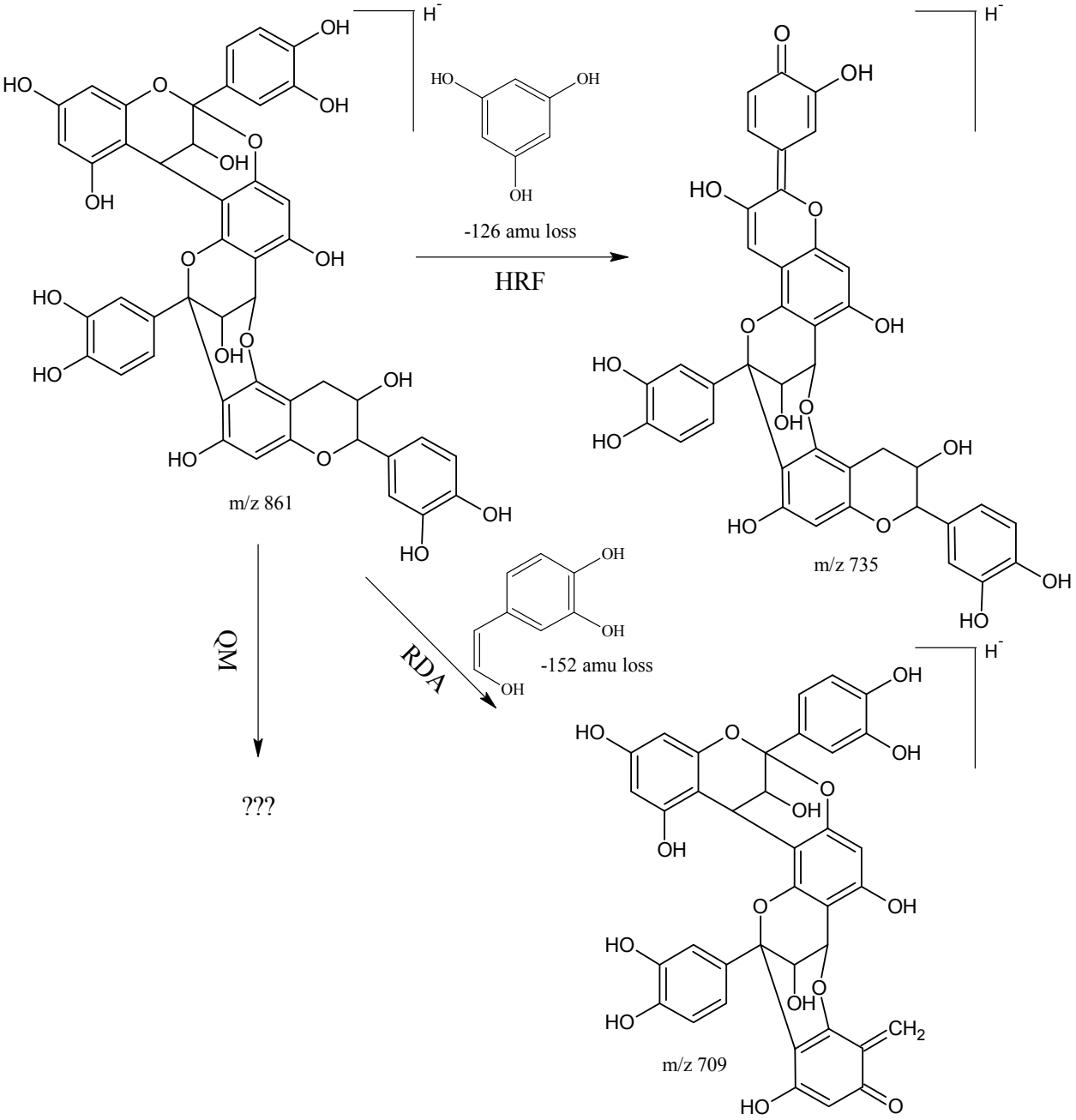

Fig. 10. Proposed fragmentation scheme for a Procyanidin trimer with two A-type linkages.

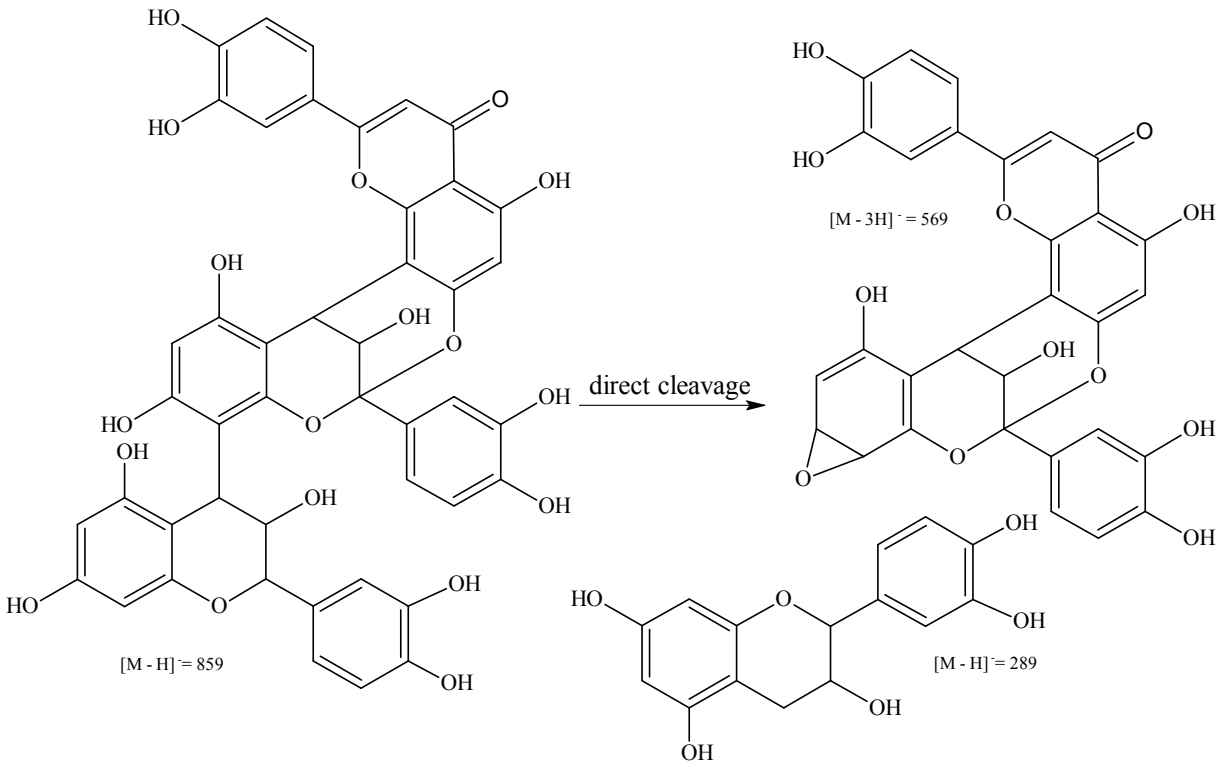

Fig. 11. Direct cleavage mechanism for a procyanidin A-type trimer containing luteolin or kaempferol. Mechanism shown with luteolin as the flavone. Again, two hydrogen atoms are lost from the middle and bottom units of the trimer during this mechanism. Site of the deprotonation is arbitrary for the product ions m/z 289 and m/z 569.

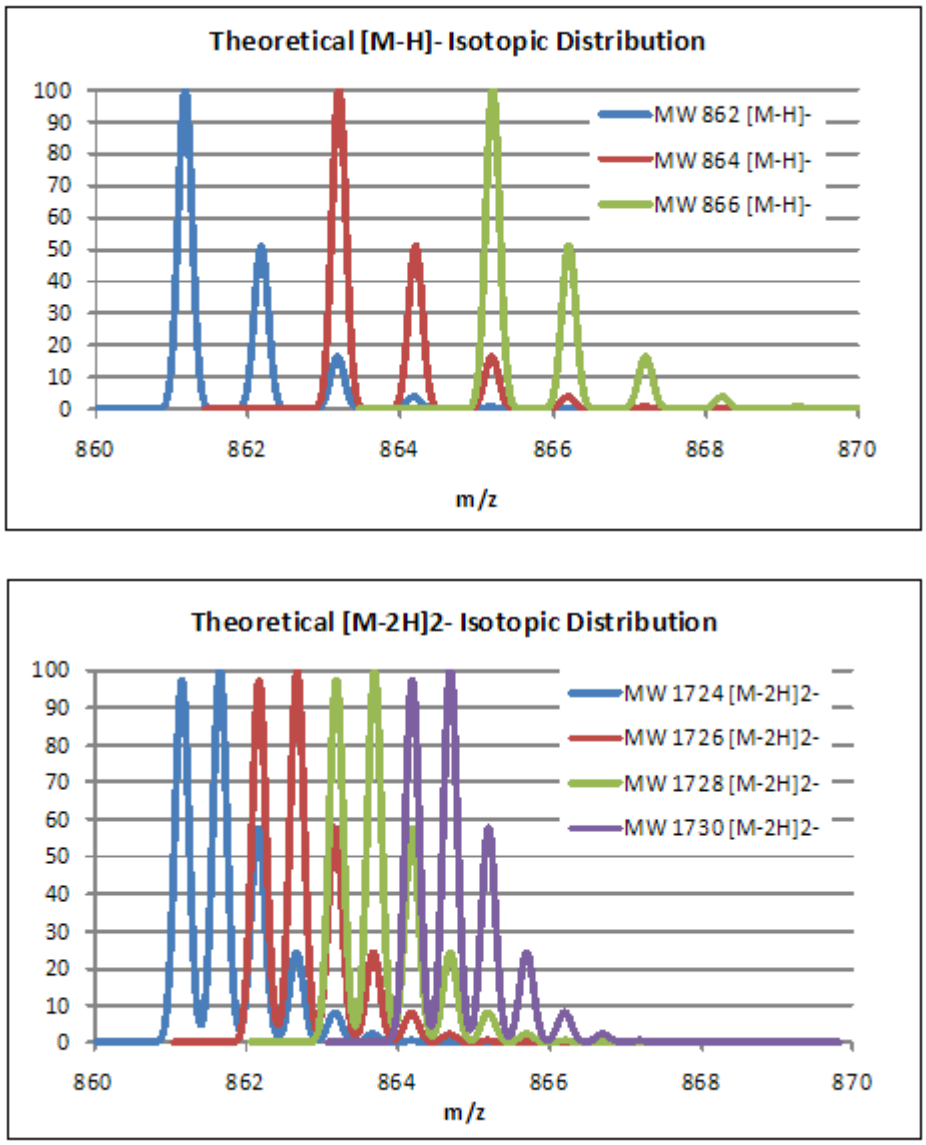

Fig. 12. Theoretical isotopic distribution for DP 3 and DP 6, singly and doubly charged respectively.

Table 1
Tentative Compound Identification.

ID	ID #	NP	C18 R_t (min)	DP	MW	$[M-H]^-$	PI-BP	Other Product Ions (*m/z*)	%
(+)-catechin	1	A	29.53	1	290	289	245	205, 179	52
(-)-epicatechin	2	A	34.32	1	290	289	245	205, 179	35
316 amu flavanoid (rhamnetin, isorhamnetin, tamarixetin, nepetin) plus glucose or galactose	3	A	45.21	-	478	477	315	300, 271, 247	6
316 amu flavanoid (rhamnetin, isorhamnetin, tamarixetin, nepetin) plus rutinoside	4	A	66.17	-	624	623	315	300, 271, 255, 243	4
Procyanidin dimer B-type [(E)C→B→ (E)C]	5	B	32.22	2	587	577	451	425, 407, 289, 287	4.3
Proanthocyanidin dimer [(E)C→luteolin or keampferol]	6	B	33.84	2	574	573	447	555, 529, 421, 403, 289, 283	2.1
Procyanidin dimer A-type [(E)C →A →(E)C]	7	B	38.86	2	576	575	449	423, 407, 289, 285	61
Procyanidin dimer A-type [(E)C →A→(E)C]	8	B	45.76	2	576	575	449	423, 407, 289, 285	26
Procyanidin dimer A-type [(E)C →A→(E)C]	9	B	48.31	2	576	575	449	423, 407, 289, 285	2.8
Procyanidin dimer A-type [(E)C→A→(E)C]	10	B	52.05	2	576	575	449	423, 407, 289, 285	1.9
Procyanidin dimer A-type [(E)C→A→(E)C]	11	B	62.29	2	576	575	449	423, 407, 289, 285	0.5
Manniflavanone (biflavanone)	12	B	66.33	2	590	589	463	445, 421, 303, 285	0.5
Proanthocyanidin A-type [(E)C →A→(E)GC]	13	C	26.7	2	592	591	439	573, 465, 451, 421, 303, 285	1.7
Procyanidin dimer B-type [(E)C→B →(E)C]	14	C	28.82	2	578	577	451	425, 407, 289, 287	22
Procyanidin dimer B-type [(E)C→B→(E)C]	15	C	30.74	2	578	577	451	425, 407, 289, 287	8.0
Proanthocyanidin B-type [(E)C→B→(E)GC]	16	C	34.5	2	594	593	575	556, 456, 449, 423, 303, 289, 285	tr
Procyanidin dimer B-type [(E)C →B→(E)C]	17	C	35.53	2	578	577	451	425, 407, 289, 287	14
Procyanidin dimer A-type [(E)C→A→(E)C]	18	C	37.5	2	576	575	449	539, 447, 435, 423, 407, 289, 287, 285	7.7
Procyanidin dimer A-type [(E)C→A→ (E)C]	19	C	38.47	2	576	575	449	423, 407, 289, 285	2.4
Proanthocyanidin dimer [(E)C→luteolin or kaempferol]	20	C	41.24	2	574	573	447	555, 529, 421, 323, 289, 283	1.3
Proanthocyanidin dimer [(E)C→luteolin or kaempferol]	21	C	42.69	2	574	573	447	555, 529, 421, 323, 289, 283	3.1

ID	ID #	NP	C18 R_t (min)	DP	MW	$[M-H]^-$	PI-BP	Other Product Ions (*m/z*)	%
Procyanidin dimer A-type [(E)C→A→(E)C]	22	C	44.94	2	576	575	449	423, 407, 289, 285	26
Procyanidin dimer A-type [(E)C→A→(E)C]	23	C	48.07	2	576	575	449	423, 407, 289, 285	12
Procyanidin dimer A-type [(E)C-(C4 →A→(E)C]	24	C	59.01	2	576	575	449	423, 407, 289, 285	1.4
Procyanidin trimer [(E)C→A→(E)C→A→(E)C]	25	D	27.78	3	862	861	735	709, 693, ms3(735)→609, 567, 447	0.4
Procyanidin trimer (C1) [(E)C→B→(E)C→B→(E)C]	26	D	29.35	3	866	865	695	739, 713, 577, 575, 451, 407, 289, 287	0.5
Procyanidin trimer A-type [(E)C→B→(E)C→A→(E)C]	27	D	30.57	3	864	863	575	737, 711, 693, 559, 449	21
Procyanidin trimer A-type [(E)C→B→(E)C→A→(E)C]	28	D	32.41	3	864	863	575	737, 711, 693, 559, 449	22
Proanthocyanidin trimer A-type [(E)C→A→(E)Af →B→(E)C]	29	D	33.95	3	847	846	411	711, 557, 435, 289	3.4
Procyanidin trimer A-type [(E)C→B→(E)C→A→(E)C]	30	D	36.42	3	864	863	575	737, 711, 693, 559, 449	39
Procyanidin trimer A-type [(E)C→B→(E)C→A→(E)C]	31	D	37.61	3	864	863	575	737, 711, 693, 559, 449	3.6
Proanthocyanidin trimer (2 A-type linkages, with one (epi)gallocatechin)	32	D	38.67	3	878	877	741	725, 589, 575	0.9
Procyanidin trimer A-type [(E)C→A→(E)C→B→ kaempferol or luteolin]	33	D	40.13	3	860	859	569	733, 707, 691, 443	1.2
Procyanidin trimer A-type [(E)C→B→(E)C→A→(E)C]	34	D	48.31	3	864	863	575	737, 711, 693, 449	5.7
Procyanidin trimer [(E)C→A→(E)C→A→(E)C]	35	D	54.11	3	862	861	571	735, 709, 693, 575, 449	2.1
Procyanidin B-type dimer	36	E	27.95	2	578	577	no frag data	-	0.9
Proanthocyanidin trimer (2 A-type linkages, with one (epi)gallocatechin)	37	E	28.52	3	878	877	725	741, 707, 573, 575, 331	1.5
Procyanidin tetramer A-type [(E)C→B→(E)C→B→(E)C→A→(E)C]	38	E	30.19	4	1152	1151	981	1025, 999, 863, 575	2.3
Procyanidin trimer A-type [(E)C→A→(E)C→B→(E)C]	39	E	31.22	3	864	863	711	693, 573, 451, 411	4.8

ID	ID #	NP	C18 R_t (min)	DP	MW	$[M-H]^-$	PI-BP	Other Product Ions (*m/z*)	%
Procyanidin trimer A-type [(E)C→A→(E)C→B→(E)C]	40	E	31.85	3	864	863	711	693, 573, 451, 411	25
Proanthocyanidin dimer [(E)C→luteolin or kaempferol]	41	E	31.85	2	574	573	411 (from 40)	447	tr
Procyanidin tetramer 2 A-type linkages	42	E	33.82	4	1150	1149	575	859, 737	23
Procyanidin trimer A-type [(E)C→B→(E)C→A→(E)C]	43	E	35.11	3	864	863	711	693, 575, 573, 451, 411	1.9
Procyanidin tetramer 2 A-type linkages	44	E	36.94	4	1150	1149	575	859, 737	23
Procyanidin dimer A-type [(E)C→A→(E)C]	45	E	36.94	2	576	575	449	423, 407, 289, 285	tr
Procyanidin tetramer A-type [(E)C→B→(E)C→B→(E)C→A→(E)C]	46	E	38.58	4	1152	1151	575	981, 863, 737, 693, 449	5.9
Procyanidin tetramer A-type [(E)C→A→(E)C→B→(E)C→B→(E)C]	47	E	44.8	4	1152	1151	981	861, 737, 573, 411	3.5
Procyanidin dimer A-type [(E)C→A→(E)C]	48	E	49.19	2	576	575	449	423, 407, 289, 285	2.8
Procyanidin dimer A-type [(E)C→A→(E)C]	49	E	64.25	2	576	575	449	423, 407, 289, 285	5.6

List of abbreviations used: Identification (ID), Normal Phase (NP), Reversed Phase Retention Time (C18 R_t), Degree of Polymerization (DP), Molecular Weight (MW), Molecular Ion ($[M-H]^-$), Product Ion Base Peak (PI-BP) (i.e. the most abundant ion in the MS), Relative percentage in the respective fraction (%), Trace (tr). Abbreviations related identification of molecular species: Epicatechin (E), Catechin (C), Gallocatechin (GC), Afzelechin (Af), A-type linkage (A) which can be (C4 →C8),(C2 →O7) or (C4 →C6),(C2 →O7), B-type linkage (B) which can be (C4 →C8) or (C4 →C6). The C4 →C6 has been found to rarely occur. The abbreviation E(C) indicates the monomeric unit is indistinguishable using mass spectrometry.

Table 2
Highly Tentative Identification of Oligomeric Species.

ID	ID #	NP	C18 R_t (min)	DP	MW	MI (NRMS)	MI CS (z)	MI (HRMS)	PI > MI (NRMS)
Procyanidin tetramer A-type [(E)C→B→(E)C→A→(E)C→B→(E)C]	50	F	29.36	4	1152	1151	1	1151	no
Procyanidin tetramer A-type [(E)C→A→(E)C→B→(E)C→B→(E)C	51	F	30.31	4	1152	1151	1	1150	no
Procyanidin tetramer A-type [(E)C→A→(E)C→B→(E)C→B→(E)C]	52	F	31.49	4	1152	1151	1	1150	no
Procyanidin pentamer A-type [(E)C→B→(E)C→A→(E)C→B→(E)C→A→(E)C]	53	F	31.49	5	1438	1437	1	1436	no
Procyanidin tetramer A-type [(E)C→A→(E)C→B→(E)C→B→(E)C]	54	F	32.64	4	1152	1151	1	1150	no
Procyanidin pentamer A-type [(E)C→B→(E)C→A→(E)C→B→(E)C→A→(E)C]	55	F	32.64	5	1438	1437	1	1436	no
Procyanidin tetramer A-type [(E)C→B→(E)C→A→(E)C→A→(E)C]	56	F	33.62	4	1150	1149	1	1149	no
Procyanidin tetramer A-type [(E)C→B→(E)C→A→(E)C→B→(E)C]	57	F	33.62	4	1152	1151	1	1150	no
Procyanidin pentamer A-type [(E)C→B→(E)C→A→(E)C→B→(E)C→A→(E)C]	58	F	33.62	5	1438	1437	1	1436	no
Procyanidin tetramer A-type [(E)C→B→(E)C→A→(E)C→A→(E)C]	59	F	34.83	4	1150	1149	1	1149	no
Procyanidin tetramer A-type	60	F	34.83	4	1152	1151	1	1151	no
Procyanidin pentamer A-type [(E)C→B→(E)C→A→(E)C→B→(E)C→A→(E)C]	61	F	34.83	5	1438	1437	1	1436	no
Procyanidin tetramer A-type [(E)C→B→(E)C→A→(E)C→A→(E)C]	62	F	37.79	4	1150	1149	1	1148	no
Procyanidin tetramer A-type [(E)C→A→(E)C→B→(E)C→B→(E)C]	63	F	37.79	4	1152	1151	1	1150	no
Procyanidin pentamer A-type [(E)C→A→(E)C→A→(E)C→B→(E)C→B→(E)C]	64	F	37.79	5	1438	1437	1	1436	no
Procyanidin tetramer A-type [(E)C→B→(E)C→A→(E)C→A→(E)C]	65	F	44.06	4	1150	1149	1	1149	no
Procyanidin tetramer A-type [(E)C→B→(E)C→B→(E)C→A→(E)C]	66	F	44.06	4	1152	1151	1	1151	no
Procyanidin pentamer A-type [(E)C→B→(E)C→A→(E)C→B→(E)C→A→(E)C]	67	F	44.06	5	1438	1437	1	1437	no
Procyanidin dimer A-type	68	G	28.34	2	576	575	1	-	no
Procyanidin hexamer [1 or 2A, 4 or 3B linkages]	69	G	28.34	6	1728, 1726	862	2	1728, 1727	yes
Procyanidin tetramer [2A, 1B]; some octamer	70	G	28.34	4	1150	1149	1, 2	1149	yes
Procyanidin hexamer [2A, 3B]	71	G	28.34	6	1726	1725	1	1725	no
Procyanidin dimer A-type	72	G	30.47	2	576	575	1	-	no
Procyanidin heptamer [3A, 3B]	73	G	30.47	7	2012	1005.6	2	2012, 2009	yes
Procyanidin tetramer [2A, 1B]; some octamer	74	G	30.47	4	1150	1149	1, 2	1148, 2297	yes
Procyanidin pentamer [2A, 2B]	75	G	30.47	5	1438	1437	1	1437, 1435	no
Procyanidin hexamer [1A, 4B]	76	G	33.96	6	1728	863	2	1728, 1727	yes
Procyanidin heptamer [3A, 3B]	77	G	33.96	7	2012	1006	2	2012, 2010	yes

ID	ID #	NP	C18 R_t (min)	DP	MW	MI (NRMS)	MI CS (z)	MI (HRMS)	PI > MI (NRMS)
Procyanidin tetramer [2A, 1B]; some octamer	78	G	33.96	4	1150	1149	2	1149, 2298	yes
Procyanidin hexamer [2A, 3B]	79	G	33.96	6	1726	1725	1	1724, 1725	no
Procyanidin A-type dimer	80	H	31.78	2	576	575	1	-	-
Procyanidin hexamer [1A, 4B]	81	H	31.78	6	1728	863	1, 2	1724, 1726	yes
Procyanidin heptamer [3A, 3B]	82	H	31.78	7	2012	1006.5	2	2011, 2012	yes
Procyanidin octamer [2A, 5B]	83	H	31.78	8	2302	1149	2	2297, 2298, 2301	yes
Procyanidin nonamer [2A, 6B]	84	H	31.78	9	2590	1293	2	2589, 2591	yes
Procyanidin pentamer [2A, 2B]	85	H	31.78	5	1438	1437	1, 2	2875?	yes
Procyanidin hexamer [2A, 3B]	86	H	31.78	6	1726	1725	1	1725 only	1841
Procyanidin A-type dimer	87	H	33.59	2	576	575	1	-	-
Procyanidin heptamer [3A, 3B]	88	H	33.59	7	2012	1006.6	2	2011, 2012	yes
Procyanidin octamer [2A, 5B]	89	H	33.59	8	2302	1149	2	2297, 2298	yes
Procyanidin nonamer [2A, 6B]	90	H	33.59	9	2590	1294	2	2588, 2592	yes
Procyanidin pentamer [2A, 2B]	91	H	33.59	5	1438	1437	1, 2	2872, 2875?	yes
Procyanidin hexamer [2A, 3B]	92	H	33.59	6	1726	1725	1	3458	no
Procyanidin A-type dimer	93	H	37.87	2	576	575	1	-	-
Procyanidin octamer [2A, 5B]	94	H	37.87	8	2302	1150	2	2297, 2298	yes
Procyanidin nonamer [2A, 6B]	95	H	37.87	9	2590	1293.5	2	2588, 2594	yes
Procyanidin pentamer [2A, 2B]	96	H	37.87	5	1438	1437	1, 2	2872	yes
Procyanidin hexamer [2A, 3B]	97	H	37.87	6	1726	1725	1	1725 only	1990
Procyanidin A-type dimer	98	H	65.7	2	576	575	1	-	-
Procyanidin nonamer [2A, 6B]	99	H	65.7	9	2590	1294.6	2	2588, 2589, 2593	yes
Procyanidin pentamer [2A, 2B]	100	H	65.7	5	2590	1438.1	1, 2	2872, 2873, 2875	yes

List of abbreviations used: Identification (ID), Normal Phase (NP), Reversed Phase Retention Time (C18 R_t), Degree of Polymerization (DP), Molecular Weight (MW), Molecular Ion (MI), Normal Range Mass Spectrometry (NRMS), Charge State (CS), High Range Mass Spectrometry (HRMS), Product Ion (PI). Abbreviations related identification of molecular species are consistent with that of Table 1.

Table 3
Identification of Linkage Site of Sepcies in Fraction F.

ID	ID #	C18 R_t (min)	DP	MW	$[M-H]^-$	PI-BP	Other Product Ions (*m/z*)
Procyanidin tetramer A-type [(E)C→B→(E)C→A→(E)C→B→(E)C]	50	29.36	4	1152	1151	863	1025, 999, 573
Procyanidin tetramer A-type [(E)C→A→(E)C→B→(E)C→B→(E)C	51	30.31	4	1152	1151	981	999, 861, 573
Procyanidin tetramer A-type [(E)C→A→(E)C→B→(E)C→B→(E)C]	52	31.49	4	1152	1151	861	999, 739, 573
Procyanidin pentamer A-type [(E)C→B→(E)C→A→(E)C→B→(E)C→A→(E)C]	53	31.49	5	1438	1437	1149	861, 691, 575
Procyanidin tetramer A-type [(E)C→A→(E)C→B→(E)C→B→(E)C]	54	32.64	4	1152	1151	861	999, 739, 573
Procyanidin pentamer A-type [(E)C→B→(E)C→A→(E)C→B→(E)C→A→(E)C]	55	32.64	5	1438	1437	1149	861, 737, 575, 573
Procyanidin tetramer A-type [(E)C→B→(E)C→A→(E)C→A→(E)C]	56	33.62	4	1150	1149	861	863, 735, 575, 573
Procyanidin tetramer A-type [(E)C→B→(E)C→A→(E)C→B→(E)C]	57	33.62	4	1152	1151	863	1025, 999, 573
Procyanidin pentamer A-type [(E)C→B→(E)C→A→(E)C→B→(E)C→A→(E)C]	58	33.62	5	1438	1437	1149	861, 737, 575, 573
Procyanidin tetramer A-type [(E)C→B→(E)C→A→(E)C→A→(E)C]	59	34.83	4	1150	1149	861	735, 575, 573
Procyanidin tetramer A-type	60	34.83	4	1152	1151	861	863, 575
Procyanidin pentamer A-type [(E)C→B→(E)C→A→(E)C→B→(E)C→A→(E)C]	61	34.83	5	1438	1437	1149	861, 737, 575, 573
Procyanidin tetramer A-type [(E)C→B→(E)C→A→(E)C→A→(E)C]	62	37.79	4	1150	1149	997	861, 575, 573
Procyanidin tetramer A-type [(E)C→A→(E)C→B→(E)C→B→(E)C]	63	37.79	4	1152	1151	981	861, 739, 573
Procyanidin pentamer A-type [(E)C→A→(E)C→A→(E)C→B→(E)C→B→(E)C]	64	37.79	5	1438	1437	1147	1149, 997, 859, 575, 573
Procyanidin tetramer A-type [(E)C→B→(E)C→A→(E)C→A→(E)C]	65	44.06	4	1150	1149	997	861, 575, 573
Procyanidin tetramer A-type [(E)C→B→(E)C→B→(E)C→A→(E)C]	66	44.06	4	1152	1151	981	863, 739, 575
Procyanidin pentamer A-type [(E)C→B→(E)C→A→(E)C→B→(E)C→A→(E)C]	67	44.06	5	1438	1437	1149	861, 737, 575, 573

Abbreviations are consistent with Table 1.

CHAPTER 4

Application of Proanthocyanidins in Peanut Skins as a Natural Yeast Inhibitory Agent

(Manuscript to be submitted to Food Microbiology)

ABSTRACT

Proanthocyanidins were extracted from peanut skins and investigated for their antimicrobial activity against *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, and *Zygosaccharomyces bisporus* in traditional growth media (Sabouraud Dextrose and Maltose broth) and a simulated apple juice beverage. Peanut skins extracts (PSE) were prepared through a multi-solvent extraction procedure. The PSE extended the lag phase at a concentration of 1 mg/mL, however 10 mg/mL was needed to totally inhibit growth for 120 hrs. The PSE treatment effects were not significantly different growth media. Once inhibition from PSE was seen, PSE was fractionated by normal phase high performance liquid chromatography (HPLC) and fractions were studied for yeast inhibition. Compounds present in active fractions were identified by liquid chromatography-mass spectrometry ($LC\text{-}MS^n$) to determine the compounds responsible for inhibition. Fractions consisting mostly of A-type proanthocyanidin dimers, trimers, and tetramers were most inhibitory towards the yeasts tested in this study. Both optical density (OD) and timed course plating methods were performed in this study. The OD method led to an overestimation of inhibitory effects of PSE, but the two methods agreed in respect to treatment effects and dependent inhibition.

Keywords: Proanthocyanidin, peanut skin, optical density, natural antimicrobial, yeast

1. Introduction

The aim of this study was to find an inexpensive natural antimicrobial agent from a current waste product. An increasing number of consumers now desire that foods contain a limited amount of chemical preservatives. For this reason the food industry has a growing interest in finding natural food additives to accomplish the same results (Gould, 1996; Tajkarimi et al., 2010). The enhanced demand for natural and minimally processed ingredients is also attributed to the legislations governing the use of current preservatives (Mandalari et al., 2010).

The need to have a safe food supply has led to much research in the area of natural antimicrobials and their use to limit food pathogens (Bisha et al., 2010; Shan et al., 2008). This has lead to a neglect of research in the area of food spoilage organisms. Relatively few studies exist on the use of natural antimicrobials against yeasts and molds. Yeasts and molds are robust microorganisms, possessing an ability to grow in a wide range of foods. This is a major issue in high acid foods since these organisms are one of the few microorganisms that can grow under these conditions. A natural antimicrobial that could be applied to foods encountering microbial food spoilage issues would be of great interest to the food industry.

Traditionally, natural antimicrobials have consisted of essential oils. These oils often have high minimum inhibitory concentrations for yeasts 800 μg/mL (Mandalari et al., 2007) and 3000 μg/ml towards fungus (Rasooli et al., 2008). A few studies have revealed that proanthocyanidins may have antimycotic action. Pine bark extract containing polymeric flavanols (proanthocyanidins) and tannic acid exhibited broad activity towards yeast strains of the genera *Candida, Cryptococcus, Filobasidiella, Issatchenkia, Saccharomyces*: MICs ranged from 200 to 4000 μg/ml of powdered extract (Romani et al., 2006). Fractionated extracts containing proanthocyanidins and their glycosides from *Ixora coccinea* (Jungle Flame) were

found to have antibacterial activity towards the gram positive microorganism *Bacillus subtilis* at 20 mg/mL by means of agar diffusion assays (Idowu et al., 2010). Epigalocatechin gallate (EGCG) was shown to have inhibitory action towards clinically pathogenic *Candida* species via visual turbidity inspection, although the concentrations of EGCG for antifungal susceptibility were slightly higher than those of commercially available antifungal agents on the whole (Park et al., 2006). An extract of *Camellia sinensis* (tea leaves) caused a rapid decrease of viable cells of *Filobasidiella neoformans* at a concentration of 25 mg/mL displaying dose dependent antimycotic activity (Turchetti et al., 2005).

The measurement of optical density (OD) has been used to provide measurements related to the growth of yeasts and antifungal drug test mixtures, the antimicrobial action of synthetic peptides, and the maximum specific growth rates of various organisms (Couto et al., 2005; Dalgaard et al., 1994; Enrique et al., 2007; Wesolowski et al., 2008). This method has also been used to screen a grape seed extract for its applicability as a produce wash (Bisha et al., 2010) and to model the growth rate of *Listeria monocytogenes* using absorbance measurements and plate counts (Valero et al., 2006). However, it always appears that there is a need for a correction factor to have absorbance measurements correlate well with plate count data (Francois et al., 2005). It often seems that OD methods result in an overestimation in the log reductions with respect to plate counts (Lambert, 1998). Lambert hypothesized that the differences between the methods were due to the level of cell injury, which is masked by OD methods, but recognized by plate counts.

The objectives of this work were to determine if an extract of peanut skins would function as a natural antimicrobial for yeasts, and determine which compounds were responsible for the inhibitory effect.

2. Materials and methods

2.1 Extract Source and Standards

Peanut skins (Virginia variety) were obtained from Tidewater Blanching (Suffolk, VA) and were frozen at -20 °C upon receipt. (+)-Catechin, (-)-epicatechin, (±)-catechin, and kaempferol standards were purchased from Sigma (St. Louis, MO). Procyanidin B2 was purchased from Extrasynthese (Genay, France). Standards and PSE were added dry to the liquid media for inhibition studies in order to eliminate the possibility of solvent related effects.

2.2 Cultures and Growth Conditions

Cultures of the spoilage yeasts *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, and *Zygosaccharomyces bisporus* used for inoculation were obtained from The American Type Culture Collection (ATCC, Manassas, VA). Before experimentation the cultures were transferred to Sabouraud Dextrose broth (Difco, Becton Dickinson, Sparks, MD) and allowed to grow at 30 °C for at least 24 hrs. It was determined through plating on 3M™ Petrifilm™ Yeast and Mold count plates (St. Paul, MN) that the cultures were at a level of approximately 10^7 CFU/mL.

Sabouraud Dextrose and Maltose broth (Difco, Becton Dickinson, Sparks, MD) were also used as the growth medium for OD (turbidity) and time course plating measurements. These growth mediums were prepared according to the instructions given on their product labels.

An Apple Juice Medium (AJM) was prepared in order to simulate a common food beverage matrix. This growth medium consisted of the following: 100 mL of preservative free Apple Juice (Motts brand 100% preservative free) purchased from the local grocery store, 46.8 g glucose, 59.4 g fructose, and 1.8 g sucrose all purchased from Fisher Scientific (Pittsburgh, PA). These ingredients were combined and brought to a volume of 1 L. The pH was adjusted to 3.5

using 1M malic acid (Fisher Scientific, Pittsburgh, PA). The degree brix content was verified at 12°. This growth media was 0.2 μm filter sterilized through sterile Whatman PTFE Puradisc™ syringe filters (Whatman Inc., Piscataway, NJ).

2.3 Multistep Peanut Skin Extraction (PSE) Procedure

The method used for extraction of proanthocyanidins was similar to a previously developed process (Masquelier, 1987). Acetone, chloroform, dichlormethane, ethanol, and methanol were HPLC-grade quality obtained from Fisher Scientific (Pittsburgh, PA). The water used for these experiments was purified using a Millipore Elix® 5 (Molsheim, France). Acetic acid, ethyl acetate, sodium chloride, and sodium sulfate were also purchased from Fisher Scientific (Pittsburgh, PA). Peanut skins (100 g) were extracted with boiling water (99.7 °C) at a ratio of 1 g to 10 mL. The liquid was decanted and then filtered (Whatman No. 4) to remove remaining solid particles. NaCl was added to the filtrate to saturation filtration repeated to remove precipitates. The phenolic compounds were then extracted in a separatory funnel using ethyl acetate and dried with sodium sulfate. The ethyl acetate extract was then concentrated via a rotary evaporator to about 1/5 the initial volume. Finally, the procyanidins were precipitated with three equal volumes of chloroform and isolated by filtration (Whatman No. 4). After air drying, the procyanidin precipitate was a tan powder. Yields of approximately 1-1.5 g of powder were obtained from 100 g of peanut skins.

2.4 Normal Phase (NP) HPLC

An Agilent (Palo Alto, CA) 1000 series high performance liquid chromatograph (HPLC) separated single solvent extracts or PSE on a Regis Technologies (Morton Grove, IL) GFF II (25 cm x 4.6 mm i.d., 5 μm, 80 Å) porous silica column. The gradient method consisted of solvent A (dichloromethane), solvent B (methanol), and solvent C (water/acetic acid, 1:1, v/v). A flow

rate of 1.0 mL/min was applied as follows: 14-28.4% B from 0 to 30 min, 28.4-50% B from 30 to 60 min, 50-96% B from 60 to 65 min, held at 96% B from 65-75 min, followed by column equilibration from 75-100 min. Solvent C was held at 1% C for the entire run. The detection conditions were 210-360 nm for the diode array detector (DAD). The specific wavelengths of 254 nm, 270 nm, 280 nm, 310 nm, and 350 nm were used for detection. The signal at 280 nm was used for quantitation purposes. Since catechin and epicatechin have similar molar extinction coefficients at 280 nm (Whiting and Coggins, 1975), and the extracts contained oligomers and polymers of these basic units, it was assumed that the same molar extinction coefficients at 280 nm could be used to estimate proanthocyanidin concentrations. A procedure similar to this was employed recently (Appeldoorn et al., 2009). Fractions from individual runs were collected and pooled to test their ability to function as yeast inhibitory agents. The fractions were evaporated to dryness and then reconstituted with the appropriate growth media before analysis.

2.5 Conditions for Reversed Phase (RP) Electrospray Ionization (ESI) Mass Spectrometry Analysis

The ESI-HPLC-UV-MS system consisted of an Agilent (Palo Alto, CA) 1100 series HPLC coupled to a ThermoFinnigan (San Jose, CA) LCQ with electrospray ionization (ESI). HPLC separations were accomplished using a Waters Atlantis dC18 (2.1 x 150 mm, 3μ) with guard column. A gradient method consisting of solvent A (5.0 mM ammonium acetate in water to aid ionization) and solvent B (methanol) was applied at a flow rate of 0.15 mL/min as follows: 0-40% from 0 to 20 min, 40-41% from 20 to 25 min, held at 41% B from 25 to 35 min, 41-45% B from 35 to 51 min, 45-95% B from 51 to 68 minutes, and held at 95% B from 68-80 min, followed by column equilibration from 80-110 min. MS and UV (280 nm) detection was performed from 0-80 min. HPLC-ESI-MS data was acquired under negative mode using

Xcalibur (Thermo Fisher Scientific, Waltham, MA) and analyzed with the same software. Zoom MS (zMS) for mass ranges of interest was done to determine multiply charged species, and isotope patterns. The optimized electrospray/ion optics parameters were as follows: spray voltage, 3.2 kV; sheath gas (nitrogen), capillary voltage, -12 V; capillary temperature, 250 °C; tube lens offset voltage, 0 V. At least one blank run consisting of 1:1 water:methanol was run between different samples to monitor sample carryover.

2.6 Interaction of PSE with Growth Medium

It is well established that tannins (proanthocyanidins) will interact and form a complex with proteins (Chung et al., 1998; Francisco and Resurreccion, 2008; Hagerman and Butler, 1981). In our experiments, the PSE extract interacted with the Sabouraud Dextrose and Maltose broths, causing a precipitate to form. No significant change in pH was observed after addition of PSE. When PSE was exposed to an amino acid mixture at the same approximate concentration as the enzymatic digest of casein present as the protein source for Sabouraud Dextrose and Maltose broths, no precipitate formed. This suggests proteins and peptides are complexed by the PSE extract. The protein-proanthocyanidin precipitate was filtered through a 0.2 μm Whatman PTFE Puradisc™ syringe filter (Whatman Inc., Piscataway, NJ) before optical density determinations. The effects of filtered and non-filtered extracts were determined during time course plating (Table 3). No precipitate was formed when the PSE was added to the AJM, but a slight color change was observed due to the addition of the proanthocyanidins in the PSE. The lack of precipitate formation is likely due to the fact that AJM contains small amounts of protein or peptides. For this reason, AJM was chosen as the growth medium for optical density studies of the PSE fractions.

2.7 Determination of Optical Density

A Bioscreen C Microbiology Reader (Growth Curves, Piscataway, NJ) a combined incubator and automated turbidimeter was used for determination of optical density (OD). Optical density was determined at 600 nm. Liquid growth medium was combined with PSE or without PSE (control) and 0.2 μm syringe filtered Whatman PTFE Puradisc™ (Whatman Inc., Piscataway, NJ) to ensure the medium was sterile. Each well was filled with 340 μL of growth medium. In addition 10 μL of 0.1% peptone water containing approximately 500 cells were added to each of the wells. Growth was measured by determining the change in the OD (600 nm) every 30 minutes for either 3 or 5 days set at 30 °C. Experiments using Sabouraud Dextrose and Maltose broths were conducted for 3 days, while experiments using AJM were conducted for 5 days due to slower growth of the yeasts to their stationary growth phase. The growth curve data was generated by using EZExperiment software (Growth Curves, Piscataway, NJ) and exported as a Microsoft Excel spreadsheet (Microsoft, Seattle, WA). Controls were also run with no microorganism added. This was done either as growth medium only, or growth medium with PSE or standards added. These samples showed no significant increase in optical density over time. All samples were run in triplicate.

2.8 Time Course Plating

The yeast inhbitory activity of PSE at different concentrations was determined as a function of time with a time course plating assay. Sterile growth media was prepared as outlined in section 2.2. The media was then combined with PSE or PSE fractions and 0.2 μm syringe filtered. For one treatment (Table 3) the media-PSE mixture was not filtered. Next, 10 μL of 0.1% peptone water containing approximately 500 cells were added to 10 mL of media in a sterile tube. For the effect of the fractionated PSE and standards (Table 5) 10 μL of 0.1%

peptone water containing approximately 500 cells was added to 1 mL of media. Tubes were then incubated at approximately 30 °C statically. Trials were performed in triplicate. At the appropriate time periods, 1 mL of tube contents were either plated directly, or diluted to obtain CFU/mL in the appropriate range for colony counting using 3M™ Petrifilm™ Yeast and Mold count plates (St. Paul, MN). Petrifilms plated with high concentrations of proanthocyanidins had backgrounds that turned blackish upon plating. It was later determined that this was due to interference of the proanthocyanidins with the phosphatase reactions (3m, 2006). Even with this interference, the colonies could be visualized. This had no impact on plate counts. This was validated by plate counts of the control and treatments only differing slightly at time zero. Yeast and Mold count plates for each tube were incubated at approximately 30 °C for at least 48 hours before yeast and molds were counted. No colonies resembling molds could be visualized.

2.9 Statistical Analysis

For determination of optical density and time course plating data from three independent replications was subjected to statistical analysis by means comparison using Tukey-Kramer Honestly Significant Difference (T-K HSD) post-ANOVA testing. Multivariate regression analysis for OD measurements were conducted on the entire dataset by comparing OD values of the control to OD values of the experimental treatments. The correlation coefficient was calculated in respect to the control by means of the Pearson product-moment correlation coefficient, which measures the strength of the linear relationship between two variables. Spearman's ρ is coefficient is given as a non-parametric measure of the dependence between two variables (control and treatment). This procedure was selected since the OD data displays a bimodal distribution due to lag and stationary phase data. Percent inhibition was calculated by comparing treatment mean for the entire experimental period to the control with no PSE added.

Percent inhibition has been used to express yeast growth inhibition due to synthetic peptides (Enrique et al., 2007). The same procedures were performed for time course plating results, with the exception that only the data points presented were utilized for treatment mean calculations. Data were analyzed using JMP 8.0 (SAS Institute, Cary, NC) using $\alpha = 0.05$.

3. Results and Discussion

3.1 Data Analysis and Statistical Procedures

Effects of PSE treatments were compared by T-K HSD at selected time periods. This is the most statistically conservative of the multiple comparison statistical tests. Therefore, the type I error is minimized. However, there are some experimental variables that must be recognized before drawing statistical conclusions. Differences between treatments during lag phase growth may be due to experimental conditions unrelated to growth. For OD measurements, a slight color change was observed upon addition of PSE to the treatments. This color change is likely responsible for the differences observed for OD measurements based upon level of addition of PSE. These practical differences are unimportant in relation to yeast growth inhibition. As yeast growth occurred, statistical differences became more pronounced and standard errors became larger, as would be expected for microbial growth. For time course plating studies, lag phase differences for group statistics may be related to low standard errors due to analysis of log transformed data. For these reasons, only logarithmic and stationary phase growth will be discussed as statistically different in respect to yeast growth as a result of treatment effects.

Curve fitting analysis was performed as another procedure for statistical comparison. Curve fitting data has been used to make sense of OD growth curves previously (Lambert, 1998).

Both parametric and non-parametric statistical data is presented. The data follows the general pattern that if the correlation between control and treatment is high, then the correlation coefficient or Spearman's ρ will be close to 1. Therefore treatments exhibiting yeast inhibitory action exhibit correlation coefficients diverging from 1. Inhibition (%) is provided as a general illustration for examining the overall treatment effects irrespective of time.

3.2 Selection of Different Types of Growth Media

Different types of growth media were selected to investigate organism growth and the effects of PSE in a particular type of medium. Sabouraud Dextrose broth has traditionally been used as a growth medium for yeasts and molds. The availability of a vast amount of D-glucose allows yeasts to reach the stationary phase quickly in this media (Table 1 & 3). Sabouraud Maltose broth was chosen to examine the possibility that PSE could interfere with the yeast's ability to utilize maltose as their carbon source. It was hypothesized that PSE may interfere with either yeast membrane transport proteins or α-glucosidase. This hypothesis was based upon preliminary data on enzyme inhibition due to PSE (Sarnoski, unpublished results). The hypothesis may be true given the bi-phasic nature of growth under conditions of maltose as the carbon source with the addition of PSE (Fig. 1). One of the aims of this study was to examine the effect of PSE in a model beverage system. For this reason, the AJM was developed. This system is at a low pH (3.5) and consists of primarily D-fructose and D-glucose as the carbon sources. The growth rate was the slowest for all organisms in this media (Fig. 2). This is to be expected given the low pH of the media, along with less nutrient availability. *Z. bailii*, and *Z. bisporus* growth characteristics were similar to that of *S. cerevisiae* (Fig. 2). For this reason, most results are reported for *S. cerevisiae* only. A previous study has reported similar observations for these yeasts (Enrique et al., 2007). AJM was chosen for high concentration of

PSE (7 & 10 mg/mL) studies and fractionation studies due to excellent solubility of proanthocyanidins in this growth medium. The exceptional solubility of proanthocyanidins in this media is likely due to the modest amount of protein present in AJM. Treatment concentrations are likely most reflective of the true concentration in this media, since it likely some PSE was lost during filtration of Sabouraud Dextrose and Maltose media.

3.3 Effect of Whole PSE

The PSE shows some potential promise as a yeast inhibitor evidenced in its ability to either extend the lag phase of *S. cerevisiae* (Fig 2) at low concentrations or flat line growth at 10 mg/mL (Table 2 & 4). Concentrations lower than 1 mg/mL of PSE were also tested, but these treatments had little effect on OD or plate counts with respect to the control. An interesting aside was the result of filtering the protein-precipitate and its effects on growth (Table 3). It is probable that the yeast may have grown better in the filtered media due to less proanthocyanidin presence due to its precipitation with protein. Another possibility is that the proanthocyanidins were effective as an inhibitor even as a protein-proanthocyanidin complex (4 mg/mL non-filtered). The whole extract was analyzed first to determine if PSE had anti-yeast capabilities. Once it was determined that PSE did have these capabilities, the extract was fractionated to determine specific compounds associated with anti-yeast activity

3.4 Effect of Fractionated PSE

Normal phase (HPLC) was used in to fractionate proanthocyanidins according to degree of polymerization. It was hypothesized that the compounds responsible for anti-yeast activity could be isolated and identified by fractionation. Our results suggest that fraction B, D, and E contain the compounds most responsible for yeast inhibition. The main constituents of these fractions were identified as procyanidin A-type dimers, trimers and tetramers, respectively

(Chapter 3). For the sake of brevity, the full characterization of the compounds contained in fractions B, D, and E are not discussed in this study. Fraction B had the most yeast inhibitory effect of the three fractions. This might be due to the compounds contained in fraction B ability to permeate through a yeast cell wall due to their relatively low molecular weight. It has been previously reported that the cell wall of *Saccharomyces cerevisiae* is freely permeable for solutes smaller than 600 Da (Van Der Rest et al., 1995).

Given the molecular diversity of these molecules using current analytical separation techniques makes it difficult to pinpoint a specific compound responsible for the yeast inhibition, since the fractions were not composed of single compounds. Currently, there is no chromatographic technique available that can easily separate such complex mixtures. Reed (2009) has identified almost 300 different phenolic compounds in peanut skin extracts. For these reasons, it can only be concluded by this study that these molecules are resulting in additive effects, synergistic effects, or one compound is responsible for yeast inhibition in the active fractions. The true answer to this question is not currently known.

In order to determine if the OD values were accurate, the normal phase fractions were dissolved in AJM, inoculated with yeast, and plated at timed increments. Again, fractions B, D, and E showed the most anti-yeast activity, albeit at a lower capacity compared to the OD measurement. The reasons for overestimation of OD measurements are discussed in the next section.

3.5 Time Course Plating Studies vs OD

There is some discrepancy in the results of the OD measurements and plate counts. The OD measurements present an overestimation of the inhibitory activity in respect to plate counts (Table 1-5). Lambert and co-workers (1998) observed this same occurrence and hypothesized

that the OD (Bioscreen) method does not account for injured cells the same as a plating method, thus leading to an overestimation of the results. In a subsequent study, Lambert concluded that increasing the concentration of test compounds or time of exposure led to a reduction in colony size for *Staphylococcus aureus* cells (Lambert and Ouderaa, 1999). It was suggested in this study that the smaller colonies originated from injured cells that needed to recover before multiplying, and therefore did not have as much time to reach the size of healthy colonies. As expected, smaller colonies will affect optical density less than larger, healthy colonies. Similarly, smaller colony sizes were given as the explanation for inhibition of *S. cerevisiae* when exposed to essential oils as a stressor (Conner and Beuchat, 1984). Cell injury due to the addition of PSE extract is given as a likely reason for the discrepancy between the OD and plate count measurements in this study. However, without microscopy data, cell clumping cannot be ruled out as the cause of the OD differences. Therefore an OD measurement is likely a useful procedure for screening for inhibitory activity, but it is not an appropriate method to determine the quantitative aspects of inhibition.

3.6 PossibleMechanisms of PSE as an Anti-Yeast Agent

The PSE could be acting as a yeast inhibitory agent through a variety of mechanisms, either synergistically or not. One of the explanations for yeast inhibition due to tannins (proanthocyanidins) is iron deprivation (Wauters et al., 2001). It was found in this study that growth medium supplementation with more nitrogen or metal ions showed that only iron ions could restore the maximal growth rate of *S. cerevisiae*. Tannnins are known transition metal chelators (Malesev and Kuntic, 2007; Rice-Evans et al., 1997). Therefore PSE inhbition of yeast growth could be related to chelation.

Tiwari and others (2009) list phenolic compounds as affecting enzyme activity, protein denaturation, cell permeability, interference with membrane function, and interaction with membrane proteins as mechanisms of antimicrobial action. Specifically, interference with membrane function can include hindrance with electron transport, nutrient uptake, protein, nucleic acid synthesis, and enzyme activity. Shan and co-workers (2008) observed extensive cell damage in pathogenic bacteria treated with proanthocyanidins. Scanning electron microscope observations of the bacteria showed deformation of the cells, and a slight decrease in the cell size for those cells exposed to proanthocyanidins. It was hypothesized that the active components of the extract may bind to the cell surface and then penetrate to the target sites, possibly the phospholipid bilayer of the cytoplasmic membrane and membrane bound enzymes. The effects could lead to inhibition of proton motive force, inhibition of the respiratory chain and electron transfer, and inhibition of substrate oxidation. This could result in the uncoupling of oxidative phosphorylation, inhibition of active transport, loss of pool metabolites, and disruption of synthesis of DNA, RNA, protein, lipid, and polysaccharides leading to cell injury or death (Shan et al., 2008).

Through computational analysis it has been ascertained that in procyanidin B-type dimers, the two (epi)catechin monomers can interact with each other, leading to a folded and packed molecule (Mackenzie et al., 2004). In contrast, the rigidity conferred by A-type dimers due to two bonds between monomers results in a more extended conformation. Thus the A-type dimers may be able to penetrate further into a bilayer surface. This property of A-type procyanidins was supported when A-type dimers (isolated from peanut skins) were determined to have more phospholipid interaction over B-type dimers (Verstraeten et al., 2005). This ability means that these compounds can interact with lipid membranes and thus can modulate

membrane fluidity. The alteration of membrane fluidity can per se affect numerous cellular processes influencing functionality of membrane-associated enzymes, intracellular transport mechanisms, and membrane receptors (Verstraeten et al., 2005).

The necessity of a rigid cell wall for yeasts is a necessity for their survival. A strategy was developed to inhibit *S. cerevisiae* and *Z. bailii* by altering cell wall structure with a combination of antimicrobial peptide and β-1,6-glucan oligosaccharides that would lead to an impaired construction of a normal glycoslyphophatidylinositol dependent cell wall (Bom et al., 2001). The resulting cell walls were the more susceptible to the action of membrane-active agents, which resulted in total inhibition or slowed growth dependent on concentration and agent. It is therefore feasible that PSE may best be employed as hurdle technology for the food and beverage industry. The PSE added at levels in the μg/mL range might work to damage the cells of spoilage yeast, which would make them more susceptible to attack by current food preservatives used in lower concentrations than are currently practiced. Currently the level of PSE needed to completely inhibit yeast growth is likely too high to incorporate into a food beverage system without affecting the sensory properties of the beverage or food.

4. Conclusions

The time course plating procedure results agree with previous studies indicating OD measurements may lead to an overestimation of antimicrobial susceptibility. The PSE did inhibit yeast growth. However, the high concentrations needed to inhibit growth may be too high for use in the food and beverage industry. For this reason, more research needs to be preformed to determine if one compound is responsible for the inhibition and its minimum inhibitory concentration, or if multiple compounds are active. Currently no chromatographic technique has

been able to separate proanthocyanidins with the degree of complexity as identified in fraction E. In order to rule out synergistic effects, a pure compound would need to be tested.

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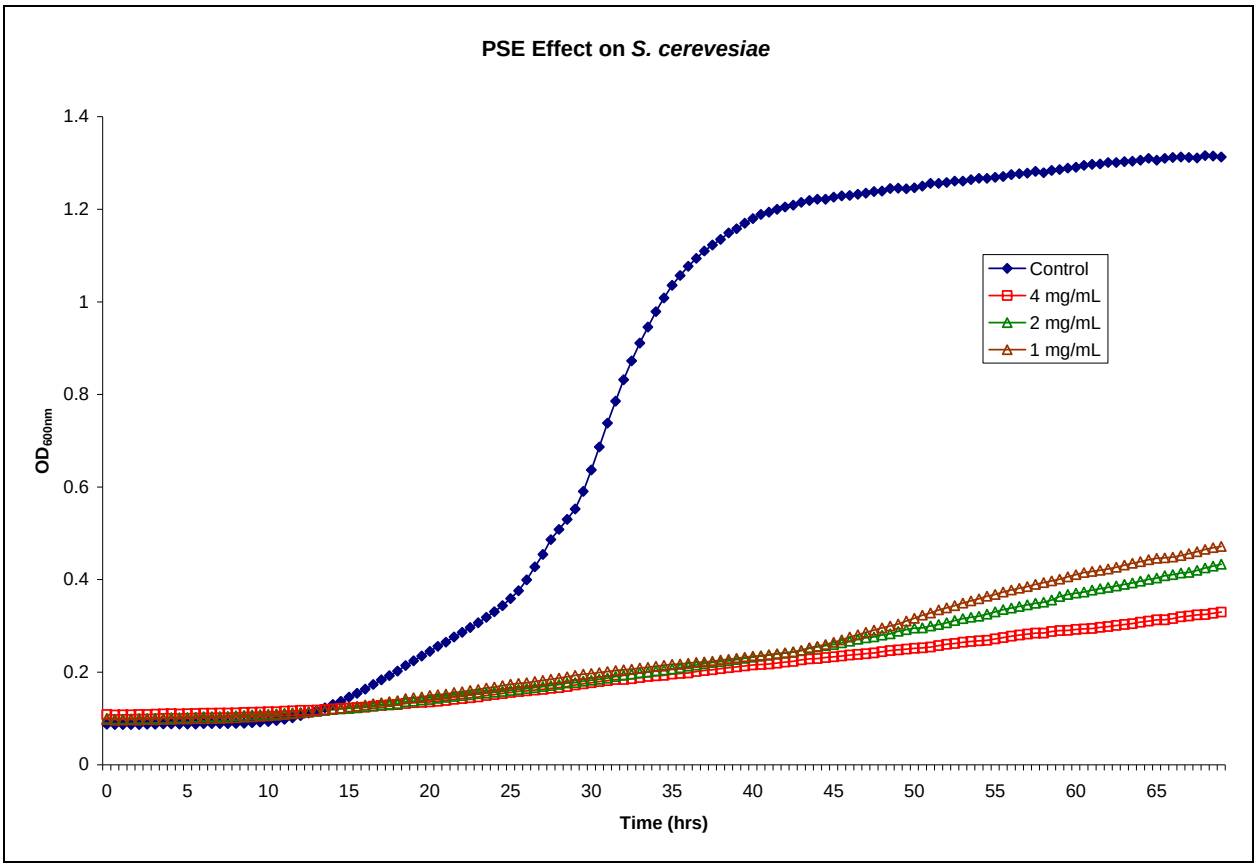

Fig. 1. PSE effect on *S. cerevesiae* in Sabouraud Maltose media over 72 hrs. The control contained no PSE.

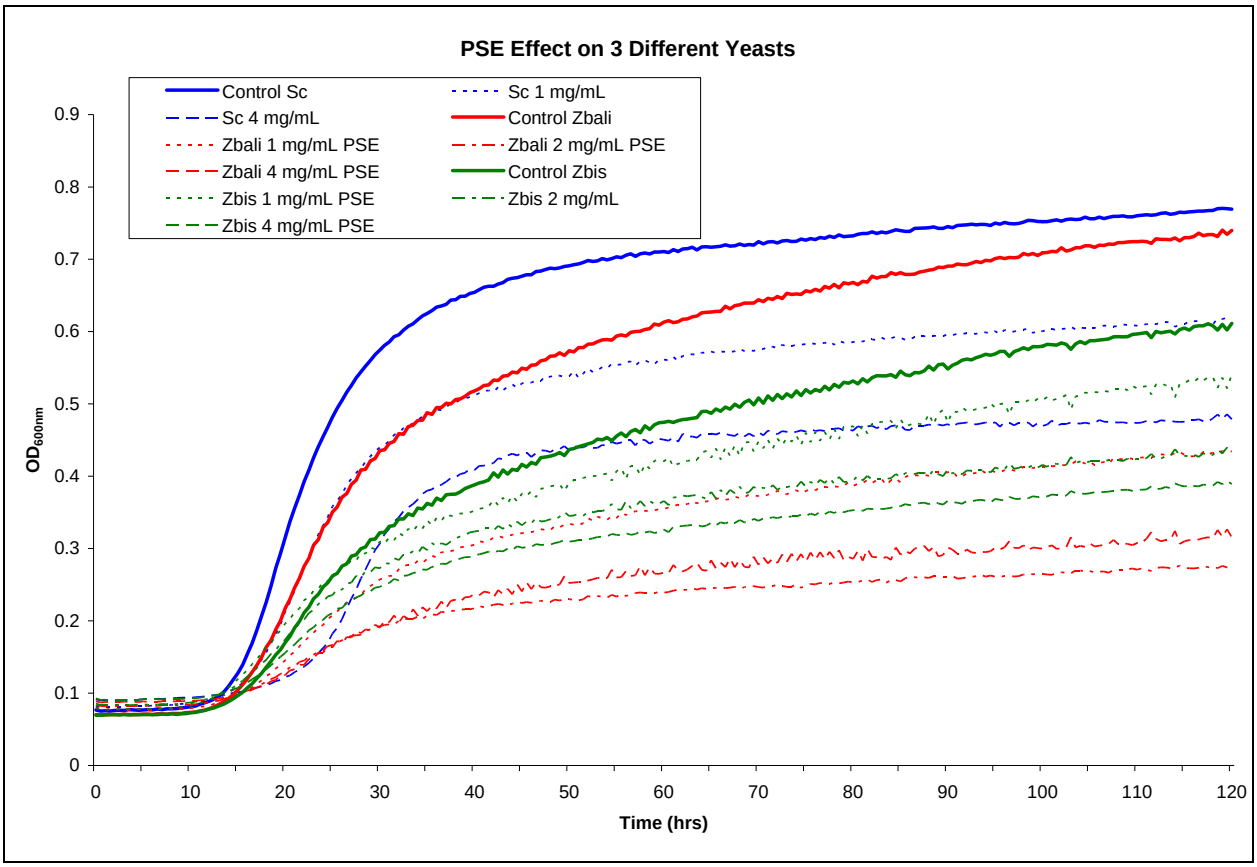

Fig. 2. Effect of PSE on different yeast organisms in Apple Juice media. Organisms are color coded.

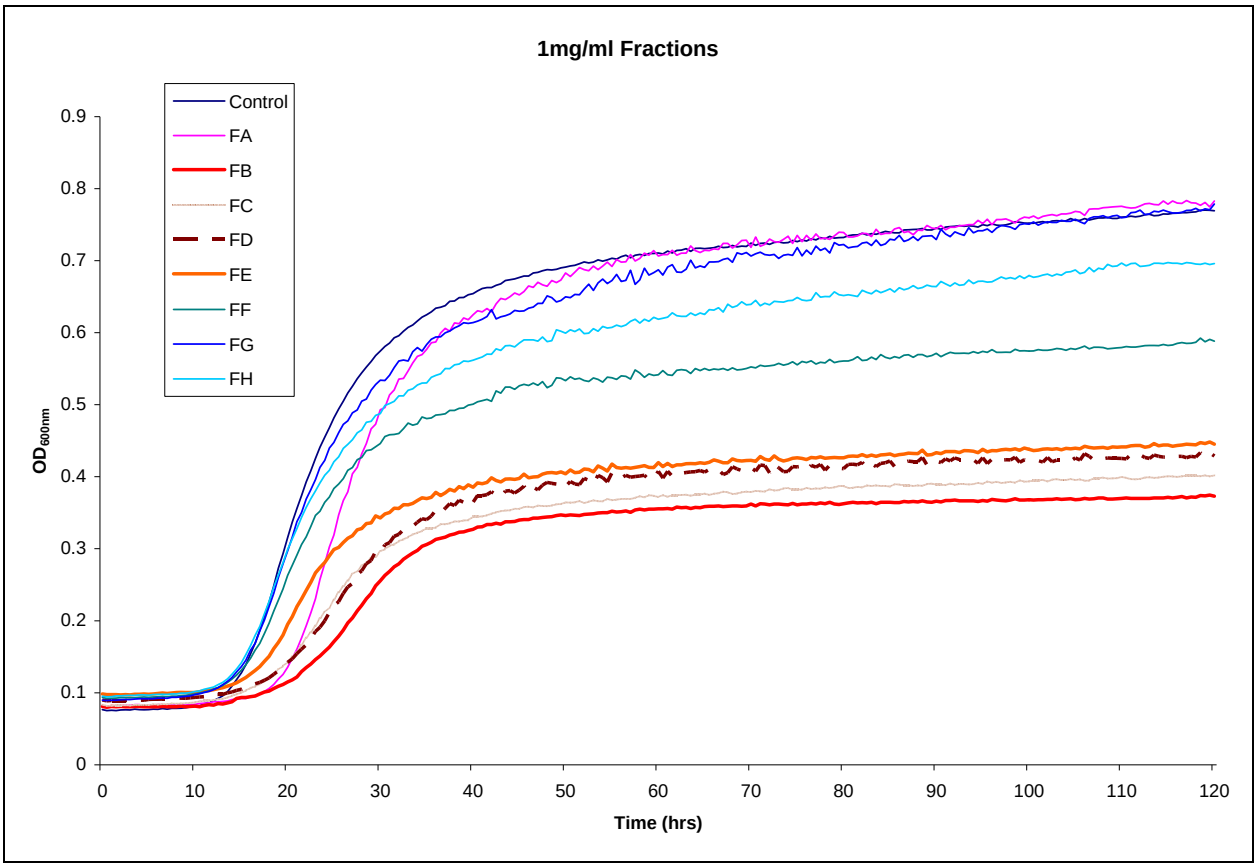

Fig. 3. Effect of 1 mg/mL fractions of PSE on *S. cerevesiae* in Apple Juice media. Abbreviations: Fraction A (FA), Fraction B (FB) etc.

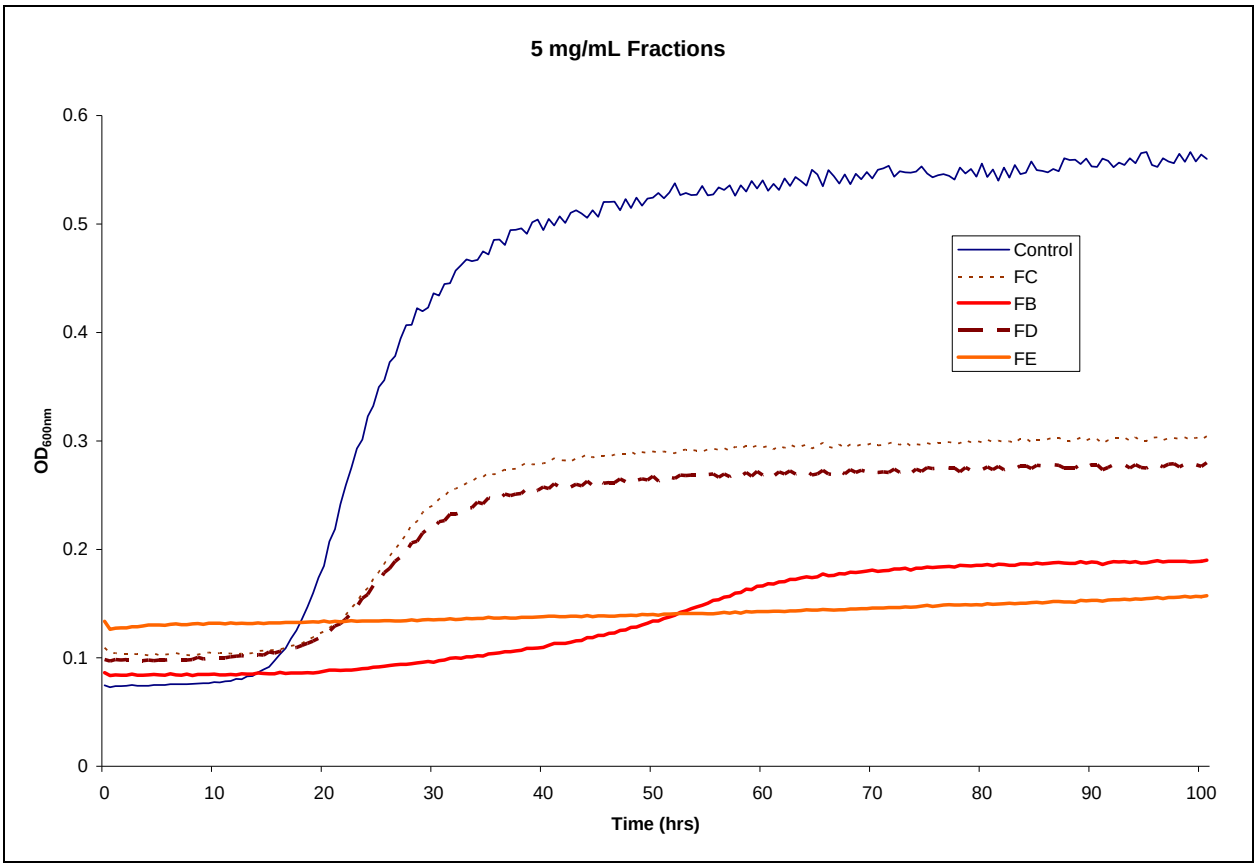

Fig 4. Effect of 5 mg/mL fractions of PSE on *S. cerevesiae* in Apple Juice media.

Table 1
Effect of PSE on *S. cerevesiae* in Dextrose media as a function of OD at 600 nm.

Time (hr)	Control (0 ppm)	1 mg/mL	2 mg/mL	4 mg/mL
0	$0.08_{b} \pm <0.01$	$0.10_{a} \pm 0.01$	$0.10_{a} \pm <0.01$	$0.11_{a} \pm <0.01$
1	$0.08_{c} \pm <0.01$	$0.09_{b} \pm <0.01$	$0.10_{ab} \pm <0.01$	$0.10_{a} \pm <0.01$
3	$0.08_{c} \pm <0.01$	$0.09_{b} \pm <0.01$	$0.10_{ab} \pm <0.01$	$0.10_{a} \pm <0.01$
8	$0.08_{c} \pm <0.01$	$0.09_{b} \pm <0.01$	$0.10_{b} \pm <0.01$	$0.11_{a} \pm <0.01$
18	$0.09_{a} \pm 0.01$	$0.12_{a} \pm 0.02$	$0.10_{a} \pm <0.01$	$0.12_{a} \pm 0.01$
24	$0.19_{a} \pm 0.10$	$0.33_{b} \pm 0.09$	$0.11_{b} \pm 0.01$	$0.13_{c} \pm 0.01$
30	$0.85_{ab} \pm 0.62$	$1.4_{a} \pm 0.08$	$0.11_{b} \pm 0.01$	$0.14_{b} \pm 0.01$
48	$1.4_{a} \pm 0.49$	$1.8_{a} \pm 0.03$	$0.29_{b} \pm 0.19$	$0.18_{b} \pm 0.01$
72	$1.7_{b} \pm 0.11$	$1.9_{a} \pm 0.02$	$1.8_{ab} \pm 0.03$	$0.25_{c} \pm 0.02$
Multivariate Statistics				
Correlation Coeff with Control	1	0.999	0.579	0.854
Spearman's ρ with Control	1	0.989	0.995	0.994
Inhibition (%)	0	- 7.41	49.1	85.2

Inhibition (%) calculated from overall treatment means.
Mean values with the same letter in a row indicate no significant differences.
Abbreviations: Coefficient (Coeff).

Table 2. Effect of PSE on *S. cerevesiae* in Apple Juice Media as a function of OD at 600 nm.

Time (hr)	Control (0 ppm)	1 mg/mL	4.5 mg/mL	7 mg/mL	10 mg/mL
0	$0.08_{d} \pm <0.01$	$0.08_{d} \pm <0.01$	$0.09_{c} \pm <0.01$	$0.11_{b} \pm <0.01$	$0.15_{a} \pm 0.01$
1	$0.08_{d} \pm <0.01$	$0.08_{d} \pm <0.01$	$0.09_{c} \pm <0.01$	$0.11_{b} \pm <0.01$	$0.12_{a} \pm <0.01$
3	$0.08_{d} \pm <0.01$	$0.08_{d} \pm <0.01$	$0.90_{c} \pm <0.01$	$0.11_{b} \pm <0.01$	$0.12_{a} \pm <0.01$
5	$0.08_{d} \pm <0.01$	$0.08_{d} \pm <0.01$	$0.09_{c} \pm <0.01$	$0.11_{b} \pm <0.01$	$0.12_{a} \pm <0.01$
18	$0.23_{a} \pm 0.02$	$0.16_{b} \pm 0.01$	$0.11_{c} \pm <0.01$	$0.11_{c} \pm <0.01$	$0.12_{bc} \pm <0.01$
24	$0.46_{a} \pm 0.05$	$0.33_{b} \pm 0.04$	$0.16_{c} \pm 0.01$	$0.12_{c} \pm <0.01$	$0.13_{c} \pm <0.01$
68	$0.72_{a} \pm 0.04$	$0.58_{b} \pm 0.04$	$0.46_{b} \pm 0.02$	$0.14_{c} \pm <0.01$	$0.14_{c} \pm <0.01$
72	$0.72_{a} \pm 0.04$	$0.58_{b} \pm 0.04$	$0.46_{b} \pm 0.02$	$0.15_{c} \pm <0.01$	$0.14_{c} \pm <0.01$
98	$0.75_{a} \pm 0.04$	$0.60_{b} \pm 0.04$	$0.47_{b} \pm 0.02$	$0.22_{c} \pm 0.03$	$0.15_{c} \pm <0.01$
102	$0.75_{a} \pm 0.04$	$0.60_{b} \pm 0.04$	$0.47_{b} \pm 0.02$	$0.23_{c} \pm 0.03$	$0.15_{c} \pm <0.01$
120	$0.77_{a} \pm 0.05$	$0.62_{ab} \pm 0.04$	$0.48_{b} \pm 0.02$	$0.27_{c} \pm 0.03$	$0.15_{c} \pm 0.01$
Multivariate Statistics					
Correlation Coeff with Control	1	0.999	0.999	0.915	0.872
Spearman's ρ with Control	1	0.999	0.999	0.998	0.958
Inhibition (%)	0	24.4	39.0	70.7	68.3

Inhibition (%) calculated from overall treatment means.
Mean values with the same letter in a row indicate no significant differences.

Table 3

Effect of PSE on *S. cerevesiae* in Dextrose media expressed as log CFU/g.

Time (hrs)	Control (0 mg/mL)	4 mg/mL filtered	4 mg/mL non-filtered
0	$1.9_{a} \pm 0.02$	$1.9_{a} \pm 0.01$	$1.8_{b} \pm 0.02$
1	$1.9_{a} \pm 0.03$	$2.0_{a} \pm 0.02$	$1.9_{a} \pm 0.06$
3	$2.2_{b} \pm 0.02$	$3.1_{a} \pm 0.01$	$2.0_{c} \pm 0.01$
8	$2.8_{a} \pm 0.08$	$2.5_{b} \pm 0.03$	$2.2_{c} \pm 0.02$
18	$5.6_{a} \pm 0.05$	$4.6_{ab} \pm 0.16$	$4.1_{b} \pm 0.39$
24	$6.2_{a} \pm 0.06$	$5.6_{b} \pm 0.14$	$4.4_{c} \pm 0.10$
30	$6.9_{a} \pm 0.07$	$6.6_{a} \pm 0.08$	$4.4_{b} \pm 0.10$
48	$7.6_{a} \pm 0.02$	$7.6_{a} \pm 0.05$	$6.3_{b} \pm 0.04$
Multivariate Statistics			
% Inhibition	0	5.67	22.02
Correlation Coeff with Control	1	0.991	0.972
Spearman's ρ	1	1	0.994

Inhibition (%) calculated from overall group means.

Mean values with the same letter in a row indicate no significant differences.

Table 4
Effect of PSE on *S. cerevesiae* in Apple Juice Media expressed as log CFU/g.

Time (hrs)	Control (0 mg/mL)	1 mg/mL	4 mg/mL	10 mg/mL
0	$1.4_b \pm 0.05$	n.d.	$1.4_b \pm 0.01$	$2.0_a \pm 0.02$
1	$1.7_b \pm 0.01$	n.d.	$1.6_b \pm 0.03$	$2.1_a \pm 0.01$
3	$2.0_b \pm 0.01$	$2.1_a \pm 0.02$	$1.6_c \pm 0.02$	$2.0_b \pm <0.01$
5	$2.2_a \pm 0.02$	n.d.	$1.8_c \pm 0.05$	$1.9_b \pm 0.04$
18	$5.0_a \pm 0.06$	$4.6_b \pm 0.02$	$3.5_c \pm 0.07$	$2.1_d \pm 0.02$
24	$6.3_a \pm 0.07$	$5.4_b \pm 0.05$	$4.3_c \pm 0.11$	$2.0_d \pm 0.04$
68	$7.0_a \pm 0.06$	$7.0_a \pm 0.03$	n.d.	$2.4_b \pm 0.02$
72	$7.1_a \pm 0.02$	$7.2_a \pm 0.01$	$6.3_b \pm <0.01$	$2.1_c \pm 0.11$
98	$7.3_a \pm 0.02$	$6.9_c \pm 0.02$	$7.0_b \pm 0.02$	$2.7_d \pm 0.03$
102	$7.2_a \pm 0.06$	n.d.	$7.0_a \pm 0.01$	$2.1_b \pm 0.18$
120	$7.3_a \pm 0.02$	$7.2_a \pm 0.01$	$6.9_b \pm 0.04$	$2.0_c \pm 0.18$
Multivariate Statistics				
% Inhibition	0	-16.3	16.6	57.0
Correlation Coeff with Control	1	0.990	0.967	0.608
Spearman's ρ	1	0.786	0.976	0.664

% Inhibition calculated from overall group means.
Mean values with the same letter in a row indicate no significant differences.
n.d. signifies no data

Table 5
Effect of fractionated PSE and flavonoid standards on *Saccharomyces cerevesiae* in Apple Juice Media.

Treatment	OD_{600nm}				Log CFU/mL		
	0 hr	72 hr	120 hr	Inhibition (%)	0 hr	120 hr	Inhibition (%)
Control (0 ppm)	$0.08_{g} \pm <0.01$	$0.60_{b} \pm 0.02$	$0.64_{ab} \pm 0.02$	0	$2.7_{a} \pm 0.09$	$7.1_{cde} \pm 0.04$	0
FA 3 mg/mL	$0.10_{defg} \pm <0.01$	$0.46_{bcd} \pm 0.05$	$0.49_{bcd} \pm 0.06$	20.4	n.d.	n.d.	n.d.
FB 5 mg/mL	$0.10_{defg} \pm <0.01$	$0.11_{g} \pm <0.01$	$0.14_{f} \pm 0.01$	77.6	$2.8_{a} \pm 0.01$	$5.1_{h} \pm 0.03$	20.7
FC 3 mg/mL	$0.11_{def} \pm <0.01$	$0.42_{cde} \pm 0.05$	$0.44_{cde} \pm 0.05$	26.5	$2.7_{a} \pm 0.04$	$7.2_{bcde} \pm 0.02$	0.49
FD 7 mg/mL	$0.12_{d} \pm <0.01$	$0.15_{fg} \pm 0.01$	$0.18_{f} \pm 0.02$	71.4	$2.8_{a} \pm 0.08$	$6.2_{g} \pm 0.02$	9.54
FE 7 mg/mL	$0.12_{de} \pm <0.01$	$0.14_{fg} \pm <0.01$	$0.18_{f} \pm 0.01$	71.4	$2.8_{a} \pm 0.07$	$6.3_{g} \pm 0.03$	7.72
FF 5 mg/mL	$0.12_{de} \pm 0.01$	$0.36_{de} \pm 0.01$	$0.38_{de} \pm 0.01$	38.8	$2.8_{a} \pm 0.04$	$6.8_{f} \pm 0.05$	3.94
FG 3 mg/mL	$0.12_{de} \pm <0.01$	$0.29_{ef} \pm 0.04$	$0.30_{ef} \pm 0.04$	49.0	$2.7_{a} \pm 0.05$	$7.1_{de} \pm 0.02$	- 0.45
FH 3 mg/mL	$0.26_{b} \pm <0.01$	$0.41_{de} \pm 0.03$	$0.45_{cde} \pm 0.03$	26.5	$2.8_{a} \pm 0.06$	$7.1_{cde} \pm 0.02$	- 0.51
ProB2 1 mg/mL	$0.09_{fg} \pm <0.01$	$0.59_{bc} \pm 0.03$	$0.65_{ab} \pm 0.04$	0	$2.8_{a} \pm 0.01$	$7.3_{abc} \pm 0.05$	- 1.03
epicat 1 mg/mL	$0.08_{fg} \pm <0.01$	$0.48_{bcd} \pm 0.02$	$0.51_{bcd} \pm 0.02$	18.4	$2.8_{a} \pm 0.02$	$7.2_{abcd} \pm 0.01$	- 0.95
epicat (2) 1 mg/mL	$0.09_{fg} \pm <0.01$	$0.53_{bcd} \pm 0.01$	$0.56_{bc} \pm 0.01$	10.2	$2.7_{a} \pm 0.02$	$7.3_{ab} \pm 0.01$	- 1.69
(±)cat 1 mg/mL	$0.17_{c} \pm 0.01$	$0.58_{bc} \pm 0.06$	$0.63_{b} \pm 0.06$	0.0	$2.6_{a} \pm 0.03$	$7.3_{a} \pm 0.04$	- 1.00
(+)-cat 3 mg/mL	$0.09_{efg} \pm <0.01$	$0.40_{de} \pm 0.03$	$0.43_{cde} \pm 0.03$	30.6	$2.8_{a} \pm 0.04$	$7.0_{e} \pm 0.01$	0.73
Kaempferol 1 mg/mL	$0.34_{a} \pm 0.02$	$0.79_{a} \pm 0.03$	$0.82_{a} \pm 0.03$	-42.9	$2.8_{a} \pm 0.01$	$7.3_{ab} \pm 0.03$	- 2.49

% Inhibition calculated from overall treatment means.
Mean values with the same letter in a column indicate no significant differences.
Abbreviations: Procyanidin B2 (ProB2), (-)-epicatechin (epicat) and epicat (2) is a duplicate, (±)-catechin ((±)-cat), (+)-catechin ((+)-cat).
n.d. signifies no data

APPENDIX

Figure A.1. Peanut skin extract.

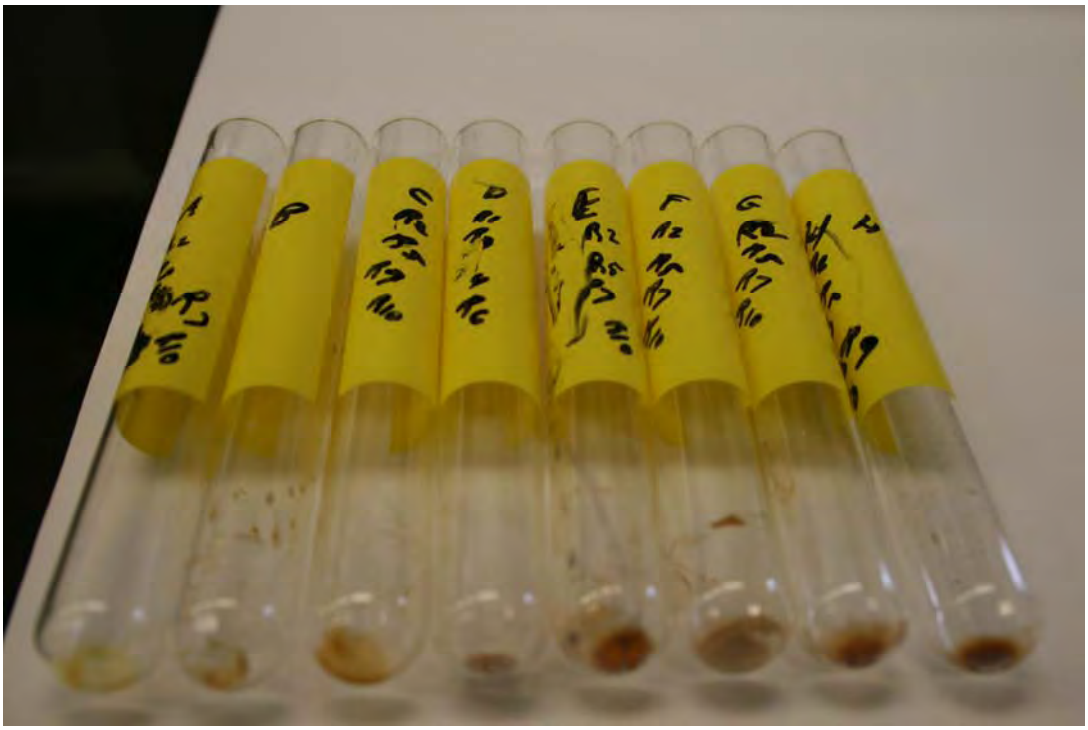

Figure A.2. Normal phase fractionated peanut skin extract.

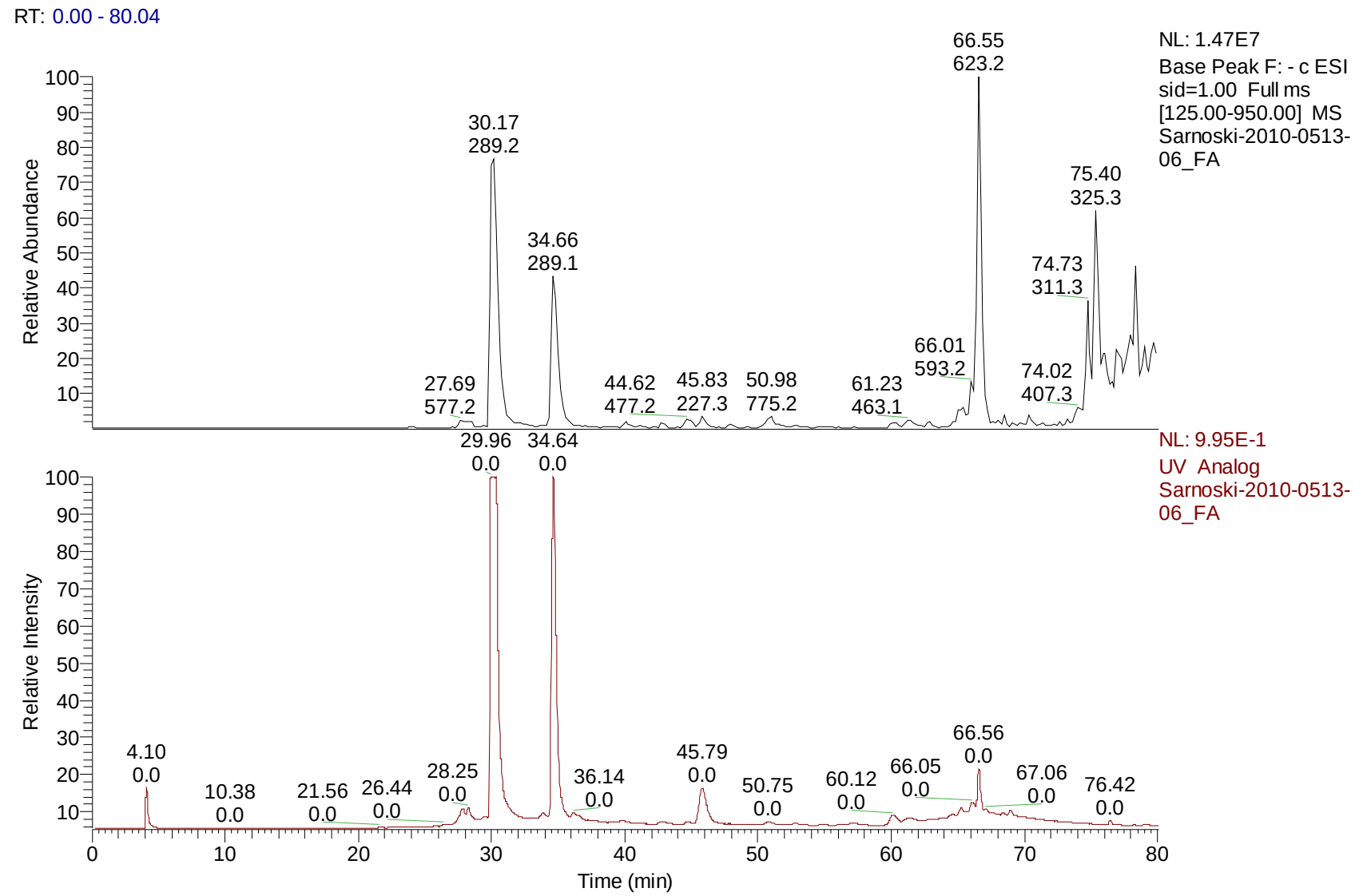

Figure A.3. MS and UV 280 nm spectra of normal phase fraction A.

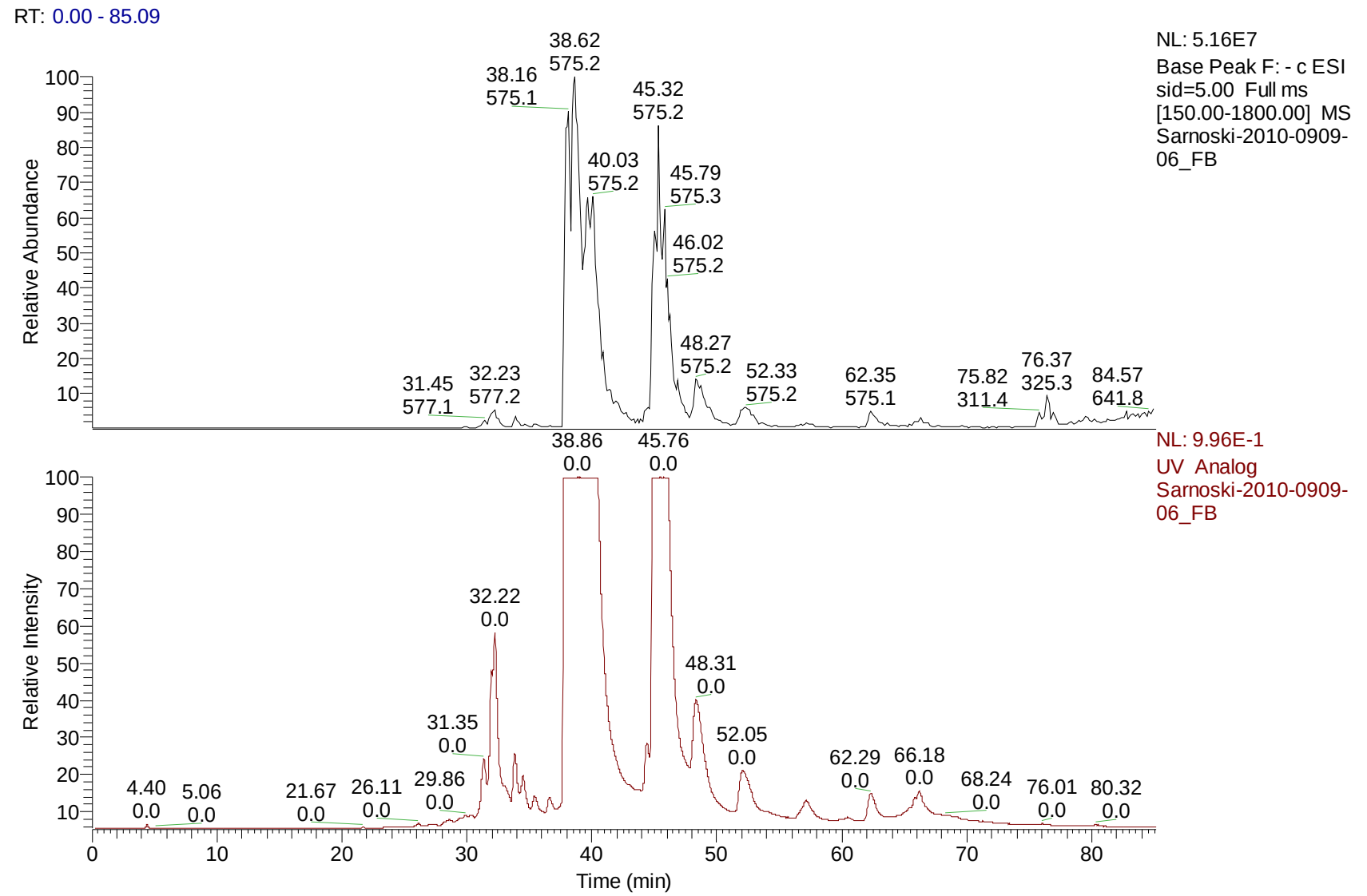

Figure A.4. MS and UV 280 nm spectra of normal phase fraction B.

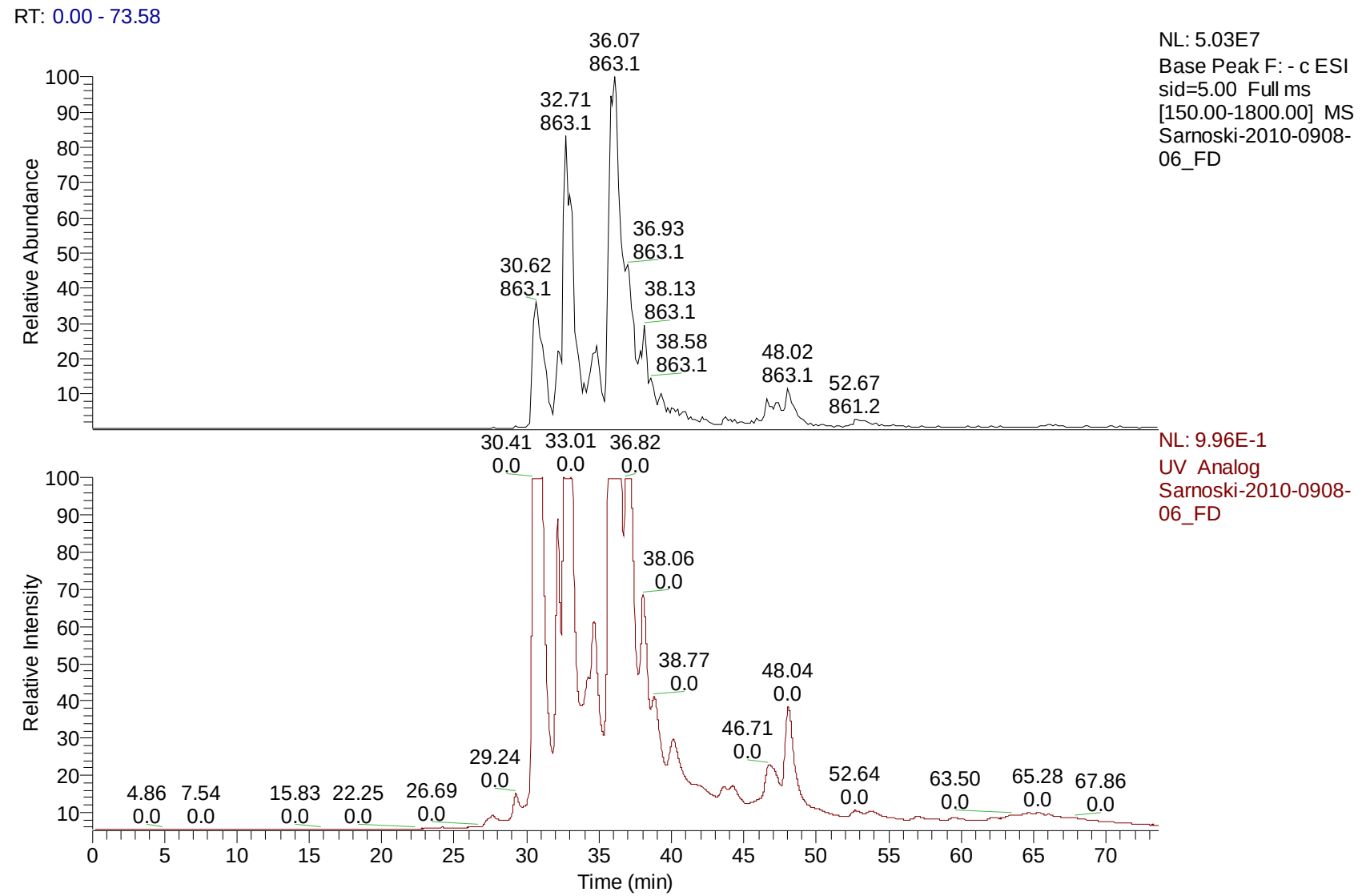

Figure A.5. MS and UV 280 nm spectra of normal phase fraction D.

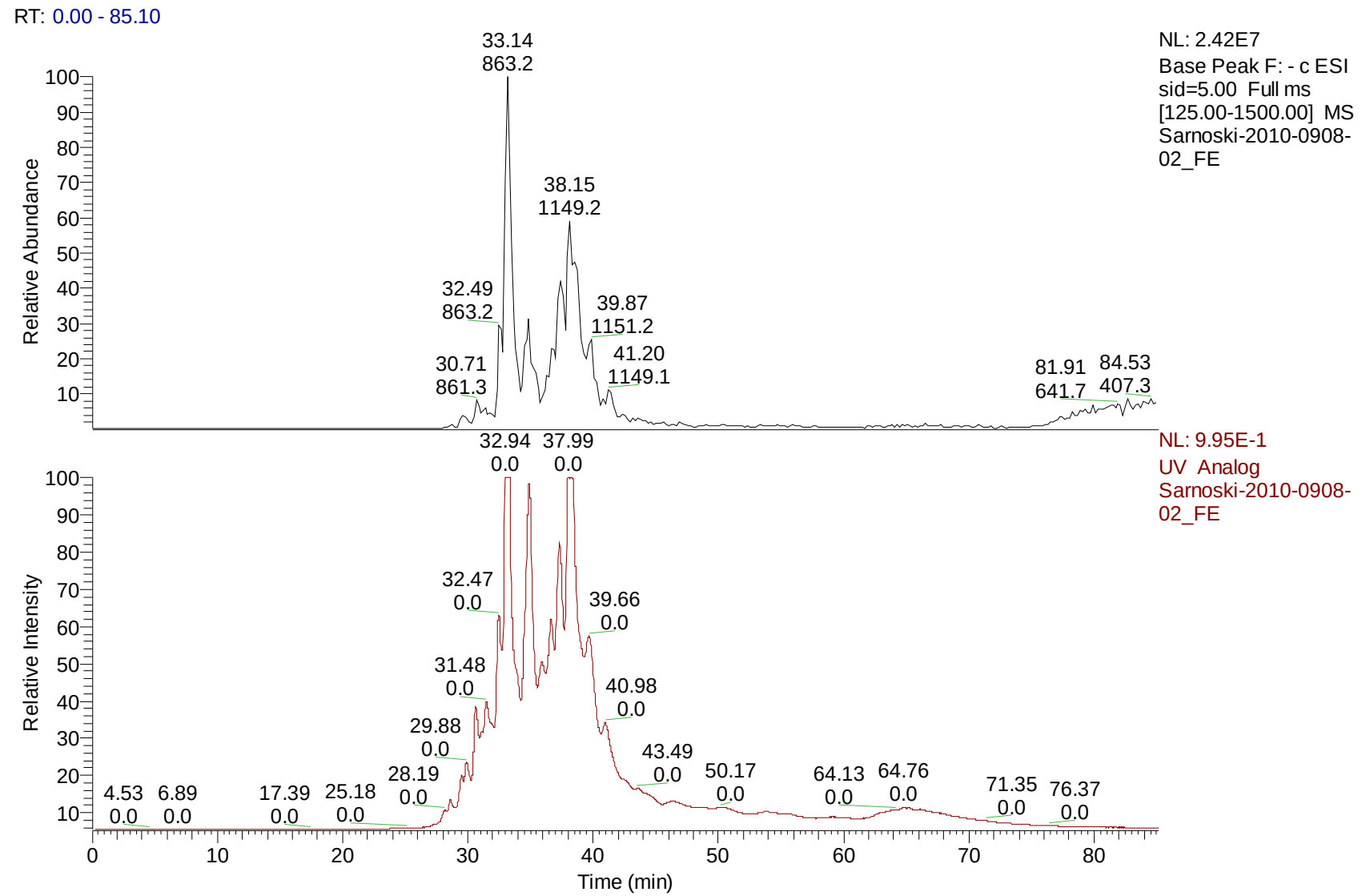

Figure A.6. MS and UV 280 nm spectra of normal phase fraction E.

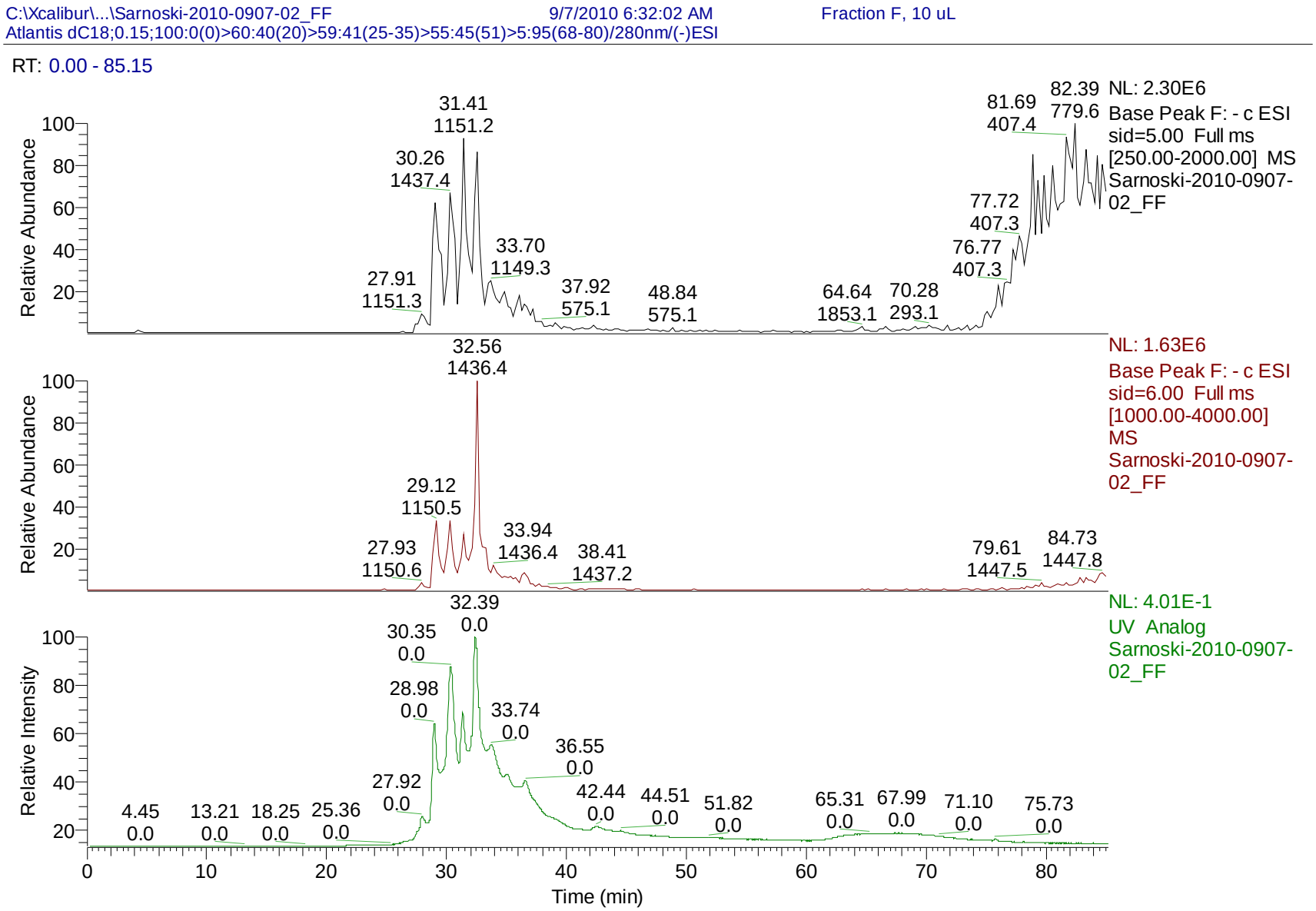

Figure A.7. MS and UV 280 nm spectra of normal phase fraction F.

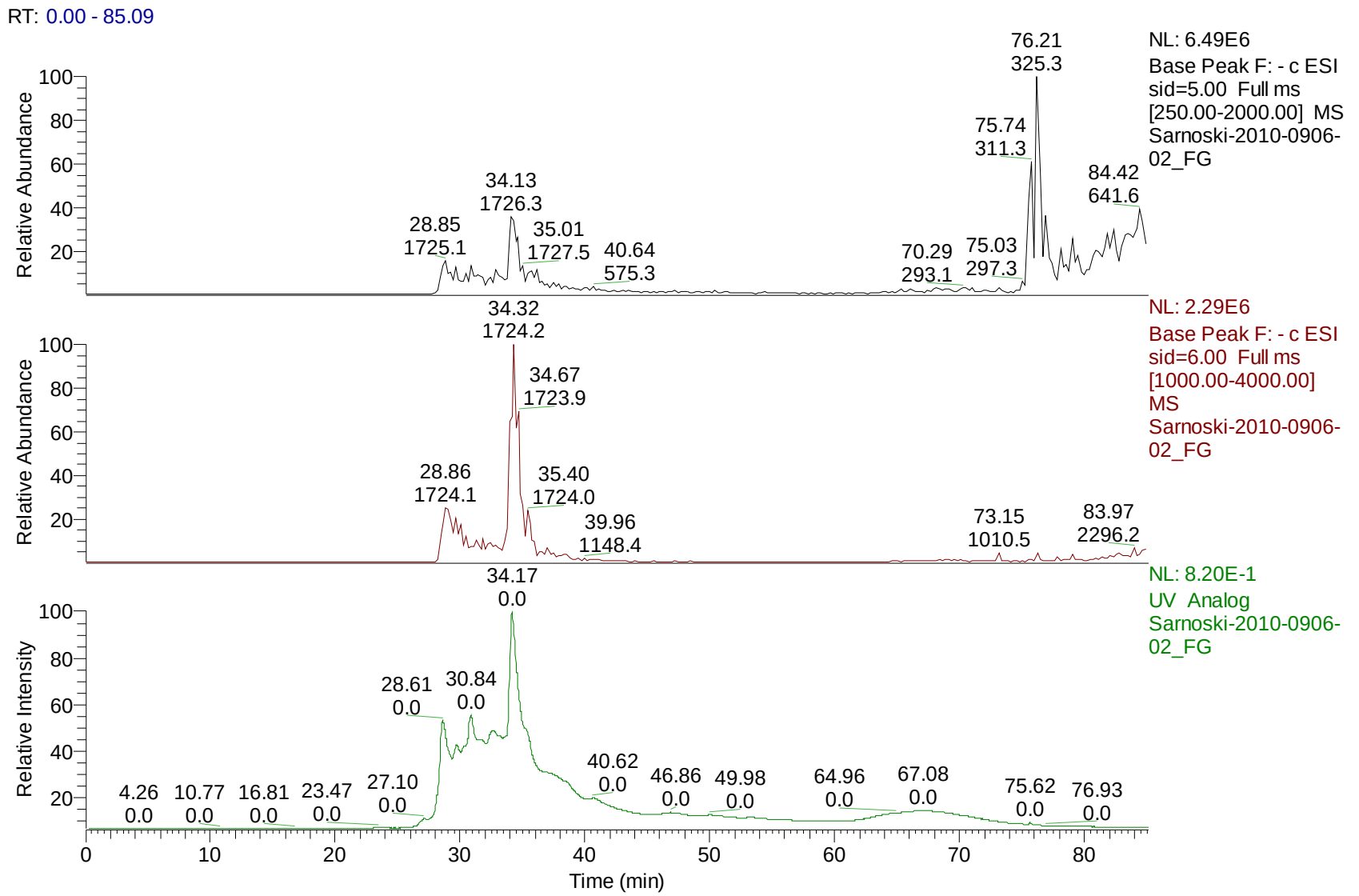

Figure A.8. MS and UV 280 nm spectra of normal phase fraction G.

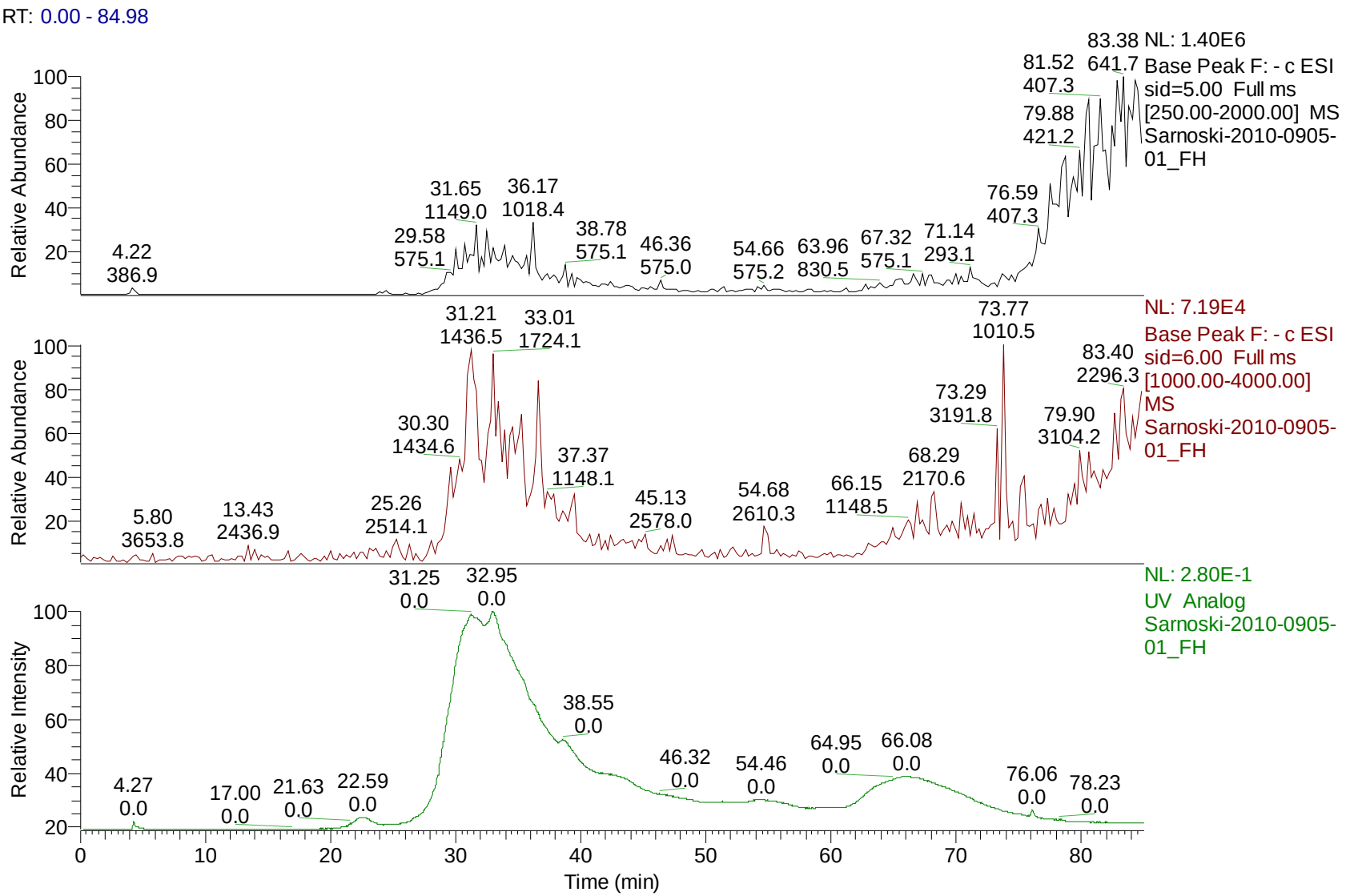

Figure A.9. MS and UV 280 nm spectra of normal phase fraction H.

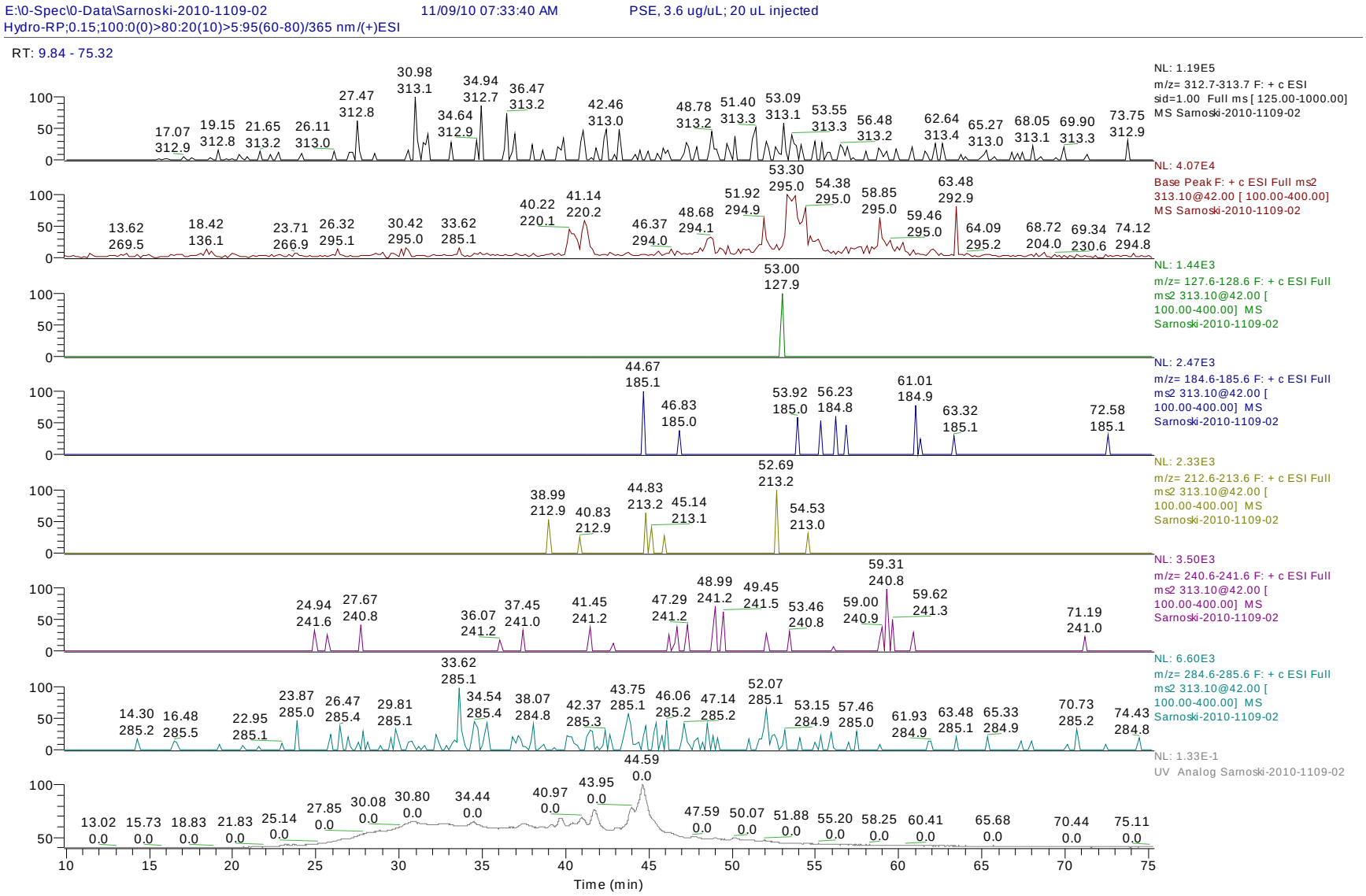

Figure A.10. Scans specific for aflatoxin B1. There were no specific ions that correlated with the detection of aflatoxin B1 in the PSE.

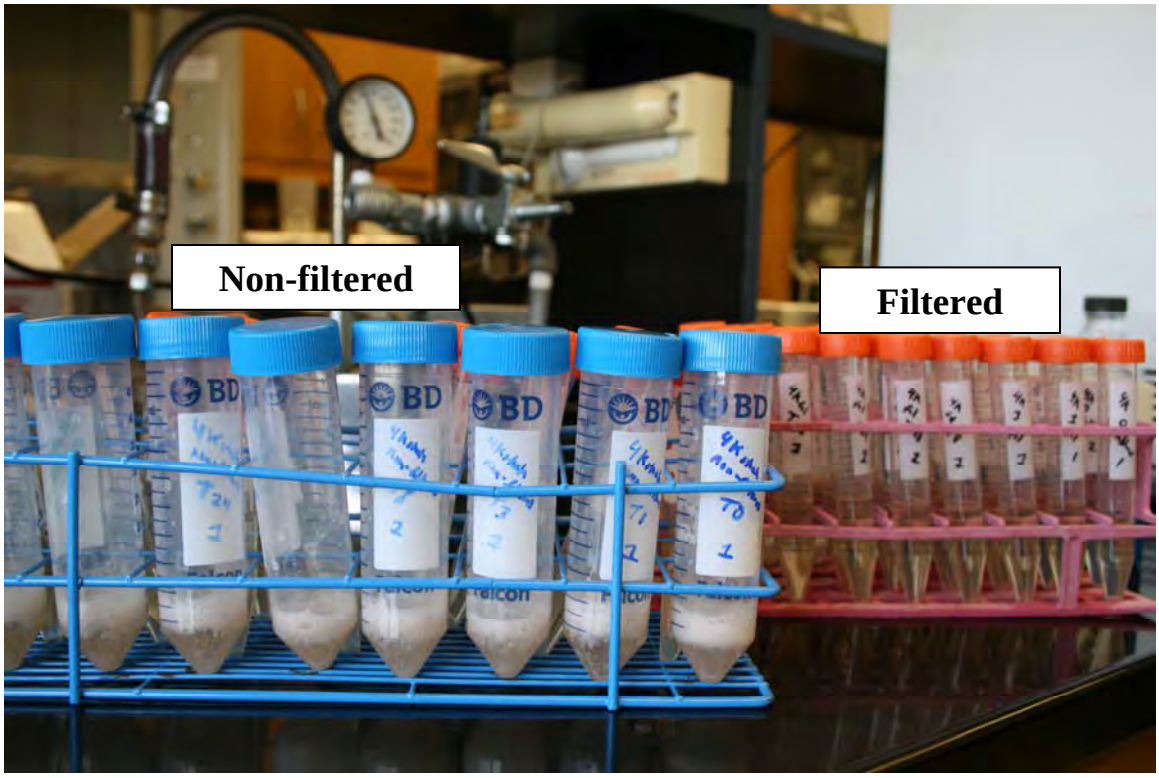

Figure A.11. Filtered and non-filtered PSE in Dextrose media.

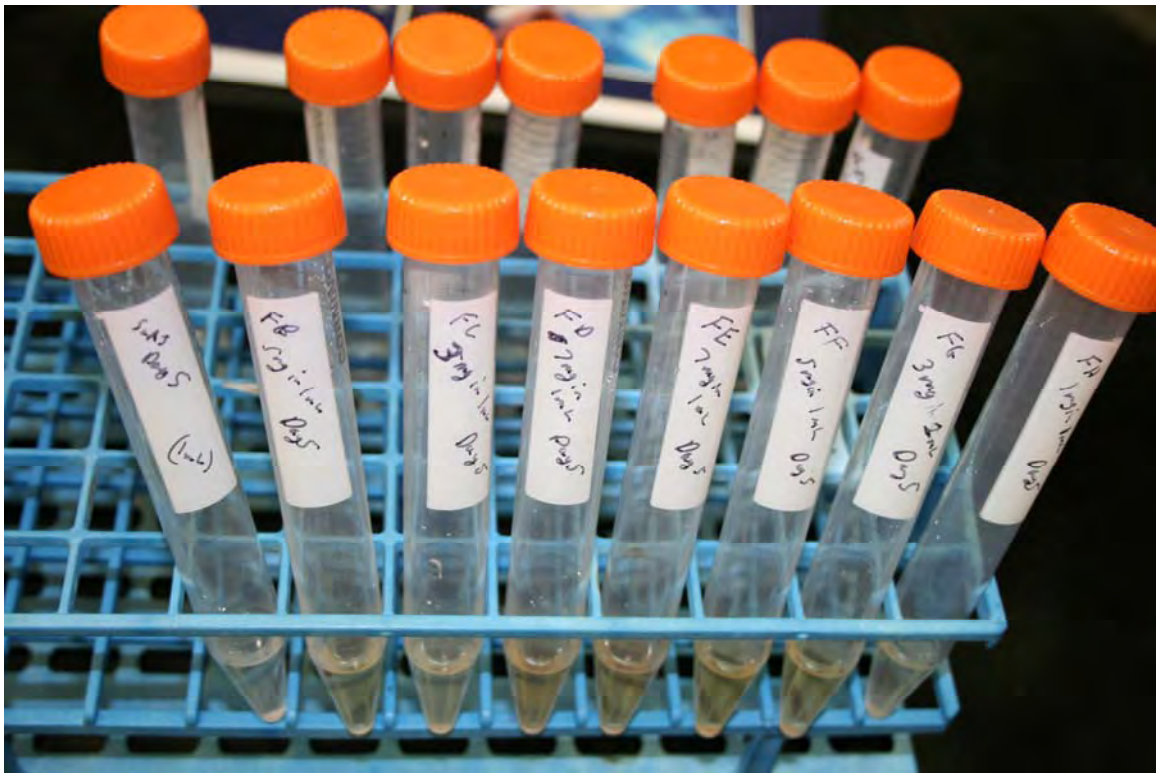

Figure A.12. PSE fractions in AJM.

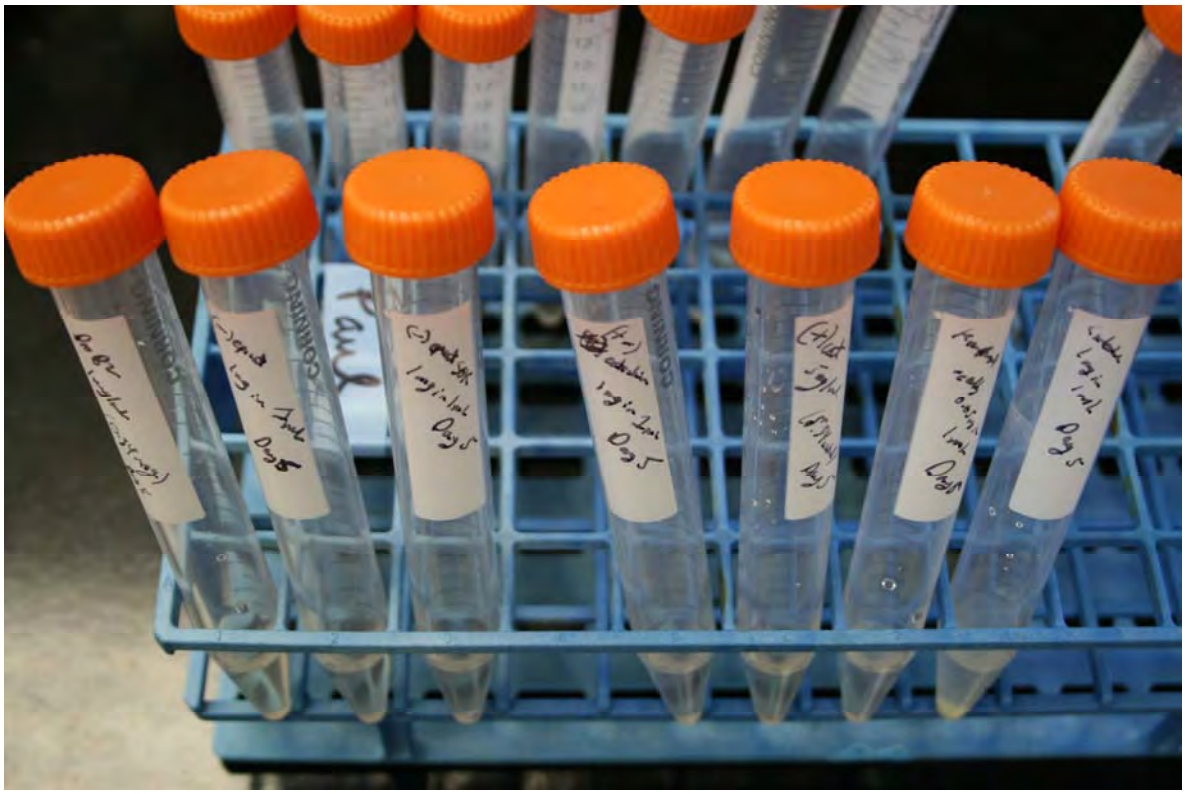

Figure A.13. Standards in AJM.

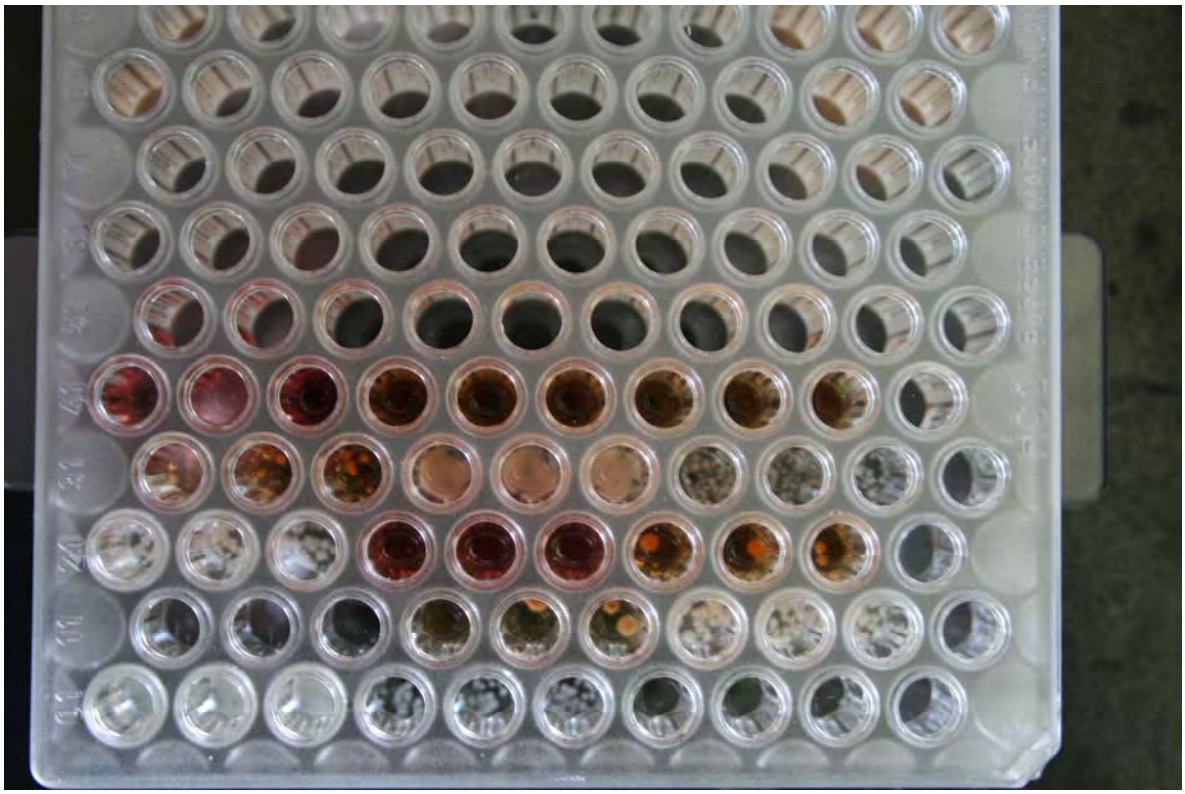

Figure A.14. Pomace extract (Purple) at different concentrations in bio-screen plate incubated for 120 hrs.

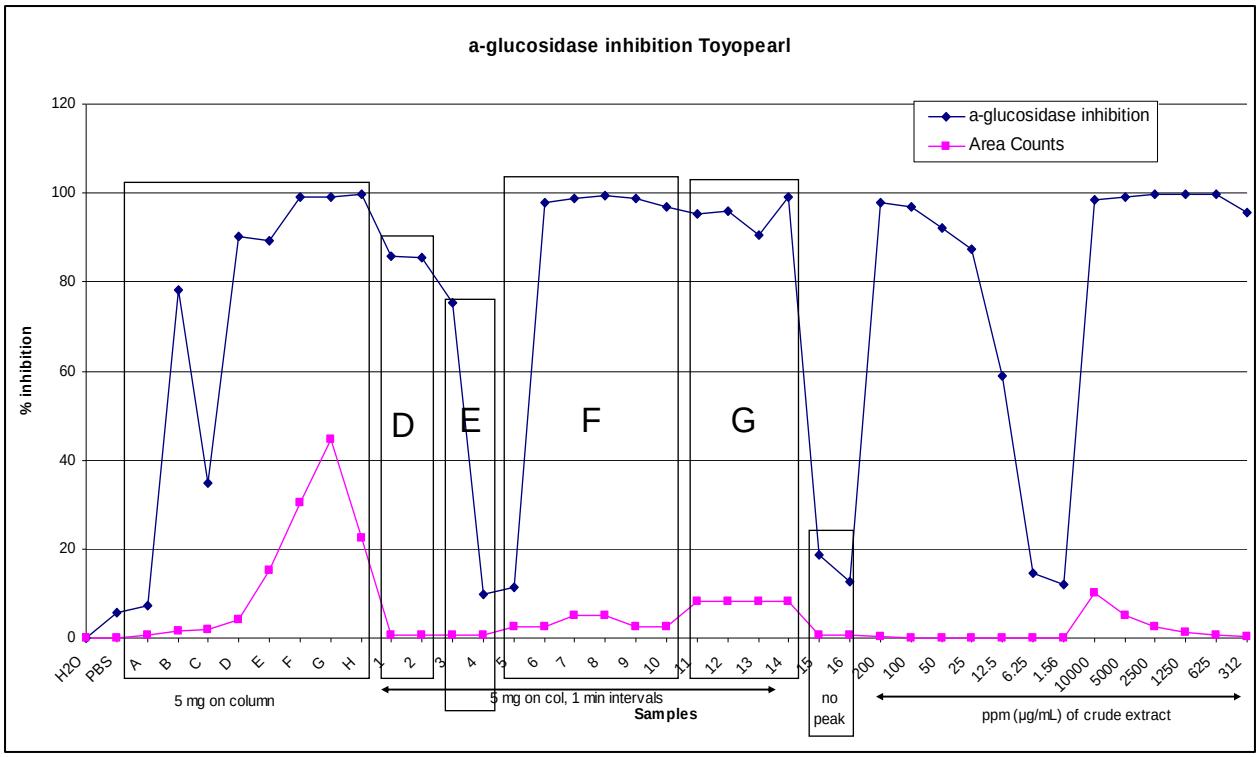

Figure A.15. SEC fractions of PSE α-glucosidase inhibition.

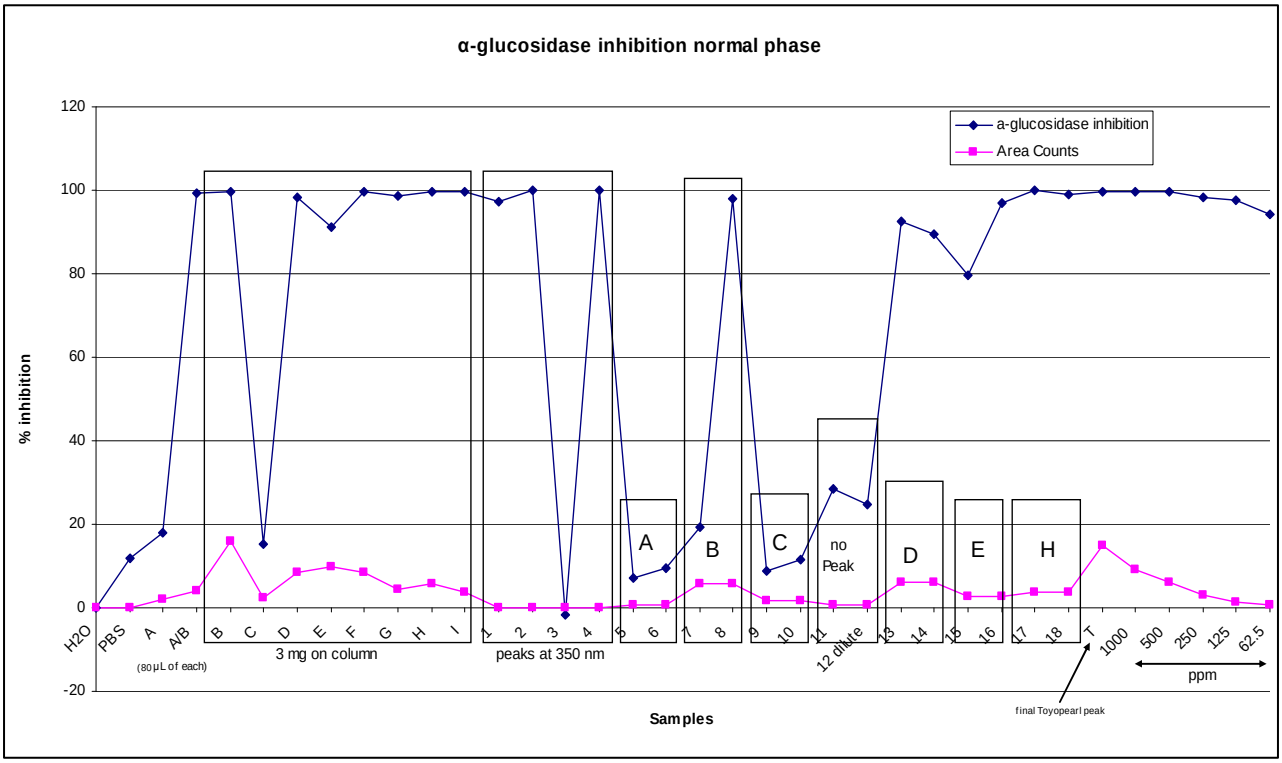

Figure A.16. Normal phase fractions of PSE α-glucosidase inhibition.

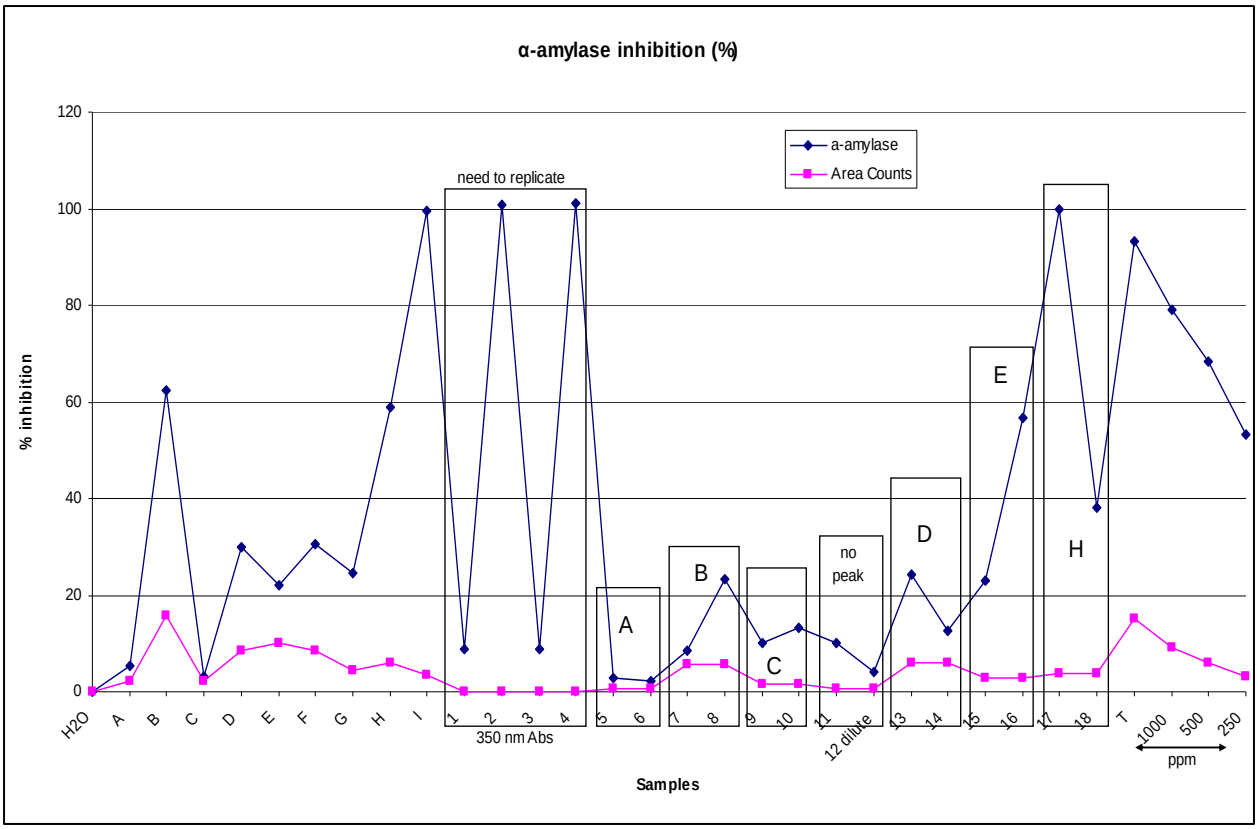

Figure A.17. Normal phase fractions of PSE α-amylase inhibition.