THE PHENOLICS OF PEANUT SKINS AND THEIR IMPACT IN PEANUT BUTTER FORMULATIONS

by

YUANYUAN MA

(Under the Direction of Ronald Bruce Pegg)

ABSTRACT

The overall aims of this work were to ascertain the primary phenolic constituents in peanut skins (PS), and to determine if their antioxidant content and capacities are conserved through industrial dry-blanching and roasting processes. The first and second studies undertaken revealed that ground PS incorporation into peanut butters effectively enhances the total phenolics and fiber contents of the product, and offers product-line diversification while still retaining the product's standard of identity. This research will aid the peanut industry via (1) by-product/waste stream utilization; and (2) diversification of product lines by creating a value-added peanut butter with potential health beneficial properties. Adding ground PS to peanut butter at 1.25, 2.5, 3.75 and 5.0% (w/w) resulted in a concentration-dependent change in the Commission Internationale de l'Éclairage L^* C* h values. Peanut butters formulated with medium- and dark-roasted PS showed an increase in hardness, and were generally more adhesive than those without PS or with dry-blanched PS added. A marked change in spreadability was found with greater than 2.5% PS fortification. Incorporation of dry-blanched PS, especially at levels below 3.75%, showed the fewest differences in terms of physical properties of the peanut butters relative to the control.

Importantly, a concentration-dependent increase in the total phenolics content (TPC) was evident with PS fortification. Dry-blanched PS possessed a TPC of ~166 mg (+)-catechin equivalents/g extract and yielded peanut butters with a 32, 33 and 38% higher TPC than that for light-, medium- and dark-roasted skin incorporation on a same mass basis, respectively. Correspondingly, dry-blanched PS addition at 1.25, 2.5, 3.75 and 5.0% (w/w) resulted in an increase in the TPC by 86, 357, 533 and 714%, respectively, compared with peanut butters devoid of PS fortification; the total proanthocyanidins content (TPACs) rose by 633%, 1,933%, 3,500%, and 5,033%, respectively. Normal-phase high-performance liquid chromatography (NP-HPLC) detection confirmed that the increase in the phenolics content was attributed to the endogenous PACs from the PS, which were characterized as dimers to nonamers by ionization mass spectrometry (NP-HPLC/ESI-MS). FRAP values increased correspondingly by 62, 387, 747, and 829%, while H-ORAC$_{FL}$ values rose by 53, 247, 382, and 415%, respectively. The dietary fiber (DF) content of dry-blanched PS was 55%, with 89 to 93% being insoluble fiber.

The third and fourth studies undertaken involved liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS^n) characterization of free, as well as soluble-ester and glycoside-bound phenolic compounds from dry-blanched PS. A large variety of phenolic compounds, including phenolic acids (hydroxybenzoic acids, hydroxycinnamic acids, and their esters), stilbenes (*trans*-resveratrol and *trans*-piceatannol), flavan-3-ols (*e.g.*, (–)-epicatechin, (+)-catechin, and their polymers {the proanthocyanidins, PACs}), other flavonoids (*e.g.*, isoflavones, flavanols, and flavones) and biflavonoids (*e.g.*, morelloflavone), were identified in dry-blanched PS by this study. PS provide an abundant and inexpensive source of natural antioxidants, especially *p*-coumaroyl species and PACs. All of these studies indicated that processing is altering the content of the phenolics in PS, but their antioxidant efficacy is retained

in the product.

INDEX WORDS: Peanut Skins (PS), Peanut Industry, Dry-blanching, Roasting, Antioxidants, Phenolics, Proanthocyanidins (PACs), high-performance liquid chromatography (HPLC), electrospray ionization mass spectrometry (ESI-MS^n).

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M.S., University of Kentucky, 2008

A Dissertation Submitted to the Graduate Faculty of the University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTORATE OF PHILOSOPHY

ATHENS, GEORGIA

2014

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DEDICATION

I am indebted to my loving parents and husband for their unconditional support and encouragement during the entire course of my graduate study. Without their care, understanding, and trust, none of this would be possible. Also, to my beautiful kittens, who bring me laughter, companion, and emotional support.

ACKNOWLEDGEMENTS

I would like to take this opportunity to express my heartfelt gratitude to many people for helping and supporting me during my research. Thanks and appreciations especially go to my advisor and mentor, Dr. Ronald B. Pegg, who has guided me through each step of the research process, inspired my scientific thinking, and offered me financial support. It is such an honor to do my doctoral degree under his tutelage. Without his encouragement and patience, my academic career could never go any further. His great understanding and professionalism in regard to science will affect my career all my life.

I must thank Drs. William L. Kerr, Ruthann B. Swanson, Phillip Greenspan, and James L. Hargrove for their willingness to serve on my committee as well as their support and contribution to my curriculum development and dissertation preparation. I would also like to thank Dr. Dennis R. Phillips from the Department of Chemistry. Thank you for your invaluable suggestions and technical support. My acknowledgements also extend to Drs. Ryszard Amarowicz and Agnieszka Kosińska-Cagnazzo of the Division of Food Science, Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Olsztyn, Poland for their technical assistance.

My many thanks must also go to my loving friends and lab mates for their willingness to share extensive knowledge of research with me and for their precious friendship. I am especially thankful to Ms. Vickie Wentzel and Marybeth Kellett for their selfless help during my research and dissertation writing.

Last but not least, I am indebted to my loving family, to whom I owe my undying gratitude; thank you for standing by me, through the good times and the bad.

Sincerely,

Yuanyuan Ma

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CHAPTER 1

INTRODUCTION

1.1 Health Benefits of Peanuts

Health benefits associated with the consumption of peanuts have been well documented, notably the possible prevention of cancer (Awad, Chan, Downie, & Fink, 2000), coronary heart disease (Kris-Etherton, Hu, Ros, & Sabaté, 2008), and type-II diabetes (Jiang *et al.*, 2002). The cardio-protective effect of nuts, including peanuts, has been demonstrated consistently from epidemiological studies (Hu & Stampfer, 1999; Kelly & Sabaté, 2006). One such study showed that women eating moderate (1-4 servings/week) and high (≥ 5 servings/week) amounts of nuts and/or peanut butter revealed their risk of CHD-related death by 18 and 19%, respectively (Kelly & Sabaté, 2006). The fatty acid profiles of peanuts have largely contributed to these benefits; peanuts are known to possess zero-*trans* fatty acids (Sanders, 2001), while being rich in monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) (Mercer, Wynne, & Young, 1990), as well as phytosterols (Awad, Chan, Downie, & Fink, 2000). Furthermore, other beneficial functional nutrients inherent to peanuts include vitamin E, L-arginine, and soluble- and insoluble-fiber, as well as water- and lipid-soluble phenolic antioxidants (Kris-Etherton *et al.*, 1999; Isanga & Zhang, 2007); these may act synergistically with the numerous protective bioactives, truly making the peanut a desirable plant food (Kris-Etherton, Hu, Ros, & Sabaté, 2008). In 2003, the U.S. Food and Drug Administration affirmed a qualified health claim for

selected tree nuts and peanut consumption concluding that there was sufficient evidence that habitual consumption of these would reduce the risk of coronary heart disease (FDA, 2003).

1.2 Peanut Production

As demonstrated by George Washington Carver more than a century ago, peanuts (*Arachis hypogaea L.*) are a valuable cash crop to the southern United States. The major peanut-producing states can be divided into three regions, namely the Southwest (Texas and Oklahoma), the Southeast (Alabama, Georgia, and Florida), and the Virginia/Carolina region (Virginia, North Carolina, and South Carolina). Revenue generated from the peanut crop in the United States alone has averaged $1 billion annually from 1996 to 2001 (Dohlman, Young, Hoffman, & Mcbride, 2004). Peanuts are consumed in many parts of the world with China, India, and the United States accounting for roughly two-thirds of the world's peanut production (USDA 2011). While in many countries the largest portion of the crop is destined for oil production, the major products fabricated from peanuts in the United States are peanut butters, salted peanuts, confections, and roasted peanuts in-the-shell (Carley, 1983). Peanut butter is considered the most important peanut-based product, with slightly more than half of the U.S. crop used for its manufacture (Woodroof, 1983). The volume of edible peanuts utilized for peanut butter significantly increased from 400 million pounds in the early 1950s (Woodroof, 1983) to over 1,000 million pounds in 2008 with steady growth through 2011 for 1,200 million pounds (USDA 2008-2009, USDA 2008-2012). These values reflect over 60% of the total edible peanut usage in the United States.

1.3 Manufacture and Uses of Peanut Butter

Peanut butter is relatively simple to manufacture: it involves the steps of shelling, dry roasting, blanching, and grinding the peanuts into a paste (Woodroof, 1983). Shelled peanuts, generally from Runner varieties, are first subjected to a uniform roasting to develop the characteristic peanut flavor. The generated flavor and color characteristics will directly impact the palatability of the resultant peanut butter. After a quick cooling, peanut skins (PS) are removed via dry blanching before the meats are ground to the desired texture (*i.e.*, smooth or chunky formulation). Additional ingredients, such as salt, sugar, and stabilizer, may be added in order to improve consumer appeal. Stabilizers (*i.e.*, hydrogenated cottonseed, canola, soybean, palm oils, or a mixture thereof) help to prevent the separation of the oils from the solid fractions. In the United States, the FDA has established a legal standard of identity (21 CFR §164.150) in the Code of Federal Regulations: it states that for a product to be called 'peanut butter,' it must contain no less than 90% peanuts by weight; otherwise, it will be referred to as a peanut spread (Merrill & Collier, 1974; FDA 2009). The remaining 10% may consist of salt, sweetener for the enhancement of flavor, emulsifier and/or stabilizer.

The profile of oil and protein constituents in peanut kernels makes peanut butter a highly nutritious end-product (Knauft, Moore, & Gorbet, 1993; Andersen, Hill, Gorbet, & Brodbeck, 1998), especially when consumed in a sandwich or on crackers where the wheat flour of bread (*i.e.*, grain source) complements the limiting amino acids of the peanuts. The appealing flavor, convenience of use, and excellent shelf-life of peanut butter contribute greatly to its popularity. According to the American Peanut Council, peanut butter ranks as one of the favorite foods of American households (Jolly *et al.*, 2005). Owing to both its nutrient profile and palatability, peanut butter is nutritious for growing children; it is often taken to schools for lunch and has

been formulated in other lunch-based foodstuffs (Woodroof, 1983).

1.4 Peanut Skins (PS) Production and their Phenolic/Dietary Fiber (DF) Profile

A major by-product of the peanut industry is the skins. Most skins are dumped into landfills, as only a small quantity is added to animal feed (Sobolev & Cole 2004). In 1999/2000, over 750,000 tons of PS were generated worldwide based on an estimated 29.1 million tons production of peanuts. The red skins of peanuts comprise 2.0 to 3.5 weight percent of the kernels. In the United States, the total volume of commercially processed shelled edible-grade peanuts used in primary products was roughly 2,000 million pounds during 2011 (USDA 2010–2012); hence, 40 to 70 million pounds of PS were generated.

PS are only now being recognized as being extremely rich in polyphenols, and therefore as having potential as a functional food ingredient. Nepote, Grosso, and Guzmán (2002) reported a content of about 159 mg total phenolics/g defatted dry skin, which had a marked antioxidant activity, as demonstrated by its capacity to inhibit oxidation of sunflower oil. A study conducted by Yu, Ahmedna, and Goktepe (2005) revealed that the phenolics from PS are abundant not only in the quantity but also in the variety of phenolics, which primarily include phenolic acids (*e.g.*, caffeic, chlorogenic, ferulic, and coumaric acids), flavonoids {*e.g.*, (*epi*)catechins and epigallocatechin (EGC), catechin gallate (CG), and epicatechin gallate (ECG)}, and stilbene (*e.g.*, *trans*-resveratrol). Free phenolic acids are not the predominant phenolic composition in PS (Yu, Ahmedna, Goktepe, & Dai, 2006) but proanthocyanidins (PACs) comprised 17% weight of PS (Karchesy & Hemingway, 1986). Because PACs are polymers of flavonoids, their phenolic nature makes them excellent antioxidant agents. Six A-type procyanidin dimers identified in PS (Lou *et al.*, 1999) were found to inhibit the inflammatory pathway mediated by

hyaluronidase-induced release of histamine. Further study by Lou *et al*. (2004) led to the isolation of five oligomeric PACs from the water-soluble fraction of PS with potential free-radical scavenging activity. Although A-type linkages are much more abundant, both A- and B-type PACs exist in PS. The degree of polymerization (DP) identified in PS was up to 12 bonds by A-type linkages; whereas, B-type structures were detected with a DP of up to only six (Monagas *et al*., 2009).

PS are also rich in dietary fiber (DF). The total DF comprises ~45% weight of roasted PS with only 2.2% as soluble fiber (Shimizu-Ibuka *et al.*, 2009). A high daily intake of DF helps lower both blood pressure and cholesterol levels, resulting in the reduced risk of coronary heart disease, stroke, hypertension, diabetes and obesity (Anderson *et al*., 2009). Unfortunately, the average fiber consumption amongst adults in the U.S. is ~15 g a day instead of the recommended 25 to 30 g. Polyphenols and DF are two well-documented dietary factors in the prevention of chronic diseases but are usually addressed as separate compounds, acting independently in the disease prevention. One study conducted by Saura-Calixto (2011) demonstrated a synergistic function of DF and dietary antioxidants, mainly in the creation of an antioxidant environment in the colon.

1.5 The Effect of Processing on Phenolics Content of Peanut Skins (PS)

Phenolic compounds are secondary metabolites in plants and they generally concentrate in the outer layers such as the peel, shell, and hull to protect inner matter. A large number of intrinsic and extrinsic factors greatly influence the types and levels of phenolics in plants; these include genetic types, environmental conditions, germination, ripening, processing, and storage (Bravo, 1998). Mild heat treatment was able to break some of the covalent bounds of phenolic

constituents, notably phenolic acids, with insoluble polymers (Peleg, Naim, Rouseff, & Zehavi, 1991) and other cell-wall components such as arabinoxylans (Hartley, Morrison, Himmelsbach, & Borneman, 1990). This liberated low-molecular-weight antioxidant compounds from the repeating subunits of high-molecular-weight polymers (Jeong *et al.*, 2004). Lee *et al.* (2006) reported that after heat treatment at 150 °C for 60 min, the TPC value of peanut hulls increased from 73 to 90 μM tannic acid equivalents. PS roasted at 175 °C for 5 min were found to contain a higher TPC than raw PS, especially when recovered by aqueous ethanolic extraction (Yu, Ahmedna, & Goktepe, 2005).

1.6 PS Fiber Content and Utilization as Dietary Fiber (DF)

Little information is currently available regarding the DF content in PS. Shimizu-Ibuka *et al*. (2009) studied the hypocholesterolemic effect of PS whereby they used a commercial roasted PS that contained ~45% of total DF with only 2.2% as soluble DF. As early as 1981, Collins and Post explored the use of peanut hull flour as a potential source of DF. The flour was prepared from either Virginia- or Runner-type peanuts, containing ~ 47% crude fiber and relatively large amounts of cellulose, hemi-cellulose, and lignin. Collins, Kalantari, and Post (1982) developed a series of wheat breads containing 0, 4 or 8% peanut hull flour in an attempt to increase the DF content. Correspondingly, the neutral detergent fiber content of the breads was increased from 4 to 6.7%. Breads containing 4% peanut hull flour were more acceptable and thus, more deemed a potential source of DF. Similarly, the sensory evaluation performed by Sanders *et al.* (2013) indicated a good acceptability of PS-formulated peanut butters, especially when incorporated at levels below 3.75%. Therefore PS, a major by-product of the peanut industry, have a potential utilization as a source of DF.

1.7 Significance of PS-fortified Peanut Butters

An increased awareness of the potential role of dietary antioxidants and fiber in health promotion and disease prevention has led to high demand for antioxidant- and fiber-enriched functional foods. PS are a concentrated source of DF and phenolics; hence, their incorporation into a variety of foods effectively enhances the fiber content and antioxidant capacity of the product in question, while providing an inexpensive and abundant source of such dietary bioactives. Incorporating PS into different food systems would maximize the availability of antioxidants and fiber to consumers. Despite the tremendous potential benefits found in PS as an alternative source of antioxidants and DF, the utilization of PS as a functional food ingredient in value-added products is not well developed.

1.8 Research Objectives

The overall objective of the research was to examine and expand on the utilization of PS as a functional food ingredient. My studies focused on three main areas: (1) the effect of PS incorporation on the color, texture, and total phenolics content of peanut butters; (2) the effect of processing on the phenolics content, fiber content and antioxidant activity of PS and resultant peanut butters; and (3) the identification of phenolics profiles in dry-blanched PS. The specific objectives of my research were to …

1) assess the effect of PS incorporation on the physical attributes (color, texture and spreadability) and total phenolics content (TPC) of the finished peanut butters compared to that void of PS (**Project I**);

2) validate and investigate the processing on the total phenolics content (TPC), PACs content, fiber content, and antioxidant activity of PS and PS-fortified peanut butters. (**Project II**); and

3) determine the phenolics profiles in dry-blanched (DB) PS by combination of high-performance liquid chromatography (HPLC) and mass spectrometry (MS) (**Project III**). Normal phase (NP)-HPLC-MS^2 was employed to determine the PACs profile in DB PS.

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CHAPTER 2

LITERATURE REVIEW

2.1 Oxidation and Health Problems

2.1.1 Reactive Oxygen Species and the Human Body

Reactive oxygen species (ROS) comprise two main categories: free radicals and non-radical derivatives (Dhalla, Temsah, & Netticadan, 2000). Free radicals, *e.g.*, $^{\bullet}OH$ and $O_2^{\bullet-}$, are highly reactive species due to the presence of one or more unpaired electrons. Non-radical derivatives, *e.g.*, H_2O_2 and HOCl, possess no unpaired electrons but as the name suggests, they are derived from a radical. They enter the oxidation chain reactions by acting as an activator or precursor of radicals and other highly oxidative molecules, such as singlet oxygen ($^1O_2$). ROS can be desirable when produced in low concentrations. For example, ROS are involved in the phagocytosis of macrophages to combat pathogens and can serve as important physiological signals to modulate the functions of cells (Forman & Torres, 2002; Finkel, 2011). Studies have found that instead of stochastic generation by cellular metabolism, ROS are synthesized purposefully by cells as secondary messengers to activate signaling pathways mediated by proinflammatory response and growth factors (Hensley, Robinson, Gabbita, & Salsman, 2000; Winterbourn & Hampton, 2008; Finkel, 2011).

As a means of regulating those signaling messengers and protecting cells from oxidative damage, the human body has both intracellular and extracellular mechanisms that can be enzymatic or non-enzymatic nature (Finkel, 2011; Birben *et al.*, 2012). For instance, superoxide dismutase (SOD) (an enzyme involved in $O_2^{\bullet-}$ dismutation to H_2O_2), catalase, and glutathione peroxidase (enzymes that detoxify H_2O_2) are dominant intracellular antioxidant enzymes. α-Tocopherol and ascorbic acid are non-enzymatic antioxidants acting as intracellular and extracellular radical scavengers. Additionally, they are synergic antioxidants, as ascorbic acid can regenerate α-tocopherol radicals to α-tocopherol (Flora, 2009).

2.1.2 Oxidative Stress and Diseases

The human body's antioxidant defense systems can be quite fragile, as they depend on a balance between oxidative stress and antioxidant defense optimums. When oxidation stress is too great, oxidation occurs. This is commonly caused by overproduction of ROS or a depleted antioxidant reserve. Oxidative stress broadly refers to an imbalance where generated ROS cannot be efficiently eliminated by the body's antioxidant defense system (Birben *et al.*, 2012).

Harman *et al.* (1956) first proposed the radical theory of aging in which aging and degenerative diseases were gradually-established consequences resulting from oxidative impairment of cell constituents and connective tissues. Since then, oxidative damage in development of aging and many other pathological conditions have been extensively studied. Growing evidence suggests that ROS-induced oxidative stresses are implicated in oxidative modification of proteins, lipids and nucleic acids, alterations of cell signaling pathways and redox conditions. Consequently, these change are responsible for cell injury or death (Dhalla, Temsah, & Netticadan, 2000; Hensley, Robinson, Gabbita, & Salsman, 2000; Waris & Ahsan,

2006; Birben *et al.*, 2012). These chronic impairments in cells and biomolecules continue to a point where various degenerative conditions and diseases start to manifest.

Although the exact mechanisms involved in ROS-mediated chronic degenerations are not fully understood, a positive association between oxidative damage and disease developments has been consistently demonstrated. ROS-induced oxidative stress has been shown to be actively involved in a variety of human diseases and chronic degeneration. These include cancer (Waris & Ahsan, 2006; Kryston, Georgiev, Pissis, & Georgakilas, 2011), cardiovascular diseases (Dhalla, Temsah, & Netticadan, 2000; Gerienling & Fitzgerald, 2003a,b), neurodegenerative diseases (Jomova, Vondrakova, Lawson, & Valko, 2010), diabetes (Rains & Jain, 2011), and ageing (Cencioni *et al.*, 2013). Mitochondrial oxidative stress, which is characterized by a pro-oxidative shift in the thiol-disulfide redox state and impaired glucose tolerance, resulting in elevated ROS production is seen in diabetes and cancer (Waris & Ahsan, 2006; Pala & Gurkan, 2008). Some diseases are associated with "inflammatory oxidative conditions," which are characterized by enhanced activity of either NADH/NADPH oxidase and/or xanthine oxidase-induced generation of ROS (Dhalla, Temsah, & Netticadan, 2000; Waris & Ahsan, 2006; Pala & Gurkan, 2008). The excessively stimulated activity of NADH/NADPH oxidase by cytokines and other factors can lead to atherosclerosis and chronic inflammation. Overproduction of ROS induced by xanthine oxidase can cause ischemia-reperfusion injury. Ageing and neurodegenerative diseases are mainly attributed to ROS-induced lipid peroxidation, DNA damage, and protein oxidation (Waris & Ahsan, 2006; Pala & Gurkan, 2008; Jomova, Vondrakova, Lawson, & Valko, 2010; Cencioni *et al.*, 2013).

2.2 ROS-induced Lipid and Protein Oxidation in Foods

Because animals and plants are the raw materials of foodstuffs, the mechanisms of oxidation in these raw materials are the sources of food oxidation. Under physiological conditions, animal and plant tissues possess endogenous antioxidant defenses; however in foods, these protective systems are depleted and oxidation processes can begin to take place. Excessive oxidation in food leads to a deterioration in food quality and can generate off-odors, off-flavors, discolorations and loss of nutrients. The production of oxidation endproducts, together with a decreased nutritional value and antioxidant content, might ultimately impair the health of consumers, resulting in disrupted antioxidant defenses in the body and an increased risk of oxidative stress-induced diseases (Kołakowska & Bartosz, 2014).

2.2.1 Lipid Oxidation

Amongst all types of biological molecules vulnerable to ROS attack, including proteins, carbohydrates, and nucleic acids, the initiation of the oxidation process in lipids requires a low energy barrier. Secondary products of lipid oxidation cannot only deplete endogenous antioxidant systems, but also interact with proteins and nucleic acids. During lipid oxidation, for example, various carbonyl compounds are generated, such as 4-hydroxynonenal, malonaldehyde, and other aldehydic scission products, which can modify the amino groups of proteins, enzymes, and DNA by intermolecular crosslinking (Frankel, 1984; Kołakowska & Bartosz, 2014). Unsaturated fatty acids in food are most susceptible to oxidative attack leading to off-flavor generation (Frankel, 1984). Typically, free radical-mediated lipid oxidation is a self-catalytic chain reaction with the following three main steps (Frankel, 1984; Sevanian & Hochstein, 1985;

Kanner & Rosenthal, 1987; Dix & Aikens, 1993; Nawar, 1996; Girrotti, 1998; Márquez-Ruiz, Holgado, & Velasco, 2014):

(1) **Initiation:** lipid free-radicals are formed by abstraction of a hydrogen atom from an allylic methylene group or attaching a radical to a double bond of the unsaturated fatty acid in the presence of ROS, transition metal ions, UV light, or singlet oxygen ($^1O_2$).

$$\mathbf{RH + initiator \rightarrow R^{\bullet}}$$

$$\mathbf{R'\text{-}CH{=}CH\text{-}R'' + O_2 \rightarrow ROOH}$$

(2) **Propagation:** lipid peroxyl radicals ($ROO^{\bullet}$) are produced by the reaction between oxygen and lipid free radicals. Once $ROO^{\bullet}$ radicals are generated, they can attack and abstract a hydrogen atom from other lipid molecules to give hydroperoxides (ROOH). These primary products of lipid oxidation are odorless and colorless, but unstable and decompose to secondary oxidation products such as aldehydes, alcohols, acids, alkyl radicals, and oxoesters.

$$\mathbf{R^{\bullet} + O_2 \rightarrow ROO^{\bullet}}$$

$$\mathbf{ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet}}$$

$$\mathbf{ROOH \rightarrow RO^{\bullet} + HO^{\bullet}}$$

$$\mathbf{2ROOH \rightarrow RO^{\bullet} + ROO^{\bullet} + H_2O}$$

(3) **Termination**: the reaction is terminated by the formation of non-radical compounds via associations and cross-linking between free radicals.

$$R^{\bullet} + R^{\bullet} \rightarrow R\text{-}R$$

$$R^{\bullet} + ROO^{\bullet} \rightarrow ROOR$$

$$ROO^{\bullet} + ROO^{\bullet} \rightarrow ROOR + O_2$$

2.2.2 Protein Oxidation

Protein oxidation refers to a situation in which amino acid side residues and/or peptide bonds are attacked by ROS or derivatives from ROS-mediated oxidation (*e.g.*, malonaldehyde from lipid oxidation), resulting in cleavage of the protein peptide backbone, formation of covalent protein cross-links, or simply the unfolding of proteins (Stadtman & Levine, 2000; Xiong, 2000; Baron, 2014). Carbonyls are characteristic products from ROS-induced protein oxidation. Protein oxidation has been reported to occur via the following mechanisms (Stadtman & Levine, 2000; Xiong, 2000; Stadtman & Levine, 2003; Lund, Heinonen, Baron & Estévez, 2011; Baron, 2014): (1) modification of amino acid side chains, such as deamination; (2) cleavage of peptide bonds; (3) interactions between sugar aldehydes or lipid carbonyls and protein amino groups; (4) Michael addition; and (5) Schiff base formations. These derived carbonyls can also react with ε–NH_2 groups of lysyl residues to form protein cross-links and ultimately, polymerization. Moreover, oxidation-induced protein cross-links can be initiated by other reactions, such as the oxidation of sulfhydryl groups in cysteine, methionine oxidation (Xiong, 2000; Stadtman & Levine, 2003; Lund, Heinonen, Baron & Estévez, 2011) and tyrosine oxidation (to dityrosine) (Giulivi, Traaseth, & Davies, 2003; Stadtman & Levine, 2003; Lund,

Heinonen, Baron & Estévez, 2011). Physicochemical changes in oxidatively-modified proteins include alterations in protein hydrophobicity, solubility, proteolytic affinity, and thermal stability (Xiong, 2000). In food systems, mild and limited protein oxidation may be desirable due to the enhancement of functionalities (Srinivasan & Xiong, 1996). However, uncontrolled and irreversible protein oxidation always leads to inactivation of functional proteins and enzymes. The accumulation of oxidatively-modified proteins is considered as one factor that contributes to decreased product qualities (Xiong, 2000).

2.3 Antioxidants

Antioxidants refer to a category of compounds, both synthetic and natural, that can retard or inhibit oxidative processes at a much lower low concentration compared to that of an oxidizing substance (Sies, 1997; Dai & Mumper, 2010). It is important to note differences in terminology: an antioxidant is a reductant, but not all reductants are antioxidants. In explanation, "reductant" and "oxidant" are chemical (redox) terms, while "antioxidant" and "pro-oxidant (a synonym for reactive species)" hold a specific reference to biological systems (Prior & Cao, 1999). For unsaturated lipids, the mechanisms involved in the antioxidant action are related to three functions; that is, (1) eliminating the presence of free radicals {*e.g.* peroxyl radicals ($ROO^{\bullet}$) and alkoxyl radicals ($RO^{\bullet}$)} generated in the propagation step by hydrogen atom donation or single electron transfer; (2) sequestering prooxidative transition metal ions, decomposing hydroperoxides, and scavenging ROS; and (3) donating hydrogen atoms to antioxidants or providing a stable antioxidant environment (Eskin & Przybylski, 2001).

According to their specific functions, antioxidants are classified into three categories, including primary chain-breaking antioxidants, secondary or preventive antioxidants, and

synergists (Eskin & Przybylski, 2001; Venskutonis, 2014). Primary antioxidants refer to hydrogen atom/electron donators, such as tocopherol isomers, phenolics and polyphenolics. After accepting a hydrogen atom or single electron from an antioxidant, active radical species are converted to neutral products while the antioxidant itself becomes a radical intermediate (albeit, a more stable one) with resonance stabilization occurring by the aromatic ring(s). Secondary or preventative antioxidants act as sequestrants to chelate metal ions as well as remove singlet oxygen, peroxides and other initiators. As their names implies, they are a series of active compounds capable of eliminating initiators and preventing oxidation chain reactions at the very beginning. For example, ethylenediamine tetraacetic acid (EDTA) is a widely-used metal ion-chelating agent for $Fe^{2+/3+}$ and Cu^{2+}. Synergists are compounds that can reinforce the antioxidative activity of primary or secondary antioxidants through developing a more suitable functional environment, inactivating metal ions, or regenerating oxidized antioxidants. A good example is α-tocopherol and ascorbic acid. The latter can reduce α-tocopherol radicals back to α-tocopherol, thereby restoring its antioxidant capacity.

2.3.1 The Role of Antioxidants in Disease Prevention

Tremendous interest in antioxidant food supplements has arisen in recent years due to the recognition of antioxidants role in fighting ROS-induced oxidative stresses and associated pathogenesis and chronic diseases. Antioxidants fall into two broad groups: nutrient antioxidants (*e.g.*, vitamin E and selenium) and natural-source antioxidants {*e.g.*, (poly)phenolics}. Vitamin E is the most well-known fat-soluble nutrient antioxidant and imparts protection against oxidative stress-induced diseases in preclinical animal models of cancers (Kline, Lawson, Yu, & Sanders, 2007), cardiovascular diseases (Galli & Azzi, 2010), and neurodegenerative disorders

(Butterfield, Castegna, Drake, & Scapagnini, 2002). Yet, the outcomes of clinical trials remain inconsistent. Selenium is another nutrient antioxidant with proven preventive benefits against oxidative impairments (Fairweather-Tait *et al.*, 2011). Unlike vitamin E, the antioxidant activity of selenium stems from being an essential constituent of selenium-containing antioxidative enzymes like glutathione peroxidase.

In addition to vitamins and minerals, (poly)phenolics (*e.g.*, flavonoids) have attracted much attention due to their favorable health benefits, which are largely attributed to their antioxidant activity. There is steadily accumulated evidence from epidemiological studies indicating an inverse association between the intake of fruits and vegetables in diets and the incidence of certain cancers and chronic diseases such as cardiovascular diseases and neurodegenerative disorders (Riboli & Norat, 2003; Barberger-Gateau *et al.*, 2007; He, Nowson, Lucas, & MacGregor, 2007). As the most abundant antioxidants of such diets, (poly)phenolics are believed to partially contribute to these beneficial effects. The preventative effects of (poly)phenolics against the aforementioned chronic diseases are well demonstrated in human cell culture and animal models (Scalbert *et al.*, 2005). However, dietary (poly)phenolics undergo extensive modifications after ingestion: (1) they are usually present in plasma at very low concentrations (nM levels) and as phase II metabolites rather than parent compounds from the dietary source; and (2) both the parent compounds and metabolites which reach the colon are degraded by local flora to smaller phenolics and aromatic catabolites and can be absorbed into the blood. Thus, more well-controlled human intervention trials and *in vitro* mechanistic studies are necessary to convince the scientific community of the protective effects and roles in the health benefits of plant-based diets (Del Rio *et al.*, 2013).

2.3.2 The Role of Natural Antioxidants in Foods

Antioxidants naturally occur in almost all agri-food material, but they are minor components compared to the macronutrients: fats, proteins and carbohydrates. After consumption, as discussed above, they play a protective role in supporting the human redox defense system against ROS and thereby help retard oxidative stress-induced pathological conditions. Likewise, when present in foods, antioxidants protect against the oxidation of food components, especially unsaturated lipids during food processing and storage. Unsaturated fatty acid-containing triacylglycerols are vulnerable to oxidation and result in food deterioration. This leads to an attempt to correlate *in vitro* antioxidant capacity with projected capabilities of antioxidants to perform in food systems. The antioxidant capacity of foods can be affected by many factors, such as the physical location of antioxidants in the food systems, interactions with other food components, and the overall food environment (*e.g.*, pH, ionic strength, hydrophilic/lipophilic balance) (Decker *et al.*, 2005); therefore, measuring this activity is much easier than assessing their possible effects *in vivo*, after consumption. On the other hand, some natural antioxidants possess colors (*e.g.*, anthocyanins), tastes (*e.g.*, PACs) and/or odors, which can negatively influence the overall sensory quality of these antioxidant-containing foods.

Some of the most well characterized antioxidant reactions in foods are enzymatic oxidation of (poly)phenolics. This process involves monophenol and diphenol oxidase activities, which result in hydroxylation to the *ortho*-position adjacent to an existing hydroxyl group of the phenolic substrate and oxidation of *ortho*-dihydroxybenzenes to ortho-benzoquinones, respectively (Oliveira, Ferreira, de Freitas, & Silva, 2011). The enzymatic oxidation of (poly)phenolics can be favorable in some situations, such as in tea fermentation, but can also cause deterioration in food quality such as the browning of apples. A strategy taken (*e.g.*, thermal

processing) to inactivate these enzymes and protect the foodstuff would also significantly affect the content and chemistry of the antioxidants and other components contained therein. Arts, van de Putte, and Hollman (2000) compared the levels of catechins in fresh as well as processed fruits and vegetables: a 25 to 60% decrease in catechin levels of the prepared foods and marked decreases in industrial canned foods were noted when compared to their raw counterparts.

2.4 Phenolic and Polyphenolic Antioxidants

Any compound containing a hydroxy-substituted aromatic ring is a phenolic compound. Polyphenols are characterized by possessing large multiples of phenolic-structural units. They are ubiquitously distributed throughout the plant kingdom as secondary metabolites (Bravo, 1998) and have become a magnet of human nutrition research due to their potent antioxidant activity. There is accumulated evidence revealing that consumption of a (poly)phenolic-rich diet can provide relief from certain physical ailments and prevention against oxidative stress-induced diseases in humans, including aforementioned cardiovascular diseases and neurodegenerative disorders. Therefore, it is not surprising that the extraction and analysis of phenolic compounds from plants and other food sources have been extensively studied (Naczk & Shahidi, 2006; Dai & Mumper, 2010).

2.4.1 Phenolics in Plants: Their Occurrence and Biosynthesis

Phenolics generally concentrate in the outer layers of plants such as the peel, shell, and hull to protect inner materials. Many phenolic compounds and mixtures thereof are prevalent in a wide variety of fruits, vegetables, grains, and other plant products (Bravo, 1998; Shahidi, 2000; Naczk & Shahidi, 2006). Amongst the wide range of phenolic compounds, dietary phenolics are

mainly comprised of phenolic acids, flavonoids, and phenolic polymers (*e.g.*, PACs) (King & Young, 1999). **Table 2.1** summarizes the current classification of dietary phenolics, including examples of food sources. Phenolic acids (*e.g.*, *p*-hydroxybenzoic acid and vanillic acid) can be present in free forms, but the more common occurrence is their corresponding methyl and ethyl esters and glycosides, which exist in both free and bound forms (Bravo, 1998). Hydroxycinnamic acids (*e.g.*, *p*-coumaric and ferulic acids) are bound to cell wall polysaccharides through covalent linkages (Bravo, 1998). In graminaceous plants, for instance, *p*-coumaric acid and ferulic acid are bound to cell walls through ester-linkages between their carboxylic groups and arabinoxylans (Hartley, Morrison, Himmelsbach, & Borneman, 1990). Flavonoids in living plants are generally present in bound forms with one or more sugar residues (Bravo, 1998; Hollman & Arts, 2000). In the case of flavan-3-ols (*e.g.*, catechin, epicatechin, and gallocatechin), free monomers are their common form (Bravo, 1998).

In plants, phenolic compounds are synthesized most commonly from the amino acid L-phenylalanine, and in fewer cases, L-tyrosine (Shahidi, 2000; Crozier, Jaganath, & Clifford, 2009). Several simple phenylpropanoids with the basic $C_6 - C_3$ carbon skeleton (*i.e.,* denoting a phenolic ring attached to a three-carbon side chain) of phenylalanine such as *p*-coumaric, caffeic, ferulic, and sinapic acids, are produced from *trans*-cinnamic acid via a series of hydroxylation (hydroxylase activity) and methoxylation (*O*-methyl transferase activity) reactions. Loss of a two-carbon moiety from the *trans*-cinnamic acid produces benzoic acid derivatives such as *p*-hydroxybenzoic acids, protocatechuic acids, and vanillic acid. An illustration of the biosynthesis pathways of these phenylpropanoids is given in **Figure 2.1(A)**. The substrates involved in the synthesis of flavonoids, isoflavonoids and stilbenes are malonyl CoA and *p*-coumaroyl CoA. Chalcone synthase (CHS) catalyzes the stepwise Claisen condensation of

three acetate units from malonyl CoA with a *p*-coumaroyl CoA to yield tetrahydroxychalcone, which further leads to the production of flavonoids and isoflavonoids. Aldol condensation of three molecules of malonyl CoA with one molecule of *p*-coumaroyl CoA via the action of trihydroxystilbene synthase brings forth stilbenes (*e.g.*, *trans*-resveratrol). The biosynthesis pathways of flavonoids, isoflavonoids and stilbenes are depicted in **Figure 2.1(B)**.

Phenolic Acids

Benzoic and hydroxycinnamic acids are two major groups of naturally-occurring phenolic acids (Teixeira *et al.*, 2013). Benzoic acids are phenolic compounds with a $C_6 - C_1$ structure (*e.g.*, *p*-hydroxybenzoic acid, protocatechuic acid, and vanillic acid) that contain one carboxylic acid functional group. Hydroxycinnamic acids (*e.g.*, *p*-coumaric acid, caffeic acid, ferulic acid, and sinapic acid) are the most important phenylpropanoids consisting of a $C_6 - C_3$ skeleton (Bravo, 1998; Crozier, Jaganath, & Clifford, 2009; Teixeira *et al.*, 2013). As depicted in **Figure 2.1(A)**, phenolic acids of the benzoic and hydroxycinnamic acid families are synthesized from L-phenylalanine (or L-tyrosine) in plants. This reaction pathway is commonly referred to as phenylpropanoid metabolism. Basic carbon skeletons and structures of benzoic and hydroxycinnamic acid families are given in **Table 2.2**. In plants, the common occurrences of hydroxycinnamic acids are esters of hydroxyacids such as quinic, shikimic, or tartaric acid (Herrmann, 1989; Crozier, Jaganath, & Clifford, 2009; Teixeira *et al.*, 2013). Other conjugated forms of hydroxycinnamic acids include amides (*e.g.*, with amino acids and peptides), esters of sugars, and glycosides (Teixeira *et al.*, 2013).

Coumarins and Stilbenes

Coumarin (1,2-benzopyrone) shares a similar $C_6 - C_3$ skeleton (**Table 2.2**) to *trans*-cinnamic acid (3-phenyl-2-propenoic acid), but it belongs to the benzopyrone family and contains a benzene ring joined to a pyrone nucleus (Jain & Joshi, 2012). It is a natural substance occurring in many plants and synthesized from *trans*-cinnamic acid via *ortho*-hydroxylation, *trans–cis* isomerization, and esterification (Jain & Joshi, 2012). Coumarin derivatives have attracted increased attention recently due to their anticarcinogenic and antithrombotic activities (Jain & Joshi, 2012; Lacy & O'Kennedy, 2004). Coumarins, especially aesculetin, are potent inhibitors of cell growth in various carcinoma cell lines (Lacy & O'Kennedy, 2004).

Stilbenes are phytoallexins with a $C_6 - C_2 - C_6$ structure (**Table 2.2)** present in plants in response to disease, injury, and stress. Red wine and peanuts (*Arachis hypogaea* L.) are dietary sources of *trans*-resveratrol (3,5,4′-trihydroxystilbene) (Crozier, Jaganath, & Clifford, 2009). Though occurring at much lower concentrations in plants compared to *trans*-resveratrol, *trans*-piceatannol (3,3′,4,5′-tetrahydroxystilbene) can be easily induced in the calluses of A. hypogaea or other related peanut tissues in a controlled environment (Ku, Chang, Cheng, & Lien, 2005). Piceid is *trans*-resveratrol-3-*O*-glucoside found in pistachio nuts (*Pistacia vera*), *Polygonum cuspidatum* (Japanese knotweed or Mexican bamboo) (Crozier, Jaganath, & Clifford, 2009), and grape juices (Romero-Pérez, Ibern-Gómez, Lamuela-Raventós, & De La Torre-Boronat, 1999).

Flavonoids

Flavonoids are distributed mostly as glycosides in plants, with some classes consisting of up to 380 variations in their chemical structure (Bravo, 1998). Flavonoids possess a basic core

structure of diphenylpropane ($C_6 - C_3 - C_6$) that consists of two aromatic rings connected by a three-carbon bridge (Bravo, 1998; Crozier, Jaganath, & Clifford, 2009). **Table 2.2** shows the basic skeleton and system employed for the carbon numbering of a flavonoid nucleus. The altered substitution and saturation divide flavonoids into several sub-classes, most notably flavones, flavonols, flavanones, flavanonols, flavan-3-ols, and isoflavones. The basic skeletons of above flavonoids/isoflavonoids are also illustrated in **Table 2.2**. There are varying arrangements of multiple hydroxyl- and methoxy-group substituents present in the structures of flavonoids along with the basic skeleton. Hydroxylation usually occurs at the C-4′ position of the B-ring and C-5, C-7 positions of the A-ring (Crozier, Jaganath, & Clifford, 2009).

Flavan-3-ols are monomeric units for PACs and are characterized by a saturated C3 element on the heterocyclic C-ring with a hydroxy or galloyl group attached at the C-3 position. Four isomers are produced by two chiral centers at C-2 and C-3 positions. (+)-Catechin and (−)-epicatechin are the most common diastereoisomeric pair found in nature, while (−)-catechin and (+)-epicatechin are somewhat rare (Crozier, Jaganath, & Clifford, 2009). As most structurally complex subclass of flavonoids, the simple monomers can further form (epi)gallocatechin by hydroxylation at the C-5′ position of the B-ring and give (epi)catechin gallate by esterification with gallic acid at the C-3 position of the C-ring. (Epi)gallocatehin-3-gallate results from the esterification of (epi)gallocatehin with gallic acid. Hence, these compounds possess hydroxy groups at the C-3′, C-4′, and C-5′ positions of the B-ring and a gallate moiety esterified at the C-3 position of the C-ring. The structures of above flavan-3-ols are depicted in **Figure 2.2**.

Phenolic Polymers

Tannins are highly hydroxylated polyphenolic compounds of intermediate to high molecular weights ranging from 500 to more than 30,000 Da (Serrano *et al.*, 2009). The term "tannin" was first introduced in 1796 and they were so-named because of their capacity to transform animal hides into leather by forming stable tannin-protein complexes with skin collagen (Bravo, 1998; Serrano *et al.*, 2009). The astringency resulting from consumption of tannin-rich foods is caused by such interaction between tannins and salivary proteins. Tannins are classified into two major groups based on their inherent chemical constituents: hydrolyzable and condensed tannins.

The hydrolyzable tannins consist of gallic acid or ellagic acid esterified with a polyol (generally D-glucose) central core; whereas, flavan-3-ol monomers compose of condensed tannins or proanthocyanidins (PACs) (Bravo, 1998; Serrano *et al.*, 2009). Hydrolyzable tannins can be further segregated into gallotannins and ellagitannins. Gallotannins contain gallic acid subunits only. Ellagitannins can be heterogeneous polymers of both ellagic and gallic acid (Serrano *et al.*, 2009). Unlike gallotannins where their galloyl groups can be linked by depside bonds to form high-molecular-weight polymers, the galloyl groups of ellagitannins are linked through C – C bonds *via* oxidative condensation reactions (Mueller-Harvey, 2001). Hydrolyzable tannins easily undergo hydrolytic dissociation in weak acid or alkali to their individual monomeric units (Bravo, 1998).

PACs are oligomers and polymers able to reach degrees of polymerization (DP) of over 50. As mentioned above, the monomeric constituent is a flavan-3-ol (*e.g.*, catechin, epicatechin, *etc.*) with oxidative condensation between C-4 position of the heterocyclic C-ring and C-6 or C-8 position of the A-ring of adjacent units (Bravo, 1998; Crozier, Jaganath, & Clifford, 2009; Serrano *et al.*, 2009). These PACs are referred to as B-type PACs. In some cases, an additional

C_2–*O*–C_7 ether linkage can be found and these PACs are often called A-type PACs. The linkage possible of A- and B-type PACs are depicted in **Figure 2.3**. PACs are usually subdivided as procyanidins, prodelphinidins and propelargonidins according to the composition of their flavan-3-ol subunits. Procyanidins formed exclusively from (epi)catechins; whereas, prodelphinidins contain (epi)gallocatehin constituents. PACs, containing (-)-epiafzelechin and (+)-afzelechin, are called propelargonidins (Crozier, Jaganath, & Clifford, 2009; Serrano *et al.*, 2009).

2.4.2 Phenolic Compounds Found in Peanuts and Peanut Skins (PS)

Peanuts

Limited quantities of isoflavones have been detected in peanuts, including daidzein (52–1753 μg/100 g d.w.), genistein (13–227 μg/100 g d.w.), and biochanin A (37–137 μg/100 g d.w.) (Chukwumah *et al.*, 2007a). The contents, however, vary depending on the extraction medium and the sample preparation techniques employed in analyses. Moreover, isoflavone levels can be significantly increased with thermal processing by releasing corresponding bound conjugates. According to Chukwumah *et al.* (2007b), boiled peanuts showed higher isoflavone content compared to raw peanuts, with increases in biochanin A and genistein of 200 and 400%, respectively. The only other flavonoid found in peanuts to date is flavonol dihydroquercetin (taxifolin), which is present in Spanish peanuts in limited amounts (Pratt & Miller, 1984). *trans*-Resveratrol is not expected to have a significant effect on the antioxidant activity of peanuts because of the small magnitude detected in raw peanut kernel (0.01–2 μg/g) (Sobolev & Cole, 1999; Sanders, McMichael & Hendrix, 2000; Tokuşoğlu, Ünal, & Yemiş, 2005). Commercial processing elicits increased *trans*-resveratrol production in the following order:

boiling > peanut butter processing > dry roasting) (Sobolev & Cole, 1999), but the levels of *trans*-resveratrol obtained from such processes are limited. In comparison, peanut roots contain 0.13-1.33 mg/g of *trans*-resveratrol (Chen, Wu, & Chiou, 2002), making the roots a much better source.

The total phenolics content of peanuts with skin are higher compared to most tree nuts, with the exception of pistachios, pecans, and walnuts (Kornsteiner, Wagner, & Elmadfa, 2006). Of the little compositional data available on peanut phenolic profiles, bound phenolics are thought to be dominant, as demonstrated by an 86% increase in TRAP values post alkaline hydrolysis (Pellegrini *et al.*, 2006). Phenolic acids and their esters have been tentatively identified in raw and roasted peanuts, including *p*-coumaric acid, *p*-coumaric acid esters, and possibly *p*-hydroxybenzoic acid esters (Talcott *et al.*, 2005). Fajardo and coworkers (1995) demonstrated a stress-elicited synthesis of free and bound phenolics in peanuts where *p*-coumaric and ferulic acid were the major compounds identified. Therefore, *p*-coumaric and ferulic acids may be contributing factors to antioxidant activity of peanuts and potential health benefits of their consumption.

Peanut Skins (PS)

Besides the kernels, PS, the other edible part of peanuts, have attracted attention because they are a rich, inexpensive source of potentially health-promoting phenolics. As mentioned before, phenolic compounds typically concentrate themselves on the outer layers of plants such as the peel, shell, and hull to protect the inner core materials. Nepote, Grosso, and Guzman (2002) reported a content of ~159 mg total phenolics/g defatted dry skin, which also exhibited a marked antioxidant activity as demonstrated by its capacity to inhibit the oxidation of sunflower oil. A

study by Yu *et al.* (2005 & 2006) revealed that PS phenolics are abundant not only in quantity but also in type, which primarily include free-, esterified- and bound-phenolic acids (*i.e.*, caffeic, chlorogenic, ferulic, and *p*-coumaric acids), flavan-3-ols (*i.e.*, epigallocatechin, catechin gallate, and epicatechin gallate) and their polymers (the proanthocyanidins, PACs), and stilbenes (*i.e.*, *trans*-resveratrol).

Free phenolic acids are not the predominant phenolics in PS (Yu, Ahmedna, Goktepe, & Dai, 2006); the PACs comprise ~17% by weight of PS (Karchesy & Hemingway, 1986). Six A-type PAC dimers were identified in PS (Lou *et al.*, 1999) and found to inhibit the inflammatory pathway mediated by hyaluronidase-induced release of histamine. A further study by Lou *et al*. (2004) led to isolation of five oligomeric PACs with potential free radical-scavenging activity from the water-soluble fraction of PS. Although A-type linkages are much more abundant, both A- and B-type PACs exist in PS. The degree of polymerization (DP) identified in PS was up to 12 bound by A-type linkages; whereas, B-type structures were detected with a DP of up to six (Monagas *et al*., 2009). PACs are polymers of flavonoids and their phenolic nature makes them excellent antioxidant agents.

2.4.3 The Effect of Processing and Extraction on Phenolics Content of Peanut Skins (PS)

Mild heat treatment was able to break some of the covalent bounds of phenolic acids with insoluble polymers (Peleg, Naim, Rouseff, & Zehavi, 1991) and other cell wall components such as arabinoxylans (Hartley, Morrison, Himmelsbach, & Borneman, 1990), liberating low-molecular-weight antioxidant compounds from the repeating subunits of high-molecular-weight polymers (Jeong *et al.*, 2004). Lee *et al.* (2006) reported that after heat treatment at 150 °C for 60 min, the TPC value of peanut hulls increased from 73 to 90 μM tannic

acid equivalents. PS roasted at 175 °C for 5 min were found to contain a higher TPC than raw PS, especially when recovered by aqueous ethanolic extraction (Yu, Ahmedna, & Goktepe, 2005). Yu and coworkers (2006) reported a significant influence of the skin removal methods on the PACs content of PS. Dry heat, like that found in dry roasting of red-skin peanuts, would increase A-type dimers and B-type trimers compared to directly peeled PS due to monomeric polymerization or the degradation of trimers and tetramers. Polymerization of monomers and B-type dimers is a plausible mechanism by which increased B-type trimer levels were detected.

In addition to processing, the extraction conditions employed such as choice of solvent system, material:solvent ratio, particle size distribution of PS, extraction times, number of extractions, and temperature (Nepote, Grosso, & Guzmán, 2005; Dai & Mumper, 2010) impact the efficiency of extraction and thereby result in different recoveries of phenolics from the source material. According to Nepote, Grosso, and Guzmán (2005) the optimum extraction conditions consist of 70% (v/v) ethanol, unground PS, a solvent/solid ratio of 20 mL/g, 10 min of shaking, and three extractions yielding a 118 mg phenol equivalents/g recovery of total phenolics. It was reported by Nepote, Grosso, and Guzmán (2002) that 159 mg phenol equivalents/g of TPC was recovered from dry blanched PS by 24 h maceration with methanol at a material to solvent ratio of 1:10 (w/v). The extraction was performed 2× at room temperature in a dark room. However, the quantities of total phenolics are difficult to compare amongst that reported in the literature because sample source, sample preparation manner, and extraction techniques are quite varied.

2.5 Extraction of Ester and Glycoside Bound Phenolic Compounds

As mentioned above, the more common occurrence for phenolic acids (*i.e.*, *p*-hydroxybenzoic acid and vanillic acid) is their corresponding methyl and ethyl esters and

glycosides which exist in free and/or bound forms (Bravo, 1998). Flavonoids found in living plants are generally in bound forms with one or more sugar residues (Bravo, 1998; Hollman & Arts, 2000). Identification of esterified phenolic derivatives is extremely difficulty due to the nearly uniform UV spectra with their unesterifed counterpart. The chemical characteristics of the new chromophore generated via esterification are not markedly different from its precursor. Sometimes there are, however, minor bathochromic shifts in the chromophoric structure such as that present in the C–O bond of caffeoyl tartrate (Robbins, 2003). One strategy to simplify and specify the analysis is using acid and/or base hydrolysis to convert the bound derivatives to a carboxylic acid. It was reported that the base hydrolysis provided phenolic acids liberated from soluble esters while phenolic acids bound to soluble glycosides were released by followed acid hydrolysis (Weidner, Amarowicz, Karamać, & Dabrowski, 1999; Amarowicz & Weidner, 2001). These liberated aglycones can be easily identified using high-performance liquid chromatography (HPLC) with diode-array detection (DAD). Likewise, the large variety of glycosides makes determination of individual flavonoid glycosides in foods formidable (Hertog, Peter, Hollman, & Venema, 1992). Therefore, flavonoids are usually analyzed after hydrolysis off their glycosides; the resulting aglycones are further confirmed by photodiode array detection or mass spectrometry (Hertog, Peter, Hollman, & Venema, 1992; Hollman & Arts, 2000; Merken & Beecher, 2000). The HPLC analysis of flavonoids in peanuts and other legume seeds subsequent an acid hydrolysis procedure was described by Wang's group (2008).

Likewise, certain percentage of PACs is "unextractable" due to confined in vacuoles or bound to cell wall material through hydrogen bonding and hydrophobic interactions (Pinelo, Arnous, & Meyer, 2006; White, Howard, & Prior, 2010). Hence, the PACs content has been underestimated because of the bound linkage between PACs and cell wall material which cannot

be broken by normal extraction methods (Arranz, Saura-Calixto, Shaha, & Kroon, 2009; White, Howard, & Prior, 2010). In order to liberate these bound derivatives, acid or base hydrolysis was applied after normal extraction (Arranz, Saura-Calixto, Shaha, & Kroon, 2009; White, Howard, & Prior, 2010). However, alkaline conditions can lead to cleavage of C–C interflavan bonds between the monomeric flavan-3-ol constituents; in addition, the A-ring of flavan-3-ols can be broken by prolonged treatment (White, Howard, & Prior, 2010).

2.6 Purification, Fractionation and Analysis of Phenolic Compounds

The concentration of the phenolic compounds in the crude plant extract could be low due to the large constitution of carbohydrates and/or lipophilic compounds, which deter simple extraction. Therefore, sequential extraction, liquid-liquid partitioning and solid phase extraction (SPE) based on polarity and acidity, are commonly used techniques to obtain polyphenol concentrated fractions before analysis (Robbins, 2003; Dai & Mumper, 2010). Lipophilic compounds can be washed away by using non-polar solvents (*i.e.*, hexane, dichloromethane, and chloroform, *etc.*); whereas, SPE is often applied to remove the polar non-phenolic substances such as sugars and organic acids. Typically, phenolic components are retained quantitatively on the SPE cartridge (*i.e.*, Amberlite XAD-16), while interfering components are washed off with water (Berardini, Knödler, Schieber, & Carle, 2005; Zhang *et al.*, 2008). The retained polyphenols are eluted from the sorbent cartridge using suitable organic solvents (*i.e.*, methanol, ethanol, *etc.*).

Among the many methodologies available for the selective separation and analysis of phenolic compounds, reversed-phase (RP)-HPLC equipped with a C_{18} column and coupled with UV-Vis photodiode array (PDA) detection is the most widely adopted and used approach

(Robbins, 2003). Phenolic acids and flavonoids show characteristic UV-range absorbance patterns from 190 to 380 nm (Merken & Beecher, 2000; Robbins, 2003). In UV/Vis diode array detection (DAD), four groups of phenolic compounds are distinguished, namely hydroxybenzoic acids (255 nm), flavan-3-ols and polymers (280 nm), hydroxycinnamic acids (320 nm) and other flavonoids (360 nm). The inherent differences in UV-spectra exhibited by the two phenolic acid families provides for their selective chromatographic identification. Nevertheless, compared to UV detection, fluorescence detection is more commonly employed for PAC analysis because of the increased selectivity and decreased interference from other absorbing species (Hümmer & Schreier, 2008). The use of mass spectrometric detection is an emerging trend, which does not require complete chromatographic separation and provides more information about structures of phenolic compounds (Robbins, 2003; Dai & Mumper, 2010).

Because a greater polarity is associated with an increased DP, theoretically PACs can be fractionated by reversed-phase (RP) or normal-phase (NP) high-performance liquid chromatography (HPLC) in an order of ascending molecular mass (Hümmer & Schreier, 2008). However, the resolution of PACs is limited to tetramers on C_{18} columns by RP-HPLC. The first successful separation of PACs was achieved on SiO_2 thin layer plates according to their DP (Lea & Arnold, 1978). Based on this thin layer chromatography method, a NP-HPLC gradient of CH_2Cl_2 and CH_3OH with traces of aqueous formic acid (1:1, v/v) was developed by Rigaud *et al.* (1993), resulting in the separation of PACs up to a DP of five in cacao bean extract. Hammerstone *et al.* (1999) identified PAC decamers in cocoa by using a gradient of similar composition, but by replacing the formic acid with acetic acid. In the above cases, chlorinated solvents were employed in the mobile phase compositions; however, these solvents are environmentally unfriendly and can cause health problems from exposure in the workplace. A

better separation of PACs in cacao with a DP up to 14 was achieved by Kelm *et al.* (2006) using a diol stationary phase with a relatively safe binary mobile phase of A (CH_3CN:CH_3COOH, 99:1, v/v) and B (CH_3OH:H_2O:CH_3COOH, 95:4:1, v/v/v).

The PAC molecules eluted *via* HPLC can be further ionized by an ion generator such as electrospray ionization (ESI), and these ions are subsequently separated according to their *m/z* ratio (Hümmer & Schreier, 2008). The HPLC-MS^n methods have been widely applied in the analysis of PACs in a variety of food matrices. Apropos PS monomers (catechin and (epi)catechin), as well as A- and B-type PACs through dimers to tetramers were identified and quantified by Yu's group (2006) with a RP-HPLC/ESI-MS system. Recently, Constanza *et al.* (2012) reported the separation and identification of PACs through monomers to hexamers in PS using NP-HPLC/ESI-MS. Analyzing A-type PACs in PS, Appeldoorn and coworkers (2009) identified 83 PAC species with DPs up to seven *via* a combination of NP-HPLC and RP-HPLC/ESI-MS^2. The fractions throughout NP-HPLC was further separated and characterized by RP-HPLC-MS^2. PS PACs up to nonamer were separated by NP-HPLC and characterized using RP-HPLC/ESI-MS^n in Sarnoski's study (2012).

2.7 Quantification of Phenolic Compounds

The nature of the sample and the purpose of the study generally determine the selection of analytical method for phenolic compounds (Dai & Mumper, 2010). The large variety of type, number and content of phenolic compounds in plants and even in different tissues of a single plant often imposes limits to the measurement (Robards, 2003; Naczk & Shahidi, 2006). For instance, traditional colorimetric methods depending on an absorbing wavelength may cause over-estimation of phenolic contents due to the overlapping of spectral responses from the

interference of UV-absorbing substances (*i.e.*, proteins, nucleic acids and amino acids). Also, the complexity of plant phenolic constitutions makes the selection of a reference standard a difficult task. Despite all these disadvantages, spectrophotometric assays are rapid, simple and reproducible and widely used for quantification of phenolic compounds. A number of methods based on different principles to determine different phenolic groups have been developed, including the Folin–Ciocalteu assay for determination of total phenolics content (TPC) (Singleton & Rossi, 1965), as well as the vanillin (Price, Van Scoyoc, & Butler, 1978), dimethylaminocinnamaldehyde (DMAC) assay (McMurrough & McDowell, 1978) and proanthocyanidin assays (acid butanol assay) (Porter, Hrtstich, & Chan, 1986) for the estimation of total proanthocyanidins (TPAC) content.

The most widely applied technique now for both separation and quantification of phenolic compounds is HPLC, which is compatible with many classes of phenolics including anthocyanins, proanthocyanidins, hydrolysable tannins, flavonols, flavan-3-ols, flavanones, flavones, and phenolic acids from different plant extracts and food samples (Naczk & Shahidi, 2006; Dai & Mumper, 2010). All phenolic components together with their possible derivatives and degradation products can be simultaneously determined by HPLC. Although RP C_{18} columns are most often employed in phenolic analysis (Robbins, 2003), its resolution of PACs is limited to tetramers (Hümmer & Schreier, 2008). In contrast, much better separation of PACs with a DP up to 14 was achieved on a diol stationary phase (Kelm *et al.*, 2006). However, it's worthy to mention the lack of complete elution of highly polymerized PACs on a silica-based NP column leads to overlapping chromatograms (Naczk & Shahidi, 2006; Hümmer & Schreier, 2008). The applications of both RP- and NP-HPLC, incidentally, become an emerging trend in phenolic analysis because of the improved separation with multidimensional techniques. A comprehensive

two-dimensional liquid chromatographic (LC × LC) technique was described by Montero *et al.* (2013), whereby both hydrophilic interaction liquid chromatography (HILIC) and RP-HPLC were applied as separation stages; this approach showed great potential in profiling the phenolic compounds of complex food systems.

The Total Phenolics Content (TPC) with Folin & Ciocalteu's Phenol Reagent

Folin & Ciocalteu's phenol reagent was used to the assessment of antioxidant contents in wine by Singleton and Rossi (1965) and resulted in the well-known total phenolics content (TPC) assay. The mechanism behind the TPC assay involves the reduction of the molybdenum component in the phosphotungstic-phosphomolybdic complexing reagent by electron transfer from phenolic compounds, which leading to the formation of blue complexes {possibly $(PMoW_{11}O_{40})^{4-}$} that is measured at 745 to 765 nm according to the following reaction scheme:

$$\mathbf{Mo^{6+}\ (yellow) + ArOH \rightarrow Mo^{5+}\ (blue\ at\ 750\ nm) + [ArOH]^{\bullet+}}$$

whereby, ArOH represents the phenolic antioxidants. However, the Folin & Ciocalteu's phenol reagent is vulnerable to a great many interferences, particularly any readily reducible component present within the assay mixture. In the case of wine and most fruits, ascorbic acid is considered as the major interfering factor (Singleton, Orthofer, & Lamuela-Raventós, 1999). In the TPC assay, mixtures of excess phenol reagent and a diluted sample or a reference standard (usually gallic acid), as well as alkaline media of pH about 10, are involved in the procedure. The blue color of resultant complex is developed over 30 to 60 min. The reaction can be accelerated by increased the alkalinity and/or temperature. However, care should be taken as the precipitation of

the phenol reagent may occur where a dense, white, crystalline material is formed by excessive heat (above 60 °C), alkalinity (above pH 10-11), or the quantity of reagent in the assay (above 5 mL/100 mL) (Rosenblatt & Peluso, 1941).

Total PACs (TPAC) Content with Dimethylaminocinnamaldehyde (DMAC)

Of different conventional methods to determine total PACs content, the PAC assay (acid butanol assay), dimethylaminocinnamaldehyde (DMAC) assay and vanillin assay are the most common specific analyses that involve particular structure properties of flavan-3-ols and PACs (Hümmer & Schreier, 2008). In the DMAC assay, the aromatic aldehyde (DMAC) reacts with specific compounds containing *meta*-oriented di- or trihydroxy substituted benzene rings such as the 5,7-dihydroxy A-ring of catechin and epicatechin, resulting in a green chromophore [**Figure 2.4(A)**] with maximum absorbance at approximately 640 nm (McMurrough & McDowell, 1978). The interference from anthocyanidins is effectively eliminated by this wavelength, which is a problem in the butanol–HCl and the vanillin assays (Payne *et al.*, 2010; Wallace & Giusti, 2010). In addition, structural properties, including a single bond between C2 and C3 and the lack of a carbonyl at C4, are also required for a positive reaction (Payne *et al.*, 2010). Therefore, compared to the acid–butanol and the vanillin assays, DMAC assay shows higher specificity and accuracy for PACs without the reaction with hydroxycinnamic acids, hydroxybenzoic acids, flavones, and flavonols (Krueger, Reed, Feliciano, & Howell, 2013).

However, The DMAC assay is more suitable for samples containing low-molecular-weight PAC oligomers for which the structure and reaction kinetics of the monomer or dimer standards are better matched. Therefore, quantification error exists when MS analyses reveal the presence of higher polymerized PACs (*i.e.*, a DP $\geq$ 4) (Krueger, Reed, Feliciano, & Howell, 2013).

Moreover, denser color is developed by monomers (catechin and epicatechin) compared to dimers, indicating the A-ring involved in intermonomer linkage is no longer available for the DMAC reaction (McMurrough & McDowell, 1978). It further turns out the reaction only takes place at the C8 terminal unit of PACs and thereby the response would decrease along with the increase of DP. As polymerization of PACs, more C8 sites would participate in the interflavan linkages and less DMAC reactive sites would be present (Krueger, Reed, Feliciano, & Howell, 2013).

2.8 Methods to Determine Total Antioxidant Capacity (TAC) of Phenolic Extracts

Because the antioxidant activity is a cooperative action in a complex sample, it's more meaningful to measure the integrated total antioxidant capacity (TAC) than study each phenolic antioxidant individually (Dai & Mumper, 2010). Generally, antioxidant assays can be roughly divided into two classes based on the antioxidant reaction involved: hydrogen atom transfer (HAT) reaction based assays and electron transfer (ET) reaction based assays (Huang, Ou, & Prior, 2005). In HAT-based assays, the free radical takes a hydrogen atom from an antioxidant compound while the antioxidant becomes a relative stable radical itself, which measures the capacity of an antioxidant to quench free radicals by hydrogen donation. Oxygen radical absorbance capacity (ORAC) and total radical-trapping antioxidant parameter (TRAP) are well-known HAT-based assays. In ET-based assays, the antioxidant donates an electron to the free radical and itself becomes relative stable radical species. A variety of assays includes trolox equivalent antioxidant capacity (TEAC), ferric ion reducing antioxidant power (FRAP) and Folin–Ciocalteu assay as a measurement of total phenolics content are ET-based assays.

HAT: $ROO^{\bullet} + ArOH \rightarrow ROOH + ArO^{\bullet}$

ET: $ROO^{\bullet} + ArOH \rightarrow ROOH + [ArOH]^{\bullet+}$

whereby, ArOH represents the phenolic compound and ArO• is the stable phenoxy radical.

2.8.1 Oxygen Radical Absorbance Capacity ($ORAC_{FL}$) Assay

The oxygen radical absorbance capacity (ORAC) assay was developed by Dr. Alexander N. Glazer in the early 1990s to determine ROS in biological systems (Glazer, 1990). The ORAC assay was adopted by Cao *et al.* (1993) for the assessment of antioxidant species in human plasma, which basically measures free radical-scavenging activity against the peroxyl radical induced by 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) [**Figure 2.4(B)**]. The antioxidant activity is determined by the ability of hydrogen donation and can be easily quantitated by the decay of fluorescent probe. Ou *et al.* (2001) improved the method and named it the $ORAC_{FL}$ assay since then, where a more stable and reproducible fluorescent probe, fluorescein (FL, 3′,6′-dihydroxyspiro[isobenzofuran-1[3*H*],9'[9*H*]-xanthen]-3-one), is used.

Briefly, radicals such as AAPH can destroy fluorescein and cause degradation of fluorescence. In the presence of antioxidant, the oxidation of fluorescent probe induced by AAPH radical is inhibited and fluorescence is thereby maintained for longer period. The radical scavenging capacity is then determined by the area of a degradation curve originating from continuous fluorescence reading of a sample in a multiple-welled fluorometer for a couple of hours. The AAPH scavenging activity is expressed by a standard curve built on different concentrations of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, a water-soluble vitamin E analogue). In short, data analysis from the $ORAC_{FL}$ assay is achieved by

(1) calculating of the area under the kinetic curve (AUC) and net AUC (AUC_{sample} - AUC_{blank}), (2) obtaining a standard curve by plotting the concentration of Trolox and the AUC (3) calculating the Trolox equivalents of a sample using the standard curve.

2.8.2 Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay (Benzie & Strain, 1996) is a simple method to determine the reduction of ferric-tripyridyltriazine complex {Fe(III)–TPTZ}. In an acidic medium, the ferric tripyridyltriazine {Fe(III)-TPTZ} complex is reduced to ferrous tripyridyltriazine {Fe(II)–TPTZ} by a reductant/antioxidant, such as a phenolic compound, with an appropriate redox potential. The resultant Fe(II)–TPTZ chromophore yields an intense blue color with a wavelength maximum of 593 nm [**Figure 2.4(C)**]. Unfortunately, the FRAP assay only detects compounds with redox potentials of < 0.7 V (Prior, Wu, & Schaich, 2005), and is indicative of the antioxidant capacity of converting radicals to stable products by electron donation.

Unfortunately the acidic pH of the FRAP assay that is necessary to maintain iron solubility, lowers the ionization potential of the reactants and increases the redox potential of the system (Prior *et al.*, 2005). An additional disadvantage of the FRAP assay is the exclusion of some polyphenols that require times for reaction that exceed 4 min (Prior, Wu, & Schaich, 2005). A slowly increasing FRAP value was found in phenolics such as caffeic acid, tannic acid, ferulic acid, *trans*-resveratrol, and quercetin even after several hours of reaction (Pulido, Bravo, & Saura-Calixto, 2000).

2.9 Dietary Fiber (DF) and Health Benefits Associated with Dietary Polyphenols

DF is the indigestible portions of plant foods, including two main components of soluble and insoluble fibers. Soluble fibers can be fermented by the florae in the colon, generating physiologically active byproducts such as short chain fatty acids; while insoluble fiber may only partially fermented in the colon but provide bulking to ease defecation. The high intake of DF lowers blood pressure and cholesterol levels, resulting in the reduced risk of coronary heart disease, stroke, hypertension, diabetes and obesity (Anderson *et al.*, 2009). Unfortunately, the average fiber consumption amongst adults in the U.S. is ~15 g a day instead of the recommended 25 to 30 g.

Polyphenols and DF are two well-documented dietary factors in the prevention of chronic diseases, but are usually addressed as separate compounds acting independently in disease prevention. A study conducted by Saura-Calixto demonstrated a synergistic function of DF and dietary polyphenols, mainly in the development of an antioxidant environment in the colon (Saura-Calixto, 2011). According to the study, DF acts as transportation for dietary polyphenols and carries them to the colon where they are released by the action of the florae and produce metabolites with potential health benefits. Nonextractable polyphenols (NEPP), largely consisting of PACs, as well as individual ferulic acid, caffeic acid, hesperidin, naringenin, catechin, epicatechin, ellagic acid, gallic acid derivatives, and *p*-hydroxybenzoic acid are major antioxidants associated with DF. Prevention effects with consumption of DF-associated NEPP on cardiovascular diseases and also on the risk colon cancer have been revealed in a great deal of *in vivo* studies (Saura-Calixto, 2011).

DF Profile of PS and Fiber Analysis

Little information is currently available on the fiber content of PS. Shimizu-Ibuka *et al.* (2009) studied the hypocholesterolemic effect of PS where they used a commercial roasted PS which contained ~45% of TDF and only 2.2% in PS was soluble DF. The determination of total DF content in food is usually accomplished by a combination of enzymatic and gravimetric methods (**Figure 2.5**) as described in AOAC Official Method 991.43 (AOAC, 2005). Sample is first digested by α-amylase to break long chain carbohydrates. Protease and amyloglucosidase are used subsequently to hydrolyze proteins and amylopectins, respectively. After precipitation by ethanol, the residue is filtered and washed. Oven dried residues are weighed. The TDF content was calculated by subtracting the mass of the crude protein, ash, and blank from the mass of the filtered and dried residues. Therefore, half of the samples are subjected to protein analysis while the others are ashed.

$$\textbf{Residue Mass (R)} = W_2 - W_1 \textbf{ (mg)}$$

$$\textbf{Ash Mass (A)} = W_3 - W_1 \textbf{ (mg)}$$

$$\textbf{Blank Mass (B)} = R_{blank} - A_{blank} - P_{blank} \textbf{ (mg)}$$

where, the blank containing only MES-TRIS buffer was processed identically as the samples. So in the above equation, R_{blank} is the residue mass of the blank, P_{blank} is the crude protein mass of the blank, and A_{blank} is the ash mass of the blank. Proteins in the blank originated from enzymes used to digest protein and carbohydrate.

$$\text{g TDF/100-g sample} = (R_{sample} - A_{sample} - P_{sample} - B)/\text{sample mass (mg)} \times 100$$

where, R_{sample} is the residue mass of the sample P_{sample} is the crude protein mass of the sample, and A_{sample} is the ash mass of the sample, all in mg.

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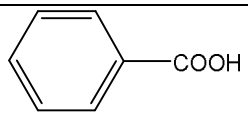
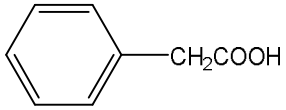
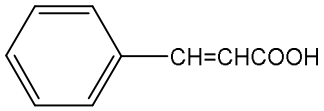
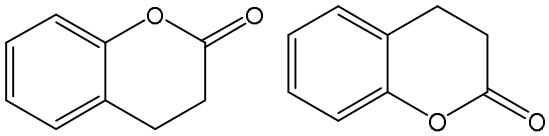
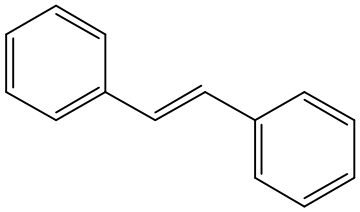
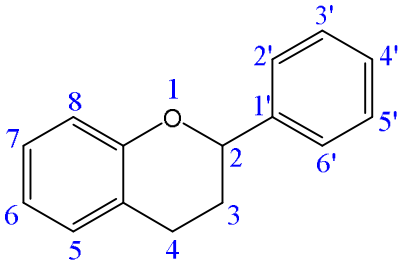
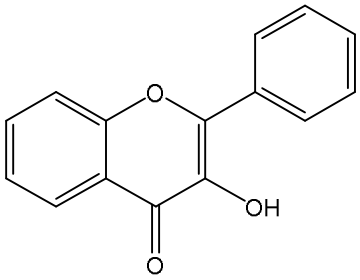
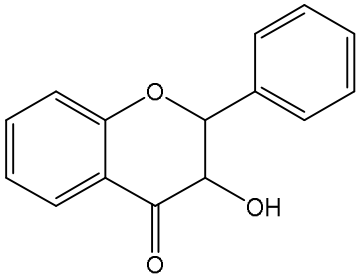
Table 2.1 Classification of major dietary phenolics and related food sources (Adapted from King & Young, 1999).

Class & subclass	Phytochemicals	Food sources
Flavonoids		
Flavonols	Quercetin, kaempferol, myricetin	Olives, onions, apples, tea, broccoli
Flavanonols	Taxifolin	Red onions
Flavones	Apigenin, luteolin	Olives, Celery, Broccoli
Flavanones	Eriodictyol, hesperetin, naringenin	Citrus fruits
Flavanols	Catechin, Epicatechin, Epigallocatechin	Tea, cocoa, chocolate, apples, grapes
Isoflavones	Genistein, daidzein	Soybeans, tofu, soy milk
Phenolic acids		
Hydroxybenzoic acid	Ellagic acids, gallic acids, *p*-hydroxybenzoic acids, protocatechuic acids	Raspberry, strawberry, grapes
Hydroxycinnamic acid	Caffeic acids, ferulic acids	Apples, pears, citrus fruits, some vegetables, whole grains, coffee
Tannins		
Condensed tannins	Procyanidins and proanthocyanidins (PACs)	Cranberry, cocoa, apples, strawberries, grapes, red wine, peanuts, tea, chocolate

Table 2.2 Basic carbon skeletons and structures of selected phenolic compounds (Adapted from Bravo, 1998).

Class & subclass	Basic skeleton	Basic structure
Phenolic acids	$C_6 - C_1$	
Phenylacetic acids	$C_6 - C_2$	
Hydroxycinnamic acids	$C_6 - C_3$	
Coumarins, isocoumarins	$C_6 - C_3$	
Stilbenes	$C_6 - C_2 - C_6$	
Flavonoids	$C_6 - C_3 - C_6$	
Flavonols		
Flavanonols		

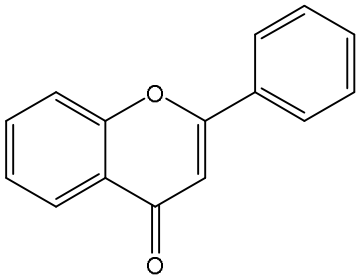
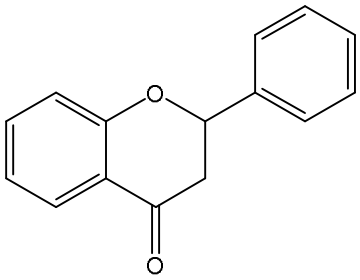
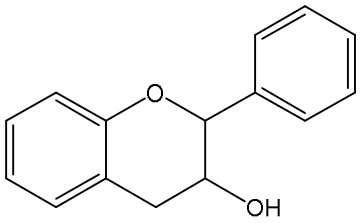
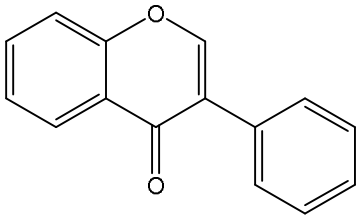
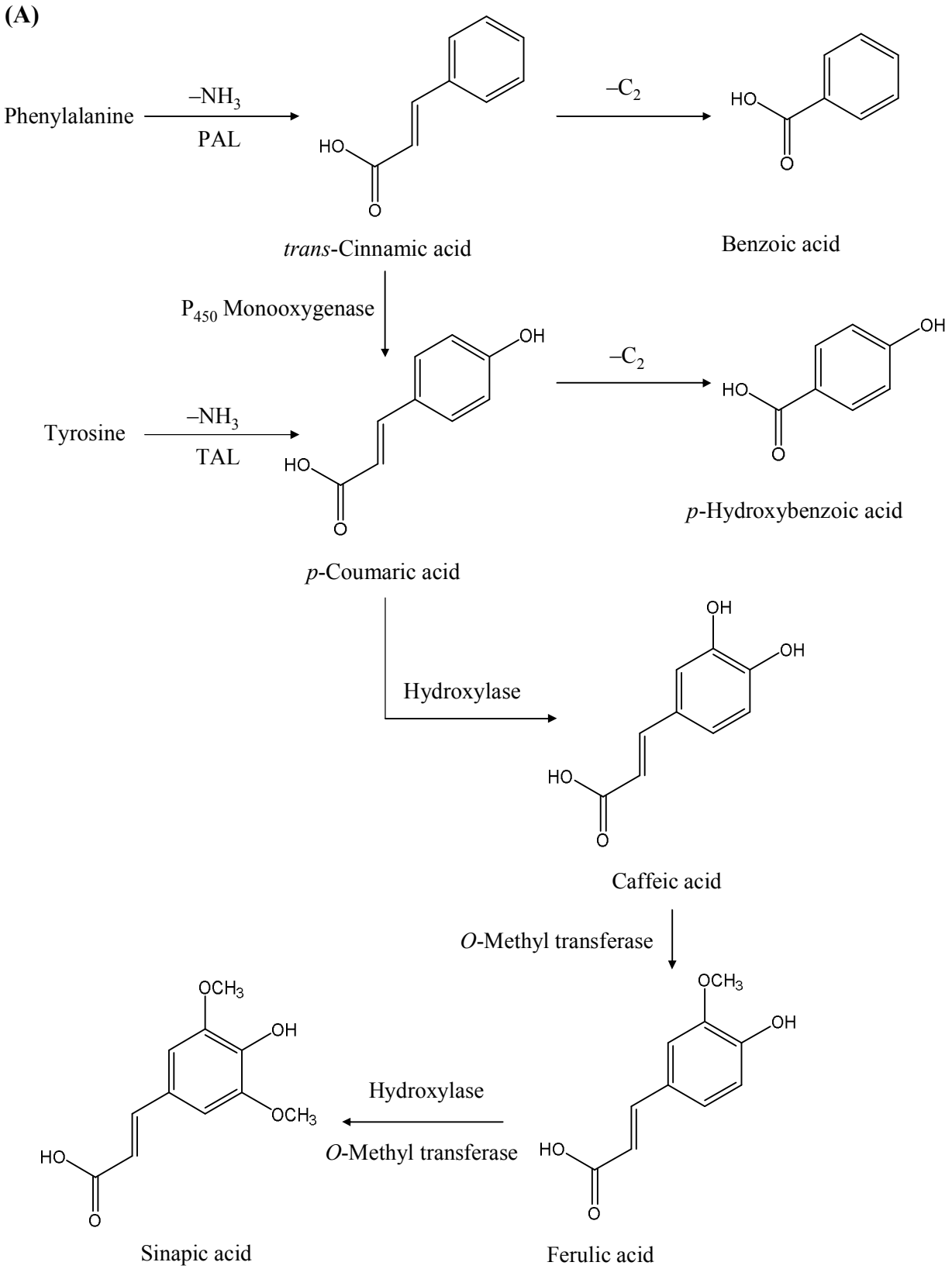
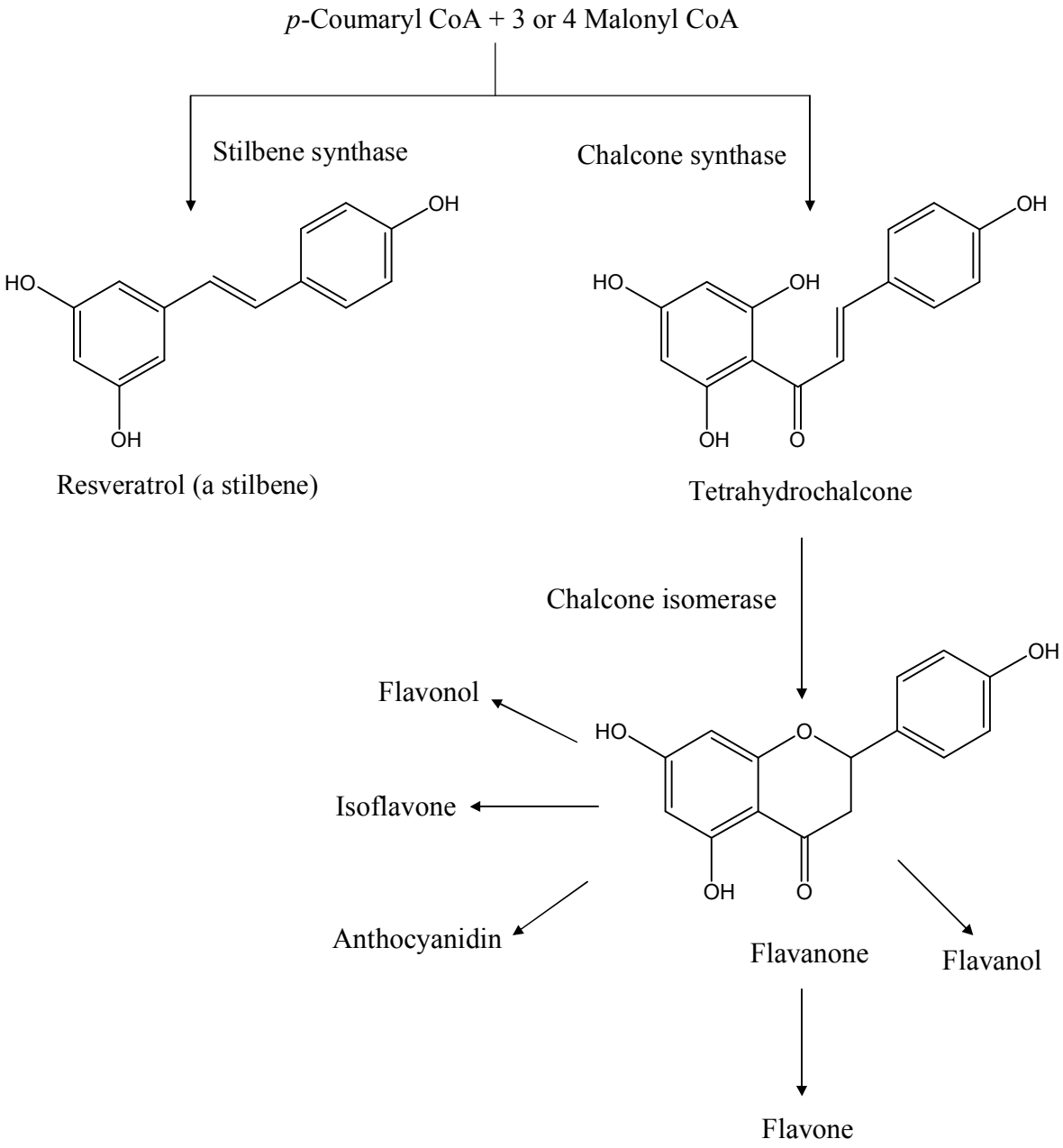
Flavones	
Flavanones	
Flavanols	
Isoflavones	
Biflavonoids	$(C_6 - C_3 - C_6)_2$
Condensed Tannins	$(C_6 - C_3 - C_6)_n$

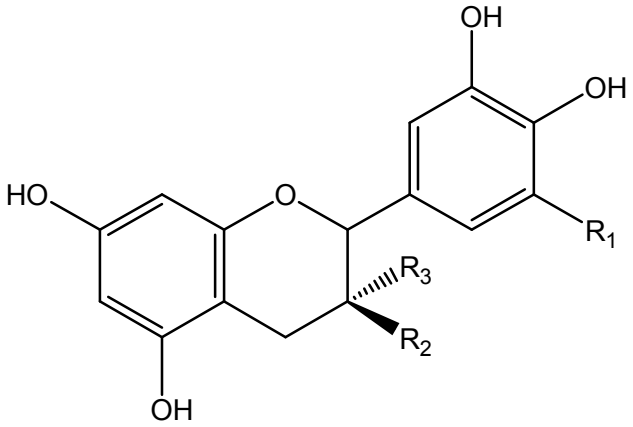
Figure 2.1 Biosynthesis of phenolic compounds: **(A)** Biosynthesis of phenylpropanoids and phenolic acids and **(B)** Biosynthesis of flavonoids (Adapted from Shahidi, 2000). Abbreviations are as follows: PAL = Phenylalanine ammonia lyase; TAL = Tyrosine ammonia lyase.

(A)


Phenylalanine
–NH3
PAL
–C2
HO
O
trans-Cinnamic acid
Benzoic acid
P450 Monooxygenase
OH
Tyrosine
–NH3
TAL
p-Hydroxybenzoic acid
p-Coumaric acid
Hydroxylase
Caffeic acid
O-Methyl transferase
OCH3
Hydroxylase
O-Methyl transferase
Sinapic acid
Ferulic acid

(B)


p-Coumaryl CoA + 3 or 4 Malonyl CoA
Stilbene synthase
Chalcone synthase
HO
OH
OH
Resveratrol (a stilbene)
HO
OH
OH
O
Tetrahydrochalcone
Chalcone isomerase
Flavonol
Isoflavone
Anthocyanidin
HO
O
OH
OH
OH
O
Flavanone
Flavanol
Flavone



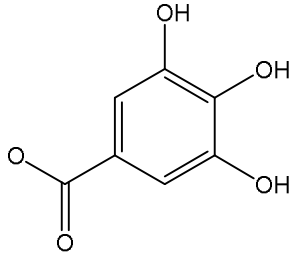
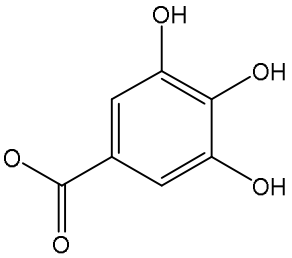
Flavanol	**R_1**	**R_2**	**R_3**
Catechin	H	OH	H
Epicatechin (EC)	H	H	OH
Gallocatechin (GC)	OH	OH	H
Epigallocatechin (EGC)	OH	H	OH
Gallocatechin gallate (GCG)	OH	 *O*-Gallate	H
Epicgallocatechin gallate (EGCG)	OH	H	 *O*-Gallate

Figure 2.2 Chemical structures of flavan-3-ols.

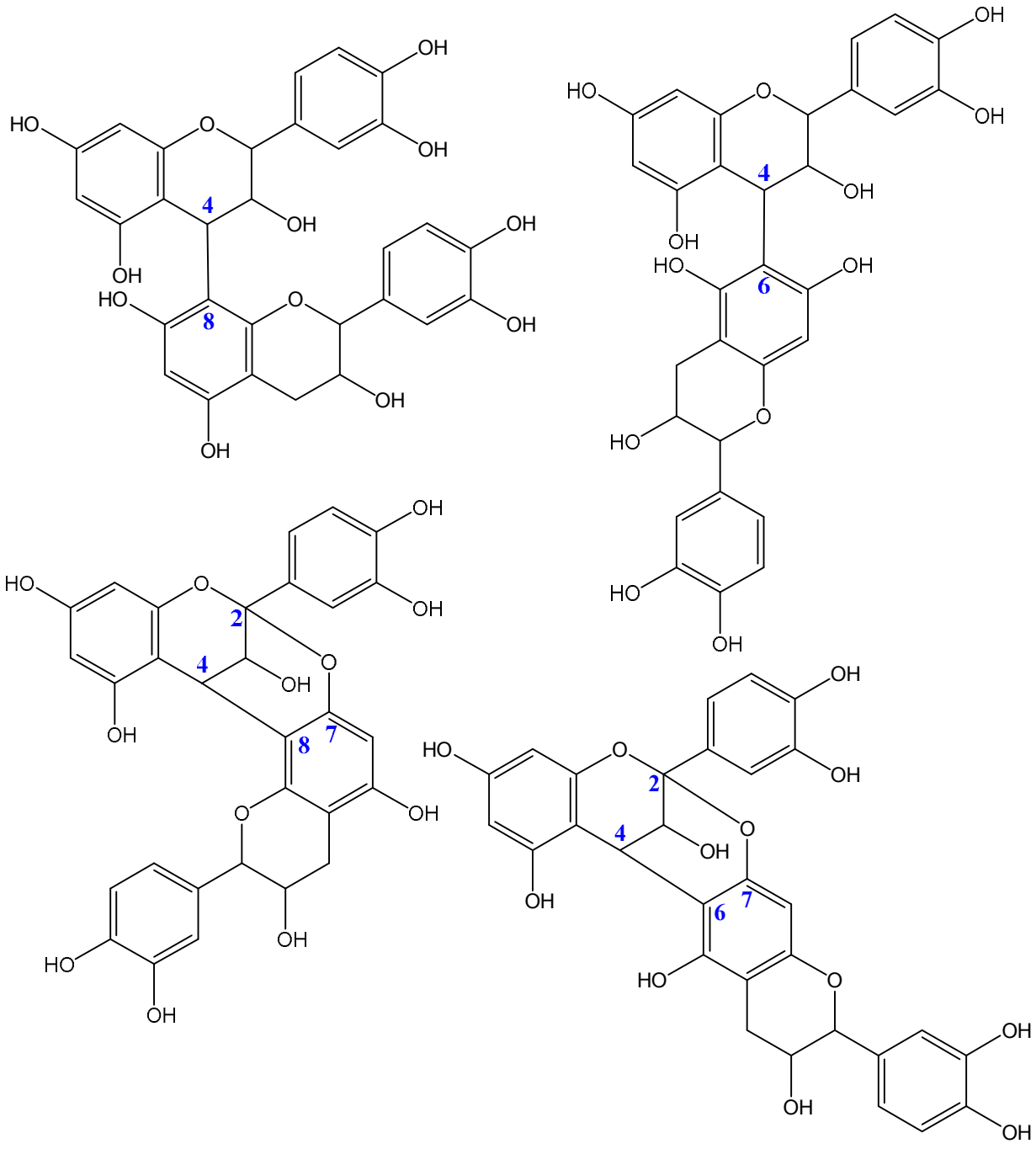
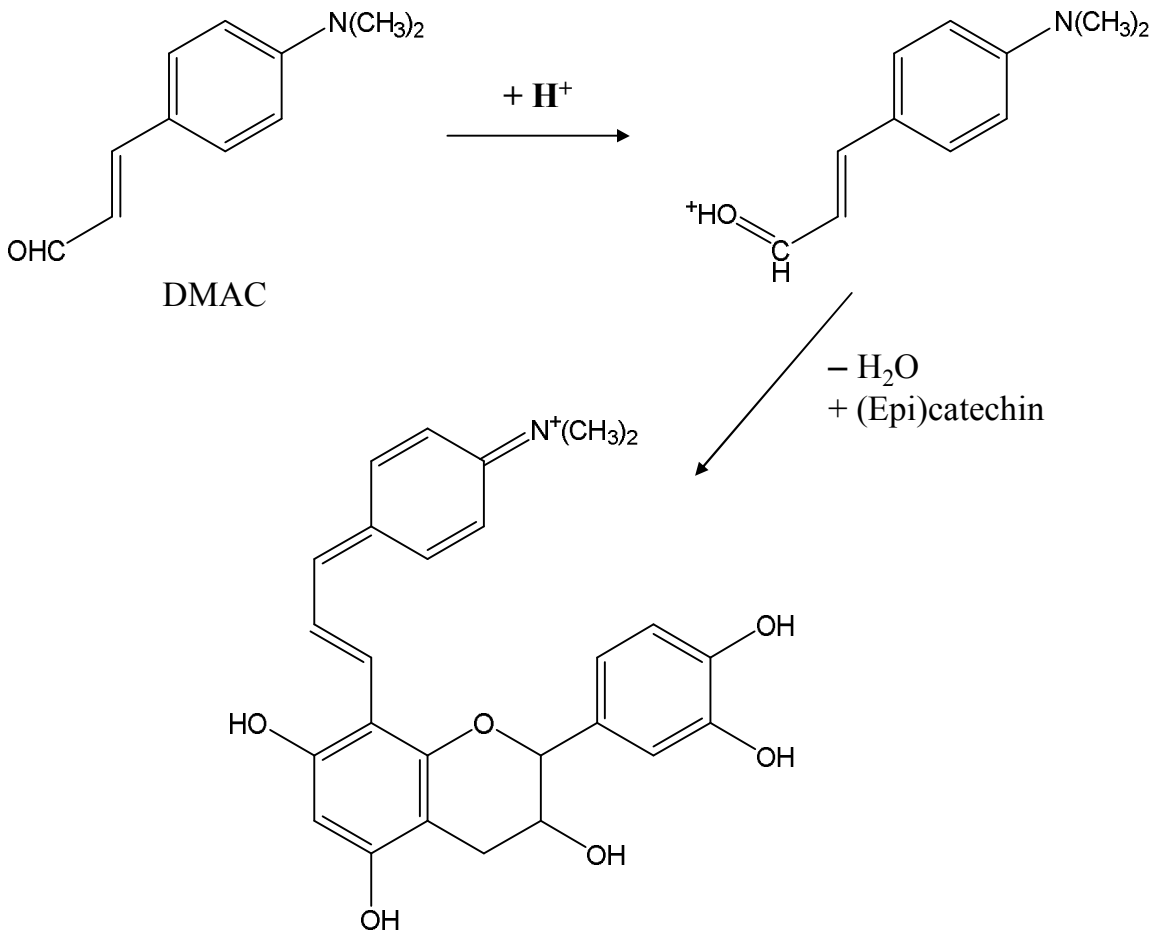


Figure 2.3 Linkages found in proanthocyanidins (PACs): A-type bond with both (C4→C8) and (C2→O→C7) linkages or (C4→C6) and (C2→O→C7) linkages, B-type bond which can be (C4→C8) or (C4→C6) linkage.

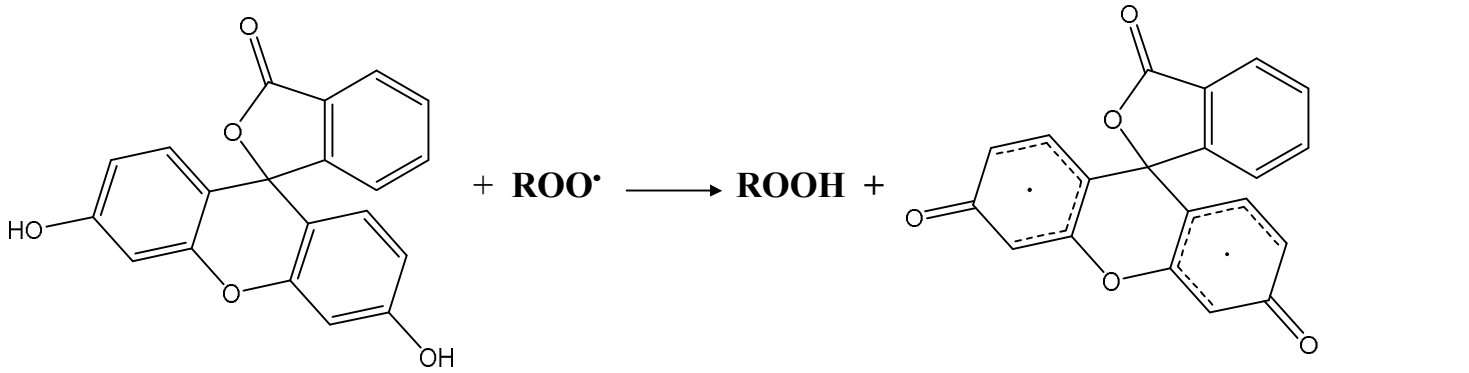
Figure 2.4 Proposed mechanisms for **(A)** Dimethylaminocinnamaldehyde (DMAC) assay (Adapted from Wallace & Giusti, 2010), **(B)** Oxygen radical absorbance capacity ($ORAC_{FL}$) assay, and **(C)** Ferric Reducing Antioxidant Power (FRAP) assay (Adapted from Huang, Ou, & Prior, 2005). Abbreviations are as follows: ArOH, antioxidant; $ArO^{\bullet}$, phenoxyl radical; $ROO^{\bullet}$, peroxyl radical; ROOH, hydroperoxide.

(A)



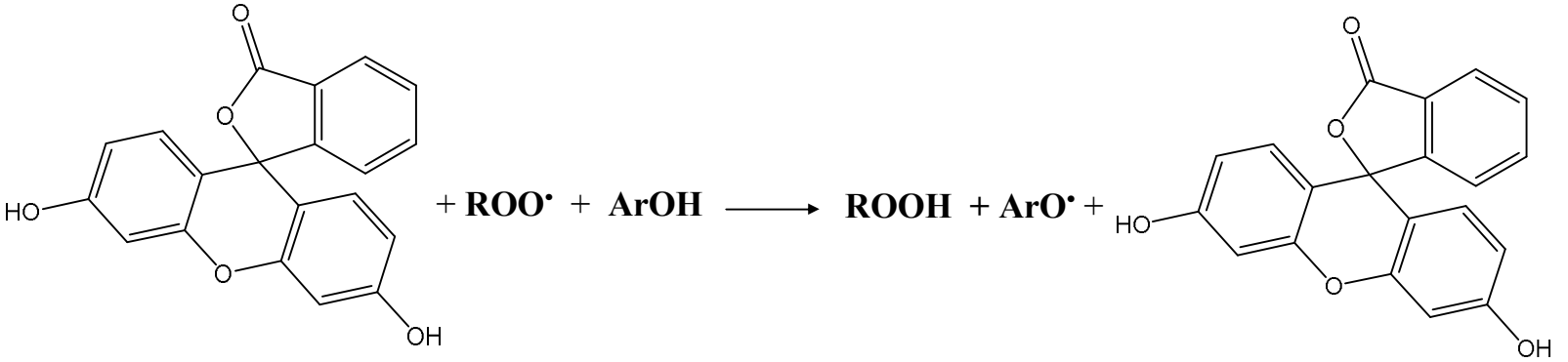
DMAC-(Epi)catechin Complex (green at 640 nm)

(B)



Fluorescein

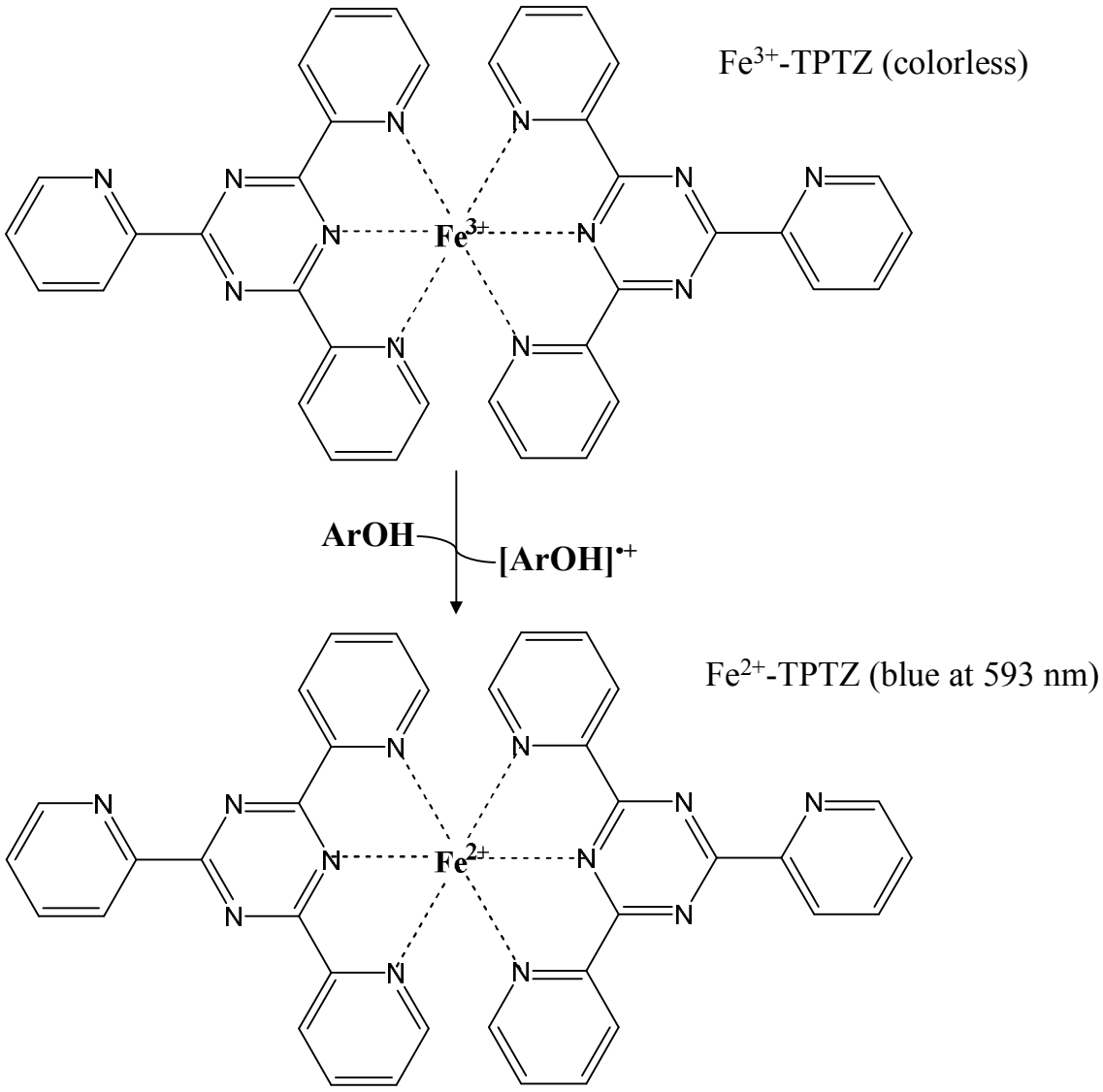
Dehydrofluorescin (no signal at 520 nm)



Fluorescein

Fluorescein (emission at 520 nm)

(C)



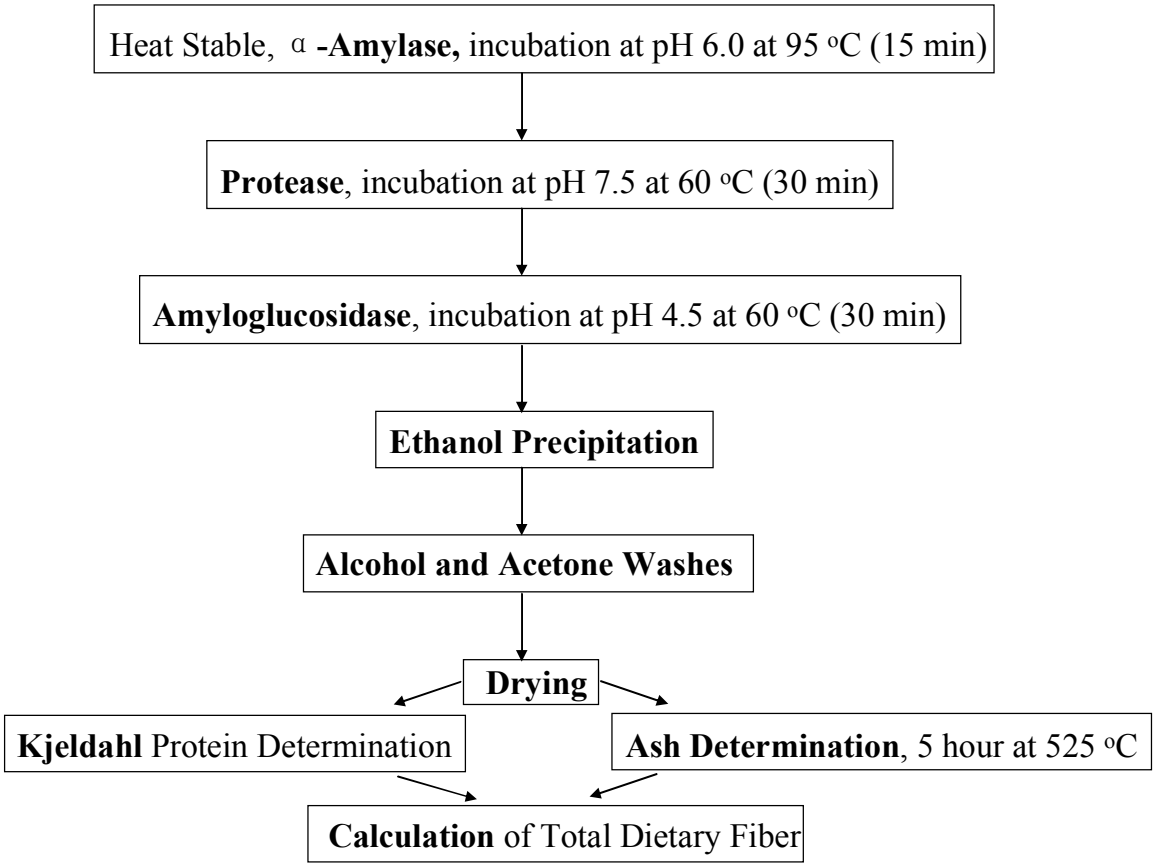


Figure 2.5 The flowchart of total dietary fiber (TDF) assay. Adapted from AOAC Official Method 991.43 (AOAC, 2005).

CHAPTER 3

EFFECT OF PEANUT SKINS INCORPORATION ON THE COLOR, TEXTURE, AND TOTAL PHENOLICS CONTENT OF PEANUT BUTTERS

Ma, Y.; Kerr, W. L.; Cavender, G. A.; Swanson, R. B.; Hargrove, J. L.; Pegg, R. B. *Journal of Food Process Engineering*. **2013**, *36,* 316–328. Reprinted here with permission of Publisher (John Wiley and Sons).

Abstract

The impact of incorporating ground dry-blanched or roasted peanut skins (PS, three types: light-, medium- and dark-roasted) into peanut butter as a functional food ingredient was examined. A desirable grind size for the PS of less than 300 mm was achieved in most cases by milling the skins with the sugar. Adding ground PS to peanut butter at 1.25, 2.5, 3.75 and 5.0% (w/w) resulted in a concentration-dependent change in the Commission Internationale de l'Éclairage L^* C^* h values. Peanut butters formulated with medium- and dark-roasted PS showed an increase in hardness, and were generally more adhesive than those without PS or with dry-blanched PS. A marked change in spreadability was found with greater than 2.5% added PS. Incorporation of dry-blanched PS, especially at levels below 3.75%, produced the fewest alterations in physical properties of the peanut butters compared with the control. Importantly, a concentrationdependent increase in the total phenolics content (TPC) was evident with PS fortification. Dry-blanched PS had a TPC of ~166 mg (+)-catechin equivalents/g extract and yielded peanut butters with a 32, 33 and 38% higher TPC than found with light-, medium- and dark-roasted skin incorporation, respectively. Correspondingly, dry-blanched PS addition at 1.25, 2.5, 3.75 and 5.0% (w/w) resulted in an increase in the TPC by 86, 357, 533 and 714%, respectively, compared with peanut butters without PS fortification.

3.1 Introduction

As demonstrated by George Washington Carver more than a century ago, peanuts (*Arachis hypogaea* L.) are a valuable cash crop to the southern U.S.A. Revenue generated from the peanut crop in the U.S.A. alone has averaged $1 billion annually from 1996 to 2001 (Dohlman, Young, Hoffman, & Mcbride, 2004). Peanuts are consumed in many parts of the world with China, India and the U.S.A. accounting for roughly two-thirds of the world's peanut production (United States Department of Agriculture [USDA 2011]). While in many countries the argest portion of the crop is destined for oil production, the major products made from peanuts in the U.S.A. are peanut butter, salted peanuts, confections and roasted peanuts in-the-shell (Carley, 1983). Peanut butter is considered the most important peanut-based product, with more than half of the U.S. crop used for its manufacture (Woodroof, 1983). The volume of edible peanuts utilized for peanut butter significantly increased from 400 million pounds in the early 1950s (Woodroof 1983) to over 1,000 million pounds in 2008 (USDA 2008–2009). According to the USDA data for the years 2008–2011 (USDA 2008–2012), the quantity of commercially-processed shelled edible-grade peanuts utilized in the production of peanut butter products has increased from ~1,000 to ~1,200 million pounds. These values reflect 65 and 60% of the total edible peanut usage in the U.S.A., respectively.

The profile of oil and protein in peanut kernels makes peanut butter a highly nutritious end-product (Knauft, Moore & Gorbet, 1993; Andersen, Hill, Gorbet & Brodbeck, 1998), especially in the form of sandwiches where the wheat flour of bread complements the limiting amino acids of peanuts. The appealing flavor, convenience of use and excellent shelf life of peanut butter contribute greatly to its popularity. According to the American Peanut Council, peanut butter ranks as one of the favorite foods of American households (Jolly *et al.* 2005).

Owing to both its nutrient profile and palatability, peanut butter is nutritious for growing children; it is often taken to schools for lunch or has been formulated in other lunch-based foodstuffs (Woodroof, 1983).

Peanut butter is relatively simple to manufacture: it involves the steps of shelling, dry roasting, blanching and grinding the peanuts into a paste (Woodroof, 1983). Shelled peanuts, generally from Runner varieties, are first subjected to a uniform roasting to develop the characteristic peanut flavor. The generated flavor and color characteristics will directly impact the palatability of the resultant peanut butter. After a quick cooling, peanut skins (PS) are removed *via* dry blanching before the meats are ground to the desired texture (*i.e.*, smooth or chunky formulation). Additional ingredients, such as salt, sugar and stabilizer, may be added in order to improve consumer appeal. Stabilizers (*i.e.*, hydrogenated cottonseed, canola, soybean, palm oils or a mixture therefrom) help to prevent the separation of the oil and solid fractions. As the peanut butter cools after processing, either naturally or by shock chilling, the drop in temperature initiates the process of crystallization of the stabilizer leading to entrapment of oil and prevention of its migration to the surface of the product. The tempering period of freshly prepared peanut butter is important to allow for proper formation of the crystal network.

In the U.S.A., the Food and Drug Administration (FDA) has established a legal standard of identity (21 CFR § 164.150) in the Code of Federal Regulations (CFR): it states that for a product to be called "peanut butter," it must contain no less than 90% peanuts by weight and no more than 55% fat; otherwise, it will be referred to as a peanut spread (Merrill & Collier, 1974; FDA 2009). The remaining 10% may consist of salt, sweetener for the enhancement of flavor, emulsifier and/or stabilizer. In 2012, market products labeled as peanut butters can be procured, which contain additives and flavorings such as dark chocolate, cinnamon and raisins, maple

syrup, and honey, even though they violate the FDA's standard of identity.

A by-product of the peanut industry is the skins. Most skins are dumped in landfills, whereas only a small quantity is added to animal feed (Sobolev & Cole, 2004). In 1999/2000, over 750,000 tons of PS were generated worldwide based on an estimated 29.1 million tons production of peanuts with an average skin content of 2.6%. In the U.S.A., the total volume of commercially processed shelled ediblegrade peanuts was roughly 2,000 million pounds during 2011 (USDA 2011–2012); hence, *ca.* 52 million pounds of PS were generated. PS are only now being recognized as extremely rich in polyphenols and fiber, and therefore with potential as a functional food ingredient. Nepote *et al.* (2002) reported a content of *ca.* 159 mg total phenolics/g defatted dry skin, which also had a marked antioxidant activity, as demonstrated by its capacity to inhibit oxidation of sunflower oil. A study conducted by Yu, Ahmedna, and Goktepe (2005) revealed that the phenolics from PS are abundant not only in the quantity but also in the variety of phenolics, which primarily include phenolic acids (*e.g.*, caffeic, chlorogenic, ferulic, and coumaric acids), flavonoids {*e.g.*, (epi)catechins and epigallocatechin (EGC), catechin gallate (CG), and epicatechin gallate (ECG)}, and stilbene (*e.g.*, *trans*-resveratrol).

An increased awareness of the potential role of antioxidants in health promotion and disease prevention has led to a demand for antioxidant-enriched foods. PS are a concentrated source of phenolics; hence, their incorporation into foods would effectively enhance the antioxidant capacity without significantly increasing the cost of the finished product. Despite the tremendous potential benefit of PS, allergy-based issues because of possible protein residue on the skins, and processing challenges, for the most part, have prevented PS utilization as a functional food ingredient in value-added products. Quite interesting is that the proanthocyanidins in PS have demonstrated anti-allergic effects both *in vitro* and *in vivo*: these are attributed to the formation

of indigestible complexes (*i.e.*, PS covalently bound to peanut allergens, Chung & Reed, 2012), the lowering of serum IgE and IgG1 levels (Takano *et al.*, 2007), and the inhibition of degranulation induced by antigen stimulation of rat basophilic leukemia (RBL-2H3) cells (Tomochika *et al.*, 2011).

The archetypal texture of peanut butter is characterized by optimum levels of firmness, high cohesiveness and spreadability, as well as sufficient adhesiveness. The variety of peanut, processing conditions and added ingredients can significantly impact the textural properties of peanut butters. As reported by Crippen *et al.* (1989) grind size, sucrose and salt levels affect sensory smoothness, hardness, spreadability, adhesiveness and cohesiveness of peanut butters. Reduced fat products are referred to as a peanut spread, not a peanut butter, because they only contain roughly 60% peanuts and do not adhere to the USDA standard of identity for peanut butter (Anon., 2002). Soy protein and corn syrup solids are often added to substitute for the missing peanuts. Most times, this results in undesirable textural and sensory properties as exhibited by a lesser desirable peanut flavor, increased grittiness and a sweeter taste. Incorporation of PS into peanut butter will maintain the standard identity of the product and should increase the product's fiber content and antioxidant activity, diversify the existing market, and provide economic returns for this by-product. Sensory acceptance of the reformulated peanut butters with PS was confirmed by Sanders *et al.* (2011); the authors noted that consumers showed a preference for food products with claims associated with high phenolics. Any alterations in textural and physical properties, important to sensory properties and processing aspects, have yet to be reported for peanut butter with added PS.

The main objectives of the present study are (1) to formulate peanut butters fortified with varying levels of PS (both dry-blanched and roasted), while maintaining the product's standard

of identity; (2) to assess the effect of PS incorporation on the physical attributes of the finished products compared with the peanut butter void of PS; and (3) to determine the impact on the total phenolics content (TPC) and potential antioxidant activity of the PS-containing peanut butters.

3.2 Materials and Methods

Materials

Dry-blanched PS were provided by Sylvester Blanching, a division of Universal Blanchers, LLC (Sylvester, GA). Roasted PS (light, medium and dark) were a gift from the Golden Peanut Company, LLC (Alpharetta, GA). Peanut paste, flour salt and hydrogenated vegetable oil (*i.e.*, stabilizer) were supplied by Seabrook Ingredients, Inc. (Edenton, NC). Domino-premium pure cane granulated sugar and Bakers & Chefs refined, bleached and deodorized (RBD) peanut oil were purchased from Sam's Club (Athens, GA). All solvents and reagents were of analytical American Chemical Society [ACS] grade, unless otherwise specified. Methanol, ethanol (95%), and hexanes were purchased from VWR International (Suwanee, GA). Folin & Ciocalteu's phenol reagent and (+)- catechin were acquired from the Sigma-Aldrich Chemical Co. (St. Louis, MO).

Physical Methods

Peanut Butter Processing

Seventeen formulations of peanut butter (~45% total fat) were prepared; the details are provided in **Table 3.1**. For each formulation, PS and sugar were ground together at different ratios prior to inclusion into the product using an electrically-driven stone grinder (Super Masscolloider CA6-3, Masuko Sangyo Co., Ltd., Kawaguchi City, Saitama, Japan) fitted with a

BA6-80 grit stone assembly. More specifically, 22.9, 45.8, 68.7 and 91.6 g of dry-blanched PS were ground in the masscolloider with 195 g of sugar; the PS–sugar powders, after sieving (see details later) were then added to peanut butter formulations to achieve PS addition levels of 1.25, 2.5, 3.75 and 5.0% PS. After grinding, the PS–sugar mixtures were sieved through a no. 20 mesh screen using a vibratory separator (Model LS18S3333, Sweco Inc., Florence, KY). Particles too large to pass through the screen were subsequently re-ground.

Commercial peanut paste, taken from a 4 °C cooler, was first softened in a steam cabinet (Model SB 24.24, Pyramid Food Processing Equipment Manufacturing Inc., Tewksbury, MA), and then warmed to ~60 °C in a 19-L steam kettle (Dover Corporation, Elk Grove Village, IL) to facilitate portioning. RBD peanut oil, flour salt (~300 mm *i.d.*) and ground PS–sugar mixtures (see **Table 3.1** for quantities) were added to the portioned peanut paste. The contents were mixed in a jacketed food processor (Model R10, Robot Coupe USA, Inc., Jackson, MS) under a 60-kPa vacuum to prevent the incorporation of air. For all trials, the vacuum was produced by a Welch vacuum pump 8910A (Welch Vacuum Technology, Inc., Skokie, IL) and the heat generated by circulating a 76.5 °C propylene glycol solution through the jacket of the food processor with a temperature-controlled bath (Neslab Instruments, Inc., Newington, NH). Stabilizer was added after the temperature of the mixture reached 70 °C; the product was blended for an additional 5 min, after which, the hot peanut butter (~450 g) was poured into 500-mL home canning jars (Hearthmark, LLC, Daleville, IN). After the capped peanut butters had cooled to room temperature, they were placed in storage at 4 °C until the time of analysis. Each batch comprised 3 kg of the final product.

Particle Size Distribution of Ground Peanut Skins (PS)

PS-sugar mixtures were placed in separate Petri dishes and were washed with deionized water to remove the sugar. The isolated ground skins were then immersed in 95% (v/v) ethanol and a picture was taken using a Leica MZ6 Stereomicroscope (Leica Biosystems Richmond, Inc., Richmond, IL). Microscopy images of the ground PS samples were analyzed by ImageJ (v. 1.44, U.S. National Institutes of Health, Bethesda, MD) to obtain a distribution of particle sizes, and then expressed as Feret's diameter.

Color Analysis of Peanut Butters

Peanut butter samples were removed from refrigerated storage and equilibrated to room temperature (~22 °C) for 24 h before analyzing. Samples of each formulation were then transferred to glass Petri dishes (55 mm *i.d.* × 17 mm, Fisher Scientific Ltd., Suwanee, GA), and carefully packed and leveled to prevent air pockets. A colorimeter with a 2-degree observer angle (Model CR-410, Konica Minolta Sensing Americas, Inc., Ramsey, NJ) was employed to assess color using the Commission Internationale de l'Éclairage L^* C* h system, which represents a color space expressed in cylindrical coordinates. Prior to measurement, the colorimeter was calibrated with a white D_{65} standard (Y = 94.7, x = 0.3156, and y = 0.3319) provided by the manufacturer. L^* indicates lightness (0 to 100) and is the same as the L^* of the L^* a^* b^* color space, C* is chroma {0 to 100; $C^* = [(a^*)^2 + (b^*)^2)^{½}]$}, which measures the color saturation or purity, and h is hue angle [$h = \arctan(b^*/a^*)$] that indicates the primary color (0 to 360°). All measurements were made from the bottom side of the Petri dishes to prevent equipment fouling. Color was measured four times for each sample and mean values are reported. Prior to each replication, the colorimeter was recalibrated against a white standard tile provided by the

manufacturer.

Texture Analysis of Peanut Butters

Each peanut butter formulation was subjected to a modified texture profile analysis according to Ahmed and Ali (1986) using a texture analyzer (Model TA-XT2i, Stable Microsystems Ltd., Godalming, Surrey, U.K.) fitted with a 5-kg load cell and a 25-mm cylindrical probe (TA-3) with rounded edges to prevent shearing. Small Petri dishes (60 × 15 mm) were filled with peanut butter and leveled by scraping a straight edge across the top of the dish. Each Petri dish was secured in place and penetrated twice by the probe to a depth of 4 mm. The crosshead speed was set at 0.8 mm/s during both the compression and return cycles with a 5-s pause between compressions. Data were collected and analyzed with the Texture Expert Exceed software package (v. 2.61, Stable Microsystems). Parameters of hardness, cohesiveness, gumminess and adhesiveness were acquired from the analysis of the force–time plots after Pons and Fiszman (1996).

Instrumental Spreadability Evaluation of Peanut Butters

Spreadability of peanut butters was assessed using the texture analyzer described earlier, but fitted with a conical spreadability rig (Model HDP/SR, Stable Microsystems). Samples of each peanut butter formulation were tightly packed into individual sample cones to exclude air pockets and then leveled off with a spatula to give a uniform upper surface. The downward crosshead speed was set at 3.0 mm/s, and each sample was penetrated to the full depth of the cone. Firmness and work of shear were determined by analysis of the force–time plot, with the former being obtained from the maximum force (g) and the latter being the area of the positive

region of the curve expressed in g•s (Raju & Pal, 2009).

Chemical Methods

Phenolic Extraction

Extraction of phenolics from PS and fortified peanut butters was carried out according to Amarowicz *et al.* (2004) with slight modification. In brief, samples were placed in Whatman cellulose extraction thimbles (43 mm *i.d.* × 123 mm *e.l.*, VWR International, Suwanee, GA), covered with a plug of glass wool and defatted in a Soxhlet extraction apparatus under reflux for 12–14 h with hexanes. Defatted PS and peanut butters were transferred to 125-mL Erlenmeyer flasks at a mass-tosolvent ratio of 1:10 (w/v) with 80% (v/v) acetone. Extractions were performed in a gyratory water bath shaker (Model G76, New Brunswick Scientific Company, Inc., New Brunswick, NJ) set at 150 rpm and a temperature of 45 °C for 30 min. The slurry was then filtered by gravity through fluted P8 filter paper (Fisher Scientific). The extraction process was repeated twice as described earlier. All filtrates were pooled and acetone was evaporated with a Büchi Rotavapor R-210 using a V-700 vacuum pump connected to a V-850 vacuum controller (Büchi Corporation, New Castle, DE) at 45 °C. Aqueous residue was frozen and then lyophilized in a FreeZone 2.5-L bench-top freeze dryer (Labconco Corporation, Kansas City, MO) to ensure all traces of moisture were removed and then stored in amber glass bottles at –20 °C until further analyzed.

Preparation of Polyphenolic Extracts (PPEs)

About 3 g of each crude peanut butter extract was dispersed in 10 mL of deionized water, sonicated to facilitate dissolution, and then applied to the top of a chromatographic column (30

mm *i.d.* × 340 mm *e.l.*, Kontes Glass Inc., Vineland, NJ) packed with Amberlite XAD-16 [(bead size: 20-60 mesh), Sigma-Aldrich] and washed with ~1000 mL of deionized water to remove sugars and organic acids. After the first 800 mL, the Brix reading of the eluent was checked using a digital PAL-1 pocket refractometer (Model 3810, Atago U.S.A., Inc., Bellevue, WA) until a zero value was reached. The polyphenolic extract (PPE) was then eluted from the column with anhydrous methanol (~300 mL) as the mobile phase. Methanol was evaporated using the Büchi Rotavapor as described earlier. The PPE was lyophilized *via* the bench-top freeze dryer to ensure all traces of moisture were removed and then stored in amber glass bottles at –20 °C until used further.

Total Phenolics Content (TPC)

The TPC value of each extract was determined spectrophotometrically using the classical Folin–Ciocalteu assay (Singleton & Rossi, 1965). Briefly, 1.0 mL of a methanolic solution of each extract was pipetted into a test tube followed by the addition of 7.5 mL of deionized water, 0.5 mL of 2 N Folin & Ciocalteu's phenol reagent, and 1.0 mL of a saturated Na_2CO_3 solution. The contents were vortexed for 15 s followed by a 60-min resting period at room temperature to allow for optimal color development. Absorbance readings of samples were taken at λ_{max} = 750 nm with an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies, Wilmington, DE). Quantification was based on a standard curve generated with (+)-catechin. Sample TPCs were expressed as mg (+)-catechin equivalents/g PS [dry weight (d.w.)] or 100 g peanut butter [fresh weight (f.w.)].

Statistical Analysis

Extractions were performed in triplicate from different batches of PS for each type and from formulated peanut butters. All physical and chemical measurements were replicated a minimum of three times. Results were then expressed as means ± standard deviations reported for each data grouping. Data were analyzed by a one-way analysis of variance (ANOVA) statistical model using the statistical analysis system (SAS, v. 9.3, SAS Institute Inc., Cary, NC). When significance was determined at $P < 0.05$, data for each treatment across the processing method were subjected to Tukey's Studentized Range test to segregate treatment means.

3.3 Results and Discussion

Particle Size Distribution of Ground PS

A distribution of particle sizes represented by Feret's diameters (*i.e.*, the maximum caliper, measured from the longest distance between any two points along the selection boundary) for ground PS preparations is depicted in **Figure 3.1**. The particle size distributions of PS samples ranged from 9 to 2,140 μm for dry-blanched PS ground with granulated sugar at a ratio of 45.8:195 (w/w). Noteworthy is that particle sizes greater than 400 μm comprised less than 1% of the PS-sugar mixture. The distribution range was similar when the PS-sugar ratio increased to 91.6:195 (w/w). For ground dry-blanched PS, greater than 75% of the particles had a size ranging from 9 to 147 μm. The particle size distribution for dark roasted PS showed a distinct difference from that of dry-blanched ones, ranging from 27 to 1,441 μm. Greater than 80% of the dark roasted PS particles were within 27 to 269 μm. Distribution of the light-roasted skins approximated that of the dry blanched skins.

One challenge in the peanut butter industry is that added salt and sugar are insoluble in the low moisture conditions, and may lead to large gritty particles in the final product (Woodroof, 1983). To avoid such grittiness, powder sugar and pulverized salt (*i.e.*, flour salt) are generally employed during peanut butter manufacturing. Based on similar concerns, the PS used in this study were ground with granulated sugar to a very fine particle size to prevent a gritty mouthfeel. We found that efficient grinding of PS to the appropriate size range could best be attained by simultaneously grinding granulated sugar and PS together.

Color Analysis

The data from the color measurements are presented in **Table 3.2**. Significant differences in color were found for both type and level of PS incorporated. In the *L** C* h system used, *L** varies from 0 to 100 (black to white) and C* from 0 to 100 (neutral grey to color purity). A hue angle (h) of 0° would correspond to a red color while 90° would correspond to yellow. Peanut butter without added PS was closer to yellow than other samples (h = 71.6°), was slightly lighter (*L** = 68.6) and had the highest color saturation (C* = 33.7). Except for 1.25% light-roasted PS, all other formulations led to a darker color in the ensuing peanut butters. Increasing PS levels also produced darker peanut butters. Dry blanched formulations were most similar in color to the control, with *L** ranging from 66.2 to 63.3 as compared to 68.6 for the no-PS control. As could be expected, samples that incorporated darker-roasted PS were somewhat darker. For example, *L** for light-roasted PS peanut butter ranged from 68.6 to 62.4, while that for dark-roasted PS peanut butter ranged from 67.4 to 61.1 In general, samples with added PS had a smaller hue angle, and again darker roasted PS led to the greatest change. The hue angle for the control was 71.6°, for dry-blanched PS 70.6 to 68.6°, for light-roasted PS 71.9 to 68.3°, for medium-roasted

PS 71.9 to 67.6°, and for dark-roasted PS 72.0 to 67.9°. All of these reflect a change from an orange-brown color to one with slightly more of a red hue. At the same time, color purity decreased with added PS. C* for the control was 33.7, for dry-blanched PS 32.4 to 29.1, for light-roasted PS 31.2 to 26.6, for medium-roasted PS 31.1 to 24.4, and for dark-roasted PS 30.7 to 24.3.

Increasing levels of PS had a similar effect on color. In general, increasing PS from 1.25 to 5.0% led to darker samples, with slightly more red hue, and lesser color purity. As an example, *L** for the dark-roasted PS peanut butter varied from 68.6, 67.4, 65.2, 62.0 and 61.1 for peanut butter with 0, 1.25, 2.5, 3.75, and 5.0% of PS incorporation, respectively. For dark-roasted PS, h ranged from 71.6 to 67.9° for 0 and 5% PS samples. Chroma values decreased in order from 33.7, 30.7, 28.2, 25.7 and 24.3 for peanut butter with 0, 1.25, 2.5, 3.75 and 5.0% dark roasted PS. Although differences in color were not large, overall samples with darker and higher levels of PS were slightly darker, brown in appearance and exhibited less color purity.

Two thermal treatments are involved in the manufacture of peanut butter, namely roasting for flavor development and dry blanching for PS removal. During the heat treatment, peanuts gradually render the characteristic golden brown color resulting from sugar-amino acid reactions with the production of melanoidins (Hodge, 1953). A higher heating temperature and longer roasting time will lead to further generation of melanoidins and thereby intensify the brown color in peanuts. In addition, at relatively high temperatures, sugar caramelization can contribute to the browning reaction of peanuts. Tannins and catechol-type compounds contribute toward the color noted in raw PS (Sobolev & Cole, 2004). Because PS contain *ca.* 16 to 18% crude protein, similar browning reactions are expected in PS during thermal processing (Sobolev & Cole, 2004). Therefore, incorporation of PS into peanut butter would be expected to change the color of the

resultant products through mixing of components with slightly different color. Thus, the darkest-color roasted PS would cause the greatest change in color of the formulated peanut butters, while dry-blanched-only or light-roasted PS would cause the least change. For instance, the dark-roasted PS were initially a dark-chocolate brown, and when added to the peanut butter created a darker, browner peanut butter. In contrast, dry-blanched PS were the lightest in color and had more of a golden-yellow hue. In general, incorporation of dry blanched PS into the peanut butters lead to the least change in the color parameters. One additional factor should be noted in that the PS and the lipid phase of peanut butter have different refractive indices. Consequently, the absorption of oil into the PS would increase the light scattering in formulated peanut butters and change the manner in which color is reflected to the observer.

Texture Analysis

The aggregate data from the texture analysis are presented in **Table 3.3**. Except for gumminess, all other investigated textural parameters were significantly impacted by the PS incorporation. Peanut butters formulated with medium- and dark-roasted PS exhibited an increase in hardness. For example, while the no-PS control peanut butter had a hardness of 127 g-force, dark roasted-PS peanut butter had a hardness between 215 and 237 g. Peanut butters containing roasted PS were generally more adhesive than those without or with dry-blanched PS. Addition of dry-blanched PS (greater than 2.5%) demonstrated, unexpectedly, a decrease in adhesiveness. All PS addition affected a decrease in the product's cohesiveness. None of the parameters examined for texture varied in a concentration-dependent manner. While the control peanut butter had an adhesiveness of –545 g•s, that for dark roasted-PS peanut butter ranged from –674 to –701 g•s. The control peanut butter was slightly more cohesive (1.6) as compared

to samples with incorporated PS (0.9–1.3).

For viscoelastic semisolid materials, an apparent increase in hardness may arise from a greater elastic component or an increase in viscosity, as both resist instantaneous compression. PS, therefore, can be considered as the elastic component incorporated into peanut butters, and this resulted in an increase of the elastic modulus. Alternatively, the PS particles may increase the resistance to flow for any viscous element of the peanut butter. Crippen *et al.* (1989) reported that a coarse grind size of peanut paste brought about significantly ($P < 0.05$) greater instrumental hardness than that of medium and fine grinds. Similarly, the size distribution of ground-roasted PS was coarser and the particles were more uniform in size compared to ground dry-blanched PS. Thus, the incorporation of roasted PS would be expected to harden the resultant peanut butters. When the texture analyzer's plunger compressed the sample, larger particles with a greater surface area should provide more resistance, thereby leading to an increased apparent hardness. The decrease in cohesiveness may be related to the disruption of the continuous oil phase in peanut butters after PS incorporation with oil–PS interfaces being more easily separated than those in oil-filled regions. Gumminess is defined as the energy involved to disintegrate a semisolid food to a state of readiness for swallowing and is calculated from hardness and cohesiveness measurements (Pons & Fiszman, 1996). Thus, the incorporation of PS might not change the energy required by disintegration due to increased hardness offset by decreased cohesiveness in the resultant peanut butters.

In this work, as a consequence of roasted PS incorporation, peanut butters were slightly more adhesive. Syarief *et al.* (1985) implied that increased adhesiveness and mouth-coating were inversely related to the ease of swallowing peanut butter impairing the sensory impression of the product, and How and Young (1985) reported that a majority of consumers dislike sticky and

overly adhesive peanut butter. Thus it would be expected that our samples would be more difficult to swallow, resulting in decreased sensory scores. However, in some food products, sensory adhesiveness does not correlate that well with instrumental adhesiveness (Truong, Walter, & Hamann, 1997). Sensory adhesiveness is evaluated in the mouth using a lateral force as well as a vertical force in the presence of saliva, while the measurement of instrumental adhesiveness employs a plunger that applies a vertical force. In addition, a metal plate is used to simulate the human palate and the measurements are also determined in the absence of saliva.

Spreadability

The firmness and spreadability data are given in **Table 3.4**. Firmness did not significantly increase with PS addition at levels below 3.75%. Only the peanut butters containing 5.0% dry-blanched PS, light-roasted PS above 2.5%, and 5.0% medium-roasted PS showed an increase in firmness. A negative correlation between firmness and spreadability was reported in creamery butters and peanut butters investigated by Syarief *et al.* (1985). The measurements of firmness conflict somewhat with the hardness (**Table 3.3**), but are likely due to the differences in the probes employed and their geometry. In the texture assay a 25 mm cylindrical probe was used, resulting in the measurement of forces primarily due to compression, while the cone rig used for spreadability analysis (employing both a positive and a negative cone) resulted in measurement of forces generated from the penetration and spreading of the sample. The latter incorporated a combination of shear and compression; the sample extruded through the annular space as the cones came closer together. The forces are generated from the puncture, and then a combination of shear and compression are needed to penetrate and spread the sample. We believe this latter geometry to result in the surface areas of distributed particles playing a less important role in the

determination of apparent hardness.

Spreadability, as measured by work of shear, decreased for some samples and increased for others. Peanut butter with 1.25% or 3.75% dry-blanched PS, 1.25% medium-roasted PS, or 5.0% dark-roasted PS took less work to spread than that of the control. Samples with greater than 3.75% dry-blanched, light-roasted or medium-roasted PS took more work to spread. In general, the work to spread increased with PS concentration for dry-blanched, light-roasted, and medium-roasted PS peanut butter, while it decreased with concentration for dark roasted-PS peanut butter. As seen in **Table 3.3**, peanut butter with dark-roasted PS was also harder and less cohesive than the control, dry-blanched or light-roasted PS peanut butter. As previously noted, hardness is measured primarily under compression. For the spreadability rig, the work is determined much more by shear force. The fact that layers of dark-roasted PS peanut butter can more easily move past each other may be related to the lower cohesiveness of these samples.

Total Phenolics Content (TPC)

The TPC, expressed as mg (+)-catechin equivalents/g PS (d.w.) or 100 g peanut butter (f.w.) are depicted in **Figure 3.2**. The classical method of Singleton and Rossi (1965) using Folin & Ciocalteu's phenol reagent was employed in this work to determine the TPC in PS and peanut butter acetonic extracts. Dry-blanched PS possessed the highest TPC of 166 mg catechin eq./g, which was 32, 33, and 38% higher than that for light-, medium-, and dark-roasted skins, respectively. Although dry-blanched PS exhibited the greatest TPC, all four PS types effectively increased the TPC of the formulated peanut butters in a concentration-dependent manner. Using dry-blanched PS as an example, the TPC increased by 86, 357, 533, and 714%, respectively after incorporating PS into peanut butter at 1.25, 2.5, 3.75, and 5.0% (w/w) addition levels, when

compared to the peanut butter devoid of PS.

Phenolic compounds are secondary metabolites in plants and they generally concentrate in the outer layers such as the peel, shell, and hull to protect inner matter. A large number of intrinsic and extrinsic factors greatly influence the types and levels of phenolics in plants; these include genetic types, environmental conditions, germination, ripening, processing, and storage (Bravo, 1998). In the case of peanut hulls, the TPC varied markedly with maturity (Yen, Duh, & Tsai, 1993) and choice of cultivars (Yen & Duh, 1995). Mild heat treatment was able to break some of the covalent bounds of phenolic acids with insoluble polymers (Peleg, Naim, Rouseff, & Zehavi, 1991) and other cell wall components such as arabinoxylans (Hartley, Morrison, Himmelsbach, & Borneman, 1990), liberating low-molecular-weight antioxidant compounds from the repeating subunits of high-molecular-weight polymers (Jeong *et al.*, 2004). Lee *et al.* (2006) reported that after far-infrared radiation treatment at 150 °C for 60 min, the TPC value of peanut hulls increased from 73 to 90 μM tannic acid equivalents. PS roasted at 175 °C for 5 min were found to contain a higher TPC than raw PS, especially when recovered by water and ethanol extraction (Yu, Ahmedna, & Goktepe, 2005). In addition to processing, the extraction conditions employed such as choice of solvent system, material:solvent ratio, particle size distribution of PS, extraction times, number of extractions, and temperature (Nepote, Grosso, & Guzman, 2005; Dai & Mumper, 2010) impact the efficiency of extraction and thereby result in different recoveries of phenolics from the source material. According to Nepote *et al.* (2005) the optimum extraction conditions consist of 70% (v/v) ethanol, unground PS, a solvent/solid ratio of 20 mL/g, 10 min of shaking, and three extractions yielding a 118 mg phenol equivalents/g recovery of total phenolics.

The TPC data for dry-blanched PS from this study were close to that reported by Nepote *et al.* (2002), where the TPC was 159 mg phenol equivalents/g recovered from dry blanched PS by 24 h maceration with methanol at a material to solvent ratio of 1:10 (w/v). The extraction was performed 2× at room temperature in subdued lighting. A much higher TPC value of 280 mg/g was detected by Francisco and Resurreccion (2009) in Runner PS processed at 135 °C for 5 min. The phenolics were recovered from PS by 3 × 10-min extractions at room temperature. Seventy percent (v/v) ethanol was applied as the extraction solvent at the sample/solvent ratio of 1: 20 (w/v). This protocol was carefully followed, but we were unable to reproduce the finding and to validate the result. None of these treatments are adopted by the peanut industry where dry blanching of PS is fulfilled by heating only at ~138 °C for up to 25 min. The roasted PS are produced by heating the shelled peanuts to ~160 °C for 40 to 60 min (Woodroof, 1983). The quantities of total phenolics are difficult to compare amongst that reported in the literature because sample source, sample preparation manner, and extraction techniques are quite varied. Though the findings of this work did not display the highest TPC value ever reported for PS, this investigation supports the employment of PS as a potential source of phenolics which can significantly increase the TPC of fortified peanut butters.

3.4 Conclusions

Simultaneous grinding of PS with sugar can produce powders with desirable grind sizes of an appropriate range for incorporation into peanut butters (less than 300 μm). Incorporation of ground PS also impacts the physical properties of resultant peanut butters with the extent depending on the type of skins used. In some cases a concentration-dependent relationship was found. In general, the addition of PS decreases the cohesiveness of the resultant peanut butters,

and in the case of medium- and dark-roasted PS increases both hardness and adhesiveness. Further, while there was poor correlation between hardness values and firmness values (measured by a spreadability rig), increases in firmness are good predictors of poor spreadability. Additionally, peanut butters formulated with PS show decreases in lightness and color saturation relative to those devoid of PS, with levels greater than 2.5% also decreasing the hue angle.

In general, dry blanched PS caused the least overall change to the physical properties of peanut butter at a given level of incorporation. Further, these peanut butters also have higher levels of potentially healthful phenolic compounds. Thus, given a similar price structure as non-fortified peanut butters, it is expected that these formulations hold the most promise as a functional food. Hence, the findings of this study have paved a way for the utilization of a low-valued industrial by-product (*i.e.*, PS) as an ingredient in functional food formulations which improve an existing product and allow diversification of the brands available in the market.

Acknowledgments

This research was supported by a grant funded by the Georgia Food Industry Partnership and is greatly appreciated. Thanks are extended to the following: Rick Boyce at Seabrook Ingredients, a division of Universal Blanchers, LLC, for the peanut paste, flour salt, and stabilizer; Bruce Kotz at Golden Peanut Corporation, LLC, for the light, medium, and dark roasted PS; Bill Paulk at Sylvester Blanching, a division of Universal Blanchers, LLC, for the dry blanched peanuts skins; and John T. Powell, President of the Peanut Institute Inc., Albany, GA.

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Table 3.1 Peanut butter formulations[1].

Peanut Skin Type	Formulation (%)							
	Lipid Content in Skins	Lipid Content in Peanut Paste	Skins[2]	Sucrose	RBD Peanut Oil[3]	Flour Salt	Stabilizer[4]	Peanut Paste
Control	0	50.0	0	6.5	0	1.5	2.0	90.0
Dry-blanched (DB)	18.1	50.0	1.25	6.5	0.49	1.5	2.0	88.75
			2.5	6.5	0.97	1.5	2.0	87.5
			3.75	6.5	1.46	1.5	2.0	86.25
			5.0	6.5	1.95	1.5	2.0	85.0
Light-roasted (LR)	27.0	50.0	1.25	6.5	0.39	1.5	2.0	88.75
			2.5	6.5	0.79	1.5	2.0	87.5
			3.75	6.5	1.18	1.5	2.0	86.25
			5.0	6.5	1.57	1.5	2.0	85.0
Medium-roasted (MR)	29.0	50.0	1.25	6.5	0.37	1.5	2.0	88.75
			2.5	6.5	0.74	1.5	2.0	87.5
			3.75	6.5	1.11	1.5	2.0	86.25
			5.0	6.5	1.48	1.5	2.0	85.0
Dark-roasted (DR)	26.9	50.0	1.25	6.5	0.39	1.5	2.0	88.75
			2.5	6.5	0.79	1.5	2.0	87.5
			3.75	6.5	1.18	1.5	2.0	86.25
			5.0	6.5	1.58	1.5	2.0	85.0

[1]Each formulation was prepared in three different batches.
[2]Skins were ground in the masscolloider with sugar at different ratios: for dry blanched, 22.9, 45.8, 68.7 and 91.6 g of PS were ground with 195 g of sucrose; for light roasted, 25.7, 51.4, 77.1 and 102.8 g of PS were ground with 195 g of sucrose; for medium roasted, 26.4, 52.9, 79.3 and 105.7 g of PS were ground with 195 g of sucrose; for dark roasted, 25.7, 51.3, 77.0 and 102.6 g of PS were ground with 195 g of sugar. All formulations resulted in peanut butters containing 1.25, 2.5, 3.75 and 5.0% of PS, respectively.
[3]Refined, bleached, deodorized peanut oil was added to balance the lipid level of each formulation at 45% fat.
[4]The stabilizer comprised hydrogenated rapeseed and cottonseed oils.

Table 3.2 Color analysis of formulated peanut butters.

Formulation	Peanut Skins (%)	*L**	C*	h
Control	0	68.6 ± 0.14^{a}	33.7 ± 0.17^{a}	71.6 ± 0.13^{b}
DB	1.25	$66.2 \pm 0.12^{c,d}$	32.4 ± 0.08^{b}	70.6 ± 0.04^{d}
	2.5	$65.7 \pm 0.13^{c,d}$	$30.8 \pm 0.17^{c,d}$	70.0 ± 0.03^{e}
	3.75	64.1 ± 0.33^{e}	30.4 ± 0.22^{d}	$69.0 \pm 0.01^{f,g}$
	5.0	$63.3 \pm 0.06^{e,f}$	29.1 ± 0.39^{e}	$68.6 \pm 0.09^{h,i}$
LR	1.25	68.6 ± 0.27^{a}	31.2 ± 0.07^{c}	$71.9 \pm 0.05^{a,b}$
	2.5	65.2 ± 0.06^{d}	29.1 ± 0.35^{e}	71.2 ± 0.19^{c}
	3.75	63.8 ± 0.57^{e}	27.7 ± 0.41^{g}	$68.9 \pm 0.22^{g,h}$
	5.0	$62.4 \pm 0.16^{f,g}$	26.6 ± 0.34^{h}	68.3 ± 0.26^{i}
MR	1.25	$66.4 \pm 0.49^{b,c}$	31.1 ± 0.18^{c}	$71.9 \pm 0.23^{a,b}$
	2.5	65.3 ± 0.46^{d}	28.4 ± 0.09^{f}	70.6 ± 0.14^{d}
	3.75	$63.2 \pm 0.16^{e,f}$	26.9 ± 0.15^{h}	69.4 ± 0.17^{f}
	5.0	$61.6 \pm 0.42^{g,h}$	24.4 ± 0.16^{j}	67.6 ± 0.02^{j}
DR	1.25	67.4 ± 0.38^{b}	$30.7 \pm 0.46^{c,d}$	72.0 ± 0.14^{a}
	2.5	65.2 ± 0.91^{d}	$28.2 \pm 0.38^{f,g}$	$70.2 \pm 0.15^{d,e}$
	3.75	$62.0 \pm 0.58^{g,h}$	25.7 ± 0.32^{i}	67.9 ± 0.18^{j}
	5.0	61.1 ± 0.67^{h}	24.3 ± 0.09^{j}	67.9 ± 0.07^{j}

[1]A Konica Minolta colorimeter (Model CR-410) was employed to assess color where *L** is lightness, C* is chroma {$C^* = [(a^*)^2 + (b^*)^2)^{½}]$} and h is hue angle {$h = \arctan (b^*/a^*)$}. The means ± standard deviations are reported based on four samplings. Values within a given column followed by the same letters are not significantly different ($P > 0.05$).

Table 3.3 Textural properties of formulated peanut butters[1].

Formulation (PS addition %)		Textural Objective Measure			
		Hardness (g)	Adhesiveness (g•s)	Cohesiveness	Gumminess
Control	0	127 ± 12.2^{e}	-545 ± 20.5^{b,c}	1.6 ± 0.24^{a}	203 ± 10.7^{a,b,c}
DB	1.25	157 ± 10.6^{c,d,e}	-515 ± 30.6^{a,b}	1.1 ± 0.06^{b,c,d}	169 ± 8.3^{c,d}
	2.5	195 ± 14.4^{a,b,c,d}	-599 ± 34.0^{b,c,d}	1.0 ± 0.10^{b,c,d}	196 ± 8.1^{a,b,c,d}
	3.75	130 ± 10.4^{e}	-400 ± 58.3^{a}	1.3 ± 0.11^{b}	169 ± 19.3^{c,d}
	5.0	169 ± 22.8^{b,c,d,e}	-400 ± 57.6^{a}	0.9 ± 0.19^{d}	148 ± 24.4^{d}
LR	1.25	145 ± 10.4^{d,e}	-687 ± 6.2^{d,e,f}	1.3 ± 0.08^{b,c}	183 ± 9.3^{b,c,d}
	2.5	154 ± 24.0^{c,d,e}	-581 ± 24.5$^{\mathbf{b,c,d}}$	1.1 ± 0.11^{b,c,d}	174 ± 18.7^{c,d}
	3.75	202 ± 13.4^{a,b,c,d}	-664 ± 39.6^{c,d,e,f}	1.1 ± 0.07^{b,c,d}	212 ± 10.4^{a,b,c}
	5.0	202 ± 26.2^{a,b,c,d}	-702 ± 79.5^{d,e,f}	1.0 ± 0.09^{b,c,d}	208 ± 11.4^{a,b,c}
MR	1.25	234 ± 29.9^{a}	-661 ± 28.6^{c,d,e,f}	0.9 ± 0.16^{d}	219 ± 25.0^{a,b,c}
	2.5	223 ± 21.5^{a,b}	-737 ± 47.7^{e,f}	1.0 ± 0.10^{b,c,d}	225 ± 12.8^{a,b}
	3.75	203 ± 17.9^{a,b,c,d}	-612 ± 50.8$^{\mathbf{b,c,d,e}}$	1.0 ± 0.07^{b,c,d}	201 ± 3.8^{a,b,c}
	5.0	210 ± 38.1^{a,b,c}	-668 ± 67.1^{c,d,e,f}	1.0 ± 0.02^{b,c,d}	214 ± 36.5^{a,b,c}
DR	1.25	226 ± 29.5^{a,b}	-696 ± 58.0^{d,e,f}	1.0 ± 0.07^{b,c,d}	236 ± 26.0^{a}
	2.5	216 ± 34.7^{a,b,c}	-701 ± 69.3^{d,e,f}	1.1 ± 0.13^{b,c,d}	241 ± 18.7^{a}
	3.75	237 ± 29.5^{a}	-786 ± 55.9^{f}	1.0 ± 0.05^{b,c,d}	237 ± 31.3^{a}
	5.0	215 ± 45.6^{a,b,c}	-674 ± 45.2^{d,e,f}	1.1 ± 0.14^{b,c,d}	225 ± 25.7^{a,b}

[1]Means ± standard deviations are reported based on four samplings. Values within a given column followed by the same letters are not significantly different ($P > 0.05$).

Table 3.4 Firmness and spreadability of formulated peanut butters[1].

Formulation (PS addition %)		Textural Objective Measure	
		Firmness (g)	Work of Shear (g•s)
Control	0	$2420 \pm 91^{c,d}$	$2040 \pm 86^{d,e,f}$
DB	1.25	$2150 \pm 44^{f,g}$	1770 ± 32^{h}
	2.5	2420 ± 94^{d}	$2090 \pm 125^{d,e}$
	3.75	$2220 \pm 75^{e,f}$	$1810 \pm 64^{g,h}$
	5.0	2990 ± 116^{a}	2730 ± 109^{a}
LR	1.25	$2380 \pm 59^{d,e}$	$2030 \pm 81^{d,e,f}$
	2.5	$2390 \pm 32^{d,e}$	$2030 \pm 88^{d,e,f}$
	3.75	2630 ± 19^{b}	$2301 \pm 42^{b,c}$
	5.0	2680 ± 39^{b}	2430 ± 93^{b}
MR	1.25	2010 ± 71^{g}	1740 ± 69^{h}
	2.5	$2160 \pm 69^{f,g}$	$1900 \pm 100^{e,f,g,h}$
	3.75	$2140 \pm 114^{f,g}$	$2350 \pm 100^{b,c}$
	5.0	$2600 \pm 38^{b,c}$	$2340 \pm 40^{b,c}$
DR	1.25	$2350 \pm 21^{d,e}$	$2150 \pm 68^{c,d}$
	2.5	$2110 \pm 78^{f,g}$	$1920 \pm 99^{e,f,g,h}$
	3.75	$2170 \pm 57^{f,g}$	$1990 \pm 39^{d,e,f,g}$
	5.0	$2080 \pm 37^{f,g}$	$1880 \pm 54^{f,g,h}$

[1]Means ± standard deviations are reported based on four samplings. Values within a given column followed by the same letters are not significantly different ($P > 0.05$).

Figure Captions

Figure 3.1 Particle size distribution histograms of ground peanut skins (PS). **(A)** on left, 45.8 g of dry blanched PS ground with 195 g of sugar & on right, 91.6 g of dry blanched PS ground with 195 g of sugar; **(B)** on left, 51.4 g of light roasted PS ground with 195 g of sugar & on right, 102.8 g of light roasted PS ground with 195 g of sugar; and **(C)** on left, 51.3 g of dark roasted PS ground with 195 g of sugar & on right, 102.6 g of dark roasted PS ground with 195 g of sugar.

Figure 3.2 Total phenolics content (TPC) of peanut skins **(A)** and peanut butters **(B)**. Means (n = 3) without a common letter differ significantly ($P < 0.05$).

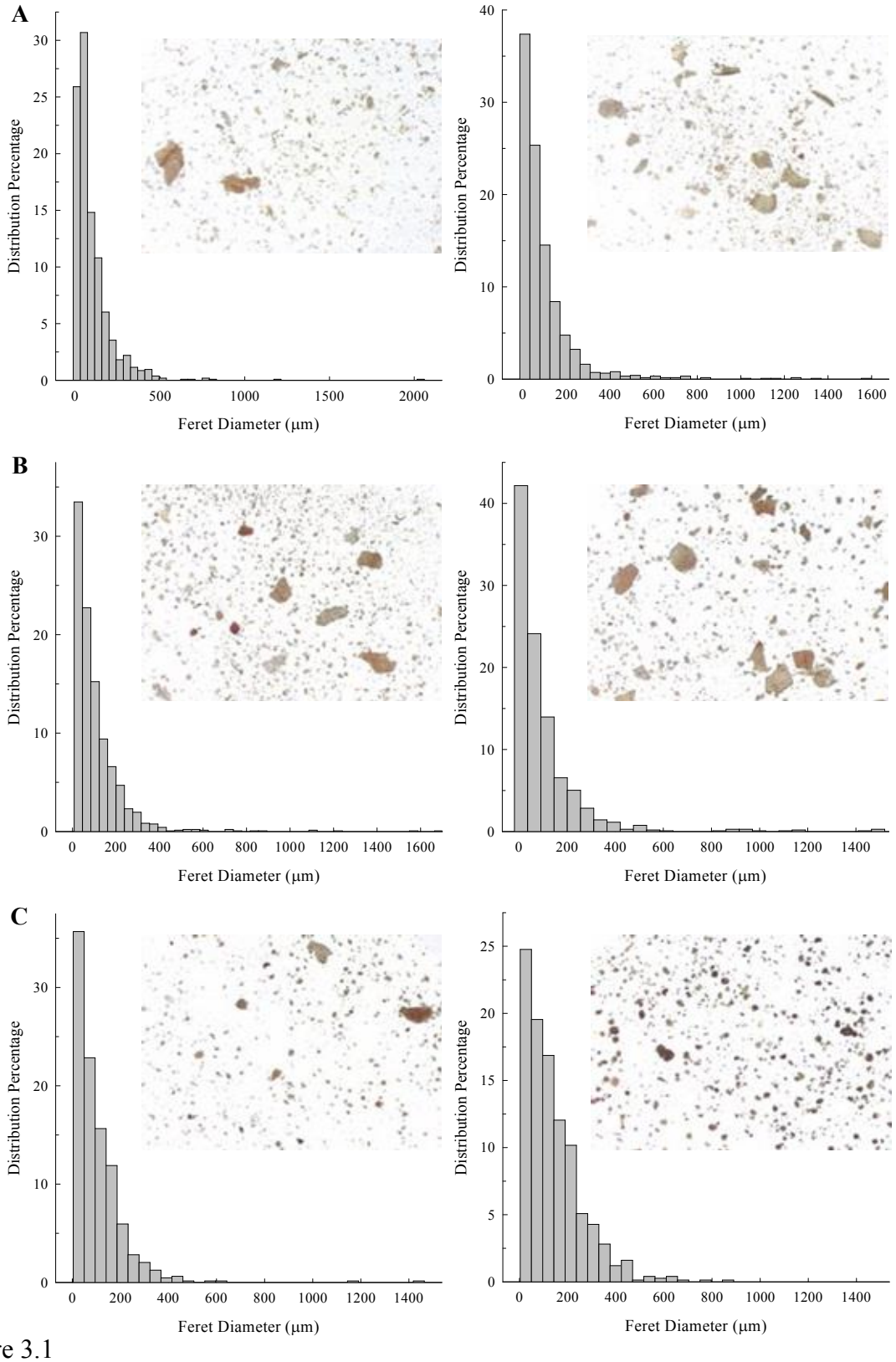

Figure 3.1

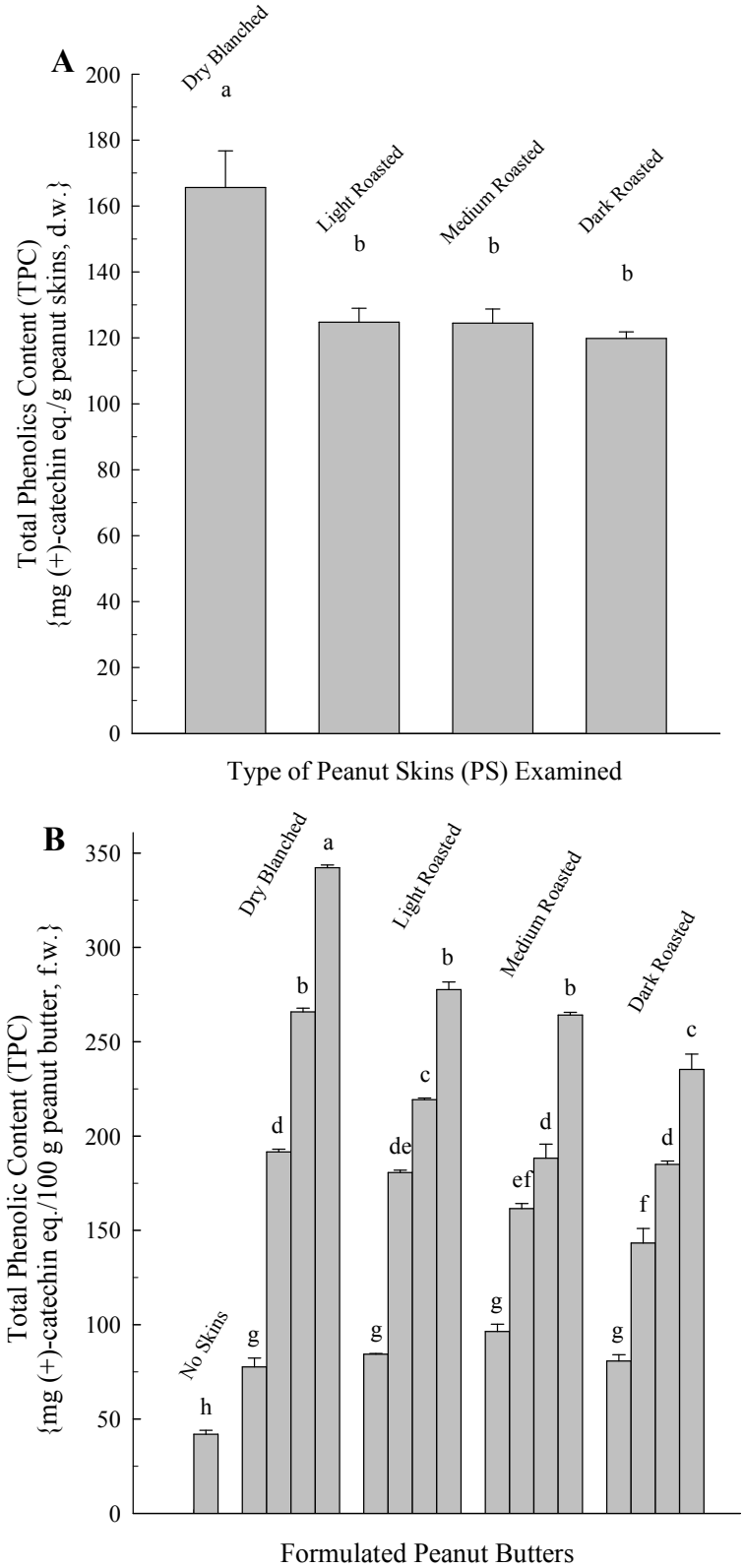
A
Dry Blanched
Light Roasted
Medium Roasted
Dark Roasted
a
b
b
b
Total Phenolics Content (TPC)
{mg (+)-catechin eq./g peanut skins, d.w.}
0
20
40
60
80
100
120
140
160
180
200
Type of Peanut Skins (PS) Examined
B
No Skins
Dry Blanched
Light Roasted
Medium Roasted
Dark Roasted
h
g
d
b
a
g
de
c
b
g
ef
d
b
g
f
d
c
Total Phenolic Content (TPC)
{mg (+)-catechin eq./100 g peanut butter, f.w.}
0
50
100
150
200
250
300
350
Formulated Peanut Butters

Figure 3.2

CHAPTER 4

PEANUT SKINS-FORTIFIED PEANUT BUTTERS: EFFECT OF PROCESSING ON THE PHENOLICS CONTENT, FIBER CONTENT AND ANTIOXIDANT ACTIVITY

Ma, Y.; Kerr, W. L.; Swanson, R. B.; Hargrove, J. L.; Pegg, R. B. *Food Chemistry*. **2014**, *145,* 883–891. Reprinted here with permission of publisher (Elsevier).

Abstract

Incorporation of ground peanut skins (PS) into peanut butter (PB) at 1.25, 2.5, 3.75, and 5.0% (w/w) resulted in a marked concentration-dependent increase in both TPC and antioxidant activity. Using dry-blanched PS to illustrate, the TPC increased by 86, 357, 533, and 714%, respectively, compared to the PB control devoid of PS; the TPACs rose by 633, 1933, 3500, and 5033%, respectively. NP-HPLC detection confirmed that the increase in the phenolics content was attributed to the endogenous PACs of the PS, which were characterized as dimers to nonamers by NP-HPLC/ESI-MS. FRAP values increased correspondingly by 62, 387, 747, and 829%, while H-ORAC$_{FL}$ values grew by 53, 247, 382, and 415%, respectively. The DF content of dry-blanched PS was ~55%, with 89-93% being insoluble fiber. Data revealed that PS addition enhances the antioxidant capacity of the PB, permits a "good source of fiber" claim, and offers diversification in the market's product line.

4.1 Introduction

As demonstrated by George Washington Carver more than a century ago, peanuts (*Arachis hypogaea L.*) are a valuable cash crop to the southern United States. China, India and the U.S.A. account for ~2/3 of the world's peanut production. In 2012 the U.S. peanut production reached 6.7 billion pounds nationwide, generating a farm gate value of $2.3 billion (USDA 2013). The potential health benefits associated with eating peanuts are well documented, notably the prevention against cardiovascular disease, type-2 diabetes, cancer, and other degenerative diseases (Awad, Chan, Downie, & Fink, 2000; Jiang *et al.*, 2002; Isanga & Zhang, 2007; Kris-Etherton, Hu, Ros, & Sabaté, 2008). Peanut lipids have largely contributed to these benefits; they possess zero *trans*-fatty acids (Sanders, 2001) and are rich in monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs) (Mercer, Wynne, & Young, 1990) and phytosterols (Shin, Pegg, Phillips, & Eitenmiller, 2010). Additional beneficial nutrients endogenous to peanuts include vitamin E, L-arginine, soluble and insoluble fiber, as well as water- and lipid-soluble phenolic antioxidants (Kris-Etherton *et al.*, 1999; Isanga & Zhang, 2007; Shin *et al.*, 2009). The nutrients act synergistically with the numerous protective bioactives making the peanut a desirable nutrient-dense plant-based food (Kris-Etherton, Hu, Ros, & Sabaté, 2008).

Besides the kernels, peanut skins (PS), as the other edible part of peanuts, have attracted attention because they are a rich, inexpensive source of potentially health-promoting phenolics and dietary fiber (DF). Phenolic compounds typically concentrate themselves on the outer layers of plants such as the peel, shell, and hull to protect the inner core materials. Nepote, Grosso, and Guzman (2002) reported a content of ~159 mg total phenolics/g defatted dry skin, which also exhibited a marked antioxidant activity as demonstrated by its capacity to inhibit the oxidation of

sunflower oil. A study by Yu, Ahmedna, and Goktepe (2005) revealed that PS phenolics are abundant not only in quantity but also in type, which primarily include phenolic acids (*e.g.*, caffeic, chlorogenic, ferulic, and coumaric acids), flavonoids {*e.g.*, (*epi*)catechins and epigallocatechin (EGC), catechin gallate (CG), and epicatechin gallate (ECG)}, and stilbene (*e.g.*, *trans*-resveratrol). Free phenolic acids are not the predominant phenolics in PS (Yu, Ahmedna, Goktepe, & Dai, 2006); the PACs comprise ~17% by weight of PS (Karchesy & Hemingway, 1986). Six A-type PAC dimers were identified in PS (Lou *et al.*, 1999) and found to inhibit the inflammatory pathway mediated by hyaluronidase-induced release of histamine. A further study by Lou *et al.* (2004) led to isolation of five oligomeric PACs with potential free radical-scavenging activity from the water-soluble fraction of PS.

PACs are complex flavonoid polymers; their phenolic nature makes them excellent candidates as food antioxidants. The health benefits associated with PACs have been documented in terms of antioxidation, anti-carcinogenesis, and cardiovascular disease prevention (Santos-Buelga & Scalbert, 2000). On the other hand, the anti-nutritional effects associated with PACs, notably inhibition on protein digestibility, are clear in animals consuming large amounts of PACs, but have no established nutritional significance in humans (Santos-Buelga & Scalbert, 2000). In the gut, PACs trigger adaptative responses, such as increasing the secretion of various endogenous proteins (especially salivary proline-rich proteins) and biliary acids, which can improve the absorption and utilization of ingested proteins. Moreover, tannins bound to proteins have been shown to retain their antioxidant activity and may provide persistent antioxidant activity in the gastrointestinal tract when consumed (Riedl *et al.*, 2002).

PS are also rich in DF: the total dietary fiber (TDF) comprises ~45% weight of roasted PS, of which roughly 2.2% is soluble fiber (Shimizu-Ibuka *et al.*, 2009). A high daily intake of DF helps

lower blood pressure and cholesterol levels, resulting in the reduced risk of coronary heart disease, stroke, hypertension, diabetes and obesity (Anderson *et al*., 2009). Unfortunately, the average fiber consumption among adults in the U.S. is ~15 g a day instead of the recommended 25-30 g. Polyphenols and DF are two well-documented dietary factors in the prevention of chronic diseases, but are usually addressed as separate compounds acting independently in disease prevention. A 2011 study by Saura-Calixto demonstrated a synergistic function of DF and dietary antioxidants, mainly in the development of an antioxidant environment in the colon.

PS have been used in traditional Chinese medicine for the treatment of chronic hemorrhage and bronchitis (Lou *et al.*, 1999). In contrast, the value of PS in the western world has not been recognized until only recently. As a major waste of peanut processing, most PS are dumped but a small quantity is added to animal feed. In 1999/2000, over 750,000 tons of PS were generated worldwide based on an estimated 29.1 million tons production of peanuts. The red skin of peanuts comprises 2.0-3.5 weight percent of the kernels. In the United States, the total volume of commercially processed shelled edible-grade peanuts used in primary products was roughly 2,000 million pounds during 2011 (USDA 2010–2012); hence, 40 to 70 million pounds of PS were generated.

An increased awareness of the role of dietary antioxidants and fiber in health promotion and disease prevention has led to a high demand for antioxidant and fiber-enriched functional foods. PS are a concentrated source of DF and phenolics; thus, their incorporation into a variety of foods would effectively enhance the fiber content and antioxidant capacity of the resultant product and further provide an inexpensive and abundant source of these dietary bioactives. Despite the tremendous potential benefit of PS as an alternative source of antioxidants and DF, their utilization as a functional food ingredient in value-added products is lacking.

The objectives of the current study are (i) to assess the effect of processing on the phenolics content (TPC and TPAC content) and antioxidant activity of PS and in value-added peanut butters fortified with PS; (ii) to determine the increased fiber content after incorporating PS into the peanut butter prototypes; and (iii) to characterize the phenolics in PS and the PS-fortified peanut butters.

4.2 Materials and Methods

Materials

Dry-blanched PS were provided by Universal Blanchers, LLC (Sylvester, GA). Roasted PS (light, medium, and dark) were a gift from the Golden Peanut Company, LLC (Alpharetta, GA). Peanut paste, flour salt, and hydrogenated vegetable oil (*i.e.*, stabilizer) were supplied by Seabrook Ingredients, Inc. (Edenton, NC). Domino premium pure cane granulated sugar and peanut oil with zero *trans*-fat were purchased from Sam's Club (Athens, GA). All solvents and reagents were of analytical (ACS) grade, unless otherwise specified. Methanol, ethanol (95%) and hexanes were purchased from VWR International (Suwanee, GA). Folin & Ciocalteu's phenol reagent and a TDF assay kit (Cat No. TDF100A-1KT) were acquired from the Sigma-Aldrich Chemical Co. (St. Louis, MO).

Peanut Butter Processing

Seventeen formulations of peanut butter (~45% total fat) were prepared; the details are described by Ma *et al.* (2013). To facilitate incorporation, PS and sugar (195 g) were ground together at different ratios prior to inclusion into the prototypes using an electrically-driven stone grinder (Super Masscolloider CA6-3, Masuko Sangyo Co., Ltd, Kawaguchi-City, Saitama, Japan)

fitted with a BA6-80 grit stone assembly. More specifically, 22.9, 45.8, 68.7, and 91.6 g of dry-blanched PS were ground with 195 g of sugar; the PS-sugar powders after sieving were then added to peanut butter formulations to achieve PS addition levels of 1.25, 2.5, 3.75, and 5.0% PS. Fortified peanut butters were placed in storage at 4 °C until analyzed.

Extraction of Phenolics

Phenolic compounds were extracted from both PS and PS-fortified peanut. Samples were placed in cellulose extraction thimbles (43 mm *i.d.* × 123 mm *e.l.*, VWR International, Suwanee, GA), covered with a plug of glass wool and defatted in a Soxhlet extraction apparatus under reflux for 12 to 14 h with hexanes. Defatted PS and peanut butters were transferred to 125-mL Erlenmeyer flasks at a mass-to-solvent ratio of 1:10 (w/v) with 80% (v/v) aqueous acetone. Extractions were performed in a gyrotary water bath shaker (Model G76, New Brunswick Scientific Company, Inc., New Brunswick, NJ) set at 150 rpm and a temperature of 45 °C for 30 min. The slurry was then filtered by gravity through P8 filter paper (Fisher Scientific Co., Suwanee, GA). The extraction process was repeated 2× as described above. All filtrates were pooled and acetone was evaporated with a Büchi Rotavapor R-210 using a V-700 vacuum pump connected to a V-850 vacuum controller (Büchi Corporation, New Castle, DE) at 45 °C. The aqueous residue was frozen and then lyophilized in a FreeZone® 2.5-L bench-top freeze dryer (Labconco Corporation, Kansas City, MO) to ensure all traces of moisture were removed and then stored in amber-glass bottles at -20 °C until further analyzed.

Preparation of Desugared Peanut Butter Extracts

The method described by Srivastava *et al.* (2010) for preparing a polyphenolic extract from

blackberries was adapted for peanut butter. Roughly 3 g of each crude peanut butter extract were dispersed in 10 mL of deionized water, sonicated to facilitate dissolution, and then applied to the top of a chromatographic column (30 mm *i.d.* × 340 mm *e.l.*, Kontes Glass Inc., Vineland, NJ) packed with Amberlite XAD-16 [(bead size: 20-60 mesh), Sigma-Aldrich] and washed with ~1000 mL of deionized water to remove sugars and organic acids. After the first 800 mL, the Brix reading of the eluent was checked using a digital PAL-1 pocket refractometer (Model 3810, ATAGO U.S.A., Inc., Bellevue, WA) until a zero value was reached. The desugared extract was then eluted from the column with anhydrous methanol (~300 mL) as the mobile phase. Methanol was evaporated using the Büchi Rotavapor as described above. The extract was lyophilized via the bench-top freeze dryer to ensure all traces of moisture were removed and then stored in amber-glass bottles at -20 °C until used further.

Total Phenolics Content (TPC)

The TPC value of each extract was determined spectrophotometrically using the classical Folin–Ciocalteu assay (Singleton & Rossi, 1965). Briefly, 1 mL of a methanolic solution of each extract was pipetted into a test tube followed by the addition of 7.5 mL of deionized water, 0.5 mL of 2 N Folin & Ciocalteu's phenol reagent, and 1.0 mL of a saturated Na_2CO_3 solution. The contents were vortexed for 15 s followed by a 60-min quiescent period at room temperature to allow for optimal color development. Absorbance readings of samples were measured at λ_{max} = 750 nm with the Agilent 8453 UV-visible spectrophotometer (Agilent Technologies, Inc., Wilmington, DE). Quantification was based on the standard curve generated with the (+)-catechin. Sample TPCs were expressed as milligrams (+)-catechin equivalents/g PS (d.w.) or 100-g peanut butter (f.w.).

Total PACs (TPAC) Content

The TPAC content was assessed by the 4-dimethylaminocinnamaldehyde (DMAC) assay taught by Payne *et al.* (2010). Briefly, 50 µL of methanol and 50 µL of standard solutions (procyanidin B2; *i.e.*, (-)-epicatechin-(4β→8)-(-)-epicatechin) or 50 µL of test samples, dissolved in methanol, were respectively added to a COSTAR® 96-well clear, non-sterile, non-treated microtiter assay plate and then mixed with 250 µL of the DMAC solution. This reagent was prepared fresh each day by dissolving 30 mg of DMAC in 30 mL of 1:9 (v/v) HCl and reagent alcohol. Absorbance readings were recorded with a FLUOstar Omega microplate reader (BMG LABTECH Inc., Cary, NC). Assay conditions comprised bottom scanning every 1 min over 12 min at λ_{max} = 640 nm at an incubation temperature of 25 °C. The plate was shaken for 3 s before measurement. The maximum absorbance during 12-min readings was used for calculation. TPAC contents were expressed as mg procyanidin B2 equivalents/g PS (d.w.) or 100-g peanut butter (f.w.).

Ferric Reducing Antioxidant Power Assay (FRAP)

The FRAP value of each extract was determined according to Pulido, Bravo, and Saura-Calixto (2000) with slight modifications. Briefly, the FRAP reagent was prepared fresh each day by combining 2.5 mL of a 10 mM TPTZ [2,4,6-tri(2-pyridyl)-*s*-triazine] solution in 40 mM HCl, 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$ and 25 mL of acetate buffer (0.3 M, pH 3.6). The FRAP reagent was kept warm at 37 °C in a 5-L Isotemp® digital-control water bath (Model 205, Fisher Scientific) until dispensed. Two hundred µL of the warmed FRAP reagent was mixed with 20 µL of deionized water and 6.66 µL of a test sample or the reagent blank (5% ethanol for PS and 5% methanol for peanut butter extracts) in a prewarmed COSTAR® 96-well clear, non-sterile,

non-treated microtiter assay plate. The measurement was carried out with the BMG LABTECH plate reader set at 37 °C. Absorbance readings were recorded by bottom scanning every 20 s over 4 min at λ_{max} = 593 nm. The plate was shaken before each cycle. The 4-min readings were used for calculation of FRAP values, which were expressed as mmol Fe^{2+} equivalents/100-g PS (d.w.) or μmol Fe^{2+} equivalents/100-g peanut butter (f.w.).

Hydrophilic-Oxygen Radical Absorbance Capacity (H-ORAC$_{FL}$) Assay

The radical-scavenging capacity of each extract was determined by the H-ORAC$_{FL}$ assay developed by Cao, Alessio, and Culter (1993). Briefly, Trolox standard aliquots with concentrations of 12.5, 25, 50, 80 and 100 μM were prepared. Fluorescein and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) were warmed at 37 °C in the 5-L Isotemp® water bath until further use. Twenty μL of sample, standards and reagent blank (5% ethanol for PS or 5% methanol for peanut butter extracts) were respectively added to a prewarmed COSTAR® 96-well clear, non-sterile, non-treated microtiter assay plate. After inserting the plate into the BMG LABTECH plate reader set at 37 °C, 200 μL of 9.57×10^{-8} M fluorescein and 20 μL of 80 μM AAPH were pumped into each well. Fluorescence intensity was recorded by bottom scanning for ~3 h at a λ_{ex} = 485 nm and an λ_{em} = 520 nm. The plate was shaken before each cycle. Data reduction was achieved by (i) calculating of the area under the kinetic curve (AUC) and net AUC ($AUC_{sample} - AUC_{blank}$), (ii) constructing a standard curve by plotting the concentration of Trolox against the AUC, and (iii) determining the Trolox equivalents of a sample using the standard curve.

Fiber Analysis

Total Dietary Fiber (TDF)

The TDF content was determined on duplicate samples of dried and defatted PS and peanut butters (also desugared) from three batches according to AOAC Official Method 991.43 (AOAC, 2005). Porcelain crucibles, that had been soaked in 10% HCl, rinsed well 3× with deionized water, and fired in a 45.3-L capacity Thermo Scientific Thermolyne tabletop muffle furnace (Fisher Scientific) overnight at 700 °C, were placed in a desiccator until used. To each crucible ~1 g of Celite was added, followed by drying at 130 °C in a forced-air convection oven to a constant mass (W_1). Finely ground samples (~1.000 ± 0.005 g) accurate to 0.1 mg and a blank (containing only MES-TRIS buffer {0.05 M of 2-(*N*-morpholino)ethanesulfonic acid and 0.05 M Tris(hydroxymethyl)aminomethane, pH 8.2}) were gelatinized in the presence of heat-stable α-amylase at 95 °C to hydrolyze the α(1→4) bonds of large α-linked polysaccharides, such as starch, yielding glucose and maltose. The subsequent enzymatic digestions with protease and amyloglucosidase at 60 °C were performed to degrade proteins and cleave α(1→6) glycosidic linkages, respectively. The final digests (samples and blank) were treated with 95% (v/v) ethanol to precipitate DF and remove depolymerized protein and glucose. The residues were filtered and sequentially washed with 78% (v/v) ethanol, 95% (v/v) ethanol, and acetone. After drying at 105 °C in the forced-air convection oven overnight, the residues plus Celite and crucible were weighed; the mass was recorded as W_2. One duplicate was subjected to Kjeldahl nitrogen analysis as specified in the AOAC Method 960.52 (AOAC, 2005) for crude protein (P) determination using N × 5.46 as the conversion factor. The other was placed in the muffle furnace at 525 °C overnight for ash determination (W_3). The TDF content was calculated by subtracting the mass of the crude protein, ash, and blank from the mass of the filtered and dried

residues.

$$\text{Residue Mass (R)} = W_2 - W_1 \text{ (mg)}$$

$$\text{Ash Mass (A)} = W_3 - W_1 \text{ (mg)}$$

$$\text{Blank Mass (B)} = R_{blank} - A_{blank} - P_{blank} \text{ (mg)}$$

where, the blank containing only MES-TRIS buffer was processed identically as the samples. So in the above equation, R_{blank} is the residue mass of the blank, P_{blank} is the crude protein mass of the blank, and A_{blank} is the ash mass of the blank. Proteins in the blank originated from enzymes used to digest protein and carbohydrate.

$$\text{g TDF/100-g sample} = (R_{sample} - A_{sample} - P_{sample} - B)/\text{sample mass (mg)} \times 100$$

where, R_{sample} is the residue mass of the sample P_{sample} is the crude protein mass of the sample, and A_{sample} is the ash mass of the sample, all in mg.

Insoluble Dietary Fiber (IDF) and Soluble Dietary Fiber (SDF) Contents

The IDF enzyme digests were filtered and residues were washed 2× with 10 mL of 70 °C deionized water. Water filtrates were combined and used for the SDF analysis. The water-washed residues were further washed with 78% (v/v) ethanol, 95% (v/v) ethanol and acetone. The mass was recorded after oven-drying at 105 °C. The water filtrates were precipitated with 95% (v/v) ethanol, filtered and dried. Both SDF and IDF residues were corrected for the contents of crude protein, ash and blank, as was the case in TDF analysis.

Normal Phase High-Performance Liquid Chromatography Electrospray Ionization Mass Spectrometry (NP-HPLC/ESI-MS)

The flavan-3-ols and PACs in the extracts of PS and peanut butters were separated by

NP-HPLC based on their degree of polymerization (DP). An Agilent 1200 Series HPLC was employed and conditions of separation involved a PrincetonSPHER DIOL column (250 mm × 4.6 mm, 5-μm particle size, 60 Å; Princeton Chromatography, Inc., Cranbury, NJ) equipped with a guard cartridge/holder system; a thermostatted column compartment set at 30 °C; a binary mobile phase of A (CH_3CN: CH_3COOH, 98:2, v/v) and B (CH_3OH:H_2O:CH_3COOH, 95:3:2, v/v/v); a 1 mL/min flow rate for 0-35 min of 0-40% B, held for 5 min, 40-45 min of 40-0% B, and then an additional 5 min hold to re-equilibrate the system; 20 μL injection; and fluorescence detection with λ_{ex} and λ_{em} set at 276 and 316 nm, respectively. All test samples and PAC standards (*i.e.*, flavan-3-ol monomers, dimers, trimers, and tetramers only) were dissolved in anhydrous CH_3OH (20 mg/mL), diluted with mobile phase A at a 1:9 (v/v) ratio, and passed through a 0.45-μm PTFE syringe filter prior to injection. Tentative identification of separated components was made by retention time mapping with authenticated standards prior to ESI-MS analysis.

NP-HPLC/ESI-MS analyses of the phenolics were carried out on an Agilent 1100 HPLC system, with the same chromatography conditions as described above, combined with a Bruker Esquire 3000^{plus} ion trap mass spectrometer (Bruker Daltonics Inc., Billerica, MA) which was equipped with an ESI source. Instrument control and data acquisition were performed with Esquire 5.3 software. The HPLC eluent flowed directly into the mass spectrometer *via* a flow splitter delivering roughly 100 μL/min. The ESI mass detector was employed in the negative-ion mode with a skimmer voltage of -40 V and a capillary exit voltage of -241 V. The voltage applied to the capillary tip was 4.0 kV. Nitrogen was used as the nebulizing gas at a pressure of 10 psi, a dry gas flow rate of 6 L/min and a dry gas temperature of 280 °C. A full scan was performed over the mass range of 200-3000 Da at a rate of 13,000 *m/z* per second. Each mass spectrum

generated was based on an average of five scans.

Statistical Analysis

All colorimetric and fluorescence assay measurements were repeated a minimum of three times. The TDF assay was carried out in duplicate. The results were expressed as mean ± standard deviation. Data for TPCs, TPAC contents, FRAP values, H-ORAC$_{FL}$ values, and TDF contents were analyzed by a one-way analysis of variance (ANOVA) using the General Linear Model (SAS, version 9.3, SAS Institute, Inc., Cary, NC) to determine significant differences at the 95% confidence interval (α=0.05). Tukey's studentized range test was used to segregate treatment means.

4.3 Results and Discussion

Total Phenolics Content (TPC)

In this study, the classical method of Singleton and Rossi (1965) using Folin & Ciocalteu's phenol reagent was employed to determine the TPC in acetonic extracts of defatted PS and PS-fortified peanut butters (with further chromatography on the Amberlite XAD-16 column). The findings are listed in **Table 4.1** and expressed as mg (+)-catechin equivalents/g PS (d.w.) or 100-g peanut butter (f.w.). The greatest TPC of ~166 mg (+)-catechin eq./g was found in the dry-blanched PS; this result was 33%, 34% and 38% higher than that for light-, medium-, and dark-roasted skins, respectively. Even though dry-blanched PS exhibited the highest TPC, all four PS types effectively increased the phenolics content of the formulated peanut butters in a concentration-dependent manner. After adding dry-blanched PS to peanut butter at 1.25, 2.5, 3.75, and 5.0% (w/w), the TPCs grew by 86, 357, 533, and 714%, respectively, relative to the peanut

butter devoid of PS. Interesting was the fact that at fortified levels below 3.75%, peanut butters formulated with dry-blanched PS showed no overall significant difference compared to those containing roasted PS.

Phenolic compounds are secondary metabolites in plants that generally concentrate in the outer layers of plants such as peel, shell and hull to protect inner core materials. A large number of intrinsic and extrinsic factors, such as genetics, environmental conditions, germination, ripening, processing and storage, influence the types and levels of phenolics found in plants. Mild heat treatment can break some covalent bounds of phenolic acids with insoluble polymers and other cell wall components such as arabinoxylans, thereby liberating low-molecular-weight antioxidant compounds from the repeating subunits (*e.g.*, flavan-3-ols) of high-molecular-weight oligomers (Jeong *et al.*, 2004). PS roasted at 175 °C for 5 min were found to contain a higher TPC than raw PS, especially when recovered by aqueous and ethanolic extraction (Yu, Ahmedna, & Goktepe 2005). In addition to processing, the extraction conditions employed like choice of solvent system, material-to-solvent ratio, particle size distribution of the PS, extraction time, number of extractions, and temperature (Nepote, Grosso, & Guzmán, 2005) all impact the efficiency of bioactive recovery and thereby result in different removal of phenolics from the source material.

Our TPC data for dry-blanched PS was similar to that reported by Nepote, Grosso, and Guzman (2002) where the TPC was ~159 mg phenol equivalents/g recovered from blanched PS by 24-h maceration with methanol at a material-to-solvent ratio of 1:10 (w/v); the extraction was performed 2× at room temperature in the dark. Using a microwave-assisted system to extract phenolic antioxidants from PS, Ballard, Mallikarjunan, Zhou and O'Keefe (2010) reported a comparable TPC result of 144 mg gallic acid equivalents/g skin under optimized conditions.

Francisco and Resurreccion (2009) reported a markedly greater TPC value of 280 mg/g in Runner PS processed at 135 °C for 5 min. The phenolics were recovered from PS by 3 × 10-min extractions at room temperature. Ethanol (70%, v/v) was the extractant used at a material-to-solvent ratio of 1:20 (w/v). We carefully followed the reported methodology but could neither reproduce the finding nor validate the result.

None of the treatment conditions described above are adopted by the peanut industry. Dry blanching of raw peanuts, to remove the seed coat (*i.e.*, testa) from the kernel, is achieved by transporting peanuts on a belt through a low-temperature heating zone (at a maximum of 96 °C) for ~45 min. Roasted PS, on the other hand, are a by-product of fluidized bed heating of raw peanuts. For light-roasted PS, the skins are subjected to fluidized bed heating at 124 °C for 10 min followed by 168 °C for an additional 10 min. For dark-roasted PS, the initial thermal processing is the same, but the final temperature in the last 10 min is 182 °C; medium-roasted PS are subjected to a final temperature between 168 and 182 °C. TPC values from the literature are difficult to compare against one another, because the sample source, manner of preparation, and extraction techniques applied are quite varied. Though our work did not show the highest TPC value ever reported for PS, it analyzed PS generated by the peanut industry as such. Moreover, this investigation supports the employment of PS in a functional food (*i.e.*, peanut butter) as a potential source of antioxidant-rich phenolics.

Profile of PACs Determined by the DMAC Assay and NP-HPLC/ESI-MS

The TPAC contents, expressed as mg procyanidin B2 equivalents/g PS (d.w.) or 100-g peanut butter (f.w.), are depicted in **Figure 4.1(A)** and **4.2(A)**, respectively. Dry-blanched PS possessed the highest TPAC content of 84 mg/g which was 42, 47, and 62% higher ($P < 0.05$) than the

light-, medium-, and dark-roasted skins. All four PS types effectively ($P < 0.05$) increased the PACs content of the formulated peanut butters in a step-wise, concentration-dependent manner. Using dry-blanched PS as an example, the TPAC contents rose by 633, 1933, 3500, and 5033%, respectively, after adding PS into peanut butter at 1.25, 2.5, 3.75, and 5.0% (w/w), compared to the peanut butter control devoid of PS. A significant difference in the TPAC content of dry-blanched PS- and roasted PS-fortified peanut butters was not evident until the PS incorporation level reached 5.0%. Based on NP-HPLC/ESI-MS, most PAC oligomers were barely evident in peanut butters devoid of PS, expect for DP2 (*i.e.,* a dimer represented by a degree of polymerization of 2) with an A-type linkage. In contrast, a wide variety of PACs (with DPs from 2 to 9 possessing both A- and B-linkages) were found in the acetonic extracts of PS as well as those from PS-fortified peanut butters (with further chromatography on the Amberlite XAD-16 column). Addition of PS contributed to the enhanced PAC profile in the formulated peanut butters, not only in quantity but also in type (**Table 4.3**).

PACs, also known as condensed tannins, are oligomeric and polymeric end products of the flavonoid biosynthetic pathway consisting of flavan-3-ol (*i.e.*, (+)-catechin and (-)-epicatechin) monomers and their galloyl derivatives. B-type PAC dimers are linked via (C4→C8) or (C4→C6) bonds, whereas A-type dimers contain an additional (C2→O→C7) linkage. The DMAC assay is considered as a rapid method to quantify the PACs content and can effectively exclude interference from other sample components. The reaction of charge delocalization on the DMAC molecule is specific for phenolic compounds where *meta*-oriented di- or tri-hydroxyphenols are found, such as in PACs (Krueger, Reed, Feliciano, & Howell, 2013). As stated above, our data revealed the highest TPC was present in dry-blanched PS at 166 mg (+)-catechin/g skin. The TPC value was not significantly different from roasted PS that showed ~123±2.6 mg/g.

Therefore, > 50% of the TPC found in PS was PACs based on this projection. A number of studies have reported that PACs are the major phenolic constituent in PS. According to Karchesy and Hemingway (1986), ~17% of the PS by weight is PACs, of which 50% were low-molecular-weight oligomers. The accurate quantification of PACs using the procyanidin B2 standard is somewhat impaired by an ascending DP. The DMAC assay is more suitable for samples containing low-molecular-weight PAC oligomers for which the structure and reaction kinetics of the B2 dimer standard are better matched. Therefore, quantification error exists when MS analyses reveal the presence of higher polymerized PACs (*i.e.*, a DP ≥ 4) (Krueger, Reed, Feliciano, & Howell, 2013) in PS and their fortified peanut butters.

Six A-type PAC dimers and five oligomeric PACs with both A- and B-type linkages were isolated and characterized by Lou's group (1999 & 2004). A higher degree of polymerized PAC trimers, tetramers, and oligomers together with B-type PAC dimers were identified in PS by Lazarus, Adamson, Hammerstone, and Schmitz (1999). According to LC-MS data reported by Yu, Ahmedna, Goktepe, and Dai (2006), the PACs were mainly of the A-type, including dimers, trimers and tetramers; they also showed much greater concentrations compared to other phenolics in the PS, such as free phenolic acids.

Yu and coworkers (2006) reported a significant influence of the skin removal methods on the PACs content of PS. Dry heat, like in roasting, would increase A-type dimers and B-type trimers compared to directly peeled PS; whereas, wet blanching would leach out most A- and B-type PACs due to the high solubility of these compounds in hot water. The increased A-type dimers might arise from monomeric polymerization or the degradation of trimers and tetramers. Polymerization of monomers and B-type dimers is a plausible mechanism by which increased B-type trimers were detected. In our study, all four types of PS were treated with dry heat of

some sort. The highest PACs content of dry-blanched PS might result from the more optimal processing conditions (*i.e.*, temperature and time) for monomeric polymerization and/or oligomer ($n > 4$) degradation.

Ferric Reducing Antioxidant Power Assay (FRAP)

In acidic medium the ferric tripyridyltriazine {Fe(III)−TPTZ} complex is reduced to ferrous tripyridyltriazine {Fe(II)−TPTZ} by a reductant/antioxidant, such as a phenolic compound, with an appropriate redox potential. The resultant Fe(II)−TPTZ chromophore yields an intense blue color with a wavelength maximum of 593 nm. Unfortunately, the FRAP assay only detects compounds with redox potentials of < 0.7 V (Prior, Wu, & Schaich, 2005), and is indicative of the antioxidant capacity of converting radicals to stable products by electron donation (*i.e.*, via a single-electron transfer {SET} and not a hydrogen-atom transfer {HAT} mechanism). The dry-blanched PS possessed the highest FRAP value [**Figure 4.1(B)**], which was 66, 55, and 38% greater ($P < 0.05$) than the light-, medium-, and dark-roasted PS, respectively. After incorporation of dry-blanched PS, the FRAP values rose by 62, 387, 747, and 829%, respectively, in the peanut butter prototypes at 1.25, 2.5, 3.75, and 5.0% (w/w) compared to the peanut butter control devoid of PS **[Figure 4.2(B)**]. Corresponding to their highest TPC and TPAC contents, dry-blanched PS exhibited the greatest FRAP value, as predicted. Interestingly, this superiority was not demonstrated in PS-fortified peanut butters when the addition level was below 3.75%.

In their review, Prior, Wu, and Schaich (2005) reported that the number of hydroxy constituents on aromatic residues correlates positively with reducing power. The high FRAP value found in dry-blanched PS is most likely due to these skins possessing the greatest TPAC content; condensed tannins are more hydroxylated than phenolic acids on an equimolar basis.

The inability of the FRAP assay to detect compounds that function by HAT narrowed down electron donation to be a possible mechanism contributing toward the potent antioxidant activities observed for phenolics, especially PACs present in PS. Even though the acidic pH of the FRAP assay is necessary to maintain iron solubility, it lowers the ionization potential of the reactants and increases the redox potential of the system (Prior, Wu, & Schaich, 2005). An additional disadvantage of the FRAP assay is the exclusion of some polyphenols that require longer times for reaction, for example, > 4 min (Prior, Wu, & Schaich, 2005). A slowly increasing FRAP value was found in phenolics such as caffeic acid, tannic acid, ferulic acid, *trans*-resveratrol, and quercetin even after several hours of reaction (Pulido, Bravo, & Saura-Calixto, 2000).

Hydrophilic-Oxygen Radical Absorbance Capacity (H-ORAC$_{FL}$) Assay

In **Figure 4.1(C)** and **4.2(C)**, the H-ORAC$_{FL}$ values of PS and peanut butters were expressed as Trolox equivalents (TE)/g PS d.w. or g peanut butter f.w., respectively. Briefly, the H-ORAC$_{FL}$ assay measures the antioxidant scavenging activity against the peroxyl radical induced by AAPH at 37 °C. Generated peroxyl radicals react with a fluorescent probe and form an oxidized nonfluorescent reaction product, which is quantitated by monitoring real-time fluorescence. In the presence of an antioxidant species, the oxidation of the fluorescent probe is inhibited: the antioxidant acts as the first defense in quenching the peroxyl radicals resulting in a longer observed period of fluorescence (Prior, Wu, & Schaich, 2005). The greater the H-ORAC$_{FL}$ value denotes an increased efficacy of HAT by the antioxidant or antioxidant constituents within a specific matrix (*e.g.*, an acetonic phenolic extract). The dry-blanched PS exhibited the highest H-ORAC$_{FL}$ value compared to roasted PS, and was 10, 25, and 23% greater ($P < 0.05$) than the

light-, medium-, and dark-roasted PS, respectively. Incorporation of dry-blanched PS increased the H-ORAC$_{FL}$ values by 53, 247, 382, and 415%, respectively, in the formulated peanut butters at 1.25, 2.5, 3.75, and 5.0% (w/w) compared to peanut butter devoid of PS. Peanut butter fortified with dry-blanched PS showed significant higher radical-scavenging capacity than those with roasted PS, but only when the incorporation level reached 3.75%.

PS have been shown efficacious in scavenging free radicals and inhibiting edible oil oxidation. The radical-scavenging capacity of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) has been well demonstrated (Nepote, Grosso, & Guzman, 2002; Lou *et al.*, 2004; Win *et al.*, 2011). Nepote, Grosso, and Guzman (2002) reported a marked antioxidant activity of PS extracts, as demonstrated by their capacity to inhibit oxidation of sunflower oil. Compared to raw peanut kernels, PS and hull methanolic extracts were more effective in the inhibition of linoleic acid peroxidation (Win *et al.*, 2011). $ABTS^{+\bullet}$, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation, quenching by the phenolics of PS has also been documented (Yu, Ahmedna, & Goktepe, 2005; Francisco & Resurreccion, 2009). Despite only moderate and low antioxidant activity in lard and rapeseed oil when compared to extracts from Labiatae plants, PS exhibited comparable activities in free radical-scavenging with other natural oxidants and satisfactory reducing power (Hoang *et al.*, 2008).

Davis, Dean, Price, and Sanders (2010) revealed a positive relationship between mild heat treatment and H-ORAC$_{FL}$ values of PS. The H-ORAC$_{FL}$ values of roasting PS at 166 °C from 0 to 77 min ranged from 1.52 to 2.10 mmol TE/g PS. The maximum radical-scavenging capacity was determined at 7-min roasting and then declined with an extended heating period. This was similar to our observations, where the H-ORAC$_{FL}$ values for dry-blanched and roasted skins ranged from 1.96 to 2.42 mmol TE/g PS. Dry-blanched PS experience a less severe thermal

treatment compared to roasted counterparts and would predictably exhibit higher H-ORAC$_{FL}$ values. Correspondingly, addition of dry-blanched PS into the peanut butter matrix resulted in a product with a tremendous increase in radical-scavenging capacity. Our data clearly demonstrates the free radical-scavenging effects of PS and that this activity is retained in the formulated peanut butters.

Fiber Analysis

The fiber contents of PS and peanut butters are presented in **Table 4.2**. Briefly, the TDF contents were ~55% in dry-blanched PS, 43% in light- and medium-roasted PS, and 45% in dark-roasted PS with 89-93% of this being IDF and 7-9% being SDF. The TDF content of the peanut butter control was ~2.3 g per serving (28.5 g). The 5.0% addition of ground dry-blanched PS enhanced the fiber content to ~2.9 g per serving, thereby permitting a "good source of fiber" claim to be made according to US FDA food labeling regulations. Even though dry-blanched PS indicated the highest content of IDF relative to roasted analogs, the superiority was not evident in PS-fortified peanut butters even at the 5.0% incorporation level.

Little information is available about the fiber content of PS. Shimizu-Ibuka *et al*. (2009) studied the hypocholesterolemic effect of PS and employed a commercial roasted PS which contained ~45% TDF, of which only 2.2% was SDF. As early as 1981, Collins and Post explored the use of peanut hull flour as a potential source of DF. The flour was prepared from either Virginia- or Runner-type peanuts containing ~47% crude fiber and relatively large amounts cellulose, hemicellulose, and lignin. Collins, Kalantari, and Post (1982) developed a series of wheat breads containing 0, 4, and 8% peanut hull flour, respectively, in an attempt to increase the DF content. Correspondingly, the neutral detergent fiber content of the breads increased from 4

to 6.7%. Breads containing 4% peanut hull flour were more acceptable than those at 8%; the flour made from peanut hull could be a potential source of DF.

Likewise, our results demonstrated a positive relationship between TDF content in the peanut butters and the step-wise addition of PS. Consumer acceptability of PS-fortified peanut butters was conducted by Sanders *et al.* (2013), who reported that with incorporation at the 2.5% level, PS addition – regardless of heat treatment – yielded peanut butters that equaled the control in acceptability. When PS were added at the 5.0% level, peanut butters containing the dry-blanched PS were most acceptable, equaling the acceptability of the formulations with 2.5% roasted PS. The increased TDF content was not an impediment to consumer acceptability. As the major by-product of the peanut industry, PS have great potential for their utilization as a source of DF in functional foods.

Correlation of PS Incorporation with TPC, Free-radical-Scavenging Activity and TDF Content

Using dry-blanched PS as an example, the correlation coefficients showing the relationships between PS incorporation, TPC, H-ORAC$_{FL}$ value, and TDF content are presented in **Figure 4.3**. The proportional increase of TPC, free radical-scavenging activity, and TDF content with PS addition indicated strong positive correlations ($R^2 \geq 0.95$) of those functional properties with the PS level in peanut butters. PS addition affected an increase in the TPC, especially TPACs, in peanut butters and thereby resulted in improved radical-scavenging/antioxidant performance. A strong correlation (R^2 ~0.98) between the TPC and $ABTS^{+\bullet}$ radical-scavenging capacity was reported in PS by Yu, Ahmedna, and Goktepe (2005). Similarly, an R^2 of ~0.84 between the TPC and DPPH• radical-scavenging capacity was reported in PS by Win *et al*. (2011). The

contribution of PS to the TPC, H-ORAC$_{FL}$ value, and TDF content in the formulated peanut butters of this study suggests that a serving of a PS value-added food is a good source of DF and may provide antioxidant benefits.

4.4 Conclusions

PS possess a wealth of natural antioxidants with marked radical-scavenging activity and a high content of DF, as determined in this study using selected *in vitro* assays. Although dry-blanched PS exhibited the greatest enhancement, all four PS types effectively increased the phenolics content, antioxidant capacity and the fiber content of the fabricated peanut butters in a concentration-dependent manner while maintaining the US FDA's standard of identity for peanut butter. Moreover, the improved TPCs in the PS-fortified peanut butters were largely attributed to the PACs, as demonstrated by the DMAC assay and NP-HPLC/ESI-MS.

The findings from a consumer acceptability study on the PS-fortified peanut butters performed by researchers of our team but communicated in an independent publication indicate a high acceptability at 2.5% level of PS incorporation. According to our findings, when incorporated at the 2.5% level, increases in DF and phenolics content did not differ significantly with PS type. Given a similar price structure as non-fortified peanut butters, these formulations hold great promise as a functional food. In other words, the findings from this study have paved the way for better utilization of a low-valued industrial by-product (*i.e.*, PS) as an ingredient in functional food formulations that can improve an existing product and allow diversification of established brands available in the market.

Acknowledgements

This research was supported by a grant funded by the Georgia Food Industry Partnership and is greatly appreciated. Thanks are extended to the following: Rick Boyce at Seabrook Ingredients, a division of Universal Blanchers, LLC, for the peanut paste, flour salt, and stabilizer; Bruce A. Kotz at Golden Peanut Corporation, LLC, for the light, medium, and dark roasted PS; Bill Paulk at Sylvester Blanching, a division of Universal Blanchers, LLC, for the dry-blanched PS; Agnieszka Kosińska of the Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences for her assistance with setting up the NP-HPLC analyses; and John T. Powell, President of the Peanut Institute Inc., Albany, GA.

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Table 4.1 Total phenolics contents (TPCs) of peanut skins (PS) and PS-fortified peanut butters (PB), expressed as (+)-catechin equivalents/g PS of 100-g peanut butter.

Type of PS	TPC[1] in PS (mg/g)	Formulation %PS added	TPC[1] in Peanut Butters (mg (+)-catechin eq./100 g)
0% PS, Control		0	42.0 ± 2.1^{h}
Dry-blanched (DB)	166 ± 11.1^{a}	1.25	77.6 ± 4.8^{g}
		2.5	192 ± 1.5^{d}
		3.75	266 ± 2.0^{b}
		5.0	342 ± 1.4^{a}
Light-roasted (LR)	125 ± 4.2^{b}	1.25	84.3 ± 0.4^{g}
		2.5	181 ± 1.3^{de}
		3.75	219 ± 0.9^{c}
		5.0	278 ± 7.1^{b}
Medium-roasted (MR)	124 ± 4.3^{b}	1.25	96.4 ± 3.9^{g}
		2.5	162 ± 2.7^{ef}
		3.75	188 ± 12.5^{d}
		5.0	264 ± 1.4^{b}
Dark-roasted (DR)	120 ± 2.0^{b}	1.25	80.8 ± 3.3^{g}
		2.5	143 ± 9.8^{f}
		3.75	185 ± 1.8^{d}
		5.0	235 ± 18.1^{c}

[1]Means ± standard deviations (n=3). Values within a given column followed by the same letters are not significantly different ($P < 0.05$) according to ANOVA and Tukey's studentized range test.

Table 4.2 Dietary fiber (DF) content (*i.e.*, total, insoluble, and soluble, g/100 g) of peanut skins (PS) and PS-fortified peanut butters.

PS type	Formulation % PS added	Total DF		Insoluble DF		Soluble DF	
		PS	Peanut butters	PS	Peanut butters	PS	Peanut butters
0% PS, Control	0		8.26±0.44^{c}		7.47±0.30^{e}		1.04±0.11^{a}
DB	2.5	54.7±0.34^{a}	8.90±0.08^{bc}	51.3±0.04^{a}	7.58±0.04^{de}	4.55±0.20^{a}	1.40±0.23^{a}
	5		10.3±0.44^{a}		9.05±0.42^{a}		1.46±0.30^{a}
LR	2.5	42.8±0.10^{c}	8.76±0.23^{bc}	38.7±0.14^{c}	7.79±0.16^{cde}	3.24±0.33^{a}	1.18±0.05^{a}
	5		9.55±0.29^{ab}		8.59±0.17^{ab}		1.21±0.14^{a}
MR	2.5	43.2±0.11^{c}	8.61±0.11^{bc}	39.7±0.01^{b}	7.34±0.01^{e}	4.15±0.18^{a}	1.11±0.06^{a}
	5		9.39±0.14^{ab}		8.37±0.19^{abcd}		1.37±0.09^{a}
DR	2.5	44.6±0.05^{b}	8.74±0.06^{bc}	39.5±0.23^{b}	7.87±0.02^{bcde}	4.04±0.50^{a}	1.15±0.01^{a}
	5		9.60±0.16^{ab}		8.44±0.11^{abc}		1.45±0.13^{a}

[1]Means ± standard deviations (n=2). Values within a given column followed by the same letters are not significantly different ($P < 0.05$) according to ANOVA and Tukey's studentized range test.

Table 4.3 Proanthocyanidins (PACs) observed in peanut skins (PS) and dry-blanched (DB) PS-fortified peanut butters (0% skins vs 5% DB) based on NP-HPLC-ESI-MS analysis.

Oligomeric PACs[1]	Ions, *m/z*		Found in[2]
	$[M-H]^-$	$[M-2H]^{2-}/2$	
PAC dimer [(E)C→luteolin or kaempferol][3]	573		PS, 5% DB
PAC A-type dimer	575		PS, PB, 5% DB
PAC B-type dimer	577		PS, 5% DB
PAC A-type trimer (2A)	861		PS, 5% DB
PAC A-type trimer (1A)	863		PS, 5% DB
PAC B-type trimer	865		PS
PAC A-type tetramer (2A)	1149		PS, 5% DB
PAC A-type tetramer (1A)	1151		PS, 5% DB
PAC A-type pentamer (2A)	1437		PS, 5% DB
PAC A-type pentamer (1A)	1439		PS, 5% DB
PAC B-type pentamer	1441		PS, 5% DB
PAC A-type hexamer (3A)	1723		PS
PAC A-type hexamer (2A, 3B)[3]	1725		PS, 5% DB
PAC A-type hexamer (1A)	1727		PS, 5% DB
PAC B-type hexamer	1729		PS, 5% DB
PAC A-type heptamer (2A)	2013		PS, 5% DB
PAC A-type heptamer (1A)	2015		PS, 5% DB
PAC A-type octamer (2A, 5B)[3]	2301		PS, 5% DB
PAC A-type nonamer (3A)	2587	1293	PS, 5% DB
PAC A-type nonamer (2A, 6B)[3]	2589	1294	PS, 5% DB
PAC A-type nonamer (1A)	2591	1295	PS, 5% DB

[1]PAC = proanthocyanidins; A = an A-type bond with both (C4→C8) and (C2→O→C7) linkages or (C4→C6) and (C2→O→C7) linkages; B = B-type bond which can be (C4→C8) or (C4→C6) linkages; (E)C = (-)-epicatechin or (+)-catechin.

[2]Denotes the presence of the PAC in either peanut skins (PS); peanut butter without skins (PB); or PS-fortified peanut butter at the 5% level with dry-blanched skins (5% DB).

[3]Compounds in peanut skins reported by Sarnoski, Johnson, Reed, Tanko & O'Keefe (2012).

Figure Captions

Figure 4.1 Total proanthocyanidins (TPAC) contents (**A**), ferric reducing antioxidant power (FRAP) values (**B**), and hydrophilic-oxygen radical absorbance capacity (H-ORAC$_{FL}$) values (**C**) of peanut skins (PS). Abbreviations for the type of PS examined are as follows: DB, dry-blanched; LR, light-roasted; MR, medium-roasted; and DR, dark-roasted. Means (n=3) without a common letter differ significantly ($P < 0.05$) according to a one-way ANOVA and Tukey's studentized range test.

Figure 4.2 Total proanthocyanidins (TPAC) contents (**A**), ferric reducing antioxidant power (FRAP) values (**B**), and hydrophilic-oxygen radical absorbance capacity (H-ORAC$_{FL}$) values (**C**) of peanut skins (PS)-fortified peanut butters. A set of clustered bars on the y-axis depicts the concentration for the identified PS type (*i.e.*, DB, LR, MR, or DR); within each cluster, PS-fortification levels increased (*i.e.*, 1.25, 2.5, 3.75, 5.0%) from the lowest to highest bar. The formulated peanut butter sample (0% PS) depicted as a single bar was the control. See the caption of Fig. 1 for details concerning the abbreviations employed and statistical treatment of the data.

Figure 4.3 Positive linear correlations between total phenolics contents (TPCs, **A**), hydrophilic-oxygen radical absorbance capacity (H-ORAC$_{FL}$) values (**B**), and total dietary fiber (TDF) contents (**C**) to peanut skins (PS) incorporation.

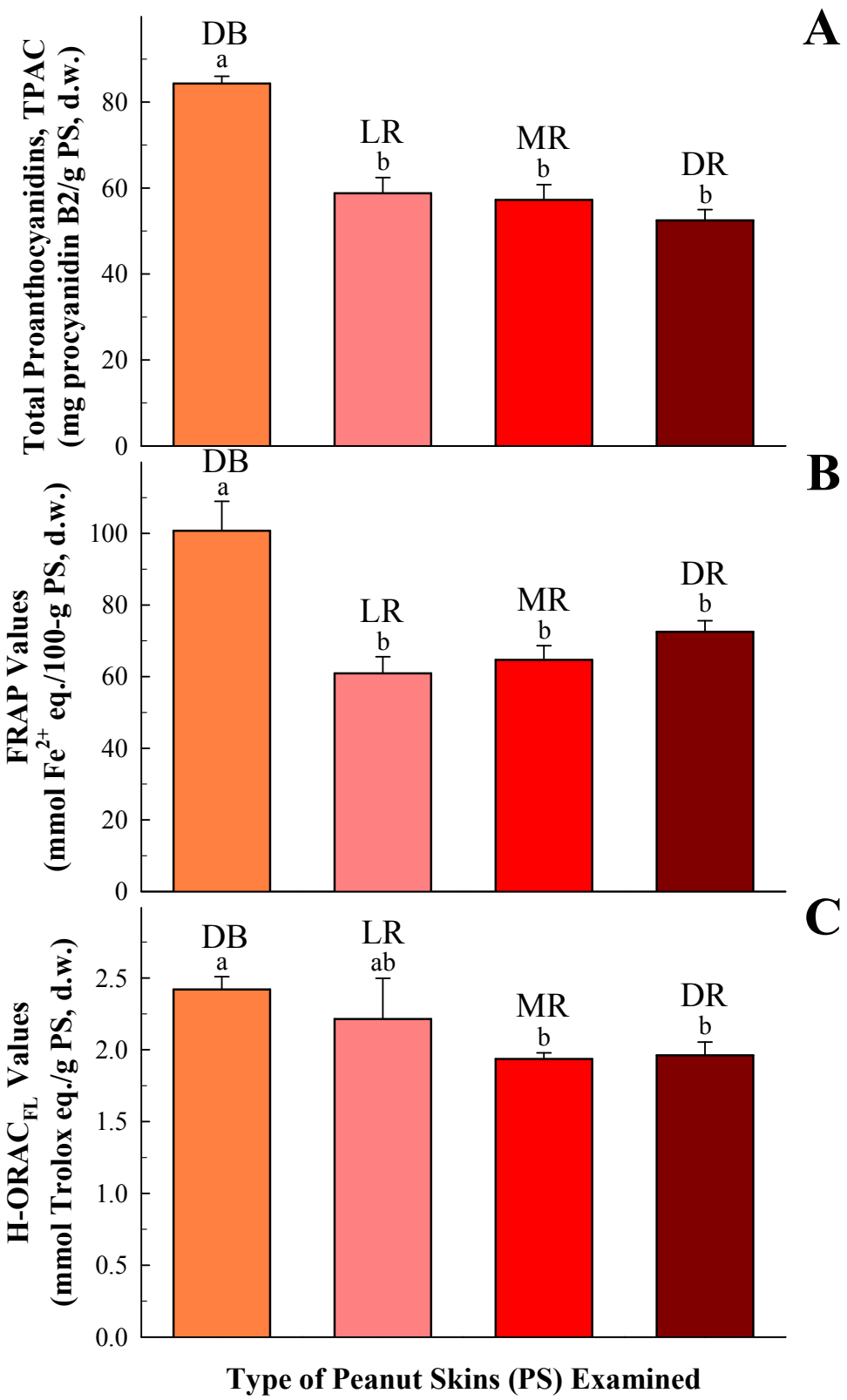
A
Total Proanthocyanidins, TPAC
(mg procyanidin B2/g PS, d.w.)
0
20
40
60
80
DB
a
LR
b
MR
b
DR
b
B
FRAP Values
(mmol Fe^{2+} eq./100-g PS, d.w.)
0
20
40
60
80
100
DB
a
LR
b
MR
b
DR
b
C
H-ORAC$_{FL}$ Values
(mmol Trolox eq./g PS, d.w.)
0.0
0.5
1.0
1.5
2.0
2.5
DB
a
LR
ab
MR
b
DR
b
Type of Peanut Skins (PS) Examined

Figure 4.1

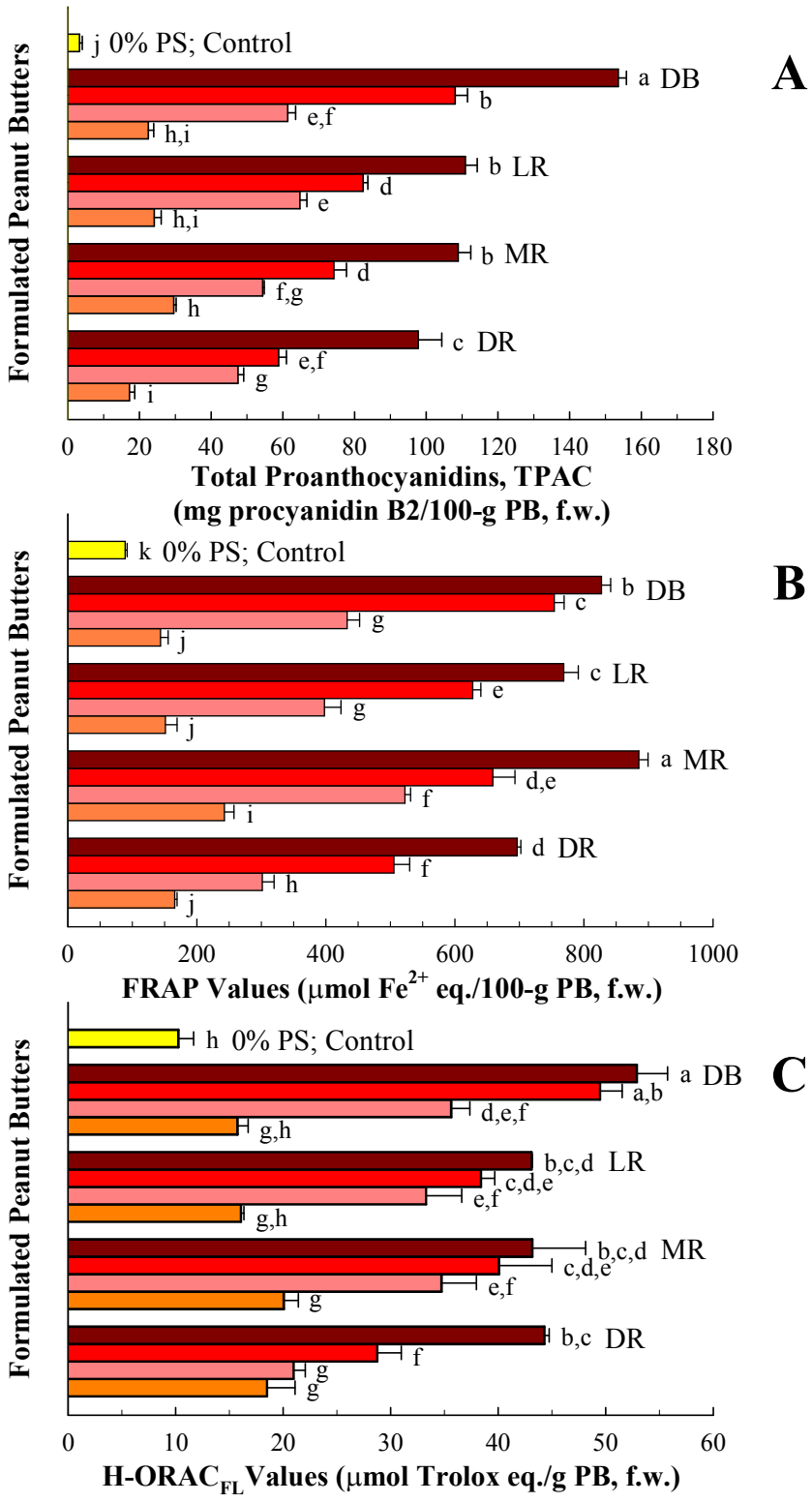
A
Formulated Peanut Butters
j 0% PS; Control
a DB
b
e,f
h,i
b LR
d
e
h,i
b MR
d
f,g
h
c DR
e,f
g
i
0
20
40
60
80
100
120
140
160
180
Total Proanthocyanidins, TPAC
(mg procyanidin B2/100-g PB, f.w.)
B
Formulated Peanut Butters
k 0% PS; Control
b DB
c
g
j
c LR
e
g
j
a MR
d,e
f
i
d DR
f
h
j
0
200
400
600
800
1000
FRAP Values (μmol Fe2+ eq./100-g PB, f.w.)
C
Formulated Peanut Butters
h 0% PS; Control
a DB
a,b
d,e,f
g,h
b,c,d LR
c,d,e
e,f
g,h
b,c,d MR
c,d,e
e,f
g
b,c DR
f
g
g
0
10
20
30
40
50
60
H-ORACFL Values (μmol Trolox eq./g PB, f.w.)

Figure 4.2

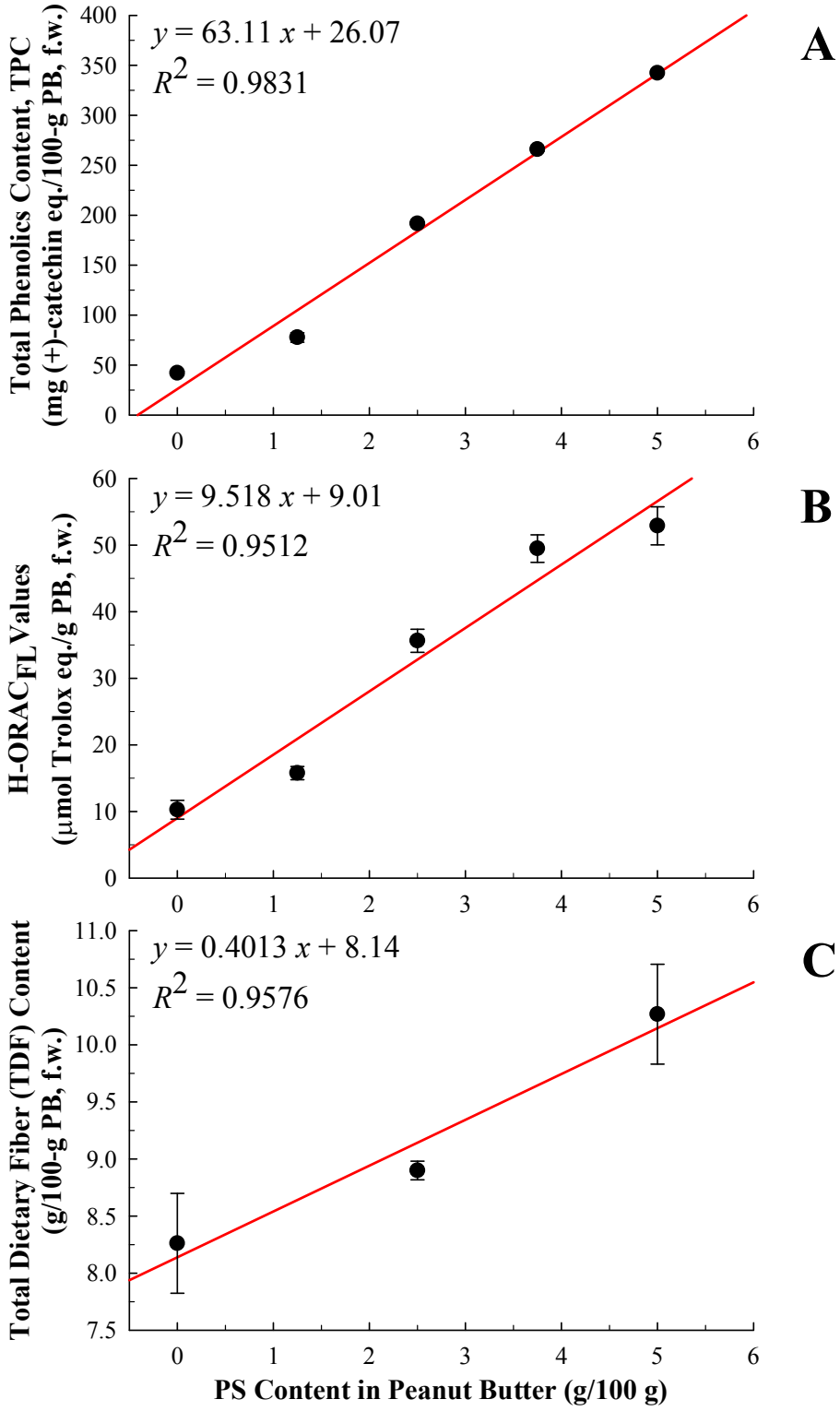
A
y = 63.11 x + 26.07
R2 = 0.9831
Total Phenolics Content, TPC
(mg (+)-catechin eq./100-g PB, f.w.)
B
y = 9.518 x + 9.01
R2 = 0.9512
H-ORACFL Values
(μmol Trolox eq./g PB, f.w.)
C
y = 0.4013 x + 8.14
R2 = 0.9576
Total Dietary Fiber (TDF) Content
(g/100-g PB, f.w.)
PS Content in Peanut Butter (g/100 g)

Figure 4.3

CHAPTER 5

SEPARATION AND CHARACTERIZATION OF FREE PHENOLIC COMPOUNDS FROM DRY-BLANCHED PEANUT SKINS BY LIQUID CHROMATOGRAPHY-ELECTROSPRAY IONIZATION MASS SPECTROMETRY

Ma, Y.; Kosińska-Cagnazzo, A.; Kerr, W. L.; Amarowicz, R.; Swanson, R. B.; Pegg, R. B. *Journal of Chromatography A*. **2014**, available on line. Reprinted here with permission of publisher (Elsevier).

Abstract

A large variety of phenolic compounds, including phenolic acids (hydroxybenzoic acids, hydroxycinnamic acids, and their esters), stilbenes (*trans*-resveratrol and *trans*-piceatannol), flavan-3-ols (*e.g.*, (-)-epicatechin, (+)-catechin, and their polymers {the proanthocyanidins, PACs}), other flavonoids (*e.g.*, isoflavones, flavanols, and flavones, *etc.*) and biflavonoids (*e.g.*, morelloflavone), were identified in dry-blanched peanut skins (PS) by this study. High-performance liquid chromatography (HPLC) coupled with electrospray ionization mass spectrometry (ESI-MS^n) was applied to separate and identify the phenolic constituents. Reversed-phase HPLC was employed to separate free phenolic compounds as well as PAC monomers, dimers, and trimers. PACs with a degree of polymerization (DP) of > 4 were chromatographed *via* hydrophilic interaction liquid chromatography (HILIC). Tentative identification of the separated phenolics was based solely on molecular ions and MS^n fragmentation patterns acquired by ESI-MS in the negative-ion mode. The connection sequence of PAC oligomers (DP < 5) could be deduced mainly through characteristic quinone methide (QM) cleavage ions. When the DP reached 6, only a proportion of the flavan-3-ols could be ascertained in the PACs because of the extremely complicated fragmentation patterns involved. The identification of free phenolic acids, stilbenes, and flavonoids was achieved by commercial standards and also by published literature data. Quantification was performed based on peak areas of the UV (free phenolic compounds) or fluorescence (PACs) signals from the HPLC chromatograms and calibration curves of commercial standards. Overall, PS contain significantly more PACs compared to free phenolic compounds.

5.1 Introduction

Phenolic compounds are ubiquitous in plants and have been classified as secondary metabolites (Bravo, 1998). Amongst the wide variety of phenolic compounds, phenolics of a dietary nature for humans typically include phenolic acids, flavonoids, and phenolic polymers (*i.e.*, tannins) (King & Young, 1999). Phenolic acids (*e.g.*, *p*-hydroxybenzoic acid and vanillic acid) can be present free; however, their common occurrence is in the form of methyl and ethyl esters, as well as glycosides being free and/or bound (Bravo, 1998). Hydroxycinnamic acids are mostly bound to cell wall polysaccharides through covalent linkages (Bravo, 1998). To illustrate, *p*-coumaric and ferulic acids are bound in graminaceous cell walls through ester-linkages between their carboxylic groups and arabinoxylans (Hartley, Morrison, Himmelsbach, & Borneman, 1990). In plants, the common occurrences of hydroxycinnamic acids are as esters of hydroxyacids like quinic, shikimic, or tartaric acid (Herrmann, 1989; Crozier, Jaganath, & Clifford, 2009; Teixeira *et al.*, 2013). Other conjugated forms of hydroxycinnamic acids include amides (*e.g.*, with L-amino acids and peptides), esters of sugars, and glycosides (Teixeira *et al.*, 2013). The majority of flavonoids found in unpicked plants is generally in bound forms with one or more sugar residues attached (Bravo, 1998; Hollman & Arts, 2000; Crozier, Jaganath, & Clifford, 2009). As for flavan-3-ols (*e.g.*, catechin, epicatechin, and gallocatechin) however, free monomers are also their common occurrence (Bravo, 1998). Mild heat treatment was found effective to cleave some of the covalent bonds of phenolic acids with insoluble polymers, and other cell wall components such as arabinoxylans in citrus peels thereby liberating low-molecular-weight antioxidant compounds from the repeating subunits of high-molecular-weight polymers (Jeong *et al.*, 2004). These released compounds were further identified using gas chromatography-mass spectrometry (MS) analysis.

Peanut skins (PS) are the outer seed coat of the peanut cotyledon, and are often removed from peanuts before processing into products such as peanut butter. They are relatively high in proanthocyanidins (PACs). PACs are oligomers and polymers of flavan-3-ols, which are primarily linked by C4→C8 bonds or less common C4→C6 bonds (B-type), whereas A-type dimers contain an additional (C2→O→C7) linkage. Despite the presence of some monomeric phenolic compounds, PACs comprise ~17% by weight of PS (Karchesy & Hemingway, 1986), and are the predominant phenolic component in PS (Yu, Ahmedna, Goktepe, & Dai, 2006). Six A-type PAC dimers identified in PS (Lou *et al.*, 1999) were found with activity to inhibit the inflammatory pathway mediated by hyaluronidase-induced release of histamine. A further study by Lou's group (2004) led to the isolation of five PAC oligomers from the water-soluble fraction of PS with potential free-radical scavenging activity. Although A-type linkages are much more abundant, both A- and B-type PACs exist in PS. The degree of polymerization (DP) identified in PS was up to 12 bound by A-type linkages; whereas, B-type structures were detected with a DP of up to only six (Monagas *et al.*, 2009).

Because a greater polarity is associated with an increased DP, theoretically PACs can be fractionated by reversed-phase (RP) or normal-phase (NP) high-performance liquid chromatography (HPLC) in an order of ascending molecular mass (Hümmer & Schreier, 2008). However, the resolution of PACs is limited to tetramers on C_{18} columns by RP-HPLC. The first successful separation of PACs was achieved on SiO_2 thin layer plates according to their DP (Lea & Arnold, 1978). Based on this thin layer chromatography method, a NP-HPLC gradient of CH_2Cl_2 and CH_3OH with traces of aqueous formic acid (1:1, v/v) was developed by Rigaud *et al.* (1993), resulting in the separation of PACs up to a DP of five in a cacao bean extract. Hammerstone *et al.* (1999) identified PAC decamers in cocoa by using a gradient of similar

composition, but by replacing the formic acid with acetic acid. In the above cases, chlorinated solvents were employed in the mobile phase compositions; these solvents are environmentally unfriendly and can cause health problems from their exposure in the workplace. A better separation of PACs in cacao with a DP up to 14 was achieved by Kelm *et al.* (2006) using a diol stationary phase with a relatively safe binary mobile phase of A (CH_3CN:CH_3COOH, 99:1, v/v) and B (CH_3OH:H_2O:CH_3COOH, 95:4:1, v/v/v).

The PAC molecules eluted *via* HPLC can be further ionized by an ion generator such as electrospray ionization (ESI), and these ions are subsequently separated according to their *m/z* ratio (Hümmer & Schreier, 2008). The HPLC-MS^n methods have been widely applied in the analysis of PACs in a variety of food matrices. Apropos PS: monomer (catechin and epicatechin), as well as A- and B-type PACs through dimers to tetramers were identified and quantified by Yu's group (2006) with a RP-HPLC-ESI-MS system. Recently, Constanza *et al.* (2012) reported the separation and identification of PACs through monomers to hexamers in PS using NP-HPLC-ESI-MS. Analyzing A-type PACs in PS, Appeldoorn *et al.* (2009) identified 83 PAC species with DPs up to seven *via* a combination of NP-HPLC and RP-HPLC-ESI-MS^2. The fractions throughout NP-HPLC were further separated and characterized by RP-HPLC-MS^2. PS PACs up to nonamer were separated by NP-HPLC and characterized using RP-HPLC-ESI-MS^n in Sarnoski's study (2012). Such applications of both RP-HPLC and NP-HPLC, incidentally, become an emerging trend in phenolic analysis because of the improved separation with multidimensional techniques (Montero, Herrero, Ibáñez, & Cifuentes, 2013).

According to Yu *et al.* (2005 & 2006), the phenolics from PS are abundant not only in quantity but also in variety, which primarily includes phenolic acids (*e.g.*, caffeic, chlorogenic, ferulic, and coumaric acids), flavan-3-ols (*e.g.*, (epi)catechin, epigallocatechin, catechin gallate,

and epicatechin gallate), stilbene (*e.g.*, *trans*-resveratrol), and PACs. However, only limited information can be found in the literature regarding phenolic profiles of PS from simple phenolic compounds to polymerized PACs using the combination technique of HPLC (both RP and HILIC) and MS^n. The objectives of the present study were as follows: (1) to assess the free phenolic compounds of the crude dry-blanched PS extracts using RP-HPLC-ESI-MS^n and (2) to assess the PACs profile of crude dry-blanched PS extracts using HILIC-ESI-MS^n.

5.2 Methods and Materials

Materials

Dry-blanched PS were a gift from Universal Blanchers, LLC (Sylvester, GA). All solvents and reagents were of analytical (ACS) grade, unless otherwise specified. Methanol, ethanol (95%), and hexanes were purchased from VWR International (Suwanee, GA). Procyanidin B2 was obtained from Indofine Chemical Company, Inc. (Hillsborough, NJ) and the PAC standards with a DP of 2 thru 10 were bought from Planta Analytica (Danbury, CT). (-)-Epiafzelechin was acquired from BOC Science (Shirley, NJ), while all other standards were purchased from either VWR International (Suwanee, GA) or the Sigma-Aldrich Chemical Co. (St. Louis, MO), depending on stock.

Extraction of Phenolics

Extraction of phenolic compounds from PS was carried out according to Amarowicz *et al.* (2004) with slight modifications. The extraction was performed in triplicate. In brief, samples were placed in Whatman cellulose extraction thimbles (43 mm *i.d.* × 123 mm *e.l.*, VWR International, Suwanee, GA), covered with a plug of glass wool and defatted in a Soxhlet

extraction apparatus under reflux for 12 h with hexanes. Defatted PS were transferred to 125-mL Erlenmeyer flasks at a mass-to-solvent ratio of 1:10 (w/v) with 80% (v/v) acetone. Extractions were performed in a gyrotary water bath shaker (Model G76, New Brunswick Scientific Company, Inc., New Brunswick, NJ) set at 150 rpm and a temperature of 45 °C for 30 min. The slurry was then filtered by gravity through fluted P8 filter paper (Thermo Fisher Scientific, Inc., Suwanee, GA). The extraction process was further repeated 2× as described above. All filtrates were pooled and acetone was evaporated with a Büchi Rotavapor R-210 using a V-700 vacuum pump connected to a V-850 vacuum controller (Büchi Corporation, New Castle, DE) at 45 °C. The aqueous residue was frozen and then lyophilized in a FreeZone® 2.5-L bench-top freeze dryer (Labconco Corporation, Kansas City, MO) to ensure all traces of moisture were removed and then stored in amber-glass bottles at -20 °C until further analyzed.

Preparation of the Free Phenolic Fraction from the Crude Extracts

Free phenolic compounds were separated from the crude extracts as taught by Weidner *et al.* (1999). Briefly, 0.4 g of the crude extract was dissolved in 20 mL of deionized water, which was acidified with 6 M HCl to pH 2.0. Subsequently, the free phenolic compounds were extracted into 20-mL diethyl ether using a separatory funnel. After extraction 5×, the pooled organic phase was evaporated with a Büchi Rotavapor R-210 using a V-700 vacuum pump connected to a V-850 vacuum controller (Büchi Corporation, New Castle, DE) at 45 °C. All triplicate extracts from dry-blanched PS were subjected to the above procedure individually. The dry residues obtained in this manner were re-dissolved in 2 mL of 80% (v/v) aqueous methanol and filtered through a non-sterile, 13-mm diameter, 0.2-µm regenerated cellulose filter membrane (Phenomenex Inc., Torrance, CA) before injection onto the C_{18} column.

Reversed-phase High-performance Liquid Chromatography Electrospray Ionization Mass Spectrometry (RP-HPLC-ESI-MS^n)

An Agilent 1200 Series HPLC system consisting of a quaternary pump with degasser, autosampler, thermostatted column compartment, UV/Vis diode array detection (DAD) with standard flow cell, and 3D ChemStation software (Agilent Technologies) was used for the chromatography. A reversed-phase Luna C_{18} column (4.6 × 250 mm, 5-µm particle size; Phenomenex Inc., Torrance, CA) was utilized. A gradient elution consisting of mobile phase A (H_2O:CH_3CN:CH_3COOH; 93:5:2, v/v/v) and mobile phase B (H_2O:CH_3CN:CH_3COOH; 58:40:2, v/v/v) from 0 to 100% B over a 50-min period at a flow rate of 1 mL/min was employed. Before subsequent injections, the system was re-equilibrated for 30 min using 100% A. The injection volume was 20 µL. Detection wavelengths employed were 255 nm (hydroxybenzoic acids), 280 nm {(+)-catechin, (-)-epicatechin, and PACs}, 320 nm (*trans*-cinnamic acids), and 360 nm (flavonoids). Tentative identification of separated components was achieved by matching UV spectra and retention time (RT) mapping with commercial standards. For quantification, calibration curves were constructed for each standard to confirm linearity based on the UV signal as well as for the determination of response factors.

RP-HPLC-ESI-MS^n analyses of the phenolics were carried out on an Agilent 1100 HPLC system, with the same chromatography conditions as described above, combined with a Bruker Esquire 3000^{plus} ion-trap mass spectrometer (Bruker Daltonics Inc., Billerica, MA), which was equipped with an ESI source. Instrument control and data acquisition were performed with Bruker Daltonics Esquire 5.3 software. The HPLC eluent flowed directly into the mass spectrometer *via* a flow splitter delivering roughly 100 µL/min. Mass spectra were acquired in the negative-ion mode with smart settings for a target mass at 800 *m/z*, compound stability at

100%, and the trap drive level at 100%. The voltage applied to the capillary was 4.0 kV with an end plate offset of -500 V. Nitrogen was used as the nebulizing gas at a pressure of 10 psi, a dry gas flow rate of 6 L/min, and a dry gas temperature of 280 °C. The phenolic compounds were fragmented using the auto-MS^2 mode with the following precursor-ion selection parameters: two precursor ions, threshold abs of 10,000, threshold rel of 5.0%, ion excluded after two spectra, ion excluded after two spectra, and exclusion release after 0.5 min. All collision-induced dissociation mass spectra were obtained with helium as the collision gas at a fragmentation voltage of 1 V after isolation of the desired precursor ion. Full scan was performed over the mass range of 100 to 1,000 *m/z* at a rate of 13,000 *m/z* per s under standard–normal scan mode. The ion-current control target was 50,000 with a maximum accumulation time of 50 ms. Each mass spectrum generated was based on an average of ten scans. Precursor ions of interest were further subjected to auto-MS^n under the same conditions as described above.

Hydrophilic Interaction Liquid Chromatography Electrospray Ionization Mass Spectrometry (HILIC-ESI-MS^n)

The PACs in the crude extracts of PS were separated based on their degree of polymerization (DP) according to Kelm *et al.* (2006) with modifications. An Agilent 1200 Series HPLC was employed and conditions of separation involved a PrincetonSPHER DIOL column (250 mm × 4.6 mm, 5-µm particle size, 60 Å; Princeton Chromatography, Inc., Cranbury, NJ) equipped with a guard cartridge/holder system; a thermostatted column compartment set at 30 °C; a binary mobile phase of A (CH_3CN:CH_3COOH, 98:2, v/v) and B (CH_3OH:H_2O:CH_3COOH, 95:3:2, v/v/v); a 1 mL/min flow rate for 0 to 35 min of 0 to 40% B, held for 5 min, 40 to 45 min of 40 to 0% B, and then an additional 5 min hold to re-equilibrate the system; 20 µL injection; and

fluorescence detection with λ_{ex} and λ_{em} set at 276 and 316 nm, respectively. All test samples and PAC standards (*i.e.*, flavan-3-ol monomers, dimers, trimers, and tetramers only) were dissolved in anhydrous CH_3OH (20 mg/mL), diluted with mobile phase A at a 1:9 (v/v) ratio, and passed through a non-sterile, 13-mm diameter, 0.45-µm PTFE syringe filter (Thermo Fisher Scientific, Inc., Suwanee, GA) prior to injection. Tentative identification of the separated components was made by RT mapping with authenticated standards prior to ESI-MS^2 analyses.

HILIC-ESI-MS^2 analyses of the PACs were carried out on an Agilent 1100 HPLC system, with the same chromatography conditions as described above, combined with a Bruker Esquire 3000^{plus} ion-trap mass spectrometer (Bruker Daltonics Inc., Billerica, MA), which was equipped with an ESI source using the same conditions as described for RP-HPLC-ESI-MS^n except: (i) the smart setting for a target mass was set to 2,000 *m/z*; (ii) full scan was performed over the mass range of 200 to 3,000 *m/z*; (iii) each mass spectrum generated was based on an average of five scans; and (iv) active exclusion was employed after three consecutive fragmentation spectra.

Statistical Analysis

For each quantified phenolic compound, the mean and standard deviation was calculated from the measurements of triplicate extractions.

5.3 Results and Discussion

Phenolic acids and flavonoids show characteristic UV-range absorbance patterns from 190 to 380 nm (Merken & Beecher, 2000; Robbins, 2003). By UV/Vis diode array detection (DAD), four groups of phenolic compounds were distinguished, namely hydroxybenzoic acids (255 nm), flavan-3-ols and polymers (280 nm), *trans*-cinnamic acids (320 nm) and other flavonoids (360

nm). These compounds were subsequently introduced into the ESI mass spectrometer and analyzed based on their *m/z* charge. The confirmed identification was fulfilled by matching RTs and/or spectral data with those of commercial standards, when available; tentative identification was based on mass spectra. The equivocality in some identifications resulted from the presence of stereoisomers, which are impossible to distinguish solely by MS. Typically, the cleavage of a characteristic functional group from a compound in MS^n analysis brings about a corresponding change in mass. For example, glucuronide and acetyl-glucosides possess a characteristic mass loss of 176 u and 204 u, respectively. *p*-Coumaroyl, caffeoyl, feruloyl, vanilloyl, and sinapoyl groups provide mass losses of 146, 162, 176, 150, and 206 u, respectively. Loss of tartaric acid and a pentose moiety would result in a mass loss of 132 u. Identification of compound classes eluted by both RP-HPLC-MS^n (**Table 5.1**) and HILIC-MS^n (**Table 5.2**) are discussed below. **Figure 5.1** depicts the chemical structures of the phenolic monomeric aglycones detected in PS.

Free Phenolic Acids and Their Esters

Hydroxybenzoic Acids

Protocatechuic acid (compound R-4) with a maximum UV absorption at 260 nm yielded a molecular ion $[M - H]^-$ at *m/z* 153 in the negative-ion mode and a fragment ion at *m/z* 109 $[M - H - 44]^-$ through loss of a CO_2 group from the carboxylic acid moiety. Compound R-8 gave a $[M - H]^-$ at *m/z* 137. Though no significant MS^2 fragment ion was observed for this compound, it was confirmed by the *p*-hydroxybenzoic acid standard *via* RT and UV spectral matching (λ_{max} = 256 nm).

Caffeic Acid and Its Tartaric Acid Esters

Caffeic acid (compound R-9) yielded a $[M - H]^-$ at *m/z* 179 and a prominent fragment at *m/z* 135 $[M - H - 44]^-$ through loss of a CO_2 group. Compound R-5 is likely its tartaric acid ester named caffeoyltartaric acid (caftaric acid), providing a $[M - H]^-$ at *m/z* 311. Further dissociation fragments of the molecular ion were present at *m/z* 179 [M – H – 132 (tartaric acid residue)]$^-$ and 149 [M – H – 162 (caffeoyl)]$^-$. Compounds R-41 and R-59 are likely dicaffeoyltartaric acid (chicoric acid) isomers, giving a $[M - H]^-$ at *m/z* 473. This precursor ion dissociated further into product ions at *m/z* 311 [M – H – 162 (caffeoyl)]$^-$ and 293 [M – H – 162 (caffeoyl) – 18 (H_2O)]$^-$. The MS^3 fragments of the product ion at *m/z* 311 were present at *m/z* 179 [caffeic acid – H]$^-$ *via* loss of a tartaric acid moiety, 149 [tartaric acid – H]$^-$ *via* loss of a caffeoyl residue, and 135 [caffeic acid – H – 44]$^-$ *via* loss of a CO_2 group from the deprotonated caffeic acid (decarboxylated ion).

p-Coumaric Acid and Its Tartaric Acid Esters

The molecular ions generated by coumaric acids were present at *m/z* 163. The prominent fragment at *m/z* 119 was formed through losing a CO_2 group from the carboxylic acid moiety. They were then assigned as *p*-coumaric acid (compound R-31) and *o*-coumaric acid (compound R-47) by RT matching of commercial standards. *p*-Coumaric acid displayed a maximum UV absorption band at 310 nm. Compounds R-14 and R-15 are tentatively identified as *p*-coumaric acid esters named *cis*-coutaric acid (*p*-coumaroyltartaric acid) and *trans*-coutaric acid, respectively, because of the $[M - H]^-$ at *m/z* 295 and the [*p*-coumaric acid – H]$^-$ fragment at *m/z* 163. The loss of 132 u is likely due to the cleavage of a tartaric acid moiety. Its decarboxylated ion [*p*-coumaric acid – H – 44 (CO_2)]$^-$ at *m/z* 119 was present in the MS^3 spectra. The

identification was further supported by UV spectra: *cis*-coutaric acid exhibited λ_{max} at 314 and 234 nm; whereas, *trans*-coutaric acid showed λ_{max} at 316 and 234 nm (Mozetič, Tomažič, Škvarč, & Trebše, 2006). Compounds R-20 and R-39 yielded the same molecular and fragment ions as compounds R-14 and R-15 did but eluted later from the column. Compound R-20 could be *p*-coumaroyl-*O*-pentoside, whereas compound R-39 is probably *o*-coumaroyl-*O*-pentoside. The loss of 132 u herein might result from a pentose moiety.

Compound R-18 is conceivably *p*-comaroylnictotinoyltartaric acid with a $[M - H]^-$ at *m/z* 400. As seen in **Figure 5.3(A)**, the MS^2 dissociation of the molecular ion resulted in a product ion at *m/z* 277, which was assigned as $[M - H - 106 \text{ (nictotinoyl)} - 18 \text{ } (H_2O)]^-$. In the MS^3 experiment [**Figure 5.3(B)**], this product ion dissociated further into secondary product ions at *m/z* 259 by loss of a H_2O molecule, 233 by loss of a CO_2 group, 203 by loss of $C_2H_2O_3$, and 163 corresponding to a deprotonated *p*-coumaric acid by loss of $C_4H_2O_4$. The MS^4 [277→203] experiment yielded characteristic fragments at *m/z* 147 and 119 corresponding to a *p*-coumaroyl residue and a [*p*-coumaric acid – H – 44 $(CO_2)]^-$ ion, respectively. The proposed candidate of compound R-65 is di-*p*-coumaroyltartaric acid. It displayed a molecular ion at *m/z* 441 and a prominent ion at *m/z* 277 $[M - H - 146 \text{ (coumaroyl)} - 18 \text{ } (H_2O)]^-$ [**Figure 5.3(A)**], which further dissociated to deprotonated *p*-coumaric acid (*m/z* 163) and its decarboxylated ion (*m/z* 119) in the MS^3 experiment. The CID fragmentation pattern of di-*p*-coumaroyltartaric acid by ESI-MS was illustrated by Ribas-Agustí *et al.* (2011). Similarly, compounds R-49, R-62, and R-63 are tentatively identified as *p*-coumaroyltartaric acid ether-linked formononetin, *trans*-resveratrol, and piceid, respectively. The proposed structures and fragmentation pathways are depicted in **Figure 5.3.** Nevertheless, further analyses are required to support the purported identifications. Compound R-84 is an unknown *p*-coumaroyltartaric acid derivative which gave a $[M - H]^-$ at

m/z 649 and fragment ions at *m/z* 503 [M – H – 146 (*p*-coumaroyl)]$^-$, 485 [M – H – 146 (*p*-coumaroyl) – 18 (H_2O)]$^-$, 277 [M – H – 354 (unknown residue) – 18 (H_2O)]$^-$, and 203 [M – H – 354 (unknown residue) – 18 (H_2O) – 74 ($C_2H_2O_3$)]$^-$.

Compound R-51, which gave a [M – H]$^-$ at *m/z* 415, is tentatively identified as *p*-coumaroyl-*p*-hydroxybenzoyltartaric acid. Further fragment ions were present at *m/z* 277 [M – H – 120 (hydroxybenzoyl) – 18 (H_2O)]$^-$, 251 [M – H – 146 (*p*-coumaroyl) – 18 (H_2O)]$^-$, 163 [*p*-coumaric acid – H]$^-$ and 137 [hydroxybenzoic acid – H]$^-$. The deprotonated hydroxybenzoic acid (*m/z* 137) was also observed in the MS^3 spectra. Compound R-57 is likely *p*-coumaroylvanilloyltartaric acid, showing a [M – H]$^-$ at *m/z* 445 and further dissociation ions at *m/z* 281 [M – H – 146 (*p*-coumaroyl) – 18 (H_2O)]$^-$, 277 [M – H – 150 (vanilloyl) – 18 (H_2O)]$^-$, 192 [M – H – 146 (*p*-coumaroyl) – 18 (H_2O) – 74 ($C_2H_2O_3$) – 15 (CH_3)]$^-$ and 167 [vanillic acid – H]$^-$. The MS^3 fragmentation of the product ion at *m/z* 281-also yielded the deprotonated vanillic acid at *m/z* 167.

p-Coumaroylcaffeoyltartaric acid (compound R-52) gave a [M – H]$^-$ at *m/z* 457 and fragment ions at *m/z* 311 [M – H – 146 (*p*-coumaroyl)]$^-$, 295 [M – H – 162 (caffeoyl)]$^-$, 277 [M – H – 162 (caffeoyl) – 18 (H_2O)]$^-$, 179 [M – H –146 (*p*-coumaroyl) – 132 (tartaric acid residue)]$^-$ and 163 [M – H – 162 (caffeoyl) – 132 (tartaric acid residue)]$^-$. The MS^3 fragment of the product ion at *m/z* 295 was present at *m/z* 163 corresponding to a deprotonated *p*-coumaric acid. The further MS^4 dissociation of the deprotonated *p*-coumaric acid yielded a typical decarboxylated ion at *m/z* 119. This CID fragmentation pattern of *p*-coumaroylcaffeoyltartaric acid was very close to that described by Ribas-Agustí *et al.* (2011). Compounds R-67 and R-74 are *p*-coumaroylsinapoyltartaric acid isomers showing [M – H]$^-$ at *m/z* 501 and fragment ions at *m/z* 337 [M – H – 146 (*p*-coumaroyl) – 18 (H_2O)]$^-$, 307 [M – H – 146 (*p*-coumaroyl) – 18 (H_2O) – 30

$(2CH_3)]^-$, 277 [M – H – 206 (sinapoyl) – 18 $(H_2O)]^-$ and 203 [M – H – 206 (sinapoyl) – 18 (H_2O) – 74 $(C_2H_2O_3)]^-$. The MS^3 fragmentation of the product ion at *m/z* 337 produced deprotonated sinapic acid (*m/z* 223) and its decarboxylated ion (*m/z* 179). This identification was further supported by the MS^4 spectra where a deprotonated sinapic acid (*m/z* 223), a sinapoyl residue (*m/z* 207), and a decarboxylated ion of the deprotonated sinapic acid (*m/z* 179) were present. Compounds R-68, R-73 and R-77 which provided $[M - H]^-$ at *m/z* 471 and fragment ions at *m/z* 307 [M – H – 146 (*p*-coumaroyl) – 18 $(H_2O)]^-$, 277 [M – H – 176 (feruloyl) – 18 $(H_2O)]^-$ and 203 [M – H – 176 (feruloyl) – 18 (H_2O) – 74 $(C_2H_2O_3)]^-$ are tentatively identified as *p*-coumaroylferuloyltartaric acid. The MS^3 fragmentation of the product ion at *m/z* 307 yielded deprotonated ferulic acid (*m/z* 193). The fragment ions corresponding to a feruloyl residue (*m/z* 177) and a decarboxylated ion [ferulic acid – H – 44 $(CO_2)]^-$ (*m/z* 149) were present in the MS^4 spectra.

Ferulic Acid and Its Esters

The proposed candidate for compound R-17 is feruloyltartaric acid (fertaric acid) with a molecular ion at *m/z* 325 and a prominent fragment at *m/z* 193 [M – H – 132 (tartaric acid residue)]$^-$ corresponding to deprotonated ferulic acid, which dissociated further into secondary product ions at *m/z* 178 [ferulic acid – H – 15 $(CH_3)]^{-\bullet}$, 149 [ferulic acid – H – 44 $(CO_2)]^-$, and 134 [ferulic acid – H – 44 (CO_2) – 15 $(CH_3)]^{-\bullet}$ by MS^3 fragmentation. Compound R-19 is likely feruloyl aspartate with a molecular ion at *m/z* 308 and a prominent fragment *m/z* of 193 corresponding to a deprotonated ferulic acid. The 115 u loss is characteristic for an aspartate moiety. Other fragment ions observed were at *m/z* 178 [ferulic acid – H – 15 $(CH_3)]^{-\bullet}$, 149 [ferulic acid – H – 44 $(CO_2)]^-$, and 132 [M – H – 176 (feruloyl)]$^-$. Compound R-79 resulted in a

[M – H]$^-$ at *m/z* 355 and fragment ions at *m/z* 220 [M – H – 135]$^-$, 219 [M – H – 135 – H]$^-$, 193 [M – H – 162 (caffeoyl)]$^-$ and 135 [caffeic acid – H – 44 (CO_2)]$^-$, which is tentatively identified as 4-*O*-caffeoylferulic acid. Compound R-81 is likely an unknown ferulic acid derivative because of its MS^3 [403→311] fragments at *m/z* 193 [ferulic acid – H]$^-$ and 149 [ferulic acid – H – 44 (CO_2)]$^-$.

Compound R-71 is likely feruloylsinapoyltartaric acid, yielding a [M – H]$^-$ at *m/z* 531 and fragment ions at *m/z* 337 [M – H – 176 (feruloyl) – 18 (H_2O)]$^-$, 307 [M – H – 206 (sinapoyl) – 18 (H_2O)]$^-$, and 233 [M – H – 206 (sinapoyl) – 18 (H_2O) – 74 ($C_2H_2O_3$)]$^-$. Deprotonated ferulic acid (*m/z* 193) and its decarboxylated ion (*m/z* 149) were revealed by MS^3 fragmentation of the product ion at *m/z* 307. MS^4 dissociation gave ions at *m/z* 193 [ferulic acid – H]$^-$ and *m/z* 162 [177 (feruloyl) – 15 (CH_3)]$^{-\bullet}$. Feruloylcaffeoyltartaric acid (compound R-72) was observed with a molecular ion at *m/z* 487. The fragment ions were present at *m/z* 325 [M – H – 162 (caffeoyl)]$^-$, 307 [M – H – 162 (caffeoyl) – 18 (H_2O)]$^-$, 293 [M – H – 176 (feruloyl) – 18 (H_2O)]$^-$, 193 [M – H – 162 (caffeoyl) – 132 (tartaric acid residue)]$^-$, and 179 [M – H – 176 (feruloyl) – 132 (tartaric acid residue)]$^-$. The further MS^3 dissociation of the product ion at *m/z* 325 revealed deprotonated ferulic acid at *m/z* 193 and its decarboxylated ion at *m/z* 149. Compound R-88 yielded a [M – H]$^-$ at *m/z* 681 and fragment ions at *m/z* 307 [M – H – 356 (unknown residue) – 18 (H_2O)]$^-$, 251 [M – H – 356 (unknown residue) – 74 ($C_2H_2O_3$)]$^-$, 233 [M – H – 356 (unknown residue) – 18 (H_2O) – 74 ($C_2H_2O_3$)]$^-$, and 193 [ferulic acid – H]$^-$, which therefore is conceivably a feruloyltartaric acid derivative.

Coumarin

Compound R-44 is tentatively identified as dihydroxycoumarin, such as aesculetin. The

molecular ion $[M - H]^-$ was present at *m/z* 177 and further dissociation ions at *m/z* 149 [M – H – 28 (CO)]$^-$, 133 [M – H – 44 (CO_2)]$^-$, and 105 [M – H – 28 (CO) – 44 (CO_2)]$^-$ were observed.

5.3.2 Catechins and Proanthocyanidins (PACs)

Monomers to Trimers

PACs, also known as condensed tannins, are oligomeric and polymeric end products of the flavonoid biosynthetic pathway consisting of flavan-3-ol monomers (*i.e.*, (+)-catechin and (-)-epicatechin) and their galloyl derivatives. The presence of each A-type bond will result in a 2-u decrease in the mass of PACs. Quinone methide fission (QM), retro-Diels-Alder fission (RDA), and heterocyclic ring fission (HRF) are three well-described fragmentation mechanisms of PACs (Gu *et al.*, 2003). The types of PAC inter-monomeric linkages and QM fragmentation patterns employed to determine positions of A-type linkages are illustrated in **Figure 5.2**.

Compounds R-7 and R-16 are identified as (+)-catechin and (-)-epicatechin, respectively, which were confirmed by RT matching with commercial standards. Classic fragments of $[M - H]^-$ at *m/z* 289 were observed at *m/z* 245 $[M - H - 44]^-$ by loss of a $–CH_2–CHOH–$ group or neutral loss of CO_2, 205 $[M - H - 84]^-$ by loss of $C_4H_4O_2$ from the A-ring, and 179 $[M - H - 110]^-$ by loss of $C_6H_6O_2$ (B-ring). The fragmentation mechanisms of (epi)catechin are described in detail by Bravo *et al.* (2006).

Compounds R-1, R-6, R-13, R-21, and N-3 are probably PAC B-type dimers with a $[M - H]^-$ at *m/z* 577; whereas, compounds R-12, R-23, R-25, R-27, R-34, R-37, R-38, R-43, R-46, and N-2 are likely PAC A-type dimers with a $[M - H]^-$ at *m/z* 575. In the negative-ion mode, an A-type dimer would reveal a product ion at *m/z* 449 $[M - H - 126]^-$ by loss of a phloroglucinol moiety ($C_6H_6O_3$) from the top unit through HRF fragmentation. The product ion at *m/z* 423 [M –

H – 152]$^-$ is formed through RDA fragmentation of the base unit resulting in loss of a $C_8H_8O_3$ moiety. Generally, the top unit is the favored site for RDA, but fission can occur at the base unit when blocked by A-type bonds (Gu *et al.*, 2003). QM cleavage fragments of A-type dimers were present at *m/z* 289 [M_T – H (290 – H)]$^-$ (QM cleavage at the terminal {T} unit) and 285 [M_E – 5H (290 – 5H)]$^-$ (QM at the extension {E} unit). In a similar manner, product ions at *m/z* 451 [M – H – 126 ($C_6H_6O_3$)]$^-$, 425 [M – H – 152 ($C_8H_8O_3$)]$^-$, 289 [M_T – H (290 – H)]$^-$, and 287 [M_E – 3H (290 – 3H)]$^-$ were generated *via* the fragmentation of the deprotonated B-type dimer. The fragment ion at *m/z* 407 was produced by further loss of a H_2O moiety from the product ion at *m/z* 425. The acquired data and characteristic MS^2 fragmentation patterns in the present work are consistent with findings reported in previous studies (Gu *et al.*, 2003; Appeldoorn *et al.*, 2009; Sarnoski *et al.*, 2012). As for PAC A-type dimers, a molecular ion at *m/z* 597 was also observed, which corresponds to [(M – H) + Na – H]$^-$.

Compounds R-2, R-3, R-10, R-11, R-24, R-26, R-29, R-30, R-32, R-33, R-36, and N-5 to N-9 (*m/z* 863) are likely PAC A-type trimers with 1 A-bond; whereas, compounds R-35, R-42, R-45, and N-4 (*m/z* 861) are probably PAC A-type trimers with 2 A-bonds. Though lack of MS^2 fragmentation information, B-type trimers with a [M – H]$^-$ at *m/z* 865 were also observed in the PS crude extract (compound N-10). Theoretically, PAC polymers ($n \geq 3$) were dissociated in a similar manner as to the dimers. There are two types of A-type trimers possible: both generate the molecular ion at *m/z* 863, but with different QM diagnostic ions caused by varied positions of the A-bonds. QM cleavage between the top and middle units produces ions at *m/z* 575 [M_T – H (576 – H)]$^-$ and 287 [M_E – 3H (290 – 3H)]$^-$, indicating that the A-type linkage falls between the middle and the base unit. The HRF and RDA fragmentation of the top unit yield product ions at *m/z* 737 [M – H – 126 ($C_6H_6O_3$)]$^-$ and 711 [M – H – 152 ($C_8H_8O_3$)]$^-$, respectively. The HRF

fragmentation of the middle unit gives a product ion at *m/z* 449. When the A-type linkage is located between the top and the middle units, QM cleavage ions are present at *m/z* 289 [M_T – H (290 – H)]$^-$ and 573 [M_E – 3H (576 – 3H)]$^-$. In this case, the fragment ion at *m/z* 711 is formed by RDA of the middle unit. The HRF fragmentation of the middle unit generates product ions at *m/z* 451 and 411. Yet, these two types of trimers containing the same number of A-type bonds were not well separated throughout the HPLC analysis. Mostly, two series of fragments were observed in one mass spectrum. The HRF fragment ion of [M – H]$^-$ at *m/z* 861 was present at *m/z* 735 [M – H – 126 ($C_6H_6O_3$)]$^-$ and the RDA fragmentation of the base unit generated a product ion at *m/z* 709 [M – H – 152 ($C_8H_8O_3$)]$^-$. The QM cleavage between the top and the middle units resulted in fragment ions at *m/z* 575 [M_T – H (576 – H)]$^-$ and 285 [M_E – 5H (290 – 5H)]$^-$; whereas, ions at *m/z* 289 [M_T – H (290 – H)]$^-$ and 571 [M_E – 5H (576 – 5H)]$^-$ were yielded when QM cleavage occurred between the middle and the base unit. The product ion at *m/z* 449 was formed by fragmentation of the QM ion at *m/z* 575 and the HRF ion at *m/z* 735 *via* the mechanism of HRF (Gu *et al.*, 2003).

PACs Containing Luteolin or Kaempferol Units

Compounds with molecular ions at *m/z* 573 (*i.e.*, compounds R-48, R-50, and compound N-1) and 859 (compound R-22) are tentatively identified as a PAC dimer and A-type trimer containing a luteolin or kaempferol unit. These are novel PACs reported by Sarnoski *et al.* (2012). The fragment ions at *m/z* 447 and 421 were produced through HRF and RDA cleavages, respectively, at the top (epi)catechin unit of the dimer. Even though there is no confirmatory data available, luteolin was the better candidate supported by subsequent MS^4 fragmentation. In the case of this dimer, the QM cleavage was likely blocked by the carbonyl group on the C-ring of

the base luteolin or kaempferol unit. The diagnostic ions at *m/z* 289 and 283 might have been generated by direct cleavage of the interflavan bond, as noted in Sarnoski's study (2012). The proposed structure and fragmentation routes of this PAC dimer are depicted in **Figure 5.4(A)**. Unfortunately, no significant MS^2 fragment was observed for the A-type trimer (*m/z* 859).

Propelargonidins

Compound R-55 (*m/z* 559) and compound R-53 (*m/z* 845) are likely a PAC dimer and trimer with an (epi)afzelechin top unit connected to a subsequent (epi)catechin unit through an A-type linkage; such a compound is referred to as a propelargonidin. The HRF and RDA fragment ions of this dimer were revealed at *m/z* 433 $[M - H - 126 (C_6H_6O_3)]^-$ and 407 $[M - H - 152 (C_8H_8O_3)]^-$, respectively. Likewise, fragments at *m/z* 719 $[M - H - 126 (C_6H_6O_3)]^-$ and 693 $[M - H - 152 (C_8H_8O_3)]^-$ were formed from the trimer *via* the HRF and RDA mechanisms, respectively. The QM cleavage of the dimer resulted in diagnostic ions at *m/z* 289 $[M_T - H (290 - H)]^-$ and 269 $[M_E - 5H (274 - 5H)]^-$; whereas, the QM cleavage of the trimer gave diagnostic ions at *m/z* 289, 575 $[M_T - H]^-$ and 269, 555 $[M_E - 5H]^-$, indicating the occurrence of two A-type linkages. The fragmentation pathways of these two PAC oligomers have been well described by Appeldoorn *et al.* (2009).

Prodelphinidin and Prorobinetidin

The molecular ion $[M - H]^-$ of *m/z* 591 (compound R-28) was formed by a prodelphinidin A-type dimer [**Figure 5.4(B)**]. The HRF fragmentation of the top (epi)catechin unit yielded a product ion at *m/z* 465 $[M - H - 126 (C_6H_6O_3)]^-$. The ion at *m/z* 423 $[M - H - 168 (C_8H_8O_4)]^-$ was produced by RDA fragmentation of the base (epi)gallocatechin unit. QM cleavage ions were

noted at *m/z* 305 $[M_T - H (306 - H)]^-$ and 285 $[M_E - 5H (290 - 5H)]^-$, corresponding to A-bond linked (epi)gallocatechin and (epi)catechin units, respectively. Compound R-56 (*m/z* 589) is tentatively identified as a prorobinetidin dimer with a base unit most likely being a C-methyl (epi)robinetinidol A-linked to an (epi)catechin top unit. The HRF fragment ion of the (epi)catechin unit and RDA fragment ion of the C-methyl (epi)robinetinidol unit were present at *m/z* 463 $[M - H - 126 (C_6H_6O_3)]^-$ and 421 $[M - H - 168 (C_8H_8O_4)]^-$, respectively. QM cleavage gave diagnostic ions at *m/z* 303 $[M_T - H (304 - H)]^-$ and 285 $[M_E - H (290 - 5H)]^-$, corresponding to C-methyl (epi)robinetinidol and (epi)catechin units, respectively. The identification was further supported by an MS^3 experiment. The proposed structure and fragmentation schemes of compound R-56 are depicted in **Figure 5.4(C)**.

Tetramers to Nonamers

PAC tetramers to nonamers were separated and identified by HILIC-ESI-MS^2. Although the identification of the bond stereochemistry is impossible by MS, the connection sequences of PACs can be deduced up to tetramers and pentamers based on their diagnostic QM fragments (**Figure 5.2**). For example, QM cleavage of a tetramer (*m/z* 1149) containing two A-type linkages produces fragments at *m/z* 575 $[M_T - H (576 - H)]^-$ and 573 $[M_E - H (576 - 3H)]^-$, indicating that a B-type linkage is located between the two middle units and that the remaining units are linked by A-type bonds. Likewise, the production of a QM fragment at *m/z* 861 indicates that the B-type linkage is between the top and its subsequent unit, while the remaining units are linked by A-type bonds. The fragment at *m/z* 859 could be observed when the B-type linkage is located between the base and its upper unit. In PS, there are three types of tetramers containing 1 A-bond: all give a $[M - H]^-$ at *m/z* 1151. QM fragments at *m/z* 863 $[M_T - H (864 -$

H)]$^-$ and 575 [M_T – H (576 – H)]$^-$ and/or [M_E – H (578 – 3H)]$^-$ indicate that the A-type linkage lies between the base and its upper unit. The A-type linkage between the top and its subsequent unit gives rise to QM fragments at *m/z* 577 [M_T – H (578 – H)]$^-$, 861 [M_E – 3H (864 – 3H)]$^-$ and 573 [M_E – 3H (576 – 3H)]$^-$. When the A-type linkage locates itself between two middle units, QM cleavage generates diagnostic ions at *m/z* 863 [M_T – H (864 – H)]$^-$ and 861 [M_E – 3H (864 – 3H)]$^-$. More details about the QM diagnostic ions employed for PAC sequence identifications were discussed by Gu *et al.* (2003). Noteworthy is that some polymers with the same DP, especially with the same number of A-bonds, co-elute together throughout HILIC, resulting in overlapping mass spectra due to a similar polarity. The identifications reached in this work were, therefore, made very cautiously based on the characteristic fragmentation patterns of each sequence.

It would be inconclusive to deduce the connection sequence when the DP reaches 6 due to the complexity of the MS^2 fragmentation patterns. The proportion of flavan-3-ol constituents were deduced chiefly by their [M – H]$^-$ or [M – 2H]$^{2-}$ ions. QM cleavage at the extension units generates fragments with similar structures (Gu *et al.*, 2003). In our study, the major ions were displayed at *m/z* 573 + 288n. The further HRF of 573 + 288n ions resulted in *m/z* 735 + 288n ions. To clarify, the cleavage of a hexamer (*m/z* 1725) gave smaller 573 + 288n ions at *m/z* 1437 (n = 3), 1149 (n = 2), and 861 (n = 1). The HRF fragments of ions at *m/z* 1437 and 861 were present at *m/z* 1311 and 735, respectively. The cleavage of a heptamer and an octamer gave major fragments at *m/z* 1725 (n = 4) and 2013 (n = 5), respectively. An octamer containing 3 A-bonds with a [M – H]$^-$ at *m/z* 2299 is a novel PAC compound first reported in PS.

Stilbenes

Compounds R-60 and R-61 are tentatively identified as *trans*-resveratrol and *trans*-piceatannol, respectively. The molecular ion $[M - H]^-$ of compound R-60 was present at *m/z* 227, while the fragments of $[M - H]^-$ were present at 209 $[M - H - 18 (H_2O)]^-$, 185 $[M - H - 42 (CHCOH)]^-$, and 143 $[M - H - 42 (CHCOH) - 42 (C_2H_2O)]^-$. The identification was further confirmed by a commercial standard of *trans*-resveratrol by means of RT matching and UV spectral analysis (λ_{max} = 306 nm). Compound R-61 revealed a molecular ion at *m/z* 243. The fragments ions were found at *m/z* 225 $[M - H - 18 (H_2O)]^-$, 201 $[M - H - 42 (CHCOH)]^-$, 199 $[M - H - 42 (CHCOH) - 2H]^-$, and 159 $[M - H - 42 (CHCOH) - 42 (C_2H_2O)$ or $M - H - 42 (CHCOH) - 2H - 40 (C_2O)]^-$. This is the collision-induced dissociation fragmentation of $[M - H]^-$ when the deprotonation reaction occurs on the catechol moiety of *trans*-piceatannol. This, together with the other fragmentation mechanisms of *trans*-resveratrol and *trans*-piceatannol, have been elucidated by Stella *et al.* (2008). Similar MS^2 fragmentation patterns of *trans*-resveratrol and *trans*-piceatannol in peanuts were reported in Ku's study (2005).

Flavonoids

The substitution pattern and subclass of the flavonoid largely dictate its fragmentation routes (Cuyckens & Claeys, 2004). Generally compared to flavones, more MS^n fragmentation schemes are proposed for flavonols because of the additional hydroxyl group in position 3. $^{ij}A^-$ and $^{ij}B^-$ ions are diagnostic fragments for flavonoid identification and generated by retro Diels-Alder (RDA) cleavage in the C-ring. The A^- and B^- are product ions in the negative-ion mode consisting of intact A- and B-rings, while the superscripts *i* and *j* indicate the positions in the C-ring where two C–C bonds have been ruptured. Besides RDA cleavage in the C-ring, the

losses of neutral molecules such as H_2O (18 u), CO (28 u), CO_2 (44 u), C_2H_2O (42 u), and C_3O_2 (68 u) can be characteristic in the negative-ion mode. The fragmentation of flavone, flavonol, and flavanone aglycones in the negative-ion mode by ESI-trap MS are well-demonstrated by Fabre *et al.* (2001). The successive loss of $CH_3^{\bullet}$ groups and then CO (28 u) or $CHO^{\bullet}$ (29 u) are predominant for methoxylated flavonoids (Justesen, 2001). Nevertheless, the exact position of the methoxy moiety could not be defined without more intricate standards or NMR analysis. (Cuyckens & Claeys, 2004).

Isoflavones

Compound R-58 is conceivably a methyl flavonoid (300 u) hexoside with a molecular ion $[M - H]^-$ at *m/z* 461 and fragment ions at *m/z* 299 $[M - H - 162 \text{ (hexoside)}]^-$ and 284 $[M - H - 162 \text{ (hexoside)} - 15 \text{ } (CH_3)]^{-\bullet}$. The most likely candidate is 3′,5,7-trihydroxyisoflavone-4′-methoxy-3′-*O*-*β*-glucopyranoside, which has been found in PS by NMR analysis (Lou *et al.*, 2001). Compound R-64 is probably biochanin A, where a molecular ion at *m/z* 283 and a prominent fragment at *m/z* 268 $[M - H - 15 \text{ } (CH_3)]^{-\bullet}$ were observed. Biochanin A is an *O*-methylated isoflavone that has long been reported in peanuts (Chukwumah *et al.*, 2007).

Flavanones

The identification of compound R-66 is confirmed by an eriodictyol standard with RT matching. The compound gave a $[M - H]^-$ at *m/z* 287 and RDA diagnostic ions of $^{1,3}A^-$ (*m/z* 151), $^{1,3}B^-$ (*m/z* 135), and $^{1,4}A^-$ (*m/z* 125). After further CO_2 loss, the $^{1,3}A^-$ ion provided an ion at *m/z* 107 noted as $^{1,3}A^- - CO_2$. Compound R-78 is tentatively identified as eriodictyol methyl ether such as homoeriodictyol (eriodictyol 3′-methyl ether). Its molecular ion $[M - H]^-$ was present at

m/z 301 and further dissociation ions were observed at *m/z* 286 $[M - H - 15\ (CH_3)]^{-\bullet}$ and 151 as a $^{1,3}A^-$ fragment.

Flavones

Compound R-69 is identified as luteolin giving a $[M - H]^-$ at *m/z* 285. Its further dissociation ions were present at *m/z* 267 $[M - H - 18\ (H_2O)]^-$, 257 $[M - H - 28\ (CO)]^-$, 241 $[M - H - 44\ (CO_2)]^-$, 217 $[M - H - 68\ (C_3O_2)]^-$, 213 $[M - H - 44\ (CO_2) - 28\ (CO)]^-$, 199 $[M - H - 42\ (C_2H_2O) - 44\ (CO_2)]^-$, and 175 $[M - H - 68\ (C_3O_2) - 42\ (C_2H_2O)]^-$. The $^{1,3}A^-$ RDA diagnostic ion was observed at *m/z* of 151. The identification was further confirmed by RT matching with a luteolin standard. Based on the identification of luteolin, compound R-83 is conceivably a luteolin methyl ether such as diosmetin (luteolin 4′-methyl ether), with a molecular ion $[M - H]^-$ of *m/z* 299, and fragment ions at *m/z* 284 $[M - H - 15\ (CH_3)]^{-\bullet}$ and 256 $[M - H - 15\ (CH_3) - 28\ (CO)]^{-\bullet}$. The $^{1,3}A^-$ ion at *m/z* 151 was present in the MS^3 spectra. The identification is further supported by its UV spectra of 254(*sh*), 266, and 348 nm (Brad & Chen, 2013).

Compound R-80 is likely a dimethyl flavone, probably cirsiliol, giving a $[M - H]^-$ at *m/z* 329. Its MS^2 fragment ions were found at *m/z* 314 $[M - H - 15\ (CH_3)]^{-\bullet}$ and 299 $[M - H - 30\ (2CH_3)]^-$. The MS^3 dissociation of the product ion at *m/z* 314 generated secondary product ions at *m/z* 299 *via* loss of $CH_3^\bullet$, 285 *via* loss of $CHO^\bullet$, and 271 *via* loss of $CH_3^\bullet$ and CO. The RDA diagnostic ions present in the MS^3 spectra were at *m/z* 177 noted as $-CH_3{}^{1,2}A^{-\bullet}$ and 165 noted as $-CH_3{}^{1,3}A^{-\bullet}$. A commercial standard and more analyses are warranted for definitive confirmation.

Flavonols and Flavanonols

Compound R-70 is identified as quercetin with a $[M - H]^-$ of *m/z* 301 and characteristic

fragments of $^{1,2}A^-$ (*m/z* 179), $^{1,2}A^-$ – CO (*m/z* 151), and $^{1,2}A^-$ – CO – CO_2 (*m/z* 107). The identification was further confirmed by a commercial standard (λ_{max} = 256 and 370 nm). Quercetin glucuronide (compound R-40) produced a $[M - H]^-$ of *m/z* 477 and a prominent fragment at *m/z* 301 $[M - H - 176]^-$ by loss of a glucuronide moiety. The MS^3 fragmentation of the product ion at *m/z* 301 gave rise to RDA diagnostic ions at *m/z* 179 assigned to a $^{1,2}A^-$ fragment and 151 attributed to a $^{1,2}A^-$ – CO fragment. Compound R-85 is likely isorhamnetin with a $[M - H]^-$ of *m/z* 315 and a fragment ion at *m/z* 300 $[M - H - 15\ (CH_3)]^{-\bullet}$. The MS^3 fragments of the product ion at *m/z* 315 were present at *m/z* 300 *via* loss of a $CH_3^\bullet$, 271 *via* loss of a $CH_3^\bullet$ and a further $HCO^\bullet$ group, and 256 *via* loss of $CH_3^\bullet$ and CO_2. The $^{1,3}A^-$ ion at *m/z* 151 produced in the MS^3 experiment is a diagnostic ion for isorhamnetin in the ESI-MS analysis [36]. Compound R-54 is conceivably isorhamnetin acetyl-glucoside showing a $[M - H]^-$ at *m/z* 519 and a prominent fragment at *m/z* 315 $[M - H - 204]^-$ by loss of an acetyl-glucoside. The MS^3 dissociation ions of the product ion at *m/z* 315 were present at *m/z* 300 by loss of a $CH_3^\bullet$, 271 by loss of a $CH_3^\bullet$ and a further $CHO^\bullet$, 255 by loss of H_2O and C_2H_2O, and 151 attributed to a $^{1,3}A^-$ fragment. Isorhamnetin glycosides have been detected in PS according to a NMR study conducted by Lou's group (2001).

Compounds R-86 and R-87 are likely dimethyl ethers of 304 u flavonoids giving a molecular ion $[M - H]^-$ at *m/z* 331 and fragment ions at *m/z* 316 $[M - H - 15\ (CH_3)]^{-\bullet}$ and 301 $[M - H - 30\ (2CH_3)]^-$. Compound R-86 is probably dihydrorobinetin dimethyl ether based on subsequent MS^3 [331→316] fragment ions at *m/z* 301 by loss of a CH_3 group likely from the B-ring, 257 by loss of $CH_3^\bullet$ and CO_2, and 177 *via* loss of the B-ring. The RDA diagnostic ions observed in the MS^3 spectra were present at *m/z* 164 attributed to a $^{1,2}A^-$ fragment, and 136 *via* loss of $CH_3^\bullet$ on the B-ring and RDA cleavage of 1 and 2 bonds (noted as $-CH_3{}^{1,2}B^{-\bullet}$). Therefore,

the two methyl groups were undoubtedly distributed on the A-ring and B-ring respectively; that is likely, 7,3′-dimethyl dihydrorobinetin. The proposed candidate for compound R-87 is 3′,4′-dimethyl dihydroquercetin; the RDA diagnostic ions produced by the $MS^3[331\rightarrow316]$ experiment were present at *m/z* 151 assigned to a $^{1,3}A^-$ fragment, and 125 attributed to a $^{1,4}A^-$ fragment. However, commercial standards and NMR analyses are warranted for a final determination.

Biflavonoids

In addition to neutral losses of H_2O (18 u), CO (28 u), CO_2 (44 u), C_2H_2O (42 u), and C_3O_2 (68 u), a similar nomenclature of fragmentation was adopted as for flavonoids. $^{ij}A^-$ and $^{ij}B^-$ ions are RDA fragments produced by cleavage of two C–C bonds in the C-ring. The flavonoid, whose C-ring is attached to the A-ring of the other flavonoid, is denoted as 'Part I' and the other flavonoid is referred to as 'Part II.'

Compound R-75 is likely a daphnodorin H-type biflavonoid composed of eriodictyol (Part I) and C-methyl (epi)robinetinidol (Part II) units. It gave a $[M - H]^-$ at *m/z* 587 and fragment ions at *m/z* 569 $[M - H - 18 (H_2O)]^-$, 461 assigned to a $^{1,4}IB^-$ fragment, and 419 attributed to a $^{1,3}IIA^-$ fragment. The direct cleavage of the interflavan bond yielded fragments at *m/z* 285 [eriodictyol unit (288 – 3H)]$^-$ and 301 [C-methyl (epi)robinetinidol unit (304 – 3H)]$^-$. The proposed candidate of compound R-76 is a 3,8-linked biflavonoid composed of homoeriodictyol (C-3) (Part I) and eriodictyol (C-8) (Part II) units. This compound revealed a molecular ion at *m/z* 587 and fragment ions at *m/z* 569 $[M - H - 18 (H_2O)]^-$, 525 $[M - H - 18 (H_2O) - 44 (CO_2)]^-$, 464 [M – H – 123 (B-ring)]$^-$, 435 assigned to a $^{1,3}IB^-$ fragment, 419 noted as $-CH_3{}^{1,3}IB^{-\bullet}$, 311 [^{1,3}IB – 123 (B-ring)]$^-$, and 175 [^{1,3}IB – ^{1,3}IIB – 123 (B-ring)]$^-$. This identification is further supported by

the MS^3 experiment. Yet, further analyses are required to confirm the above identifications unequivocally. Compound R-82 is conceivably another biflavonoid characterized by 3,8-linked flavonoid monomers. The tentative candidate is a biflavonoid containing naringenin (C3) linked to luteolin (C8), namely morelloflavone. This compound gave a molecular ion of *m/z* 555. Further fragment ions of $[M - H]^-$ were present at *m/z* 493 $[M - H - 18 (H_2O) - 44 (CO_2)]^-$, 445 $[M - H - 68 (C_3O_2) - 42 (C_2H_2O)]^-$, 433 assigned to a $^{1,2}IIA^-$ fragment, 389 probably attributed to a $^{1,2}IIA^- - C_2H_4O$ fragment, and 283 $[^{1,3}IB - ^{1,2}IIB]^-$. The prominent ion at *m/z* 403 was a $^{1,3}IB^-$ fragment. The proposed structures and fragmentation schemes of above three biflavonoids are illustrated in **Figure 5.5(A)**, **(B)**, and **(C)**, respectively.

Quantification

Phenolic compounds were quantified based on peak areas of the UV (free phenolic compounds) or fluorescence (PACs) signals from the HPLC chromatograms and calibration curves of commercial standards; the mean phenolic contents are reported in **Tables 5.3** and **5.4**. Compared to UV detection, fluorescence detection is more commonly employed for PAC analysis because of the increased selectivity and decreased interference from other absorbing species (Hümmer & Schreier, 2008).

All values are expressed in mg respective phenolic compound/100-g dry weight (d.w.) of PS for the free phenolic compounds, and /g d.w. for the PACs. *p*-Coumaroyl derivatives, most notably tartaric acid esters, are extremely dominant in the non-PAC phenolics profile, and constituted a 73% proportion of the free phenolic compounds. Unfortunately, quantification of selected compounds was not possible due to co-elution with major *p*-coumaroyl derivatives. To illustrate, the UV peak of eriodictoyl was shielded by a band from di-*p*-coumaroyltartaric acid.

Quercetin and its methyl ether (isorhamnetin) are detected in PS primarily as flavonoid aglycones and account for ~6% of the total non-tannin constituents. As aforementioned, PACs possess the lion's share of the phenolics in PS. The PAC content comprises 99.4% of the total phenolics, as established by both RP-HPLC and HILIC. The total PAC content of 91.8 mg/g dry-blanched PS, d.w., ± 2.56 analyzed by HILIC is close to the reported value in our previous study of 84.3 mg/g dry-blanched PS, d.w., ± 1.68, where the 4-(dimethylamino)cinnamaldehyde (DMAC) colorimetric assay was used for quantification (Ma *et al.*, 2014). Trimers, tetramers, hexamers, and heptamers are the most abundant PACs and account for ~75.8% of the total content.

Noteworthy is that the quantification can be easily affected by many factors involved in the details of the extraction and fractionation protocols selected. A certain percentage of phenolics, including the PACs, are "unextractable" because these constituents are bound to cell wall material such as polysaccharides *via* hydrogen bonding and hydrophobic interactions (Pinelo, Arnous, & Meyer, 2006). As such, the true phenolics content has been underestimated because of the bound linkages between phenolics and cell wall material that cannot be disrupted by usual extraction procedures. Moreover, PACs can be modified by thermal treatment. Yu *et al.* (2006) reported a significant influence of the skin removal methods on the PACs content of PS. Dry heat, like that found in dry roasting of red-skin peanuts, would increase A-type dimers and B-type trimers compared to directly peeled PS due to monomeric polymerization or the degradation of trimers and tetramers. Polymerization of monomers and B-type dimers is a plausible mechanism by which increased B-type trimer levels were detected.

5.4 Conclusions

A large number and variety of phenolic compounds were separated and detected in PS by HPLC-ESI-MS^n, including PACs (monomers to nonamers), free phenolic acids and their esters, stilbenes, flavonoids, and biflavonoids. The novel compounds determined in this study were identified chiefly by their mass spectra and observed fragmentation pathways. The identification is still somewhat tentative due to a lack of commercial standards. The quantification was accomplished by a combination of RP-HPLC and HILIC with UV and fluorescence detection, respectively. Overall, PS contain significantly more PACs compared to free phenolic compounds. *p*-Coumaroyl derivatives account for roughly ¾ of the non-PAC phenolics found in PS. We believe that the findings reported here will greatly enrich the phenolics database of PS. PS provide an abundant and inexpensive source of natural antioxidants, especially *p*-coumaroyl species and PACs.

Acknowledgements

This research was supported by a grant funded by the Georgia Food Industry Partnership and is greatly appreciated. Use of the Proteomics and Mass Spectrometry (PAMS) core facility under the direction of Dr. Dennis Phillips of UGA's Department of Chemistry is greatly appreciated. Thanks are also extended to Bill Paulk at Sylvester Blanching, a division of Universal Blanchers, LLC, for the dry-blanched PS and John T. Powell, President of the Peanut Institute Inc., Albany, GA.

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Table 5.1 Tentative identification of phenolic compounds in dry-blanched peanut skins (PS) by C_{18} RP-HPLC-ESI-MS^n.

Cmpd No.	Tentative identification[a]	UV λ_{max} (nm)[b]	RT[c] (min)	$[M-H]^-$	Product ions[d]
R-1	PAC B-type dimer [(E)C→B→(E)C]	234, 280	4.3	577	559, 451, **425**, 407, 289, 287
R-2	PAC A-type trimer [(E)C→B→(E)C→A→(E)C]		4.2	863	737, 711, 693, 575, 559, 449, 287
R-3	PAC A-type trimer [(E)C→A→(E)C→B→(E)C]		4.2	863	737, 711, 693, 573, 451, 411, 289
R-4	Protocatechuic acid	260, 294	10.0	153	**109**
R-5	Caffeoyltartaric acid (Caftaric acid)	244, 302*sh*, 328	11.7	311	179, **149**
R-6	PAC B-type dimer [(E)C→B→(E)C]		12.1	577	559, 451, **425**, 407, 289, 287
R-7	(+)-Catechin	234, 280	13.3	289	**245**, 205, 179
R-8	*p*-Hydroxybenzoic acid	256	13.5	137	
R-9	Caffeic acid		14.5	179	**135**
R-10	PAC A-type trimer [(E)C→B→(E)C→A→(E)C]		14.9	863	737, 711, 693, 575, 559, 449, 287
R-11	PAC A-type trimer [(E)C→A→(E)C→B→(E)C]		14.9	863	737, 711, 693, 573, 451, 411, 289
R-12	PAC A-type dimer [(E)C→A→(E)C]		15.2	575	557, **449**, 423, 407, 289, 285
R-13	PAC B-type dimer [(E)C→B→(E)C]		17.0	577	559, 451, **425**, 407, 289, 287
R-14	*cis*-*p*-Coumaroyltartaric acid (*cis*-Coutaric acid)	234, 314	15.5	295	**163** MS^3[295→163]: **119**
R-15	*tans*-*p*-Coumaroyltartaric acid (*trans*-Coutaric acid)	234, 316	16.9	295	**163** MS^3[295→163]: **119**
R-16	(-)-Epicatechin		17.1	289	**245**, 205, 179
R-17	Feruloyltartaric acid (Fertaric acid)		17.3	325	**193** MS^3[325→193]: 178, **149**, 134
R-18	*p*-Coumaroylnicotinoyltartaric acid		17.7	400	**277** MS^3[400→277]: 259, 233, **203**, 163 MS^4[277→203]: **175**, 147, 119
R-19	Feruloyl asparate		18.9	308	**193**, 178, 149, 132
R-20	*p*-Coumaroyl-*O*-pentoside	234, 314	19.3	295	**163**

					MS^3[295→163]: **119**
R-21	PAC B-type dimer [(E)C→B→(E)C]	234, 280	19.9	577	559, 451, **425**, 407, 289, 287
R-22	PAC A-type trimer [(E)C→B→(E)C→A→Luteolin or Kaempferol]		20.1	859	
R-23	PAC A-type dimer [(E)C→A→(E)C]	234, 280	20.3	575	557, **449**, 423, 407, 289, 285
R-24	PAC A-type trimer [(E)C→B→(E)C→A→(E)C]	234, 280	20.9	863	737, 711, 693, **575**, 559, 449, 287
R-25	PAC A-type dimer [(E)C→A→(E)C]	234, 280	22.0	575	557, **449**, 423, 407, 289, 285
R-26	PAC A-type trimer [(E)C→A→(E)C→B→(E)C]	234, 280	22.5	863	737, **711**, 693, 573, 451, 411, 289
R-27	PAC A-type dimer [(E)C→A→(E)C]	234, 280	23.1	575	557, **449**, 423, 407, 289, 285
R-28	Prodelphinidin A-type dimer [(E)C→A→(E)GC]	234, 280	23.2	591	573, **465**, 423, 305, 285
R-29	PAC A-type trimer [(E)C→B→(E)C→A→(E)C]	234, 280	23.4	863	737, 711, 693, **575**, 559, 449, 287
R-30	PAC A-type trimer [(E)C→A→(E)C→B→(E)C]	234, 280	23.6	863	737, **711**, 693, 573, 451, 411, 289
R-31	*p*-Coumaric acid	234, 310	23.9	163	**119**
R-32	PAC A-type trimer [(E)C→B→(E)C→A→(E)C]	234, 280	23.7	863	737, 711, 693, 575, 559, 449, 287
R-33	PAC A-type trimer [(E)C→A→(E)C→B→(E)C]	234, 280	23.7	863	737, 711, 693, 573, 451, 411, 289
R-34	PAC A-type dimer [(E)C→A→(E)C]	234, 280	24.0	575	557, **449**, 423, 407, 289, 285
R-35	PAC A-type trimer [(E)C→A→(E)C→A→(E)C]	234, 280	24.6	861	**735**, 709, 693, 575, 571, 449, 289, 285
R-36	PAC A-type trimer [(E)C→B→(E)C→A→(E)C]	234, 280	25.6	863	737, 711, 693, **575**, 559, 449, 287
R-37	PAC A-type dimer [(E)C→A→(E)C]	234, 280	25.8	575	557, **449**, 423, 407, 289, 285
R-38	PAC A-type dimer [(E)C→A→(E)C]	234, 280	26.1	597	**445**
R-39	*o*-Coumaroyl-*O*-pentoside		26.2	295	**163** MS^3[295→163]: **119**
R-40	Quercetin glucuronide		26.2	477	**301**, 179, 151 MS^3[477→301]: **179**, 151
R-41	Dicaffeoyltartaric acid (Chicoric acid)	246, 304*sh*, 328	26.8	473	**311**, 293, 179, 149, 135 MS^3[473→311]: 179, **149**, 135
R-42	PAC A-type trimer [(E)C→A→(E)C→A→(E)C]	234, 280	27.5	861	**735**, 709, 693, 575, 571, 449, 289, 285
R-43	PAC A-type dimer [(E)C→A→(E)C]	234, 280	27.8	575	557, **449**, 423, 407, 289, 285

R-44	Dihydroxycoumarin		27.6	177	149, **133**, 105, 107
R-45	PAC A-type trimer [(E)C→A→(E)C→A→(E)C]	234, 280	28.0	861	**735**, 709, 693, 575, 571, 449, 289, 285
R-46	PAC A-type dimer [(E)C→A→(E)C]	234, 280	29.0	575	557, **449**, 423, 407, 289, 285
R-47	*o*-Coumaric acid		29.3	163	**119**
R-48	PAC dimer [(E)C (C4)→Luteolin or Kaempferol (C8) or (C6)]		30.4	573	555, 529, **447**, 421, 323, 289, 283 MS^3[573→447]: 429, 325, **285**, 243 MS^4[447→285]: 285, 267, 243, 241, **217**, 213, 199, 177
R-49	Formononetin-7-*O*-*p*-coumaroyltartaric acid		30.7	545	527, 399, **277**, 259, 203, 163 MS^3[545→277]: 259, 233, **203**, 175, 163, 147 MS^4[277→203]: **175**, 147, 119
R-50	PAC dimer [(E)C (C4)→Luteolin or Kaempferol (C8) or (C6)]		31.5	573	555, 529, 511, **447**, 421, 323, 289, 283 MS^3[573→447]: 429, 325, **285**, 243, 175 MS^4[447→285]: 285, **241**, 243, 217, 213, 199, 177
R-51	*p*-Coumaroyl-*p*-hydroxybenzoyltartaric acid		31.6	415	277, **251**, 203, 163, 137 MS^3[415→251]: 207, **137**, 131
R-52	*p*-Coumaroylcaffeoyltartaric acid	234, 318	32.0	457	311, **295**, 277, 179, 163 MS^3[457→295]: **163** MS^4[295→163]: **119**
R-53	Propelargonidin A-type trimer [(E)Afz→A→(E)C→A→(E)C]		32.5	845	**719**, 693, 575, 555, 449, 433, 289, 269
R-54	Quercetin methyl ether (Isorhamnetin) acetyl-glucoside		32.9	519	**315**, 300 MS^3[519→315]: 315, **300**, 272, 255, 151

R-55	Propelargonidin A-type dimer [(E)Afz→A→(E)C]		32.9	559	**433**, 407, 289, 269
R-56	Prorobinetidin A-type dimer [(E)C→A→C-methyl (Epi)robinetinidol]		33.1	589	571, **463**, 445, 421, 393, 303, 285 MS^3[589→463]: 445, **435**, 341, 301, 295, 285, 198
R-57	*p*-Coumaroylvanilloyltartaric acid		33.2	445	**281**, 277, 192, 167 MS^3[445→281]: 237, 192, **167**, 131
R-58	3′,5,7-trihydroxyisoflavone-4′-methoxy-3′-*O*-β-glucopyranoside		33.6	461	**299**, 284 MS^3[461→299]: **284**
R-59	Dicaffeoyltartaric acid (Chicoric acid)	244, 304*sh*, 330	33.9	473	**311**, 293, 179, 149, 135 MS^3[473→311]: 179, **149**, 135
R-60	*trans*-Resveratrol	306	34.6	227	227, 209, **185**, 183, 143
R-61	*trans*-Piceatannol		35.3	243	243, **225**, 199, 159
R-62	*trans*-Resveratrol-3-*O*-*p*-coumaroyltartaric acid		35.7	505	447, 341, **277**, 203, 163, 147 MS^3[505→277]: 259, 233, **203**, 163 MS^4[277→203]: **175**, 147
R-63	Piceid-6″-*p*-coumaroyltartaric acid		36.6	667	521, 485, 441, **277**, 203 MS^3[667→277]: 259, 233, **203**, 163 MS^4[277→203]: **175**, 147
R-64	Biochanin A		36.8	283	**268**
R-65	di-*p*-Coumaroyltartaric acid	234, 316	37.0	441	295, **277**, 203 MS^3[441→277]: 259, 233, **203**, 163, 147, 119 MS^4[277→203]: **175**, 147, 119
R-66	Eriodictyol		37.3	287	**151**, 135, 125, 107
R-67	*p*-Coumaroylsinapoyltartaric acid	236, 318	37.9	501	**337**, 307, 277, 203 MS^3[441→337]: 319, 293, 275, **263**, 223, 179

					MS^4[337→263]: 248, 235, **223**, 207, 192, 179
R-68	*p*-Coumaroylferuloyltartaric acid	236, 318	38.6	471	**307**, 277, 203 MS^3[471→307]: 289, 263, **233**, 193 MS^4[307→233]: 218, **205**, 177, 149
R-69	Luteolin		38.9	285	**285**, 267, 257, 241, 217, 213, 199, 175, 151
R-70	Quercetin	256, 370	39.0	301	301, 257, **179**, 151, 107
R-71	Feruloylsinapoyltartaric acid		39.2	531	337, **307**, 233 MS^3[531→307]: 289, 263, **233**, 193, 149 MS^4[307→233]: 218, **205**, 193, 162
R-72	Feruloylcaffeoyltartaric acid		39.3	487	**325**, 307, 293, 193, 179 MS^3[487→325]: **193**, 149
R-73	*p*-Coumaroylferuloyltartaric acid	314	39.9	471	**307**, 277, 203 MS^3[471→307]: 289, 263, **233**, 193 MS^4[307→233]: 218, **205**, 177, 149
R-74	*p*-Coumaroylsinapoyltartaric acid	318	40.1	501	**337**, 307, 277, 233, 203 MS^3[441→337]: 319, 293, 275, **263**, 223, 179 MS^4[337→263]: 248, 235, **223**, 207, 192, 179
R-75	Biflavonoid [Eriodictyol→C-methyl (Epi)robinetinidol]		41.4	587	569, 551, **461**, 419, 301, 285 MS^3[587→461]: **443**, 301, 285, 293, 198
R-76	Biflavonoid		41.9	587	569, 525, **464**, 435, 419, 375, 311,

	[Homoeriodictyol (C3)→Eriodictyol (C8)]				313, 175 MS^3[587→464]: 355, **311**, 301, 194
R-77	*p*-Coumaroylferuloyltartaric acid	240, 320	44.1	471	**307**, 277, 233, 203 MS^3[471→307]: 289, 263, **233**, 193 MS^4[307→233]: 218, **205**, 177, 149
R-78	Homoeriodictyol		44.3	301	301, **286**, 151
R-79	4-*O*-Caffeoylferulic acid		44.6	355	338, 220, **219**, 193, 135
R-80	Cirsiliol		44.7	329	**314**, 299 MS^3[329→314]: **299**, 285, 271, 177, 165
R-81	Ferulic acid derivative		45.1	403	329, **311**, 211 MS^3[403→311]: **293**, 249, 211, 193, 171, 149
R-82	Morelloflavone [Naringenin (C3)→Luteolin (C8)]		45.7	555	493, 445, 433, **403**, 389, 283
R-83	Luteolin methyl ether (Diosmetin)	254*sh*, 266, 348	46.8	299	299, **284** MS^3 [299→284]: **284**, 256, 216, 151
R-84	*p*-Coumaroyltartaric acid derivative		47.5	649	503, 485, **277**, 203 MS^3[649→277]: 259, 233, **203**, 163 MS^4[277→203]: **175**, 147
R-85	Quercetin methyl ether (Isorhamnetin)	256, 372	47.6	315	**315**, 300 MS^3 [315→300]: **300**, 271, 256, 151
R-86	Dihydrorobinetin 7,3′-dimethyl ether		47.9	331	**316**, 301, 211, 136, 124 MS^3 [331→316]: **301**, 257, 177, 164, 136
R-87	Dihydroquercetin 3′,4′-dimethyl ether		48.1	331	**316**, 301, 125 MS^3 [331→316]: **301**, 257, 151,

				125
R-88	Feruloyltartaric acid derivative	48.7	681	351, **329**, 307, 251, 233, 193 MS^3 [681→329]: 311, 293, **229**, 211, 171, 125

[a] The A represents an A-type bond with both (C4→C8) and (C2→O→C7) linkages or (C4→C6) and (C2→O→C7) linkages, B represents a B-type bond which can be (C4→C8) or (C4→C6) linkage; (E)C represents (epi)catechin, (E)GC represents (epi)gallocatechin and (E)Afz represents (epi)afzelechin.

[b] *sh*, shoulder in the spectrum.

[c] Retention time (RT) in total ion chromatograms.

[d] The most abundant ions observed in mass spectra are shown in bold; quinone methide (QM) fission diagnostic ions are underlined.

Table 5.2 Tentative identification of proanthocyanidins (PACs) in dry-blanched peanut skins (PS) by HILIC-ESI-MS/MS[a].

Cmpd No.	Oligomeric PACs[b]	RT[c] (min)	Ions, m/z $[M-H]^-$	$[M-2H]^{2-}/2$	Product ions[d]
N-1	PAC dimer [(E)C (C4)→Luteolin or Kaempferol (C8) or (C6)]	5.5	573		555, 529, **447**, 421, 405, 289, 283
N-2	PAC A-type dimer [(E)C→A→(E)C]	6.4–6.8	575		**449**, 423, 407, 289, 285
N-3	PAC B-type dimer [(E)C→B→(E)C]	7.6	577		451, **425**, 407, 289, 287
N-4	PAC A-type trimer [(E)C→A→(E)C→A→(E)C]	8.1	861		**735**, 709, 693, 575, 571, 449, 289, 285
N-5	PAC A-type trimer [(E)C→A→(E)C→B→(E)C]	8.5	863		737, **711**, 573, 451, 411, 289
N-6	PAC A-type trimer [(E)C→B→(E)C→A→(E)C]	8.8	863		737, 711, 693, **575**, 559, 449, 287
N-7	PAC A-type trimer [(E)C→B→(E)C→A→(E)C]	9.0	863		737, 711, 693, **575**, 559, 449, 287
N-8	PAC A-type trimer [(E)C→B→(E)C→A→(E)C]	9.2	863		737, 711, 693, **575**, 559, 449, 287
N-9	PAC A-type trimer [(E)C→A→(E)C→B→(E)C]	9.3	863		737, **711**, 573, 451, 411, 289
N-10	PAC B-type trimer [(E)C→B→(E)C→B→(E)C]	10.1	865		
N-11	PAC A-type tetramer [(E)C→B→(E)C→A→(E)C→A→(E)C]	10.4	1149		997, 979, 861, 845, 449
N-12	PAC A-type tetramer [(E)C→A→(E)C→A→(E)C→B→(E)C]	10.5	1149		997, 979, 859
N-13	PAC A-type tetramer [(E)C→A→(E)C→B→(E)C→A→(E)C]	10.4–10.6	1149		997, 979, 573, **575**
N-14	PAC A-type tetramer [(E)C→B→(E)C→A→(E)C→A→(E)C]	11.2	1149		997, 979, 861, 845
N-15	PAC A-type tetramer [(E)C→A→(E)C→B→(E)C→A→(E)C]	11.2	1149		997, 979, 573, **575**
N-16	PAC A-type tetramer [(E)C→A→(E)C→A→(E)C→B→(E)C]	11.3	1149		997, 979, 859
N-17	PAC A-type tetramer	11.3	1151		863, 861, 573, 411

	[(E)C→B→(E)C→A→(E)C→B→(E)C]			
N-18	PAC A-type tetramer [(E)C→B→(E)C→B→(E)C→A→(E)C]	11.3	1151	863, **575**, 449
N-19	PAC A-type tetramer [(E)C→B→(E)C→B→(E)C→A→(E)C]	11.5	1151	863, **575**
N-20	PAC A-type tetramer [(E)C→B→(E)C→A→(E)C→B→(E)C]	11.8	1151	999, 981, 863, 861, 711, 573, 411
N-21	PAC A-type tetramer [(E)C→B→(E)C→B→(E)C→A→(E)C]	11.8	1151	999, 981, 863, 711, **575**
N-22	PAC A-type tetramer [(E)C→B→(E)C→B→(E)C→A→(E)C]	12.1	1151	999, 981, 863, 711, **575**, 449
N-23	PAC A-type tetramer [(E)C→B→(E)C→B→(E)C→A→(E)C]	12.3–12.6	1151	999, 981, 863, 711, **575**, 449
N-24	PAC A-type tetramer [(E)C→B→(E)C→A→(E)C→B→(E)C]	12.4	1151	999, 981, 863, 861, 711, 573, 451, 411
N-25	PAC A-type tetramer [(E)C→A→(E)C→B→(E)C→B→(E)C]	12.6	1151	999, 981, 861, 577, 573
N-26	PAC A-type pentamer [(E)C→A→(E)C→A→(E)C→B→(E)C→B→(E)C]	12.7	1437	1285, 1147, 859, 577, 573
N-27	PAC A-type pentamer [(E)C→B→(E)C→A→(E)C→A→(E)C→B→(E)C]	12.8	1437	1285, **1149**, 1147, 859, 733
N-28	PAC A-type pentamer [(E)C→B→(E)C→A→(E)C→B→(E)C→A→(E)C]	12.9	1437	1285, **1149**, 997, 861, 575, 573
N-29	PAC A-type pentamer [(E)C→B→(E)C→B→(E)C→A→(E)C→A→(E)C]	13.0	1437	1285, **1149**, 861, 709, 691, 449
N-30	PAC A-type pentamer [(E)C→B→(E)C→A→(E)C→B→(E)C→A→(E)C]	13.1	1437	1285, **1149**, 997, 861, 575, 573
N-31	PAC A-type pentamer [(E)C→B→(E)C→A→(E)C→A→(E)C→B→(E)C]	13.2	1437	1285, 1267, **1149**, 1147, 997, 859
N-32	PAC A-type pentamer [(E)C→A→(E)C→B→(E)C→A→(E)C→B→(E)C]	13.2	1437	1147, 863, 845, 737, 711, 573, 451, 411

N-33	PAC A-type pentamer [(E)C→A→(E)C→B→(E)C→B→(E)C→A→(E)C]	13.3	1437	1285, 1267, 863, 861, 845, 575, 573, 449
N-34	PAC A-type pentamer [(E)C→B→(E)C→A→(E)C→A→(E)C→B→(E)C]	13.3	1437	1285, 1267, 1149, 859
N-35	PAC A-type pentamer [(E)C→B→(E)C→A→(E)C→B→(E)C→A→(E)C]	13.4–13.6	1437	1285, **1149**, 861, 735, 709, 691, 575, 573, 449
N-36	PAC A-type pentamer [(E)C→B→(E)C→A→(E)C→B→(E)C→A→(E)C]	13.8	1437	1285, **1149**, 861, 735, 709, 575
N-37	PAC A-type pentamer [(E)C→B→(E)C→A→(E)C→B→(E)C→A→(E)C]	13.9	1437	1285, **1149**, 861, 575, 573
N-38	PAC A-type pentamer [(E)C→B→(E)C→A→(E)C→B→(E)C→A→(E)C]	14.2	1437	1285, **1149**, 861, 575, 573
N-39	PAC A-type pentamer [(E)C→B→(E)C→A→(E)C→B→(E)C→A→(E)C]	14.3	1437	1285, **1149**, 861, 709, 575, 449
N-40	PAC A-type pentamer [(E)C→A→(E)C→B→(E)C→A→(E)C→B→(E)C]	14.4	1437	1285, 1267, 1147, 863, 573
N-41	PAC A-type pentamer [(E)C→B→(E)C→B→(E)C→A→(E)C→B→(E)C]	14.5	1439	1313, 1151, 1149, 1025, 997, 863, 575, 573
N-42	PAC A-type pentamer [(E)C→B→(E)C→A→(E)C→B→(E)C→B→(E)C]	15.0	1439	1313, 1287, **1151**, 1149, 1025, 861, 577, 575, 573
N-43	PAC A-type pentamer [(E)C→A→(E)C→B→(E)C→B→(E)C→B→(E)C]	15.1	1439	1313, 1287, 1269, 865, 861, 577, 573
N-44	PAC A-type pentamer [(E)C→B→(E)C→B→(E)C→B→(E)C→A→(E)C]	15.1	1439	1313, 1287, 1269, **1151**, 863, 711, 575, 449
N-45	PAC A-type pentamer [(E)C→B→(E)C→B→(E)C→A→(E)C→B→(E)C]	15.2	1439	1313, 1287, 1269, **1151**, 1149, 863, 575, 411
N-46	PAC A-type pentamer [(E)C→B→(E)C→A→(E)C→B→(E)C→B→(E)C]	15.2–15.3	1439	1313, 1287, **1151**, 1149, 861, 577, 575, 573
N-47	PAC A-type pentamer [(E)C→B→(E)C→B→(E)C→A→(E)C→B→(E)C]	15.4	1439	1313, 1287, **1151**, 1149, 863, 575, 573
N-48	PAC B-type pentamer	15.6	1441	

	[(E)C→B→(E)C→B→(E)C→B→(E)C→B→(E)C]				
N-49	PAC A-type hexamer (3A, 2B)	15.7–16.3	1723		
N-50	PAC A-type hexamer (2A, 3B)	15.7–17.0	1725		1573, 1555, **1437**, 1311, 1149, 861, 735, 573
N-51	PAC A-type hexamer (1A, 4B)	17.4	1727		1557, **1437**, 1311, 1149, 861
N-52	PAC B-type hexamer	17.7	1729		
N-53	PAC A-type heptamer (2A, 4B)	18.1	2013		1843, **1725**, 1599, 1437, 1149, 861, 573
N-54	PAC A-type heptamer (1A, 5B)	19.2	2015		**1725**, 1437, 1149, 861, 573
N-55	PAC octamer (3A, 4B)	19.5	2299		
N-56	PAC octamer (2A, 5B)	19.5–20.3	2301	1150	**2013**, 1725, 1599, 1437, 1311, 1149
N-57	PAC A-type nonamer (3A, 5B)	21.1		1293	
N-58	PAC A-type nonamer (2A, 6B)	20.9–21.0		1294	
N-59	PAC A-type nonamer (2A, 6B)	21.8		1294	
N-60	PAC A-type nonamer (1A, 7B)	22.1		1295	

[a] HILIC-ESI-MS/MS = hydrophilic interaction liquid chromatography-electronspray ionization mass spectrometry.

[b] The A represents an A-type bond with both (C4→C8) and (C2→O→C7) linkages or (C4→C6) and (C2→O→C7) linkages, B represents a B-type bond which can be (C4→C8) or (C4→C6) linkage, (E)C represents (epi)catechin.

[c] Retention time (RT) in total ion chromatograms.

[d] The most abundant ions observed in mass spectra are shown in bold; quinone methide (QM) fission diagnostic ions are underlined.

Table 5.3 Content of selected phenolics quantified in dry-blanched peanut skins (PS) by C_{18} RP-HPLC.

Free phenolic compounds[a]	Quantification λ (nm)	Content[b] (mg/100 g)
Protocatechuic acid	260	3.43 ± 0.04
p-Hydroxybenzoic acid	255	1.03 ± 0.06
Caftaric acid	320	1.51 ± 0.12
cis-Coutaric acid	320	10.1 ± 0.52
trans-Coutaric acid	320	2.11 ± 0.08
p-Coumaroyl-*O*-pentoside	320	5.52 ± 0.23
p-Coumaric acid	320	0.53 ± 0.06
Chicoric acid	320	3.44 ± 0.12
p-Coumaroylcaffeoyltartaric acid	320	2.26 ± 0.13
Chicoric acid	320	3.12 ± 0.13
di-*p*-Coumaroyltartaric acid	320	13.8 ± 1.53
p-Coumaroylsinapoyltartaric acid	320	6.32 ± 0.94
p-Coumaroylferuloyltartaric acid	320	5.87 ± 0.71
trans-Resveratrol	320	0.36 ± 0.05
Quercetin	360	2.11 ± 0.27
Isorhamnetin	360	1.51 ± 0.02
Diosmetin	360	0.40 ± 0.01

[a] Caftaric acid and chicoric acids are quantified as caffeic acid equivalents; coutaric acids and other *p*-coumaroyl derivatives are quantified using *p*-coumaric acid equivalents; isorhamnetin and diosmetin are quantified using corresponding flavonoid aglycone equivalents.

[b] Values are means of triplicate analyses ± standard deviation. Findings are reported as mg respective phenolic/100-g dry weight (d.w.) of dry-blanched PS.

Table 5.4 Quantification of proanthocyanidin (PAC) compounds in dry-blanched peanut skins (PS) by HILIC[a].

PAC compounds[b]	mg/g[c]
PAC A-type dimers	6.28 ± 0.21
PAC B-type dimers	0.74 ± 0.02
PAC trimers	21.1 ± 0.68
PAC tetramers	19.5 ± 0.75
PAC pentamers	8.30 ± 0.13
PAC hexamers	15.4 ± 0.61
PAC heptamers	13.6 ± 0.21
PAC octamers	6.90 ± 0.46

[a] HILIC = hydrophilic interaction liquid chromatography.

[b] PAC A-type dimers and B-type dimers are quantified using procyanidin B_2 equivalents. PAC heptamers and octamers are quantified using hexamer equivalents.

[c] Values are means of triplicate analyses ± standard deviation. Findings are reported as mg respective phenolic/g dry weight (d.w.) of dry-blanched PS.

Figures Captions

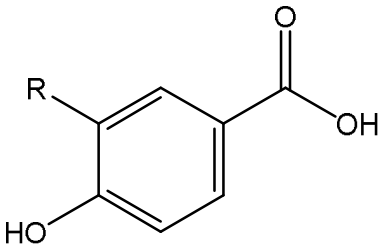
Figure 5.1 Structures of monomeric phenolic aglycones found in dry-blanched peanut skins (PS).

Figure 5.2 Linkages found in proanthocyanidins (PACs) and quinone methide (QM) cleavage of dimers to tetramers to identify the connection sequence. E represents the extension units and T the terminal unit of the PAC oligomers. A-type bond with both (C4→C8) and (C2→O→C7) linkages or (C4→C6) and (C2→O→C7) linkages, B-type bond which can be (C4→C8) or (C4→C6) linkage.

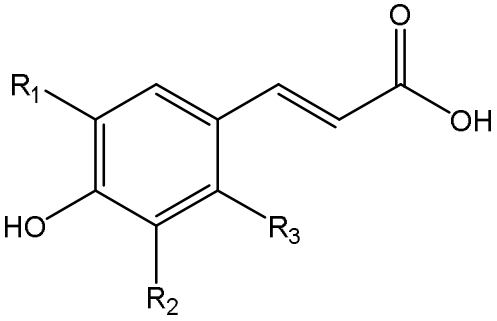
Figure 5.3 Tentative structures and fragmentation schemes (**A** is MS^2 and **B** is MS^3) of *p*-Coumaroyltartaric acid ester-linked nictotinoyl (R-18, *m/z* 400); Formononentin (R-49, *m/z* 545); *trans*-Resveratrol (R-62, *m/z* 505); Piceid (R-63, *m/z* 667); and a second *p*-Coumaroyl moiety (R-65, *m/z* 441). See **Table 5.1** for formal designations.

Figure 5.4 Tentative structures and fragmentation schemes of (**A**) PAC dimer [(E)C (C4)→Luteolin or Kaempferol (C8) or (C6)] (R-48 and R-50, *m/z* 573); (**B**) Prodelphinidin A-type dimer [(E)C→A→(E)GC] (R-28, *m/z* 591); and (**C**) Prorobinetidin A-type dimer [(E)C→A→C-methyl (Epi)robinetinidol] (R-56, *m/z* 589). See **Table 5.1** for formal designations.

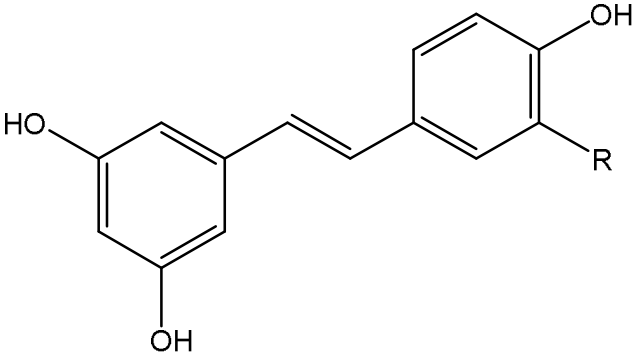
Figure 5.5 Tentative structures and fragmentation schemes of (**A**) Biflavonoid [Eriodictyol→C-methyl Robinetinidol] (R-75, *m/z* 587); (**B**) Biflavonoid [Homoeriodictyol (C3)→Eriodictyol (C8)] (R-76, *m/z* 587); and (**C**) Morelloflavone [Naringenin (C3)→Luteolin (C8)] (R-82, *m/z* 555). See **Table 5.1** for formal designations.



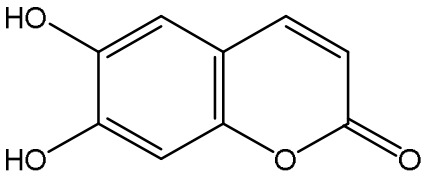
Hydoxybenzoic acids	R
p-Hydroxybenzoic acid	H
Protocatechuic acid	OH
Vanillic acid	OCH_3



Hydroxycinnamic acids	R_1	R_2	R_3
p-Coumaric acid	H	H	H
o-Coumaric acid	H	H	OH
Caffeic acid	OH	H	H
Ferulic acid	OCH_3	H	H
Sinapic acid	OCH_3	OCH_3	H



Stilbenes	R
trans-Resveratrol	H
trans-Piceatannol	OH

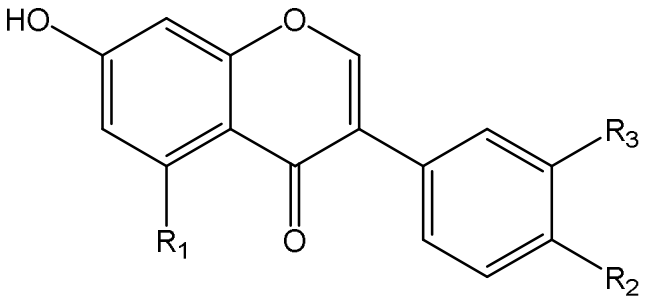


Dihydroxycoumarin

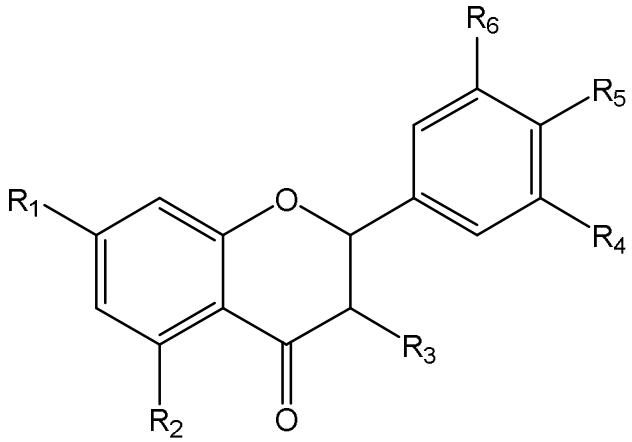
Aesculetin

Figure 5.1

\

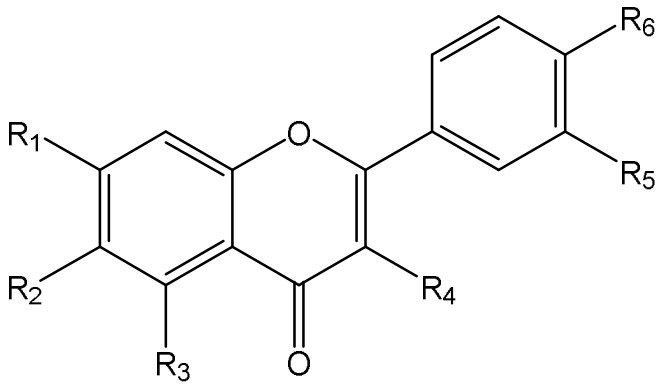


Isoflavones	R_1	R_2	R_3
Biochanin A	OH	OCH_3	H
Formononetin	H	OCH_3	H
3′,5,7-Trihydroxy-4′-methoxyisoflavone	OH	OCH_3	OH

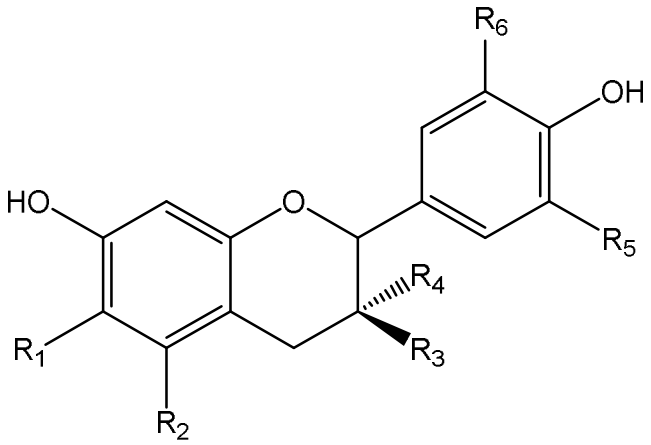


Flavanonols	R_1	R_2	R_3	R_4	R_5	R_6
Dihydrorobinetin-7,3′-dimethyl ether	OCH_3	H	OH	OCH_3	OH	OH
Dihydroquercetin-3′,4′-dimethyl ether	OH	OH	OH	OCH_3	OCH_3	H
Flavanones						
Naringenin	OH	OH	H	H	OH	H
Eriodictyol	OH	OH	H	OH	OH	H
Homoeriodictyol	OH	OH	H	OCH_3	OH	H

Figure 5.1 Continued



Flavones	R_1	R_2	R_3	R_4	R_5	R_6
Luteolin	OH	H	OH	H	OH	OH
Diosmetin	OH	H	OH	H	OH	OCH_3
Cirsiliol	OCH_3	OCH_3	OH	H	OH	OH
Flavonol						
Quercetin	OH	H	OH	OH	OH	OH
Isorhamnetin	OH	H	OH	OH	OCH_3	OH
Kaempferol	OH	H	OH	OH	H	OH



Flavan-3-ols	R_1	R_2	R_3	R_4	R_5	R_6
Catechin	H	OH	OH	H	OH	H
Epicatechin	H	OH	H	OH	OH	H
Afzelechin	H	OH	OH	H	H	H
Epiafzelechin	H	OH	H	OH	H	H
Gallocatechin	H	OH	OH	H	OH	OH
Epigallocatechin	H	OH	H	OH	OH	OH
C-methyl Robinetinidol	CH_3	H	OH	H	OH	OH
C-methyl Epirobinetinidol	CH_3	H	H	OH	OH	OH

Figure 5.1 Continued

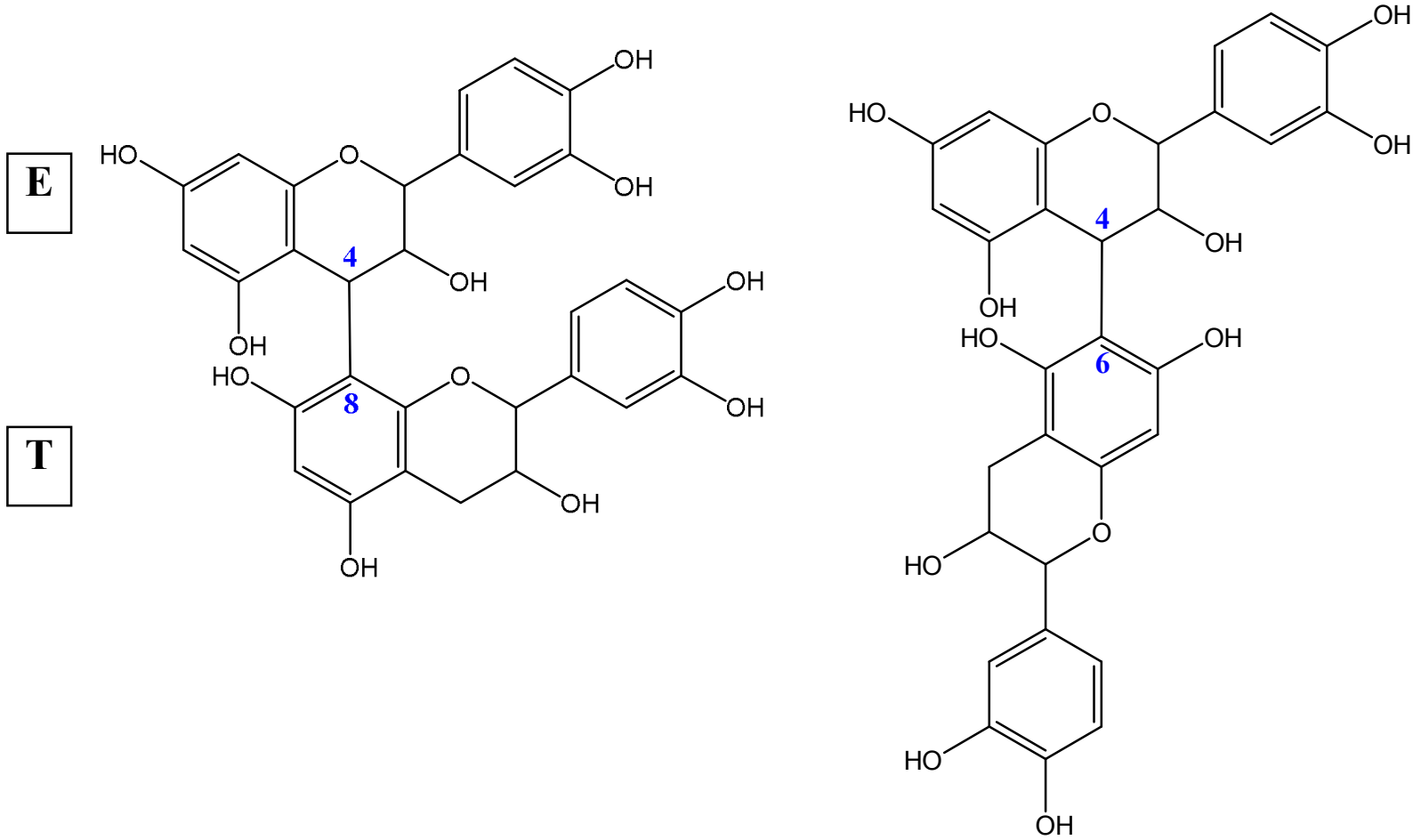


Figure 5.2

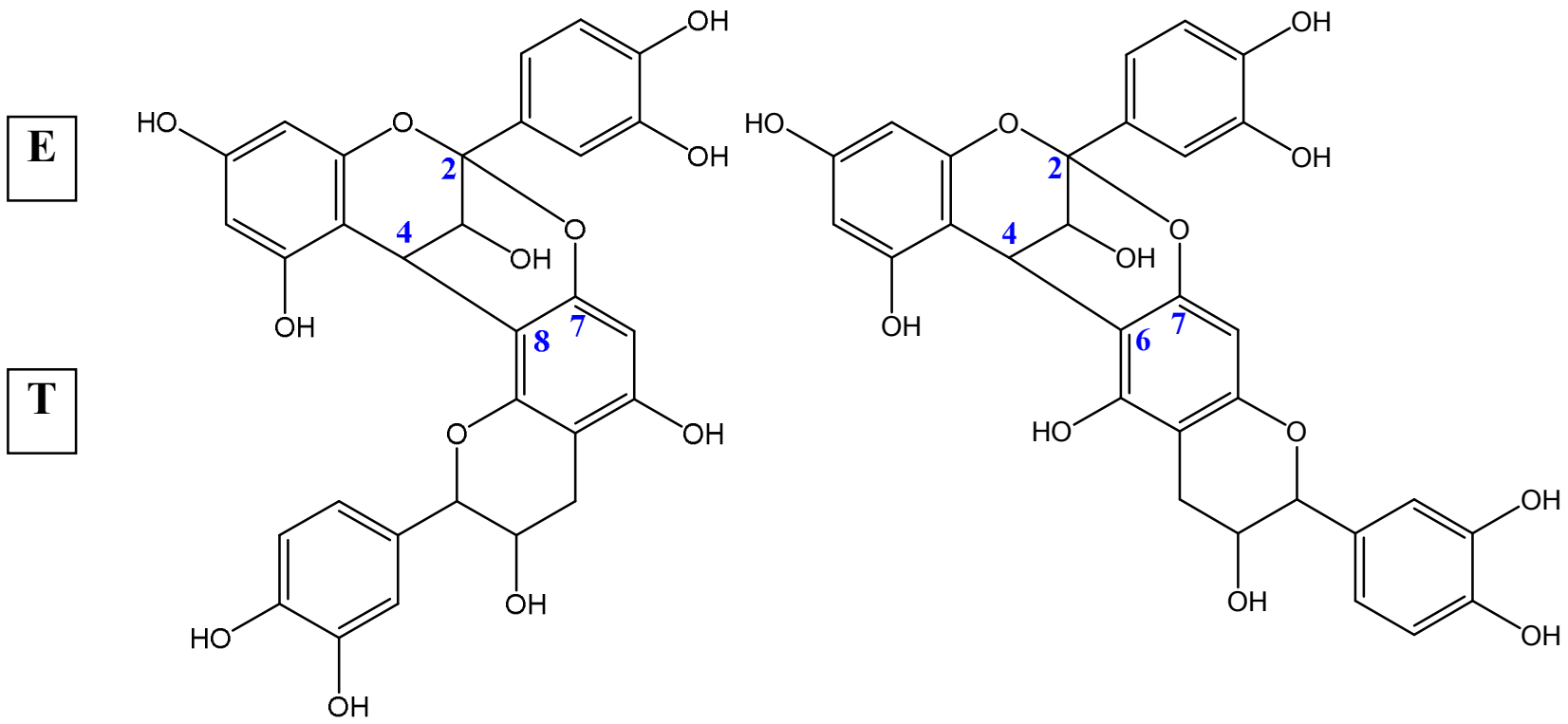


Figure 5.2 Continued

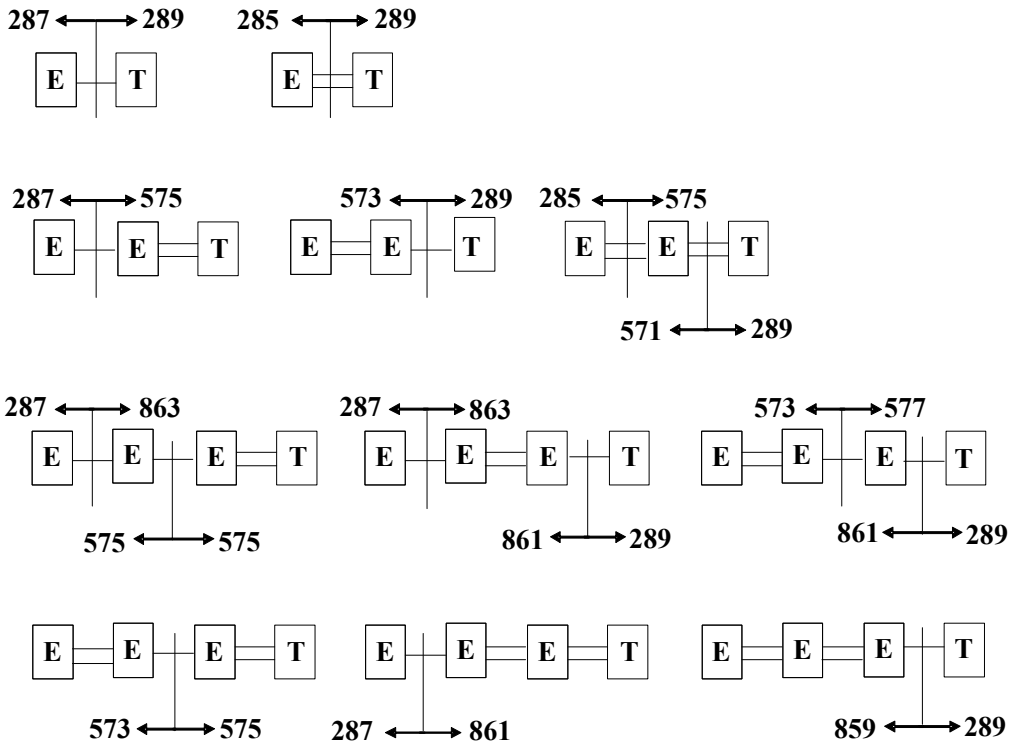


Figure 5.2 Continued

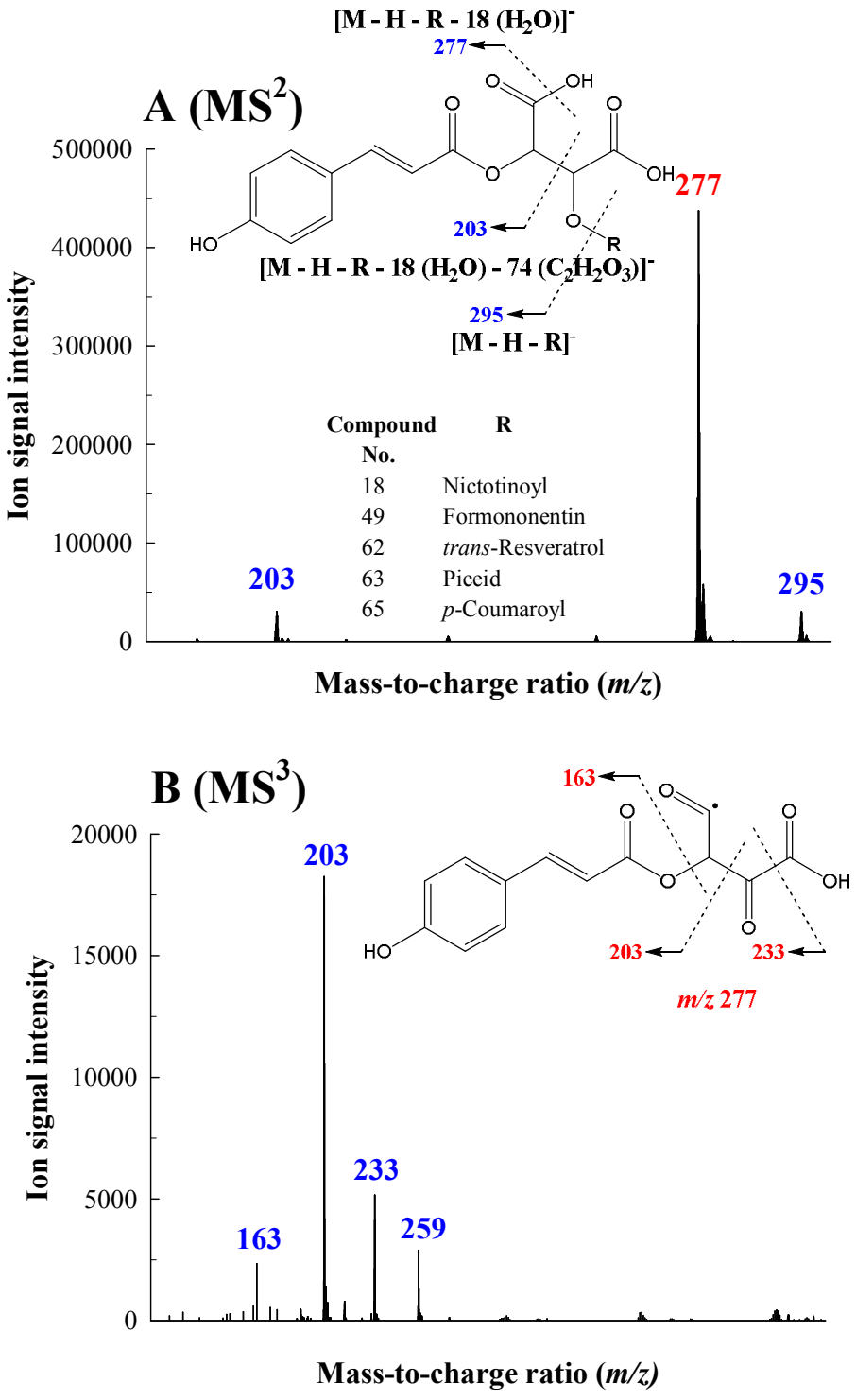

A (MS2)
[M - H - R - 18 (H2O)]-
277
203
[M - H - R - 18 (H2O) - 74 (C2H2O3)]-
295
[M - H - R]-
Ion signal intensity
Mass-to-charge ratio (m/z)
Compound No.
R
18 Nictotinoyl
49 Formononentin
62 trans-Resveratrol
63 Piceid
65 p-Coumaroyl
B (MS3)
163
203
233
259
m/z 277
Ion signal intensity
Mass-to-charge ratio (m/z)

Figure 5.3

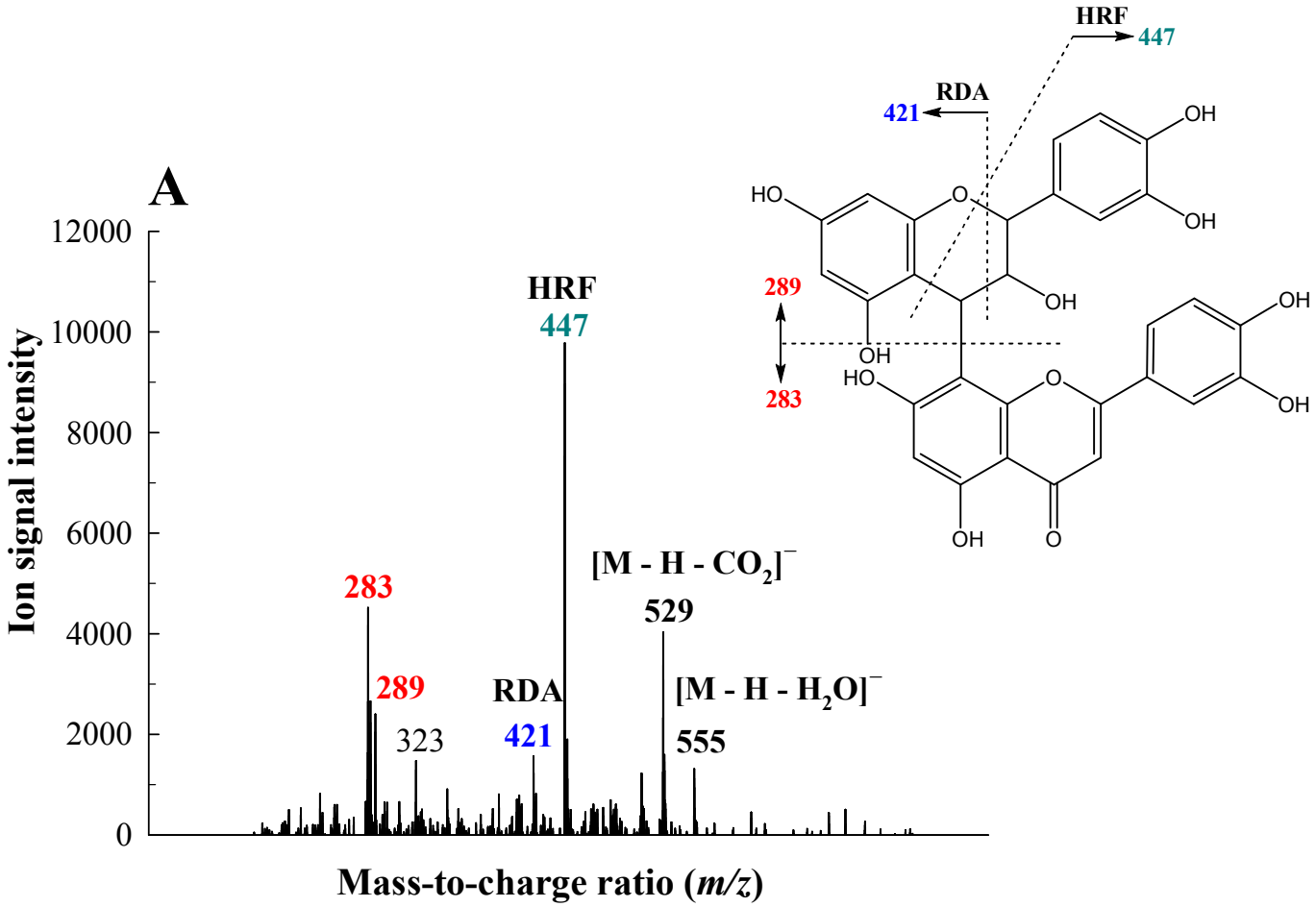

A
Ion signal intensity
12000
10000
8000
6000
4000
2000
0
HRF
447
283
289
323
RDA
421
[M - H - CO2]−
529
[M - H - H2O]−
555
Mass-to-charge ratio (m/z)
HRF
447
RDA
421
289
283
HO
O
OH
OH
OH
OH
HO
OH
OH
O
OH
O

Figure 5.4 (A)

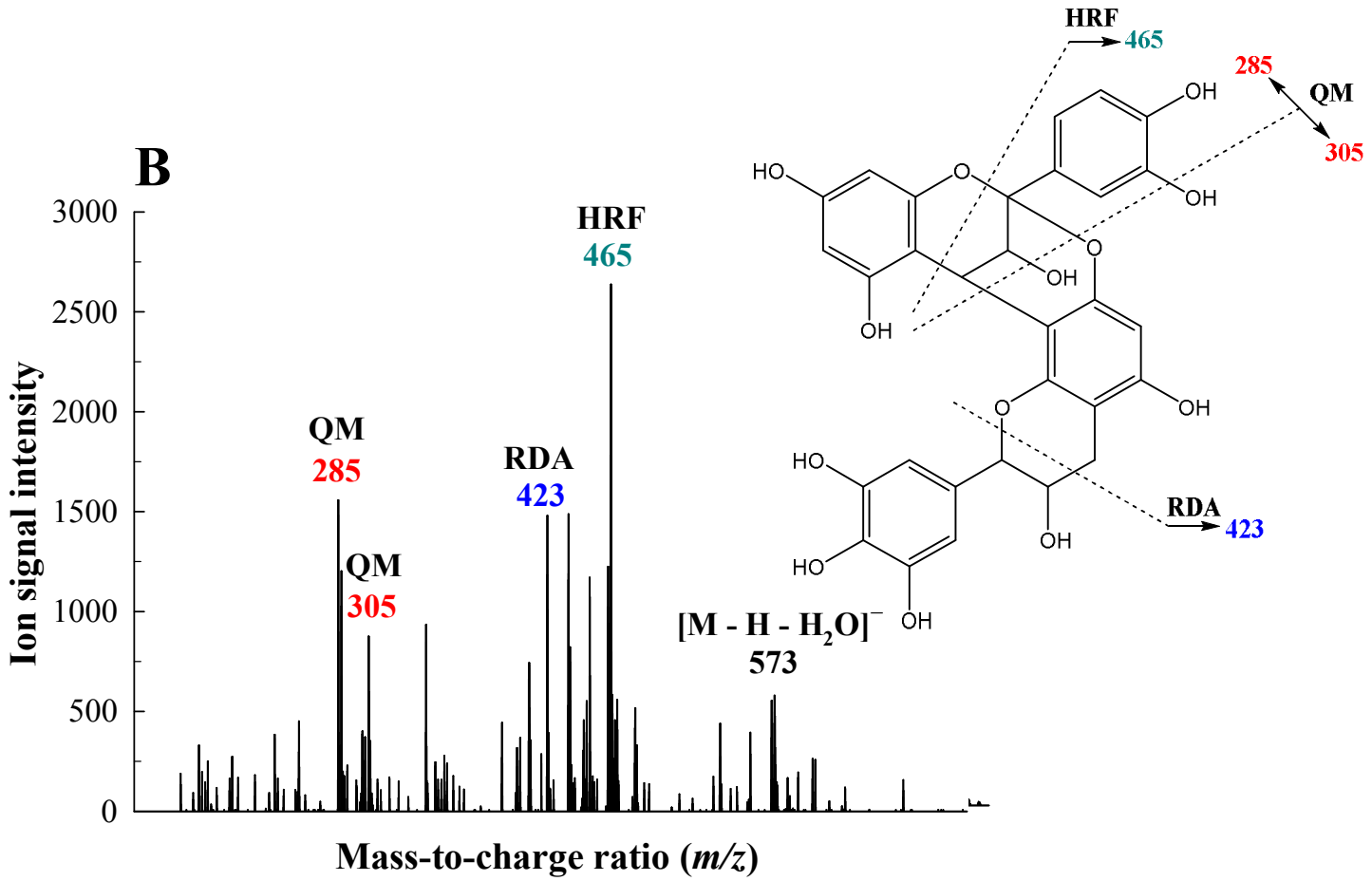

B
Ion signal intensity
3000
2500
2000
1500
1000
500
0
HRF
465
QM
285
QM
305
RDA
423
[M - H - H2O]−
573
Mass-to-charge ratio (m/z)
HRF
465
285
QM
305
RDA
423
HO
OH
O

Figure 5.4 (B)

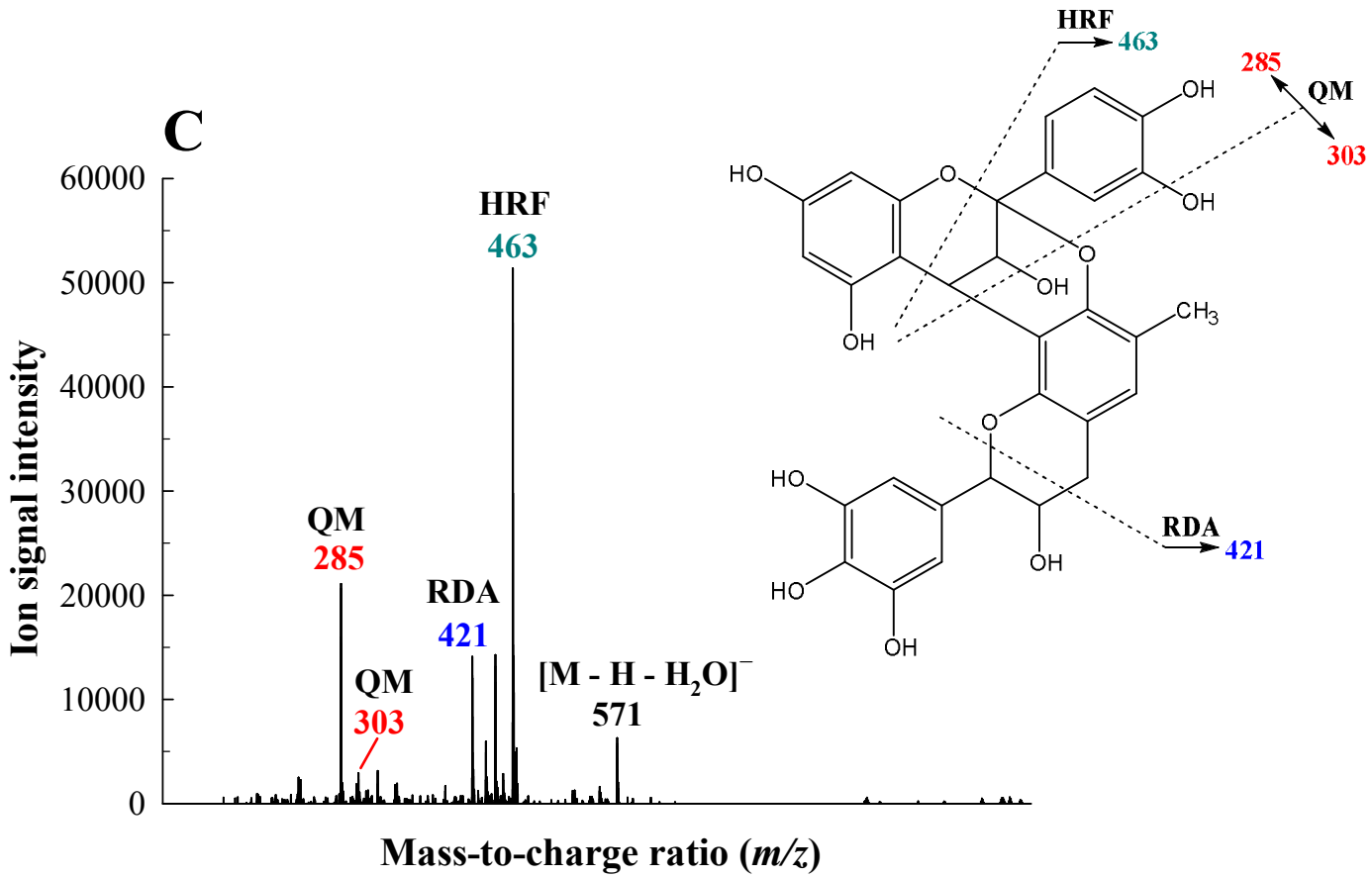

C
Ion signal intensity
60000
50000
40000
30000
20000
10000
0
HRF
463
QM
285
RDA
421
QM
303
[M - H - H2O]−
571
Mass-to-charge ratio (m/z)
HRF
463
285
QM
303
RDA
421
HO
OH
O
CH3

Figure 5.4 (C)

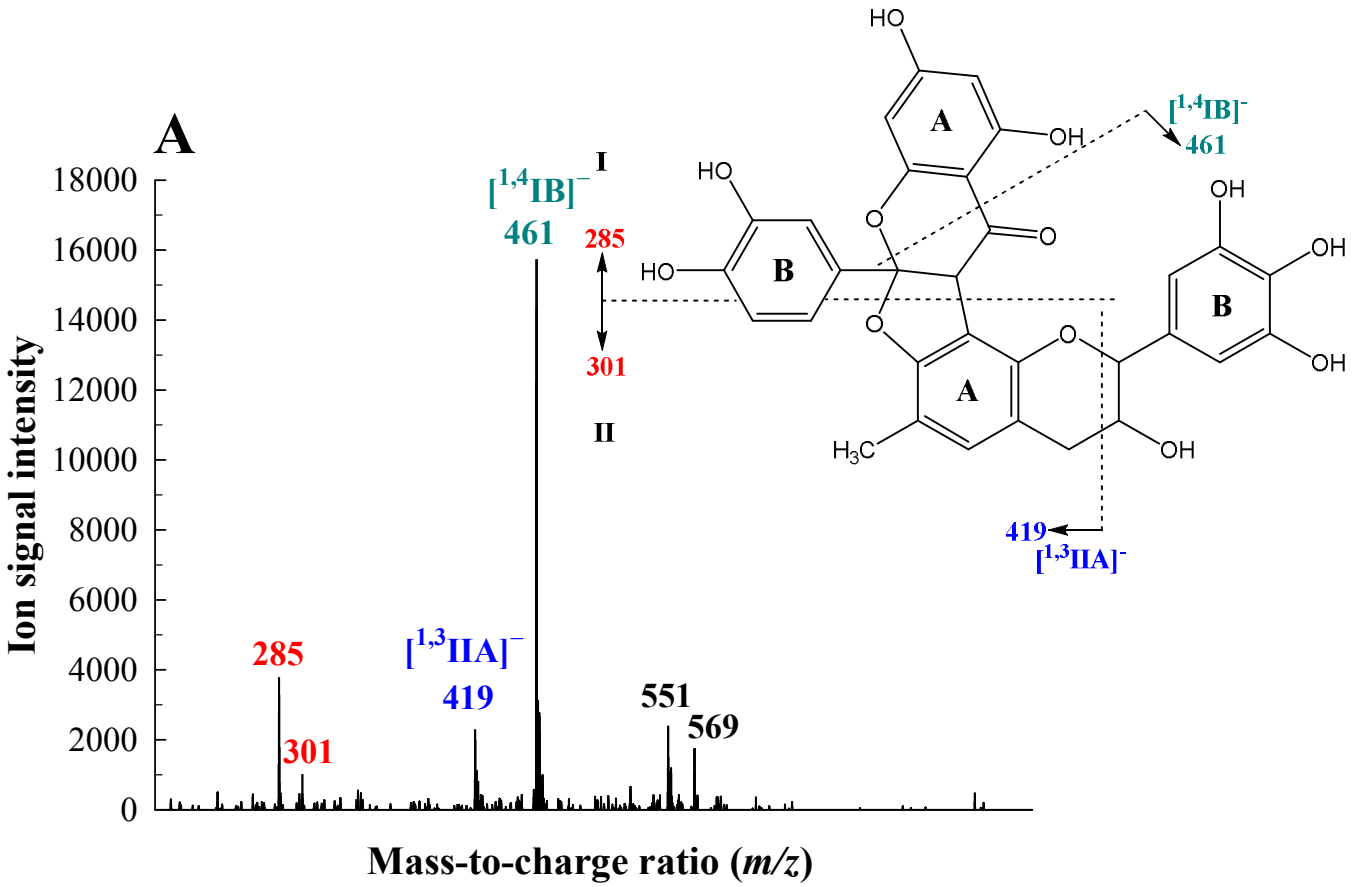

A
Ion signal intensity
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Mass-to-charge ratio (m/z)
285
301
[1,3IIA]−
419
[1,4IB]−
461
551
569
I
285
301
II
HO
A
OH
[1,4IB]-
461
HO
B
O
O
OH
B
OH
OH
O
A
H3C
OH
419
[1,3IIA]-

Figure 5.5 (A)

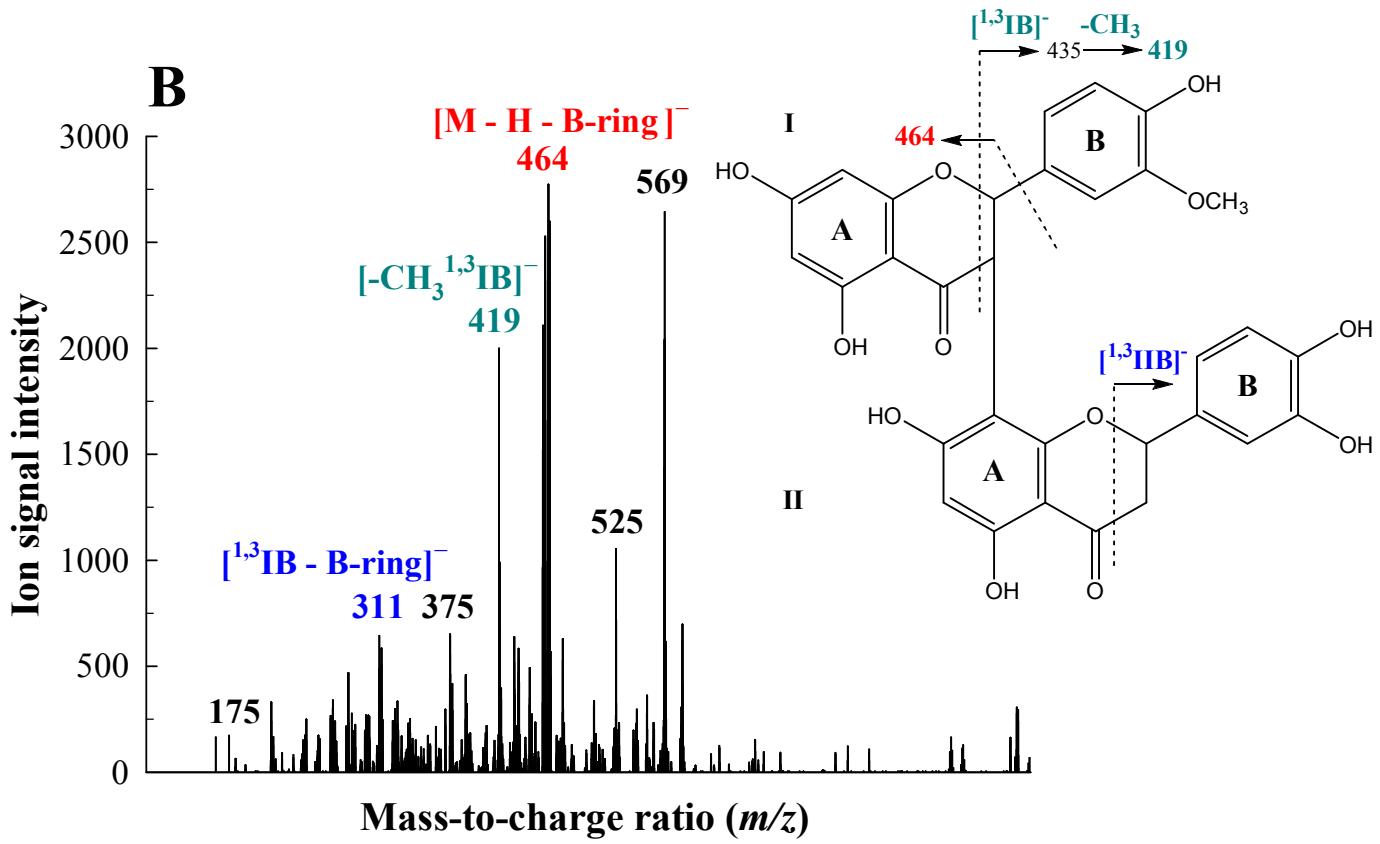

B
Ion signal intensity
Mass-to-charge ratio (m/z)
3000
2500
2000
1500
1000
500
0
[M - H - B-ring]⁻
464
569
[-CH3 1,3IB]⁻
419
525
[1,3IB - B-ring]⁻
311
375
175
I
II
[1,3IB]⁻ -CH3
435
419
464
HO
OH
O
A
B
OCH3
[1,3IIB]⁻

Figure 5.5 (B)

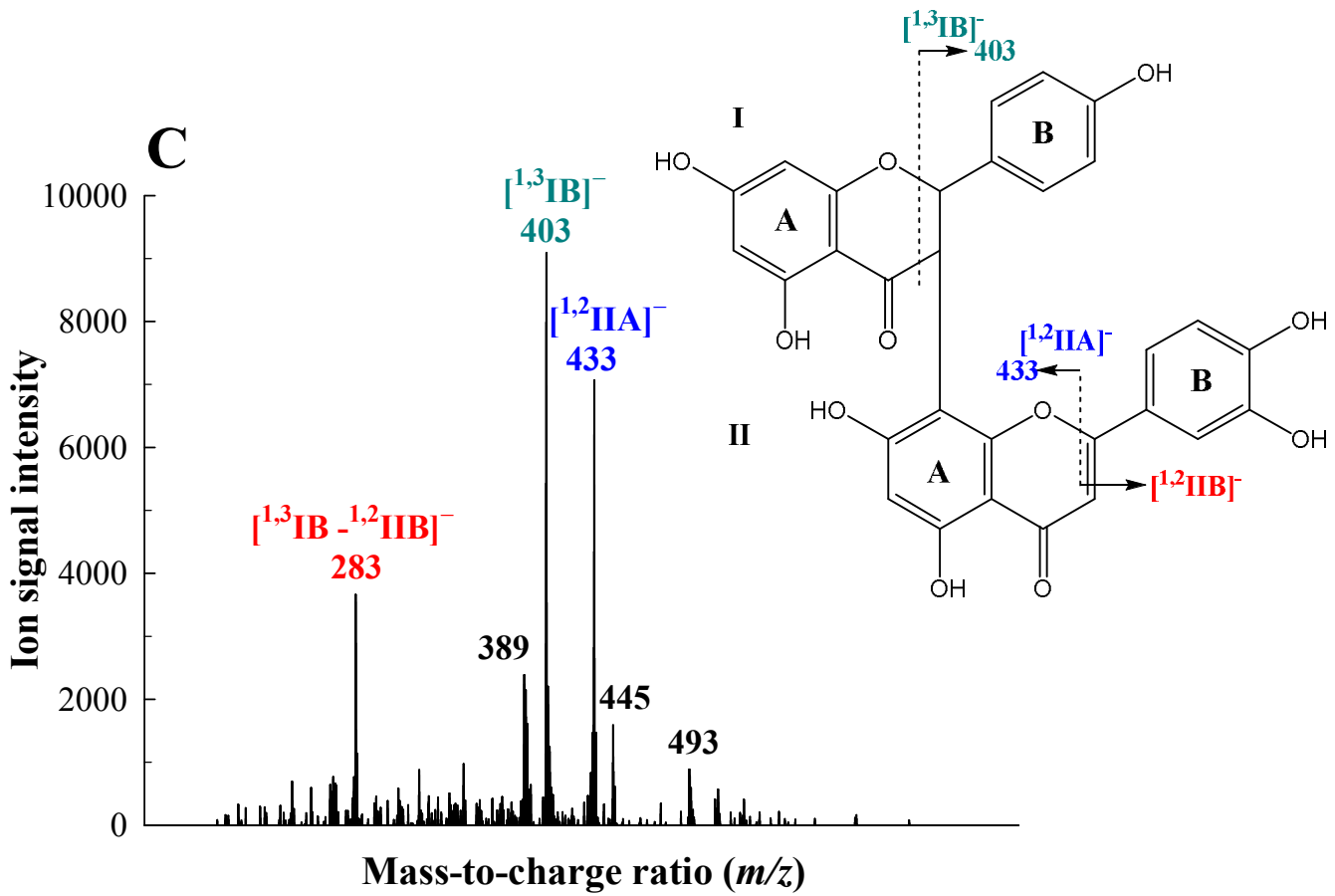

C
Ion signal intensity
Mass-to-charge ratio (m/z)
10000
8000
6000
4000
2000
0
[1,3IB]- 403
[1,3IB]− 403
[1,2IIA]− 433
[1,3IB -1,2IIB]− 283
389
445
493
I
II
A
B
HO
OH
O
[1,2IIA]- 433
[1,2IIB]-

Figure 5.5 (C)

CHAPTER 6

SEPARATION AND CHARACTERIZATION OF ESTER AND GLYCOSIDE-BOUND PHENOLIC COMPOUNDS FROM DRY-BLANCHED PEANUT SKINS BY LIQUID CHROMATOGRAPHY-ELECTROSPRAY IONIZATION MASS SPECTROMETRY

Ma, Y.; Kosińska-Cagnazzo, A.; Kerr, W. L.; Amarowicz, R.; Swanson, R. B.; Pegg, R. B.
To be submitted to the *Journal of Agricultural and Food Chemistry*, **2014**.

Abstract

A large variety of phenolic compounds, including phenolic acids (hydroxybenzoic acids, ethyl protocatechuate, hydroxycinnamic acids, as well as phenylacetic acid and phenyllactic acid), stilbenes (*trans*-piceatannol and *trans*-3,3′,5,5′-tetrahydroxy-4′-methoxystilbene), flavan-3-ols (*e.g.*, (–)-epicatechin, (+)-catechin, (–)-epiafzelechin, and their polymers {the proanthocyanidins, PACs}), other flavonoids (*e.g.*, isoflavones, flavanols, and flavones) and biflavonoids, were identified in dry-blanched peanut skins (PS). Reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with electrospray ionization tandem mass spectrometry (ESI-MS^n) was applied to separate and identify the phenolic constituents. Tentative identification of the separated phenolics was based on molecular ions and MS^n fragmentation patterns acquired by ESI-MS in the negative-ion mode. Identification of free phenolic acids, stilbenes, and flavonoids was also achieved by commercial standards and by published literature data. Quantification was performed based on peak areas of the UV signals from the HPLC chromatograms and calibration curves of the commercial standards. The flavonoids of PS exist mostly in glycoside-bound forms, but the agylcone can be easily released upon acid hydrolysis. PS contain significantly more PACs compared to free phenolic compounds. PAC monomers to tetramers constituted 92.0% of ester bound phenolic compounds. The PAC monomer {(+)-catechin} and dimers are primary phenolics released from glycosides and account for 31.7% and 59.1%, respectively, of total glycoside-bound phenolic compounds.

6.1 Introduction

Phenolic compounds are secondary metabolites ubiquitously found in plants (Bravo, 1998). Amongst the plethora found in nature, phenolics in foodstuffs comprise primarily phenolic acids, flavonoids, and tannins (King & Young, 1999). Free phenolic acids, such as *p*-hydroxybenzoic acid and vanillic acid, can be present as such, but more common is their occurrence as analog methyl and ethyl esters as well as glycosides, which can also exist in the free and bound forms (Bravo, 1998). Hydroxycinnamic acids are typically bound to cell wall polysaccharides via covalent linkages (Bravo, 1998). To illustrate, in graminaceous plants *p*-coumaric acid and ferulic acid are bound to cell walls through an ester-linkage between their carboxylic groups and arabinoxylans (Hartley, Morrison, Himmelsbach, & Borneman, 1990). Flavonoids present in living plants are generally in bound forms with one or more sugar residues attached (Bravo, 1998; Hollman & Arts, 2000; Crozier, Jaganath, & Clifford, 2009). As for flavan-3-ols (*i.e.*, (–)-epicatechin, (+)-catechin, and gallocatechin), they exist chiefly as free monomers (Bravo, 1998). The diastereoisomeric pair of (+)-catechin and (−)-epicatechin are widespread in nature, whereas relatively rare are the (−)-catechin and (+)-epicatechin forms (Crozier, Jaganath, & Clifford, 2009).

Identification of esterified phenolic derivatives is extremely difficult due to the nearly uniform UV spectra with their unesterifed counterpart. The chemical characteristics of the new chromophore generated via esterification are not markedly different from its precursor. Sometimes there are, however, minor bathochromic shifts in the chromophoric structure such as that present in the C–O bond of caffeoyl tartrate (Robbins, 2003). One strategy to simplify and specify the analysis is to employ acid and/or base hydrolysis to convert the ester derivatives to their carboxylic acid analogs. It has been reported that alkaline hydrolysis yields phenolic

compounds liberated from soluble esters, if present, while phenolic compounds bound to glycosides are released by a subsequent acid hydrolysis (Weidner, Amarowicz, Karamać, & Dabrowski, 1999; Amarowicz & Weidner, 2001). The liberated aglycones are easily identified by HPLC with diode-array detection (DAD). Likewise, the determination of individual flavonoid glycosides present in foods is formidable, due to the numerous varieties that exist (Hertog, Peter, Hollman, & Venema, 1992). Therefore, flavonoids are usually analyzed after hydrolysis of their glycosides; the resulting aglycones are further confirmed by UV DAD detection or mass spectrometry (Hertog, Peter, Hollman, & Venema, 1992; Hollman & Arts, 2000; Merken & Beecher, 2000). The HPLC analysis of flavonoids in peanuts and other legume seeds after acid hydrolysis was described by Wang's group (2008).

Proanthocyanidins (PACs) are oligomers and polymers of flavan-3-ols, which are primarily linked by C4→C8 bonds or less common C4→C6 bonds (B-type), whereas A-type dimers contain an additional (C2→O→C7) linkage. Despite some free phenolic acids, procyanidins comprised ~17% by weight in peanut skins (PS) (Karchesy & Hemingway, 1986) and are predominant in the phenolic composition (Yu, Ahmedna, Goktepe, & Dai, 2006). Although A-type linkages are much more abundant, both A- and B-type PACs occur in PS. The degree of polymerization (DP) identified in PS was up to 12 units by A-type linkages, whereas B-type structures were detected with a DP up to only 6 (Monagas *et al.*, 2009). A certain percentage of PACs are 'unextractable' because these PACs are bound to cell wall material such as polysaccharides via covalent and hydrogen bonding as well as hydrophobic interactions (Pinelo, Arnous, & Meyer, 2006; White, Howard, & Prior, 2010). Consequently, the PACs content in any analytical determination is underestimated because of the bound PAC–cell wall material, which is not disturbed by normal extraction protocols. In order to liberate these bound derivatives, acid

or base hydrolysis is required prior to conventional extraction (Arranz, Saura-Calixto, Shaha, & Kroon, 2009; White, Howard, & Prior, 2010). Severe alkaline conditions can, nonetheless, lead to cleavage of C–C interflavan bonds between the monomeric flavan-3-ol constituents. The A-ring of flavan-3-ols can also be broken by prolonged alkaline treatment (White, Howard, & Prior, 2010).

As previously reported, the phenolic compounds of PS include simple phenolic acids (*e.g.*, caffeic, ferulic, and coumaric acids), flavan-3-ols (*e.g.*, (epi)catechin, epigallocatechin, catechin gallate, and epicatechin gallate), stilbenes (*e.g.*, *trans*-resveratrol), and polymerized PACs (Yu, Ahmedna, & Goktepe, 2005; Yu, Ahmedna, Goktepe, & Dai, 2006; Ma *et al.*, 2014), but limited information is available concerning the hydrolyzable phenolics and PACs of PS. Therefore, the objectives of this study were to liberate soluble-ester and glycoside-bound phenolic compounds from dry-blanched PS extracts using alkaline and acid hydrolyses and then to characterize them by RP-HPLC-ESI-MS^n.

6.2 Materials and Methods

Materials

Dry-blanched PS were a gift from Universal Blanchers, LLC (Sylvester, GA). All solvents and reagents were of analytical (ACS) grade, unless otherwise specified. Methanol, ethanol (95%), and hexanes were purchased from VWR International (Suwanee, GA). Procyanidin B2 was obtained from Indofine Chemical Company, Inc. (Hillsborough, NJ). (–)-Epiafzelechin was acquired from BOC Science (Shirley, NJ), while all other phenolic standards were bought from either VWR International (Suwanee, GA) or the Sigma-Aldrich Chemical Co. (St. Louis, MO).

Phenolic Extraction

Extraction of phenolics from PS was carried out according to Amarowicz *et al.* (2004) with slight modification. The extraction was performed in triplicate. In brief, samples were first placed in Whatman cellulose extraction thimbles (43 mm *i.d.* × 123 mm *e.l.*, VWR International, Suwanee, GA), covered with a plug of glass wool and defatted in a Soxhlet extraction apparatus under reflux for 12 to 14 h with hexanes. Defatted PS were transferred to 125-mL Erlenmeyer flasks at a mass-to-solvent ratio of 1:10 (w/v) with 80% (v/v) acetone. Extractions were performed in triplicate in a gyrotary water bath shaker (Model G76, New Brunswick Scientific Company, Inc., New Brunswick, NJ) set at 150 rpm and a temperature of 45 °C for 30 min. The slurry was then filtered by gravity through fluted P8 filter paper (Fisher Scientific). The extraction process was repeated 2× as described above. All filtrates were pooled and acetone was evaporated with a Büchi Rotavapor R-210 using a V-700 vacuum pump connected to a V-850 vacuum controller (Büchi Corporation, New Castle, DE) at 45 °C. The aqueous residue was frozen and then lyophilized in a FreeZone® 2.5-L bench-top freeze dryer (Labconco Corporation, Kansas City, MO) to ensure all traces of moisture were removed, and then stored in amber-glass bottles at –20 °C until further analyzed.

Hydrolysis of Phenolic Compounds from Crude Extracts

Phenolic compounds were fractionated into three classes (*i.e.,* free, soluble-ester, and glycoside-bound) from the acetonic PS extracts by the method described by Amarowicz & Weidner (2001) with slight modifications. Briefly, a 400-mg portion of each crude extract was dissolved in 20 mL of deionized water followed by acidification to pH 2 with 6 M HCl. Using a separatory funnel, free phenolic compounds were then extracted into 20 mL of diethyl ether. The

extraction process was repeated 4× more; the ether layers were transferred intermittently to a 100-mL round-bottom flask and removed with a Büchi Rotavapor R-210 using a V-700 vacuum pump connected to a V-850 vacuum controller (Büchi Corporation, New Castle, DE, USA) at 30 °C. Traces of water, when present, were removed from the contents in the round-bottom flask by a nitrogen evaporator (N-EVAP™ 111 with an aluminum bead dry bath set at ~50 °C, Organomation Associates, Inc., Berlin, MA, USA). This sediment is referred to as fraction 1 (F1), and was characterized by Ma *et al.* (2014). The aqueous-phase residue in the separatory funnel was then mixed with 20 mL of 2 M NaOH, hydrolyzed under a N_2 atmosphere for 4 h at room temperature, and then acidified to pH 2 with 6 M HCl. Using 30-mL aliquots of diethyl ether, liberated phenolic compounds from soluble esters were extracted a total of 5×. The ether layers were transferred intermittently to a 100-mL round-bottom flask and removed with the Rotavapor. Traces of water, when present, were removed from the contents in the round-bottom flask by the N-EVAP. This sediment is referred to as fraction 2 (F2). The aqueous-phase residue was then finally combined with 15 mL of 6 M HCl and hydrolyzed under a N_2 atmosphere for 1 h at 100 °C in a forced-air convection oven. Using 45-mL aliquots of diethyl ether, liberated phenolic compounds from glycosides were extracted a total of 5×. The ether layers were transferred intermittently to a 100-mL round-bottom flask and removed with the Rotavapor. Traces of water, when present, were removed from the contents in the round-bottom flask by the N-EVAP. This sediment is referred to as fraction 3 (F3).

Reversed-Phase High-Performance Liquid Chromatography Electrospray Ionization Mass Spectrometry (RP-HPLC/ESI-MS^n)

An Agilent 1200 Series HPLC system consisting of a quaternary pump with degasser,

autosampler, thermostatted column compartment, UV–vis diode array detection (DAD) with standard flow cell, and 3D ChemStation software (Agilent Technologies, Santa Clara, CA, USA) was employed for the chromatography. A reversed-phase Luna $C_{18}(2)$ column (4.6 × 250 mm, 5-µm particle size; Phenomenex, Torrance, CA, USA) was utilized. A gradient elution consisting of mobile phase A ($H_2O/CH_3CN/CH_3COOH$, 93:5:2, v/v/v) and mobile phase B ($H_2O/CH_3CN/CH_3COOH$, 58:40:2, v/v/v) from 0 to 100% B over a 50-min period at a flow rate of 1 mL/min was employed. Before subsequent injections, the system was re-equilibrated for 30 min using 100% A. A 10-mg portion of the PS crude extract, F2 and F3 were dissolved in 5, 4, and 2 mL of 80% (v/v) methanol, respectively, and then passed through a Phenex™-RC, 15 mm, non-sterile, 0.2-µm syringe filter (Phenomenex Inc., Torrance, CA, USA) before determination of their phenolics; the injection volume was 20 µL. Detection wavelengths employed were λ = 255 and 260 nm (hydroxybenzoic acids, as well as ellagic acid and ellagic acid derivatives), 280 nm ((+)-catechin, (–)-epicatechin, and PACs), 320 nm (phenolic acids notably of the *trans*-cinnamic acid family), and 360 nm (flavonols and general flavonoids). Tentative identification of separated components was made by matching UV–vis spectra and retention time (t_R) mapping with commercial standards. For quantification, calibration curves were constructed for each standard to confirm linearity based on the UV–vis signal as well as for the determination of response factors.

RP-HPLC-ESI-MS^n analyses of the phenolics were performed on an Agilent 1100 HPLC system, with the same chromatography conditions as described above, coupled with a Bruker Esquire 3000^{plus} ion-trap mass spectrometer (Bruker Daltonics Inc., Billerica, MA, USA), which was equipped with an ESI source. Instrument control and data acquisition were performed with Bruker Daltonics Esquire 5.3 software. The HPLC eluent flowed directly into the mass

spectrometer via a flow splitter delivering roughly 100 μL/min. Mass spectra were acquired in the negative-ion mode with smart settings for a target mass at an *m/z* of 800, compound stability at 100%, and the trap drive level at 100%. The voltage applied to the capillary was 4.0 kV with an end plate offset of –500 V. Nitrogen was used as the nebulizing gas at a pressure of 10 psi, a dry gas flow rate of 6 L/min, and a dry gas temperature of 280 °C. The phenolic compounds were fragmented using the auto-MS^2 mode with the following precursor-ion selection parameters: two precursor ions, threshold abs of 10,000, threshold rel of 5.0%, ion excluded after two spectra, and exclusion release after 0.5 min. All collision-induced dissociation mass spectra were obtained with He as the collision gas at a fragmentation voltage of 1 V after isolation of the desired precursor ion. A full scan was performed over the mass range of 100 to 1,000 *m/z* at a rate of 13,000 *m/z* per second under standard–normal scan mode. The ion current control target was 50,000 with a maximum accumulation time of 50 ms. Each mass spectrum generated was based on an average of ten scans. Precursor ions of interest were further subjected to auto-MS^n under the same conditions as described above.

Statistical Analysis

For each quantified phenolic compound, the mean and standard deviation was calculated from the measurements of triplicate extractions.

6.3 Results and Discussion

Hydrolysis

Alkaline and acid hydrolyses of the PS crude extract were performed prior to chromatographic analysis in order to liberate phenolic aglycones from soluble esters and glycosides, respectively. Even though the HPLC profile of hydrolyzed samples may not reflect

the structure of each phenolic ester and glycoside present in PS, hydrolysis does simplify the identification of phenolic compounds and provides important information concerning the profile of the individual aglycones. Moreover, it allowed further compounds to be detected via chromatography. In the present study few phenolic species were isolated from the PS crude phenolic extracts (CE1 of **Table 6.1**), unlike those first liberated via base and acid hydrolyses followed by RP-HPLC separation and detection by ESI-MS^n (**Tables 6.2** & **6.3**). **Figure 6.1** depicts the chemical structures of the phenolic monomeric aglycones detected in PS.

Identification of Phenolic Compounds using RP-HPLC-ESI-MS^n.

Prior to hydrolysis, only PACs and rutin were detectable in the PS crude phenolic extracts. In contrast, a large variety of phenolic compounds, including PACs (monomer to pentamer), hydroxybenzoic acids, *trans*-cinnamic acids, stilbenes, and flavonoids, were eluted in fractions F2 (*i.e.*, phenolics liberated from soluble esters) and F3 (*i.e.,* phenolics liberated from glycosides). Noteworthy is that flavonoids were liberated primarily from glycosides, and recovered in F3.

Phenolic acids and flavonoids show characteristic UV range absorbance patterns from 190 to 380 nm (Merken & Beecher, 2000; Robbins, 2003). From UV–vis DAD, four groups of phenolic compounds were characterized, namely hydroxybenzoic acids (255 and 260 nm), (+)-catechin, (–)-epicatechin and their polymers (280 nm), hydroxycinnamic acids (320 nm) and flavonols (360 nm). These compounds was subsequently introduced to the ESI mass spectrometer and analyzed based on their *m/z* values. Tentative identification of separated compounds was based on mass spectrometry alone; positive identification was achieved by the matching of t_Rs and spectral data with those of commercial standards, when available. Possible misinterpretations of

spectra are due to the presence of stereoisomers, which are impossible to distinguish by mass spectra. Typically, the cleavage of a distinctive moiety from a compound in MS^n analysis brings about a corresponding change in mass. To illustrate, rutinoside possesses a characteristic mass of 308 Da; so, loss of 18 and 15 Da typically indicates the cleavage of a H_2O molecule and a $CH_3^{\bullet}$ group, respectively. A-type PACs (*m/z* 575, 861, 863, 1151, 1149, 1437, 1725, *etc.*) display a 2-Da difference in $[M - H]^-$ from B-type polymers (*m/z* 577, 865, 1153, 1441, and 1729, *etc.*). Quinone methide fission (QM), retro-Diels-Alder fission (RDA), and heterocyclic ring fission (HRF) are three major fragmentation routes of PACs well-described in the literature (Gu *et al.*, 2003a).

$^{ij}A^-$ and $^{ij}B^-$ ions are diagnostic fragments for flavonoid identification and produced by the RDA cleavage in the C-ring. A^- and B^- are product ions in the negative-ion mode consisting of intact A- and B-rings, while the superscript i and j indicate the positions in the C-ring where two C–C bonds have been broken. Besides RDA cleavage in the C-ring, losses of neutral molecules such as H_2O (18 Da), CO (28 Da), CO_2 (44 Da), C_2H_2O (42 Da), and C_3O_2 (68 Da) can be characteristic in the negative-ion mode. The fragmentations of flavone, flavonol, and flavanone aglycones in the negative-ion mode by ESI trap mass spectrometry are well-demonstrated by Fabre *et al.* (2001). The successive loss of $CH_3^{\bullet}$ and then CO (28 Da) or $CHO^{\bullet}$ (29 Da) were characteristic in methoxylated flavonoids (Justesen, 2001). However, the position of the methoxy group must be identified by the comparison with commercial standards or by NMR analysis (Cuyckens & Claeys, 2004). A similar nomenclature of fragmentation was adopted as for flavonoids. $^{ij}A^-$ and $^{ij}B^-$ ions are RDA fragments produced by cleavage of two C–C bonds in the C-ring. The flavonoid, whose C-ring is attached to the A-ring of the other flavonoid, is denoted as ‘Part I’ and the other flavonoid is referred to as ‘Part II.’ Identification of compounds detected

in both PS crude extracts and each base- and acid-hydrolytic fraction is discussed as follows.

Crude PS extracts.

Proanthocyanidins (PACs)

Compounds providing a molecular ion $[M - H]^-$ at *m/z* 575 (compounds CE-1-13, 17, 21, 24, 26, and 27) and 577 (compounds CE-1-6 and 10) are likely PAC A- and B-type dimers, respectively. In the negative-ion mode, A-type dimers yield product ions at *m/z* 449 [M – H – 126 $(C_6H_6O_3)]^-$ and 423 [M – H – 152 $(C_8H_8O_3)]^-$ via HRF fragmentation at the top unit and RDA fragmentation at the base unit, respectively. Generally, the top unit is the favored site for RDA, but fission can occur at the base unit when blocked by A-type bonds (Gu *et al.*, 2003a). QM ions were present at *m/z* 289 $[M_T - H\ (290 - H)]^-$ (QM cleavage at the terminal {T} unit) and 285 $[M_E - 5H\ (290 - 5H)]^-$ (QM cleavage at the extension {E} unit). Likewise, product ions at *m/z* 451 [M – H – 126 $(C_6H_6O_3)]^-$, 425 [M – H – 152 $(C_8H_8O_3)]^-$, 289 $[M_T - H\ (290 - H)]^-$ and 287 $[M_E - 5H\ (290 - 5H)]^-$ were present in the MS^2 fragmentation of B-type dimer. The fragment ion at *m/z* 407 was generated by further loss of a $–H_2O$ moiety from the ion at *m/z* 425. These data and characteristic MS fragmentation patterns are consistent with the findings of previous studies (Gu *et al.*, 2003a; Appeldoorn *et al.*, 2009; Sarnoski *et al.*, 2012).

Molecular ions present at *m/z* 863 (compounds CE-1-1, 3, 4, 7, 8, 9, 11, 12, 18, 19, 22, and 23) and 865 (compound CE-1-2) were likely produced by PAC A- and B-type trimers, respectively. Theoretically, PAC trimers dissociate in a similar way to dimers: there are two types of A-type trimers both possessing a molecular ion at *m/z* 863, but with different QM diagnostic ions caused by varied positions of the A-bond. QM cleavage between the top and middle units produced ions at *m/z* 575 $[M_T - H\ (576 - H)]^-$ and 287 $[M_E - 3H\ (290 - 3H)]^-$, indicating that the

A-type linkage is located between the middle and base units (compounds CE-1-1, 3, 7, 11, 18, and 22). The HRF and RDA fragmentation of the top unit yielded product ions at *m/z* 737 [M – H – 126 ($C_6H_6O_3$)]$^-$ and 711 [M – H – 152 ($C_8H_8O_3$)]$^-$, respectively. The HRF fragmentation of the middle unit gave a product ion at *m/z* 449. When the A-type linkage is located between the top and middle units (compounds CE-1-4, 8, 9, 12, 19, and 23), QM cleavage ions are present at *m/z* 573 [M_E – 3H (576 – 3H)]$^-$ and 289 [M_T – H (290 – H)]$^-$. In this case, the product ion at *m/z* 711 was formed by RDA fragmentation of the middle unit. The HRF fragmentation of the middle unit yielded product ions at *m/z* 451 and 411. However, these two types of trimer, containing the same number of A-type bond, were not well resolved by HPLC. Two series of fragments were observed in one mass spectrum. HRF and RDA fragmentation of the B-type trimer gave rise to dissociation ions at *m/z* 739 [M – H – 126 ($C_6H_6O_3$)]$^-$ and 713 [M – H – 152 ($C_8H_8O_3$)]$^-$, respectively. The HRF fragmentation of the middle unit resulted in a product ion at *m/z* 451. The prominent fragment ion at *m/z* 695 was generated by further loss of a –H_2O moiety from the RDA ion at *m/z* 713. QM diagnostic ions were present at *m/z* 577 [M_T – H (578 – H)]$^-$, 575 [M_E – 3H (578 – 3H)]$^-$ and 287 [M_E – 3H (290 – 3H)]$^-$.

[M – 2H]$^{2-}$ at *m/z* 719 (compounds CE-1-5, 14, and 15) and 574 (compounds CE-1-16 and 20) are doubly charged ions generated from a PAC A-type pentamer (*m/z* 1439) and a PAC A-type tetramer (*m/z* 1149), respectively. Apropos the pentamer: the diagnostic QM ions present at *m/z* 863 [M_T – H (864 – H)]$^-$, 575 [M_E – 3H (578 – 3H)]$^-$, and 573 [M_E – 3H (576 – 3H)]$^-$ indicate a connection sequence such as (epi)catechin→B→(epi)catechin→B→(epi)catechin→ A→(epi)catechin→B→(epi)catechin, where A and B represent A- and B-type linkages, respectively. The A-type pentamer gave rise to QM cleavage fragments at *m/z* 861 [M_E – 3H (864 – 3H)]$^-$, 577 [M_T – H (578 – H)]$^-$, and 575 [M_T – H (576 – H)]$^-$, being assigned to a sequence

such as (epi)catechin→B→(epi)catechin→A→(epi)catechin→B→(epi)catechin→B→(epi)catechin. Vis-à-vis the tetramer: the QM fragment at *m/z* 859 $[M_E - 3H\ (862 - 3H)]^-$ were observed when the B-type linkage is located between the base and its upper unit (CE-1-16). The production of a QM fragment at *m/z* 861 $[M_T - H\ (862 - H)]^-$ indicates that the B-type linkage is located between the top and its subsequent unit, while the remaining units are linked by A-type bonds (compound CE-1-20).

Flavonoid.

A 316-Da *O*-methylated flavonoid with a rutinoside attached was determined as compound CE-1-25. It yielded a $[M - H]^-$ at *m/z* 623 and fragment ions at *m/z* 315 $[M - H - 308]^-$ by loss of rutinoside moiety and *m/z* 300 $[M - H - 308 - 15]^-$ by further loss of a $CH_3^{\bullet}$ group from the deprotonated flavonoid aglycone. Although there is a wide range of candidates for 316-Da *O*-methylated flavonoids such as rhamnetin, isorhamnetin, tamarixetin, and neptin, the compound is likely to be isorhamnetin according to an NMR study reported by Lou's group (2001).

Ester-bound Phenolics

Phenylacetic Acid

Compound F-2-3 yielded a molecular ion at *m/z* 167 and a fragment ion at *m/z* 123 by loss of a CO_2 group from the carboxylic acid moiety. Among several candidates such as 3,4-dihydroxyphenylacetic acid (DOPAC), vanillic acid, orsellinic acid and homogentisic acid, the most likely compound is DOPAC, as evidenced by t_R matching with a commercial standard. Compound F-2-6 is likely a derivative of DOPAC because of the fragment ion at *m/z* 167

corresponding to the deprotonated form of DOPAC and its decarboxylated ion at *m/z* 123.

Hydroxycinnamic Acids

Compound F-2-11 yielded a molecular ion at *m/z* 179 and its decarboxylated ion at *m/z* 135. It is further confirmed as *trans*-caffeic acid by t_R matching with a commercial standard. Compound F-2-22 was identified as *p*-coumaric acid by the standard (λ_{max} = 310 nm), resulting in a $[M - H]^-$ at *m/z* 163 and a classic fragment at *m/z* 119 through neutral loss of CO_2. Compound F-2-25 displayed a maximum UV absorption band at 324 nm and was further confirmed as ferulic acid by a commercial standard. It gave a $[M - H]^-$ at *m/z* 193 together with fragment ions at *m/z* 178 $[M - H - 15\ (CH_3)]^{-\bullet}$, 149 $[M - H - 44\ (CO_2)]^-$ and 134 $[M - H - 44\ (CO_2) - 15\ (CH_3)]^{-\bullet}$.

Hydroxybenzoic acids, their esters and phenyllactic acid

Compounds F-2-4 and -7 both yielded a molecular ion at *m/z* 181. Further dissociation ions of compound F-2-4 were present at *m/z* 163 $[M - H - 18\ (H_2O)]^-$, 153 $[M - H - 28\ (CH_2CH_2)]^-$, 137 $[M - H - 44\ (C_2H_4O)]^-$, and 109 $[M - H - 72\ (COOCH_2CH_2)]^-$. The tentative candidate was ethyl protocatechuate. This compound in peanut seed testa was identified by NMR analysis performed by Huang *et al.* (2003). Compound F-2-7 gave fragment ions at *m/z* 163 $[M - H - 18\ (H_2O)]^-$ and 137 $[M - H - 44\ (CO_2)]^-$, and is likely DL-3-(4-hydroxyphenyl)lactic acid. Compound F-2-5 was confirmed by a commercial standard to be protocatechuic acid (λ_{max} = 260 nm), producing a molecular ion at *m/z* 153 and a fragment ion at *m/z* 109 by loss of CO_2 from the carboxylic acid moiety. *p*-Hydroxybenzoic acid (compound F-2-9) was recovered in F2 and showed a molecular ion at *m/z* 137. Although no significant MS^2 fragments were observed, this

compound's identity was further confirmed by t_R matching with a commercial standard.

Procyanidins (Monomers to Tetramers)

Compounds F-2-1 and 2 are likely gallocatechin and a derivative, respectively. The molecular ion $[M - H]^-$ of gallocatechin was present at *m/z* 305, which further fragmented to product ions at *m/z* 261 $[M - H - 44]^-$ by loss of a $–CH_2–CHOH–$ group or neutral loss of CO_2, 219 $[M - H - 86]^-$ by loss of a $C_4H_4O_2$ from the A-ring plus 2H, and 179 $[M - H - 126]^-$ attributed to a $^{1,4}B^-$ ion. The conjugate $^{1,4}A^-$ fragment at *m/z* 125 represents the intact A-ring with two phenolic groups and is formed through a pathway as demonstrated by Miketova *et al*. (2006). Compounds F-2-8, 10 and 12 are stereoisomers with the same molecular ions at *m/z* 289. Classic fragments were observed at *m/z* 245 $[M - H - 44]^-$ by loss of a $–CH_2–CHOH–$ group or neutral loss of CO_2, 205 $[M - H - 84]^-$ by loss of $C_4H_4O_2$ from the A-ring, and 179 $[M - H - 110]^-$ by loss of $C_6H_6O_2$ (B-ring). The fragmentation mechanisms of (epi)catechin were described by Bravo *et al.* (2006). The compounds were further confirmed by commercial standards as (–)-catechin (compound F-2-8), (+)-catechin (compound F-2-10) and (–)-epicatechin (compound F-2-12), respectively. Compound F-2-13 was found to be (–)-epiafzelechin with a $[M - H]^-$ at *m/z* 273, which dissociated further into ions at *m/z* 229 $[M - H - 44]^-$ by loss of a $–CH_2–CHOH–$ group or neutral loss of CO_2, 187 $[M - H - 86]^-$ by loss of $C_4H_4O_2$ from A-ring plus 2H, and 137 $[M - H - 136]^-$ attributed to a $^{1,3}A^-$ ion. The identification was confirmed by t_R matching with a commercial standard.

Compounds F-2-19, 21, 26, 27, 28, 30 and 31 are probably PAC A-type dimers, yielding molecular ions at *m/z* 575; whereas, compound F-2-20 is likely a PAC B-type dimer with a $[M - H]^-$ at *m/z* 577. As aforementioned, PAC A-type trimers (*m/z* 863) can further be divided into two

categories according to the location of the A-bond. Compounds F-2-14, 29, 33, 36 and 44 are likely PAC A-type trimers with an A-type linkage between the middle and bottom units. Trimers with an A-type linkage between the top and middle units are candidates for compounds F-2-15 and 45. Compound F-2-42, yielding a $[M - H]^-$ at *m/z* 861, is likely a PAC trimer with two A-type linkages. The HRF fragment ions of the top unit were present at *m/z* 735; whereas, the RDA fragmentation of the base unit generated an ion at *m/z* 709. The QM cleavage between the top and middle units gave ions at *m/z* 575 $[M_T - H\ (576 - H)]^-$ and 285 $[M_E - 5H\ (290 - 5H)]^-$. The QM cleavage between the middle and base unit produced ions at *m/z* 289 $[M_T - H\ (290 - H)]^-$ and 571 $[M_E - 5H\ (576 - 5H)]^-$. The fragment at *m/z* 449 was generated by fragmentation of the QM ion at *m/z* 575 and HRF ion at *m/z* 735 via the mechanism of HRF (Gu *et al.*, 2003a).

Compounds F-2-32, 38, 39, 43, and 46 are PAC A-type tetramers, displaying doubly charged ions $[M - 2H]^{2-}$ at *m/z* 574. Noteworthy is that no significant fragments were observed for the determination of the flavan-3-ol connection sequence. Compound F-2-47 yielded a $[M - 2H]^{2-}$ at *m/z* 507. Though no significant MS^2 fragments were evident, the proposed candidate is a PAC A-type trimer with (epi)catechin gallate as its base unit.

Propelargonidin

Compound F-2-35 (*m/z* 559) is tentatively identified as a propelargonidin A-type dimer with (epi)afzelechin as its base unit. HRF fragmentation of the (epi)catechin top unit and the RDA fragmentation of the (epi)afzelechin base unit gave product ions at *m/z* 433 $[M - H - 126\ (C_6H_6O_3)]^-$ and 423 $[M - H - 136\ (C_8H_8O_2)]^-$, respectively. Diagnostic ions at *m/z* 273 $[M_T - H\ (274 - H)]^-$ and 285 $[M_E - 5H\ (290 - 5H)]^-$ were produced by QM fission. The fragmentation pathways of a similar propelargonidin A-type dimer, only with an (epi)afzelechin as its top unit,

was well described by Appeldoorn *et al.* (2009).

Prodelphinidins

Compound F-2-16 is likely a prodelphinidin dimer containing a B-bond linked to (epi)gallocatechin (top) and (epi)catechin (base) units. It yielded a $[M - H]^-$ of *m/z* 593 and further fragments at *m/z* 467 $[M - H - 126 (C_6H_6O_3)]^-$ by HRF at the top unit, 425 $[M - H - 168 (C_8H_8O_4)]^-$ by RDA at the top unit, 303 $[M_E - 3H (306 - 3H)]^-$, and 289 $[M_T - H (290 - H)]^-$. The fragmentation scheme of this B-type prodelphinidin dimer has been documented (Gu *et al.*, 2003b). Compound F-2-17 showed a $[M - H]^-$ at *m/z* 591 and is tentatively identified as a prodelphinidin dimer containing an A-bond linked to (epi)gallocatechin (top) and (epi)catechin (base) units. The HRF fragmentation of the (epi)gallocatechin unit and RDA fragmentation of the (epi)catechin unit yielded product ions at *m/z* 465 $[M - H - 126 (C_6H_6O_3)]^-$ and 439 $[M - H - 152 (C_8H_8O_3)]^-$, respectively. The QM cleavage ions were present at *m/z* 289 $[M_T - H (290 - H)]^-$ and 301 $[M_E - 5H (306 - 5H)]^-$. On the other hand, compound F-2-23 is a similar A-type prodelphinidin dimer but with (epi)catechin as its top unit and (epi)gallocatechin as its base unit. QM diagnostic ions were present at *m/z* 305 $[M_T - H (306 - H)]^-$ and 285 $[M_E - 5H (289 - 5H)]^-$. A product ion at *m/z* 423 $[M - H - 168 (C_8H_8O_4)]^-$ was generated by the RDA fragment of the base (epi)gallocatechin unit.

A molecular ion $[M - H]^-$ at *m/z* 605 was provided by compound F-2-18. It is likely a prodelphinidin A-type dimer with a (epi)catechin top unit connected to a 5-*O*-methyl (epi)gallocatechin. The HRF fragmentation of top (epi)catechin unit gave an anion at *m/z* 479 $[M - H - 126 (C_6H_6O_3)]^-$. The $^{0,2}IIA^-$ fragment from the base methyl (epi)gallocatechin was present at *m/z* 451 $[M - H - 154 (C_7H_6O_4)]^-$, which indicates that the methoxy group is attached to the 6

position on the A-ring. QM diagnostic ions were found at *m/z* 319 $[M_T - H\ (320 - H)]^-$ and 285 $[M_E - 5H\ (290 - 5H)]^-$. The tentative structure and fragmentation pathways for compound F-2-18 are depicted in **Figure 6.2A**. Similarly, the proposed candidates for compound F-2-41, which yielded a $[M - H]^-$ at *m/z* 607, are a prodelphinidin B-type dimer containing a (epi)catechin unit and a methyl (epi)gallocatechin unit or a prodelphinidin A-type dimer with two (epi)gallocatechin units. Unfortunately, the identification cannot be unequivocally assigned because of the lack of significant MS^2 fragments.

Compound F-2-34 yielded a $[M - H]^-$ at *m/z* 877, which was tentatively identified as a prodelphinidin trimer with a connection sequence of (epi)gallocatechin→A→(epi)catechin →A→(epi)catechin; here, A represents an A-type linkage. The HRF fragment from the (epi)gallocatechin top unit and the RDA fragment from the base (epi)catechin unit were present at *m/z* 751 $[M - H - 126\ (C_6H_6O_3)]^-$ and 725 $[M - H - 152\ (C_8H_8O_3)]^-$, respectively. An ion at *m/z* 599 was generated by RDA fragmentation of the HRF ion at *m/z* 751. QM diagnostic ions were present at *m/z* 575 $[M_T - H\ (576 - H)]^-$, 289 $[M_T - H\ (290 - H)]^-$, and *m/z* 301 $[M_E - 5H\ (306 - 5H)]^-$, 587 $[M_E - H\ (592 - 5H)]^-$. HRF fragmentation of QM cleavage ions at *m/z* 587 and 575 yielded anions at *m/z* 461 and 449, respectively. The tentative structure and fragmentation pathways for compound F-2-34 is depicted in **Figure 6.2B**. The tentative candidate for compound F-2-40 is likely a prodelphinidin trimer containing a luteolin or kaempferol base unit. Further fragmentation of $[M - H]^-$ at *m/z* 875 yielded ions at *m/z* 749 $[M - H - 126\ (C_6H_6O_3)]^-$ and 723 $[M - H - 152\ (C_8H_8O_3)]^-$ by HRF and RDA fragmentation, respectively, indicating a top (epi)catechin unit with a B-type linkage to the following (epi)gallocatechin unit. Unfortunately, no significant diagnostic QM ions were observed in the mass spectra to further support the assignment. The $[M - 2H]^{2-}$ at *m/z* 581 (compound F-2-48) was a doubly charged ion yielded by

a prodelphinidin tetramer with a molecular mass of 1164 Da. The diagnostic QM fragments were present at *m/z* 877 $[M_T - H\ (878 - H)]^-$, 575 $[M_T - H\ (576 - H)]^-$, and *m/z* 873 $[M_E - 5H\ (878 - 5H)]^-$, 285 $[M_E - 5H\ (290 - 5H)]^-$, indicating a connection sequence of (epi)catechin→A→(epi)gallocatechin→A→(epi)catechin→A→(epi)catechin. HRF of the QM ions at *m/z* 877 and 575 gave further dissociation ions at *m/z* 751 and 449, respectively. The proposed structure and fragmentation pathways for compound F-2-48 are shown in **Figure 6.2 C**.

Flavonoid

The proposed candidate for compound F-2-37 is myricetin, which was further confirmed by t_R matching with a commercial standard. Its $[M - H]^-$ at *m/z* 317 dissociated into fragments at *m/z* 271 $[M - H - 18\ (H_2O) - 28\ (CO)]^-$, 243 $[M - H - 18\ (H_2O) - 56\ (2CO)]^-$ and 227 $[M - H - 18\ (H_2O) - 28\ (CO) - 44\ (CO_2)]^-$. RDA diagnostic ions at *m/z* 179, 151, and 137 were noted as $^{1,2}A^-$, $^{1,3}A^-$, and $^{1,2}B^-$, respectively.

Stilbene

Compound F-2-24 was tentatively identified as *trans*-piceatannol by a molecular ion $[M - H]^-$ at *m/z* 243. Further fragment ions were present at *m/z* 225 $[M - H - 18\ (H_2O)]^-$, 201 $[M - H - 42\ (CHCOH)]^-$, 199 $[M - H - 42\ (CHCOH) - 2H]^-$, 175 $[M - H - 68\ (C_3O_2)]^-$, 173 $[M - H - 42\ (CHCOH) - 28\ (CO)]^-$ and 159 $[M - H - 42\ (CHCOH) - 42\ (C_2H_2O)$ or $M - H - 42\ (CHCOH) - 2H - 40\ (C_2O)]^-$. This is the CID fragmentation of $[M - H]^-$ when the deprotonation reaction occurs on the catechol moiety of *trans*-piceatannol. These fragmentation mechanisms have been elucidated and described by Stella *et al.* (2008). Similar MS^2 fragments for *trans*-piceatannol in peanuts were reported by Ku *et al.* (2005).

Glycoside-bound Phenolics

Phenolic Acids

Compound F-3-3 with a molecular ion $[M - H]^-$ at *m/z* 167 and a fragment ion at *m/z* 123 is likely DOPAC, as discussed for F2. Compound F-3-5 was identified as protocatechuic acid by its $[M - H]^-$ at *m/z* 153 and the decarboxylated ion at *m/z* 109 $[M - H - 44\ (CO_2)]^-$. Compound F-3-6 may be a protocatechuic acid derivative: it yielded a $[M - H]^-$ at *m/z* 233 and a prominent fragment ion at *m/z* 153, corresponding to deprotonated protocatechuic acid. Compound F-3-8, giving a molecular ion at *m/z* 137, was identified as *p*-hydroxybenzoic acid by UV spectra (λ_{max} = 256 nm) and t_R matching with a commercial standard.

Compound F-3-11 is confirmed as vanillic acid by UV spectra (λ_{max} = 260 nm) and t_R matching with a commercial standard. It yielded a $[M - H]^-$ at *m/z* 167 and a fragment ion at *m/z* 123 by loss of CO_2 from the carboxylic acid moiety. Other dissociation ions were present at *m/z* 152 $[M - H - 15\ (CH_3)]^{-\bullet}$ and 108 $[M - H - 44\ (CO_2) - 15\ (CH_3)]^{-\bullet}$. F-3-22 is likely homovanillic acid with a $[M - H]^-$ at *m/z* 181, which dissociated further into fragments at *m/z* 137 $[M - H - 44\ (CO_2)]^-$ and 122 $[M - H - 44\ (CO_2) - 15\ (CH_3)]^{-\bullet}$.

Proanthocyanidins (PAC) (Monomers to Dimers)

Compounds F-3-1 and F-3-2 are identified as gallocatechin and a derivative, as in F2. Compound F-3-4 is likely methyl gallate, yielding a $[M - H]^-$ at *m/z* 183 and a fragment ion at 124 $[M - H - 44\ (CO_2) - 15\ (CH_3)]^{-\bullet}$. Compounds F-3-9 and F-3-14 are (+)-catechin and (–)-epicatechin, respectively, as described for F2 and further confirmed by t_R matching with commercial standards. Compounds F-3-21, 23, 27, 32, 35, 36 and 40 are likely PAC A-type dimers with molecular ions at *m/z* 575 and QM ions at *m/z* 289 $[M_T - H\ (290 - H)]^-$ and 285 $[M_E$

– 5H (290 – 5H)]$^-$.

Propelargonidins

Compound F-3-24 (*m/z* 847) is likely a propelargonidin A-type trimer with a (epi)afzelechin middle unit. QM cleavage ions were present at *m/z* 289 [M_T – H (290 – H)]$^-$ and 557 [M_E – 3H(560 – 3H)]$^-$, indicating the presence of a B-type linkage between the middle (epi)afzelechin unit and the base (epi)catechin unit. Compound F-3-52 yielded a [M – H]$^-$ at *m/z* 845 and was tentatively identified as a propelargonidin trimer with one (epi)afzelechin top unit and two A-type linkages. QM cleavage of this trimer resulted in fragments at *m/z* 289 [M_T – H (290 – H)]$^-$, 575 [M_T – H (576 – H)]$^-$ and 269 [M_E – 5H (273 – 5H)]$^-$, 555 [M_E – 5H (560 – 5H)]$^-$. The fragmentation pathways for these two propelargonidin oligomers are well documented (Gu *et al*., 2003a; Appeldoorn *et al.*, 2009;).

Proanthocyanidins (PAC) Containing an Additional Phloroglucinol Unit.

Compounds F-3-10, 12, 15, 17, 18, 20, 25, 26, and 37 are likely A-type PAC dimers containing an additional phloroglucinol unit, which was reported in cranberry by Foo *et al.* (2000). These compounds yielded molecular ions at *m/z* 699, which dissociated further into fragments at *m/z* 573 [M – H – 126]$^-$ by losing a phloroglucinol moiety ($C_6H_6O_3$) from the top (epi)catechin unit via the HRF function, 547 [M – H – 152]$^-$ by losing $C_8H_8O_3$ from the base (epi)catechin-phloroglucinol unit via the RDA function, and 531 [M – H – 168]$^-$ by losing $C_8H_8O_4$ from the base (epi)catechin-phloroglucinol unit. Product ions at *m/z* 411 and 287 were generated by HRF fragmentation at the base (epi)catechin-phloroglucinol unit. A QM diagnostic ion was present at *m/z* 285 [M_E – 5H (290 – 5H)]$^-$. Interesting is that the conjugate ion at *m/z* 413

was not observed. Our identification is further supported by the MS^3 experiment. Compounds F-3-28, 29, and 31 (*m/z* 657) appear to be generated by losing a C_2H_2O group (42 Da) from the additional phloroglucinol unit of the above described PAC dimers. Compounds F-3-33 and 50 (*m/z* 547) are also likely fragments from the aforementioned dimers by RDA at the base (epi)catechin-phloroglucinol unit. Furthermore, compounds F-3-39, 44, 48, and 51 (*m/z* 531) are tentatively identified as fragments from this dimer after loss of $C_8H_8O_4$ from the base (epi)catechin-phloroglucinol unit. Such cleavages can be induced by heat and/or acid hydrolysis applied to release phenolics bound to glycosides.

Compounds F-3-13, 16, and 19 are tentatively identified as a similar PAC dimer, but the top (epi)catechin and the base (epi)catechin-phloroglucinol units are connected by a B-type linkage. The $[M - H]^-$ was present at *m/z* 701. Diagnostic ions were evident at *m/z* 289 $[M_T - H (290 - H)]^-$ and 411 $[M_B - 3H (414 - 3H)]^-$, indicating a fragmentation pathway as described by Sarnoski *et al.* (2012) whereby QM cleavage is blocked by a carbonyl group on the C-ring of the base luteolin or kaempferol group. In the present study, the presence of an additional phloroglucinol unit, which is connected to the C-ring of the base (epi)catechin unit, inhibits QM fission. Our identification is further supported by the MS^3 experiment. Compounds F-3-42 and 47 (*m/z* 549) are likely fragments from the aforementioned B-type dimers by RDA at the base (epi)catechin-phloroglucinol unit.

The proposed candidate for compounds F-3-34 and 41 (*m/z* 683) is a PAC dimer with an (epi)catechin unit connected to a (epi)afzelechin-phloroglucinol unit through an A-type bond. HRF fragmentation ions of this dimer were present at *m/z* 557 $[M - H - 126 (C_6H_6O_3)]^-$ and 411 $[M - H - 272 (C_{15}H_{12}O_5)]^-$, which were assigned to HRF at the top (epi)catechin unit and the base (epi)afzelechin-phloroglucinol unit, respectively. Other product ions were observed at *m/z*

515 $[M - H - 168]^-$ by loss of $C_8H_8O_4$ from the base (epi)afzelechin-phloroglucinol unit and 423 $[M - H - 126 (C_6H_6O_3) - 136 (C_8H_8O_2)]^- + 2H$ by combination of HRF (at the top (epi)catechin unit) and RDA (at the base (epi)afzelechin-phloroglucinol unit) fragmentations. QM fission resulted in fragments at *m/z* 397 $[M_T - H (398 - H)]^-$ and 285 $[M_E - 5H (290 - 5H)]^-$. Our assignment is further supported by the MS^3 experiment. Compounds F-3-45, 66, and 69 (*m/z* 557) are likely fragments from the aforementioned dimers by HRF at the top (epi)catechin unit. Further dissociation ions were present at *m/z* 431 $[M - H - 126 (C_6H_6O_3)]^-$ by loss of the additional phloroglucinol group, 389 $[M - H - 168 (C_8H_8O_4)]^-$ and 285 $[M - H - 272 (C_{15}H_{12}O_5)]^-$ by HRF at the base (epi)afzelechin-phloroglucinol unit. These type of fragments can be caused by heat and/or acid applied in deglycosylation. The proposed structures and fragmentation pathways of the above three types of PACs containing an additional phloroglucinol unit are depicted in **Figure 6.3A**, **B**, and **C**, respectively.

Proanthocyanidin (PAC) Containing a novel Flavan-3-ol Unit.

Compound F-3-38 is likely a PAC dimer with a catechin connected to a novel flavan-3-ol unit through an A-type linkage. It yielded a $[M - H]^-$ at m/z 589, which further dissociated into ions at *m/z* 463 $[M - H - 126 (C_6H_6O_3)]^-$ by HRF at the top catechin unit and 453 $[M - H - 136 (C_8H_8O_2)]^-$ by RDA at the base unit. QM diagnostic ions were present at *m/z* 303 $[M_T - H (304 - H)]^-$, and 285 $[M_E - 5H (290 - 5H)]^-$. Our identification is further supported by the MS^3 experiment; nevertheless, further analyses are necessary to support the assignment. The tentative structure and fragmentation scheme are shown in **Figure 6.4A**. Compound F-3-74 is probably an unknown PAC derivative due to the product ions at *m/z* 441 and 285, which could be assign to a HRF ion and a QM ion, respectively.

Prorobinetidin and Prodelphinidin

As shown in **Figure 6.4B**, compounds F-3-55, 57, 62, 64 and 68 (*m/z* 589) are tentatively identified as a prorobinetidin dimer with a base unit most likely being a C-methyl (epi)robinetinidol A-linked to an (epi)catechin top unit. The HRF fragment ion of the (epi)catechin unit and RDA fragment ion of the C-methyl (epi)robinetinidol unit were present at *m/z* 463 $[M - H - 126 (C_6H_6O_3)]^-$ and 421 $[M - H - 168 (C_8H_8O_4)]^-$, respectively. QM cleavage gave rise to diagnostic ions at *m/z* 303 $[M_T - H (304 - H)]^-$ and 285 $[M_E - H (290 - 5H)]^-$, corresponding to C-methyl (epi)robinetinidol and (epi)catechin units, respectively. Our identification was further supported by the MS^3 experiment. The proposed candidate for compound F-3-58 is a prodelphinidin B-type dimer, as discussed in F2.

Flavonols and Flavanonols

Compound F-3-7 yielded a molecular ion $[M - H]^-$ at *m/z* 301 and fragments at *m/z* 283 $[M - H - 18 (H_2O)]^-$, 257 $[M - H - 44 (CO_2)]^-$, and 229 $[M - H - 44 (CO_2) - 28 (CO)]^-$. $^{1,2}B^-$ and $^{1,2}A^-$ ions were present at *m/z* 163 and 137, respectively. The likely candidate is robinetin. Compound F-3-78 yielded a $[M - H]^-$ at *m/z* 343 which further dissociated into ions at *m/z* 328 $[M - H - 15 (CH_3)]^{-\bullet}$, 313 $[M - H - 30 (2CH_3)]^-$, 299 $[M - H - 15 (CH_3) - 29 (HCO)]^-$, 285 $[M - H - 30 (2CH_3) - 28 (CO)]^-$, and 135 attributed to a $^{1,3}A^-$ fragment. The proposed candidate is 3′,4′,5′-trimethylrobinetin.

Compound F-3-63 is identified as quercetin with a $[M - H]^-$ at *m/z* 301 that fragmented into ions at *m/z* 273 $[M - H - 28 (CO)]^-$, 257 $[M - H - 44 (CO_2)]^-$, and 229 $[M - H - 44 (CO_2) - 28 (CO)]^-$. The $^{1,2}A^-$ ion at *m/z* 179 further yielded ions at *m/z* 151 noted as $^{1,2}A^- - CO$ and 107 noted as $^{1,2}A^- - CO - CO_2$. This compound was further confirmed by UV spectra (λ_{max} = 256 and

370 nm) and t_R matching with a commercial standard. Compound F-3-77 is likely a quercetin methyl ether such as isorhamnetin with a molecular ion $[M - H]^-$ at *m/z* 315 and a fragment at *m/z* 300 $[M - H - 15\ (CH_3)]^{-\bullet}$. The MS^3 fragments of the ion at *m/z* 315 were present at *m/z* 300 $[M - H - 15\ (CH_3)]^{-\bullet}$, 271 $[M - H - 15\ (CH_3) - 29\ (HCO)]^-$, and 256 $[M - H - 44\ (CO_2) - 15\ (CH_3)]^{-\bullet}$. The $^{1,3}A^-$ ion at *m/z* 151 is a diagnostic ion observed in CID fragmentation of isorhamnetin by ESI-MS (Justesen, 2000 & 2001).

Compound F-3-75 is kaempferol, which gave a $[M - H]^-$ at *m/z* 285 and fragments at 257 $[M - H - 28\ (CO)]^-$, 241 $[M - H - 44\ (CO_2)]^-$, 229 $[M - H - 56\ (2CO)]^-$, 213 $[M - H - 44\ (CO_2) - 28\ (CO)]^-$, 199 $[M - H - 42\ (C_2H_2O) - 44\ (CO_2)]^-$, and 151 assigned to a $^{1,3}A^-$ fragment. Identification of kaempferol was confirmed by its UV absorption spectrum and t_R matching with a commercial standard. Fabre *et al.* (2001) described the fragmentation pathways of kaempferol by ESI-MS. Compound F-3-43 is tentatively identified as fisetin. Fragments produced from $[M - H]^-$ at *m/z* 285 were present at *m/z* 257 $[M - H - 28\ (CO)]^-$, 241 $[M - H - 44\ (CO_2)]^-$, 229 $[M - H - 56\ (2CO)]^-$, 213 $[M - H - 44\ (CO_2) - 28\ (CO)]^-$, 177 $[M - H - \text{ring B}]^-$. RDA cleavage at the 1/2 position on the C-ring generated ions $^{1,2}A^-$ (*m/z* 163) and $^{1,2}B^-$ (*m/z* 121), which are highly consistent with the dissociation routes described by Fabre *et al.* (2001). The loss of a CO molecule from $^{1,2}A^-$ ion leads to a fragment at *m/z* 135 noted as $^{1,2}A^- - CO$. Compound F-3-49 yielded a $[M - H]^-$ at *m/z* 317 and was identified as myricetin as described in F2.

Compound F-3-30 yielded a $[M - H]^-$ at *m/z* 303 that further fragmented into ions at *m/z* 285 $[M - H - 18\ (H_2O)]^-$, 259 $[M - H - 44\ (CO_2)]^-$, 241 $[M - H - 18\ (H_2O) - 44\ (CO_2)]^-$, and 217 $[M - H - 18\ (H_2O) - 68\ (C_3O_2)]^-$. RDA cleavage at the 1/4 position on the C-ring gave rise to ions $^{1,4}B^-$ (*m/z* 177) and $^{1,4}A^-$ (*m/z* 125). The proposed candidate is taxifolin.

Flavanones, Flavones, and Isoflavones

Compound F-3-65 is tentatively identified as hesperetin. It yielded a $[M - H]^-$ at *m/z* 301 and further dissociation fragments at *m/z* 286 $[M - H - 15\ (CH_3)]^{-\bullet}$, 283 $[M - H - 18\ (H_2O)]^-$, 257 $[M - H - 15\ (CH_3) - 29\ (HCO)]^-$, 242 $[M - H - 44\ (CO_2) - 15\ (CH_3)]^{-\bullet}$, and 215 $[M - H - 42\ (C_2H_2O) - 44\ (CO_2)]^-$. RDA diagnostic ions were present at *m/z* 164, 151 and 125, which were ascribed to $^{1,2}A^-$, $^{1,3}A^-$, and $^{1,4}A^-$, respectively. Further loss of CO_2 from $^{1,3}A^-$ generated an ion at *m/z* 107, noted as $^{1,3}A^- - CO_2$. It undergoes the similar fragmentation route of hesperetin as reported by Justesen (2000). Compound F-3-71 (*m/z* 301) is likely homoeriodictyol, producing RDA diagnostic ions at *m/z* 151 assigned to $^{1,3}A^-$. Compound F-3-61 is likely isosakuranetin with a $[M - H]^-$ at *m/z* 285 and further dissociation ions at *m/z* 270 $[M - H - 15\ (CH_3)]^{-\bullet}$, 243 $[M - H - 42\ (C_2H_2O)]^-$, 226 $[M - H - 44\ (CO_2) - 15\ (CH_3)]^{-\bullet}$, and 199 $[M - H - 42\ (C_2H_2O) - 44\ (CO_2)]^-$. RDA cleavage at the 0/4 position of the C-ring after loss of $CH_3^\bullet$ gave a diagnostic ion at *m/z* 164. This $-CH_3{}^{0,4}B^{-\bullet}$ ion lost a further CO molecule, leading to a fragment noted as $-CH_3{}^{0,4}B^{-\bullet} - CO$ (*m/z* 136).

The proposed candidate of compound F-3-53 is 4′,3′,5,6-trihydroxy-7-methoxyflavone. It yielded a $[M - H]^-$ at *m/z* 315 that dissociated further into fragments at 300 $[M - H - 15\ (CH_3)]^{-\bullet}$ and 191 $[M - H - 109\ (B\text{-ring}) - 15\ (CH_3)]^{-\bullet}$. Compound F-3-74 is likely a flavone dimethyl ether such as 3′,5,6-trihydroxy-4′,7-dimethoxyflavone, which gave a $[M - H]^-$ at *m/z* 329. It dissociated further into fragments at *m/z* 314 $[M - H - 15\ (CH_3)]^{-\bullet}$, 299 $[M - H - 30\ (2CH_3)]^-$, and 191 $[M - H - 15\ (CH_3) - 123\ (B\text{-ring})]^-$. Unfortunately, the actual position of the methoxy group for the above two compounds cannot be confirmed without a commercial standard or NMR analysis. Compound F-3-54 yielded a $[M - H]^-$ at *m/z* 283 and a prominent fragment at *m/z* 268 $[M - H - 15\ (CH_3)]^{-\bullet}$. The proposed candidate is genkwanin. An RDA diagnostic ion

$-CH_3{}^{1,3}A^{-\bullet}$ was present at *m/z* 151, with the identification being further confirmed by a UV λ_{max} at 338 nm (Skoula, Grayer, Kite & Veitch, 2008). Compound F-3-76 is likely a luteolin methyl ether such as diosmetin with a $[M - H]^-$ at *m/z* 299 and a prominent fragment *m/z* 284 [M – H – 15 $(CH_3)]^{-\bullet}$. MS^3 [299→284] fragments were observed at *m/z* 256 [M – H – 15 (CH_3) – 28 $(CO)]^{-\bullet}$, 240 [M – H – 15 (CH_3) – 44 $(CO_2)]^{-\bullet}$, 216 [M – H – 15 (CH_3) – 68 $(C_3O_2)]^{-\bullet}$, and 151 attributed to a $^{1,3}A^-$ fragment. The identification is supported by its UV spectra of 254(*sh*), 266, and 348 nm (Brad & Chen, 2013). The fragmentation pathways for diosmetin were described by Justesen (2001).

Compound F-3-60 is likely biochanin A. It yielded a $[M - H]^-$ at *m/z* 283 and a prominent fragment at *m/z* 268 [M – H – 15 $(CH_3)]^{-\bullet}$. Further MS^3 [283→268] fragmentation generated ions at *m/z* 267 [M – H – 15 (CH_3) – H]$^{-\bullet}$, 239 [M – H – 15 (CH_3) – H – 28 $(CO)]^-$, 224 [M – H – 15 (CH_3) – H – 44 $(CO_2)]^-$, and 135 [A-ring fragment]$^-$, which are consistent with the MS^3 spectra of biochanin A obtained by Kang *et al.* (2007). Biochanin A is an *O*-methylated isoflavone and it has long been reported in peanuts (Chukwumah *et al.*, 2007). Compound F-3-72 exhibited a $[M - H]^-$ at *m/z* 299. Further fragment ions were observed at *m/z* 284 [M – H – 15 $(CH_3)]^{-\bullet}$, 255 [M – H – 15 (CH_3) – 29 $(CHO)]^-$, 177 [M – H – B-ring]$^-$, and 151 attributed to a $^{1,3}A^-$ ion. Proposed candidate is pratensein; that is, 3′,5,7-trihydroxyisoflavone-4′-methoxy-3′-*O*-β-glucopyranoside, which has been found in peanut skin according to NMR (Lou *et al.*, 2001).

Biflavonoid

Compounds F-3-46, 56, 59, and 67 are likely a daphnodorin H-type biflavonoid composed of eriodictyol (Part I) and C-methyl (epi)robinetinidol (Part II) units. They gave a $[M - H]^-$ at *m/z* 587 and fragment ions at *m/z* 569 [M – H – 18 $(H_2O)]^-$, 461 assigned to a $^{1,4}IB^-$ fragment,

and 419 attributed to a $^{1,3}IIA^-$ fragment. The direct cleavage of the interflavan bond generated fragments at *m/z* 285 [eriodictyol unit (288 – 3H)]$^-$ and 301 [C-methyl (epi)robinetinidol unit (304 – 3H)]$^-$. This identification is further supported by the MS^3 experiment; however, NMR analysis is required to support the assignment. The tentative structure and fragmentation are present in **Figure 6.4C**.

Stilbene.

Compound F-3-70 is tentatively identified as *trans*-3,3′,5,5′-tetrahydroxy-4′-methoxystilbene. It yielded a $[M - H]^-$ at *m/z* 273 and further dissociation ions at *m/z* 258 $[M - H - 15 (CH_3)]^{-\bullet}$, 163 $[M - H - 68 (C_3O_2) - 42 (C_2H_2O)]^-$, 136 $[M - H - 137 (C_7H_5O_3)]^-$, and 109 $[M - H - 165 (C_9H_9O_3)]^-$.

Quantification.

Phenolic compounds were quantified based on peak areas of the observed UV signals from the HPLC chromatograms and calibration curves of commercial standards. Mean phenolic contents for the identified compounds are reported in **Table 6.4**. All values are expressed in mg respective phenolic compound/100-g dry weight (d.w.) of PS.

Protocatechuic acid was determined to be the most dominant in the non-PAC phenolics profile, and comprised 6.62% and 6.48% of the soluble-ester and glycoside-bound phenolic compounds, respectively. Kaempferol, fisetin, quercetin, isorhamnetin (flavonols), genkwanin, and dosmetin (flavones) are quantifiable flavonoids aglycones liberated from glycosides, and account for 1.54% of the total glycoside-bound phenolic compounds. Quercetin and its methyl ether (isorhamnetin) are primary flavonoid aglycones and constituted 57.1% of the total

recovered flavonoids aglycones. As aforementioned, PACs possess the lion's share of the phenolics in PS (Ma *et al.*, 2014) and are the major phenolics released from esters and glycosides. PAC monomers {(+)-catechin and (–)-epicatechin}, dimers, trimers, tetramers constituted 9.65%, 60.0%, 6.38%, and 15.9% of soluble-ester phenolic compounds, respectively. The PAC monomer {(+)-catechin} and dimers are primary phenolics released from glycosides and account for 31.7% and 59.1% of total glycoside-bound phenolic compounds.

It should be noted that the quantification can be easily affected by many factors involved in the details of the extraction and fractionation protocols selected. As for flavonoids, strong acid hydrolysis may degrade unstable aglycones, while on the other hand, some of the cell wall-bound glycosides may not be hydrolyzed completely yielding inconsistent results (Hertog, Peter, Hollman, & Venema, 1992; Nuutila, Kammiovirta & Oksman-Caldentey, 2002). The cleavage of the C–C interflavan bond between monomeric flavan-3-ol constituents can result from severe alkaline conditions (White, Howard, & Prior, 2010). This may possibly explain why a large portion of PAC monomers and dimers were found in the profile of soluble-ester and glycoside-bound phenolic compounds. According to our MS data, there are also a number of fragments resulting from PACs containing an additional phloroglucinol unit due to heat and/or acid treatment, which would lead to underestimation of their quantity. PACs can be modified by thermal treatment. Yu *et al.* (2006) reported a significant influence of the skin removal methods on the PACs content of PS. Dry heat, like that imparted during blanching of red-skin peanuts, would increase A-type dimers and B-type trimers compared to directly peeled PS due to monomeric polymerization or the degradation of trimers and tetramers.

6.4 Conclusions

In conclusion, a large variety of compounds were liberated from soluble esters and glycosides in PS. They were further separated and detected by HPLC-ESI-MS^n. Compounds identified included PACs (monomers to tetramers), free phenolic acids (*e.g.*, hydroxybenzoic acids and *trans*-cinnamic acids), stilbenes, and flavonoids. To our knowledge this is the first reporting of PACs in PS containing an additional phloroglucinol unit. Although further analytical techniques are required for the final confirmation of some of the compounds identified, our data enriches the phenolic database of PS and perhaps provides a justification as to why PS might be considered as a valuable functional food ingredient.

Acknowledgements

This research was supported by a grant funded by the Georgia Food Industry Partnership and is greatly appreciated. Use of the Proteomics and Mass Spectrometry (PAMS) core facility under the direction of Dr. Dennis Phillips of UGA's Department of Chemistry is acknowledged. Thanks are also extended to Bill Paulk at Sylvester Blanching, a division of Universal Blanchers, LLC, for the dry-blanched PS and John T. Powell, President of the Peanut Institute Inc., Albany, GA.

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Table 6.1 Tentative identification of phenolic compounds in dry-blanched peanut skins (PS) crude extracts (CE) by C_{18} RP-HPLC-ESI-MS^2.

Cmpd No.	Tentative identification[a]	t_R[b] (min)	$[M-H]^-$	$[M-H]^{2-}/2$	Product ions[c]
CE-1-1	PAC A-type trimer [(E)C→B→(E)C→A→(E)C]	3.6	863		737, 711, 693, **575**, 559, 449, 287
CE-1-2	PAC A-type trimer [(E)C→B→(E)C→B→(E)C]	3.7	865		739, 713, **695**, 577, 575, 451, 287
CE-1-3	PAC A-type trimer [(E)C→B→(E)C→A→(E)C]	3.8	863		711, 693, 575, 559, 287
CE-1-4	PAC A-type trimer [(E)C→A→(E)C→B→(E)C]	3.8	863		711, 693, 573, 451, 411, 289
CE-1-5	PAC A-type pentamer [(E)C→B→(E)C→B→(E)C→A→(E)C→B→(E)C]	3.9	1439	719	863, 575, 573
CE-1-6	PAC B-type dimer [(E)C→B→(E)C]	11.6	577		559, 451, **425**, 407, 289, 287
CE-1-7	PAC A-type trimer [(E)C→B→(E)C→A→(E)C]	14.6	863		845, 711, 693, **575**, 559, 449, 287
CE-1-8	PAC A-type trimer [(E)C→A→(E)C→B→(E)C]	14.6	863		711, 693, 451, 411, 289
CE-1-9	PAC A-type trimer [(E)C→A→(E)C→B→(E)C]	16.2	863		**711**, 693, 573, 531, 451, 411, 289
CE-1-10	PAC B-type dimer [(E)C→B→(E)C]	19.3	577		559, 451, **425**, 407, 289, 287
CE-1-11	PAC A-type trimer [(E)C→B→(E)C→A→(E)C]	20.9	863		845, 737, 711, 693, 575, 559, 449, 287
CE-1-12	PAC A-type trimer [(E)C→A→(E)C→B→(E)C]	20.9	863		737, **711**, 693, 573, 411, 289
CE-1-13	PAC A-type dimer [(E)C→A→(E)C]	21.0	575		557, 539, **449**, 423, 407, 289, 285
CE-1-14	PAC A-type pentamer [(E)C→B→(E)C→B→(E)C→A→(E)C→B→(E)C]	21.2	1439	719	863, 575, 573, 287
CE-1-15	PAC A-type pentamer [(E)C→B→(E)C→A→(E)C→B→(E)C→B→(E)C]	21.3	1439	719	861, 577, 575, 287
CE-1-16	PAC A-type tetramer [(E)C→A→(E)C→A→(E)C→B→(E)C]	21.5	1149	574	997, 859
CE-1-17	PAC A-type dimer [(E)C→A→(E)C]	22.1	575		557, 539, **449**, 423, 407, 289,

					285
CE-1-18	PAC A-type trimer [(E)C→B→(E)C→A→(E)C]	22.5	863		711, 693, 575, 559, 449, 287
CE-1-19	PAC A-type trimer [(E)C→A→(E)C→B→(E)C]	22.5	863		**711**, 693, 573, 531, 451, 411, 289
CE-1-20	PAC A-type tetramer [(E)C→B→(E)C→A→(E)C→A→(E)C]	24.7	1149	574	997, 861
CE-1-21	PAC A-type dimer [(E)C→A→(E)C]	25.2	575		557, 539, **449**, 423, 289, 285
CE-1-22	PAC A-type trimer [(E)C→B→(E)C→A→(E)C]	25.7	863		737, 711, 693, **575**, 559, 449, 287
CE-1-23	PAC A-type trimer [(E)C→A→(E)C→B→(E)C]	26.1	863		737, **711**, 693, 573, 531, 451, 411, 289
CE-1-24	PAC A-type dimer [(E)C→A→(E)C]	27.4	575		**449**, 423, 289, 285
CE-1-25	316-Da flavonoid such as Isorhamnetin + rutinoside sugar side-group	27.7	623		356, **315**, 300, 271, 255 MS^3 [315→300]: **300**, 271, 151
CE-1-26	PAC A-type dimer [(E)C→A→(E)C]	28.4	575		557, 539, **449**, 423, 407, 289, 285
CE-1-27	PAC A-type dimer [(E)C→A→(E)C]	29.6	575		557, 539, **449**, 423, 407, 289, 285

[a]The A represents an A-type bond with both (C4→C8) and (C2→O→C7) linkages or (C4→C6) and (C2→O→C7) linkages, B represents a B-type bond which can be (C4→C8) or (C4→C6) linkage; (E)C represents (epi)catechin.

[b]Retention times (t_R) of the total ion chromatograms.

[c]The most abundant ions observed in the mass spectra are indicated in bold; quinone methide (QM) fission diagnostic ions are underlined.

Table 6.2 Tentative identification of soluble-ester-bound phenolic compounds in dry-blanched peanut skins (PS) crude extracts by C_{18} RP-HPLC-ESI-MS^2.

Cmpnd No.	Tentative identification[a]	UV λ_{max} (nm)[b]	t_R[c] (min)	$[M-H]^-$	$[M-H]^{2-}/2$	Product ions[d]
F-2-1	Gallocatechin		3.2	305		261, 219, **179**, 125
F-2-2	Unknown (likely a Gallocatechin derivative)		5.8	305		261, 219, **179**, 125
F-2-3	3,4-Dihydroxyphenylacetic acid (DOPAC)		8.2	167		**123**
F-2-4	Ethyl protocatechuate		8.7	181		163, **153**, 137, 109
F-2-5	Protocatechuic acid	260, 294	9.7	153		**109**
F-2-6	Unknown DOPAC derivative		10.5	357		315, 273, 249, **189**, 167, 123
F-2-7	DL-3-(4-Hydroxyphenyl)lactic acid		11.9	181		163, **137**
F-2-8	(–)-Catechin	234, 280	12.5	289		**245**, 205, 179, 125
F-2-9	*p*-Hydroxybenzoic acid		13.5	137		
F-2-10	(+)-Catechin	234, 280	13.9	289		**245**, 205, 179, 125
F-2-11	*trans*-Caffeic acid		17.3	179		**135**
F-2-12	(–)-Epicatechin	234, 280	17.7	289		**245**, 205,179, 125
F-2-13	(–)-Epiafzelechin		19.3	273		255, **229**, 187, 166, 137, 123
F-2-14	PAC A-type trimer [(E)C→B→(E)C→A→(E)C]	234, 280	19.6	863		737, 711, 693, **575**, 559, 449, 287
F-2-15	PAC A-type trimer [(E)C→A→(E)C→B→(E)C]		19.9	863		737, **711**, 693, 573, 531, 451, 411, 289
F-2-16	Prodelphinidin B-type dimer [(E)GC→B→(E)C]		20.4	593		575, 467, **425**, 407, 303, 289
F-2-17	Prodelphinidin A-type dimer [(E)GC→A→(E)C]		20.8	591		573, **465**, 439, 423, 301, 289
F-2-18	Prodelphinidin A-type dimer [(E)C→A→5-*O*-methyl (E)GC]	230, 282	21.6	605		587, 571, **479**, 451, 353, 319, 301, 285
F-2-19	PAC A-type dimer [(E)C→A→(E)C]	234, 280	22.2	575		557, 539, **449**, 423, 407,

						289, 285
F-2-20	PAC B-type dimer [(E)C→B→(E)C]		22.6	577		559, **451**, 425, 407, 289, 287
F-2-21	PAC A-type dimer [(E)C→A→(E)C]	234, 280	22.9	575		557, 539, **449**, 423, 407, 289, 285
F-2-22	*p*-Coumaric acid	234, 310	23.2	163		**119**
F-2-23	Prodelphinidin A-type dimer [(E)C→A→(E)GC]		23.2	591		573, **465**, 423, 305, 285
F-2-24	*trans*-Piceatannol		24.2	243		243, 215, **225**, 201, 199, 175, 173, 159
F-2-25	Ferulic acid	238, 292*sh*,322	26.0	193		178, 149, **134**
F-2-26	PAC A-type dimer [(E)C→A→(E)C]	234, 280	26.3	575		557, 539, **449**, 423, 407, 289, 285
F-2-27	PAC A-type dimer [(E)C→A→(E)C]	234, 280	27.6	575		557, 539, **449**, 423, 407, 289, 285
F-2-28	PAC A-type dimer [(E)C→A→(E)C]	234, 280	28.3	575		557, 539, **449**, 423, 407, 289, 285
F-2-29	PAC A-type trimer [(E)C→B→(E)C→A→(E)C]	234, 280	28.8	863		737, 711, 693, **575**, 559, 449, 287
F-2-30	PAC A-type dimer [(E)C→A→(E)C]	234, 280	28.9	575		557, 539, **449**, 423, 407, 289, 285
F-2-31	PAC A-type dimer [(E)C→A→(E)C]	234, 280	29.7	575		557, 539, **449**, 423, 407, 289, 285
F-2-32	PAC A-type tetramer [1B, 2A]	234, 280	30.9	1149	574	
F-2-33	PAC A-type trimer [(E)C→B→(E)C→A→(E)C]	234, 280	31.8	863		845, 737, 711, 693, **575**, 449, 287
F-2-34	Prodelphinidin A-type trimer [(E)GC→A→(E)C→A→(E)C]		32.1	877		859, 841, **751**, 725, 599, 587, 575, 559, 461, 449, 301, 289
F-2-35	Propelargonidin A-type dimer [(E)C→A→(E)Afz]		32.1	559		541, **433**, 423, 391, 285, 273

F-2-36	PAC A-type trimer [(E)C→B→(E)C→A→(E)C]	234, 280	32.2	863		737, 711, 693, **575**, 559, 449, 287
F-2-37	Myricetin		32.3	317		317, 271, 243, 227, **179**, 151, 137, 109
F-2-38	PAC A-type tetramer [1B, 2A]	234, 280	33.2	1149	574	
F-2-39	PAC A-type tetramer [1B, 2A]	234, 280	34.2	1149	574	
F-2-40	PAC A-type trimer [(E)C→B→(E)GC→A→Luteolin or Kaempferol]		34.2	875		857, 749, **723**, 597, 555, 301
F-2-41	Prodelphinidin A-type dimer [(E)C→B→methyl (E)GC] or [(E)GC→A→(E)GC]		34.3	607		
F-2-42	PAC A-type trimer [(E)C→A→(E)C→A→(E)C]	234, 280	34.5	861		843, 825, **735**, 709, 693, 575, 571, 449, 289, 285
F-2-43	PAC A-type tetramer [1B, 2A]		34.6	1149	574	
F-2-44	PAC A-type trimer [(E)C→B→(E)C→A→(E)C]		34.9	863		737, 711, 693, **575**, 559, 449, 287
F-2-45	PAC A-type trimer [(E)C→A→(E)C→B→(E)C]		35.0	863		737, **711**, 693, 573, 531, 451, 411, 289
F-2-46	PAC A-type tetramer [1B, 2A]	234, 280	35.4	1149	574	
F-2-47	PAC A-type trimer [(E)C→A→(E)C→A→(E)CG]		35.6	1015	507	
F-2-48	Prodelphinidin A-type tetramer [(E)C→A→(E)GC→A→(E)C→A→(E)C]	234, 280	37.4	1163	581	877, 873, 751, 575, 449, 411, 289, 285

[a]The A represents an A-type bond with both (C4→C8) and (C2→O→C7) linkages or (C4→C6) and (C2→O→C7) linkages, B represents a B-type bond which can be (C4→C8) or (C4→C6) linkage; (E)C represents (epi)catechin, (E)GC represents (epi)gallocatechin, (E)CG represents (epi)catechin gallate, and (E)Afz represents (epi)afzelechin.

[b]*sh*, shoulder in the spectrum.

[c]Retention times (t_R) of the total ion chromatograms.

[d]The most abundant ions observed in the mass spectra are indicated in bold; quinone methide (QM) fission diagnostic ions are underlined.

Table 6.3 Tentative identification of glycoside-bound phenolic compounds in dry-blanched peanut skins (PS) crude extracts by C_{18} RP-HPLC-ESI-MS^n.

Cmpnd No.	Tentative identification[a]	UV λ_{max} (nm)[b]	t_R[c] (min)	$[M-H]^-$	Product ions[d]
F-3-1	Gallocatechin		3.2	305	261, 219, **179**, 125
F-3-2	Unknown (likely a Gallocatechin derivative)		5.8	305	261, 219, **179**, 125
F-3-3	3,4-Dihydroxyphenylacetic acid (DOPAC)		8.2	167	**123**
F-3-4	Methyl gallate		9.4	183	**124**
F-3-5	Protocatechuic acid	260, 294	9.7	153	**109**
F-3-6	Protocatechuic acid derivative		9.8	233	213, 203, 167, **153**, 123, 109
F-3-7	Robinetin		12.8	301	301, 283, **257**, 229, 163, 137
F-3-8	*p*-Hydroxybenzoic acid	256	13.1	137	
F-3-9	(+)-Catechin	234, 280	13.8	289	**245**, 205, 179, 125
F-3-10	PAC A-type dimer [(E)C→A→(E)C→Phloroglucinol]		15.9	699	681, 573, 547, 531, 453, **411**, 287, 285 MS^3[699→411]: 411, 349, 301, **285**, 257, 243, 215, 189, 163, 149, 125
F-3-11	Vanillic acid	260, 292	16.1	167	152, **123**, 108
F-3-12	PAC A-type dimer [(E)C→A→(E)C→Phloroglucinol]	238, 278	16.7	699	681, 573, 547, 531, 453, **411**, 285, 245
F-3-13	PAC B-type dimer [(E)C→B→(E)C→Phloroglucinol]		17.1	701	**411**, 289, 245 MS^3[701→411]: 393, 383, 341, 307, **283**, 242
F-3-14	(–)-Epicatechin		17.3	289	**245**, 205, 179, 125
F-3-15	PAC A-type dimer [(E)C→A→(E)C→Phloroglucinol]	238, 278	17.6	699	681, 573, 531, 453, **411**, 287, 285, 245
F-3-16	PAC B-type dimer [(E)C→B→(E)C→Phloroglucinol]		17.9	701	**411**, 289, 245
F-3-17	PAC A-type dimer [(E)C→A→(E)C→Phloroglucinol]	238, 278	18.6	699	681, 573, 531, 453, **411**, 287, 285, 245
F-3-18	PAC A-type dimer [(E)C→A→(E)C→Phloroglucinol]	238, 278	19.5	699	681, 573, 531, 453, **411**, 287,

					285, 245
F-3-19	PAC B-type dimer [(E)C→B→(E)C→Phloroglucinol]		20.3	701	**411**, 289, 245
F-3-20	PAC A-type dimer [(E)C→A→(E)C→Phloroglucinol]	238, 278	20.6	699	681, 573, 531, 453, **411**, 287, 285, 245
F-3-21	PAC A-type dimer [(E)C→A→(E)C]	234, 280	20.8	575	557, 539, **449**, 423, 407, 289, 285
F-3-22	Homovanillic acid		20.8	181	137, **122**
F-3-23	PAC A-type dimer [(E)C→A→(E)C]	234, 280	21.7	575	557, 539, **449**, 423, 407, 289, 285
F-3-24	Propelargonidin A-type trimer [(E)C→A→(E)Afz→B→(E)C]		22.2	847	711, 557, 435, **411**, 289
F-3-25	PAC A-type dimer [(E)C→A→(E)C→Phloroglucinol]	238, 278	24.3	699	681, 573, 531, 453, **411**, 287, 285, 245
F-3-26	PAC A-type dimer [(E)C→A→(E)C→Phloroglucinol]	238, 278	24.9	699	681, 573, 531, 453, **411**, 287, 285, 245
F-3-27	PAC A-type dimer [(E)C→A→(E)C]		25	575	557, 539, **449**, 423, 407, 289, 285
F-3-28	PAC A-type dimer [(E)C→A→(E)C→Phloroglucinol] – C_2H_2O (42 Da)		25.2	657	597, 547, 531, 411, 285, **245**
F-3-29	PAC A-type dimer [(E)C→A→(E)C→Phloroglucinol] – C_2H_2O (42 Da)		25.8	657	597, 547, 531, 411, 285, **245**
F-3-30	Taxifolin		26.2	303	303, **285**, 257, 177, 125
F-3-31	PAC A-type dimer [(E)C→A→(E)C→Phloroglucinol] – C_2H_2O (42 Da)		26.3	657	597, 547, 531, 411, **245**
F-3-32	PAC A-type dimer [(E)C→A→(E)C]	234, 280	26.5	575	557, 539, **449**, 423, 407, 289, 285
F-3-33	PAC A-type dimer [(E)C→A→(E)C→Phloroglucinol] – $C_8H_8O_3$ (152 Da)		26.6	547	531, **437**, 423, 411, 285, 245
F-3-34	PAC A-type dimer [(E)C→A→(E)Afz→Phloroglucinol]		27.0	683	665, 647, **557**, 515, 423, 411, 397, 285 MS^3[683→557]: 539, 421, 395, **285**, 271

F-3-35	PAC A-type dimer [(E)C→A→(E)C]	234, 280	27.5	575	557, 539, **449**, 423, 407, 289, 285
F-3-36	PAC A-type dimer [(E)C→A→(E)C]	234, 280	28.2	575	557, 539, **449**, 423, 407, 289, 285
F-3-37	PAC A-type dimer [(E)C→A→(E)C→Phloroglucinol]	238, 278	28.4	699	681, 573, 531, 453, **411**, 287, 285, 245
F-3-38	PAC A-type dimer [(E)C→A→a novel Flavan-3-ol]		28.5	589	571, 553, 463, **453**, 409, 303, 285 MS^3[589→453]: 409, 367, 344, **327**, 303, 176, 165
F-3-39	PAC A-type dimer [(E)C→A→(E)C→Phloroglucinol] – $C_8H_8O_4$ (168 Da)		30.1	531	513, 499, **405**, 393, 363, 309, 285, 245
F-3-40	PAC A-type dimer [(E)C→A→(E)C]	234, 280	30.3	575	557, 539, **449**, 423, 407, 289, 285
F-3-41	PAC A-type dimer [(E)C→A→(E)Afz→Phloroglucinol]		31.1	683	665, 647, **557**, 515, 423, 411, 397, 285
F-3-42	PAC A-type dimer [(E)C→B→(E)C→Phloroglucinol] – $C_8H_8O_3$ (152 Da)		32.2	549	531, 489, 423, **411**, 285
F-3-43	Fisetin	248, 362	32.2	285	285, 257, 241, 229, 213, 177, **163**, 135, 121
F-3-44	PAC A-type dimer [(E)C→A→(E)C→Phloroglucinol] – $C_8H_8O_4$ (168 Da)		32.4	531	513, 499, **405**, 393, 363, 309, 285, 245
F-3-45	PAC A-type dimer [(E)C→A→(E)Afz→Phloroglucinol] – $C_6H_6O_3$ (126 Da)		32.6	557	539, **431**, 389, 285
F-3-46	Biflavonoid [Eriodictyol→C-methyl (Epi)robinetinidol]		33.4	587	569, **461**, 419, 301, 285 MS^3[587→461]: **443**, 339, 324, 301, 285, 293, 198
F-3-47	PAC A-type dimer [(E)C→B→(E)C→Phloroglucinol] – $C_8H_8O_3$ (152 Da)		33.5	549	531, 489, 423, **411**, 285
F-3-48	PAC A-type dimer [(E)C→A→(E)C→Phloroglucinol] – $C_8H_8O_4$ (168 Da)		33.9	531	513, 499, **405**, 393, 363, 309, 285, 245

F-3-49	Myricetin		34.1	317	317, 289, 271, 191, 179, **151**, 137, 125
F-3-50	PAC A-type dimer [(E)C→A→(E)C→Phloroglucinol] – $C_8H_8O_3$ (152 Da)		34.2	547	527, 489, 437, **421**, 360, 302, 245, 177
F-3-51	PAC A-type dimer [(E)C→A→(E)C→Phloroglucinol] – $C_8H_8O_4$ (168 Da)		34.3	531	469, **405**, 393, 363, 309, 285, 245
F-3-52	propelargonidin A-type trimer [(E)Afz→A→(E)C→A→(E)C]		34.4	845	**719,** 693, 575, 555, 449, 433, 289, 269
F-3-53	4′,3′,5,6-Trihydroxy-7-methoxyflavone		35.6	315	315, **300**, 232, 191
F-3-54	Genkwanin	338	37.2	283	**268**, 151
F-3-55	Prorobinetidin A-type dimer [(E)C→A→C-methyl (Epi)robinetinidol]		37.5	589	571, 553, **463**, 421, 303, 285 MS^3[589→463]: 445, **435**, 341, 301, 295, 285, 198
F-3-56	Biflavonoid [Eriodictyol→C-methyl (Epi)robinetinidol]		37.7	587	569, 551, **461**, 447, 419, 301, 285 MS^3[587→461]: **443**, 301, 285, 293, 198
F-3-57	Prorobinetidin A-type dimer [(E)C→A→C-methyl (Epi)robinetinidol]		38.1	589	571, 553, **463**, 421, 303, 285
F-3-58	Prodelphinidin A-type dimer [(E)C→B→methyl (E)GC] or [(E)GC→A→(E)GC]		38.2	607	
F-3-59	Biflavonoid [Eriodictyol→C-methyl (Epi)robinetinidol]		38.3	587	569, 551, **461**, 421, 301, 285
F-3-60	Biochanin A		38.5	283	**268** MS^3[283→268]: 267, **239**, 224, 135
F-3-61	Isosakuranetin		38.9	285	285, **270**, 243, 226, 199, 164, 136
F-3-62	Prorobinetidin A-type dimer [(E)C→A→C-methyl (Epi)robinetinidol]		39.0	589	571, 553, **463**, 421, 303, 285
F-3-63	Quercetin	256, 370	39	301	301, 273, 257, 229, **179**, 151, 107

F-3-64	Prorobinetidin A-type dimer [(E)C→A→C-methyl (Epi)robinetinidol]		39.7	589	571, 553, **463**, 421, 303, 285
F-3-65	Hesperetin		40.4	301	**301**, 286, 283, 257, 242, 215, 164, 151, 125, 107
F-3-66	PAC A-type dimer [(E)C→A→(E)Afz→phloroglucinol] – $C_6H_6O_3$ (126 Da)		41.1	557	539, 521, **431**, 389, 285
F-3-67	Biflavonoid [Eriodictyol→C-methyl (Epi)robinetinidol]		41.4	587	569, 551, **461**, 419, 301, 285
F-3-68	Prorobinetidin A-type dimer [(E)C→A→C-methyl (Epi)robinetinidol]		41.7	589	571, 553, **463**, 421, 303, 285
F-3-69	PAC A-type dimer [(E)C→A→(E)Afz→Phloroglucinol] – $C_6H_6O_3$ (126 Da)		42.7	557	539, 521, **431**, 389, 285
F-3-70	*trans*-3,3′,5,5′-tetrahydroxy-4′-methoxystilbene		43.9	273	**258**, 163, 136, 109
F-3-71	Homoeriodictyol		44.4	301	**301**, 286, 151
F-3-72	Pratensein		45.6	299	**284**, 255, 177, 151, 122
F-3-73	Unknown		45.6	567	549, 505, **441**, 399, 325, 285
F-3-74	3′,5,6-Trihydroxy-4′,7-dimethoxyflavone		46	329	**314**, 299, 191
F-3-75	Kaempferol	266, 326*sh*, 368	46.7	285	**285**, 257, 241, 229, 213, 199, 151
F-3-76	Luteolin methyl ether (Diosmetin)	254*sh*, 266, 348	47.1	299	299, **284**, 217 MS^3 [299→284]: **284**, 256, 216, 151
F-3-77	Quercetin methyl ether (Isorhamnetin)	256, 370	47.3	315	**315**, 300 MS^3 [315→300]: **300**, 271, 256, 151
F-3-78	3′,4′,5′-Trimethylrobinetin		49	343	328, **313**, 299, 285, 135

[a]The A represents an A-type bond with both (C4→C8) and (C2→O→C7) linkages or (C4→C6) and (C2→O→C7) linkages, B represents a B-type bond which can be (C4→C8) or (C4→C6) linkage; (E)C represents (epi)catechin, (E)GC represents (epi)gallocatechin, and (E)Afz represents (epi)afzelechin.

[b]*sh*, shoulder in the spectrum.

[c]Retention times (t_R) of the total ion chromatograms.
[d]The most abundant ions observed in mass spectra are indicated in bold; quinone methide (QM) fission diagnostic ions are underlined.

Table 6.4 Content of selected phenolics quantified in dry-blanched peanut skins (PS) by C_{18} RP-HPLC.

Ester-bound phenolic compounds[a]	Quantification λ(nm)	Content (mg/100 g)[b]
Protocatechuic acid	260	41.4 ± 1.72
(+)-Catechin	280	52.0 ± 2.66
(–)-Epicatechin	280	8.33 ± 0.40
Prodelphinidin A-type dimer [(E)C→A→5-*O*-methyl (E)GC]	280	22.2 ± 0.58
PAC A-type dimer [(E)C→A→(E)C]	280	74.9 ± 2.84
p-Coumaric acid	320	6.88 ± 0.22
Ferulic acid	320	2.03 ± 0.12
PAC A-type dimer [(E)C→A→(E)C]	280	44.3 ± 3.32
PAC A-type dimer [(E)C→A→(E)C]	280	152 ± 7.09
PAC A-type dimer [(E)C→A→(E)C]	280	81.2 ± 1.74
PAC A-type tetramer [1B, 2A]	280	32.1 ± 2.03
PAC A-type tetramer [1B, 2A]	280	29.5 ± 0.86
PAC A-type trimer [(E)C→B→(E)C→A→(E)C]	280	15.2 ± 0.37
PAC A-type tetramer [1B, 2A]	280	10.2 ± 0.13
PAC A-type tetramer [1B, 2A]	280	15.1 ± 0.35
PAC A-type trimer [(E)C→A→(E)C→A→(E)C]	280	24.8 ± 0.75
PAC A-type tetramer [1B, 2A]	280	8.96 ± 0.56
Prodelphinidin tetramer [(E)C→A→(E)GC→A→(E)C→A→(E)C]	280	3.66 ± 0.13
Glycoside-bound phenolic compounds		
Protocatechuic acid	260	21.5 ± 0.66
(+)-Catechin	280	105 ± 4. 57
p-Hydroxybenzoic acid	255	1.72 ± 0.00
Vanillic acid	260	2.38 ± 0.10
PAC A-type dimer [(E)C→A→(E)C→Phloroglucinol]	280	86.2 ± 6.55
PAC A-type dimer [(E)C→A→(E)C]	280	110 ± 1.27
Kaempferol	360	0.89 ± 0.67
Fisetin	360	0.75 ± 0.03
Genkwanin	320	0.08 ± 0.00
Quercetin	360	1.62 ± 0.12
Diosmetin	360	0.46 ± 0.00
Isorhamnetin	360	1.29 ± 0.00

[a]PACs are quantified as catechin equivalents; Fisetin, genkwanin, isorhamnetin and diosmetin are quantified using corresponding flavonoid aglycone equivalents.
[b]Values are means of triplicate analyses ± standard deviation. Findings are reported as mg respective phenolic/100-g dry weight (d.w.) of dry-blanched PS.

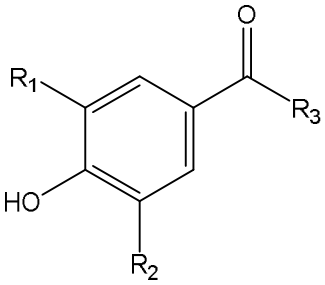
Figure Captions

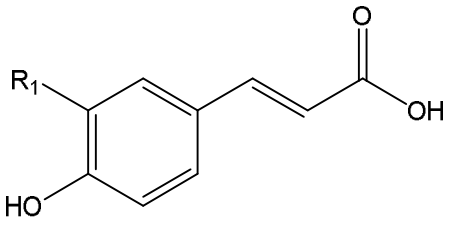
Figure 6.1 Structures of monomeric phenolic aglycones released from soluble-ester and glycoside in dry-blanched peanut skins.

Figure 6.2 Tentative structures and fragmentation schemes of (**A**) Prodelphinidin A-type dimer [(E)C→A→6-*O*-methyl (E)GC] (F-2-18, *m/z* 605); (**B**) Prodelphinidin A-type trimer [(E)GC→A→(E)C→A→(E)C] (F-2-34, *m/z* 877); and (**C**) Prodelphinidin A-type tetramer [(E)C→A→(E)GC→A→(E)C→A→(E)C] (F-2-48, *m/z* 1163). See **Table 6.3** for formal designations

Figure 6.3 Tentative structures and fragmentation schemes of (**A**) PAC B-type dimer [(E)C→B→(E)C→Phloroglucinol] (F-3-13, 16, and 19, *m/z* 701); (**B**) PAC A-type dimer [(E)C→A→(E)C→Phloroglucinol] (F-3-10, 12, 15, 17, 18, 20, 25, 26, and 38, *m/z* 699); and (**C**) PAC A-type dimer [(E)C→A→(E)Afz→Phloroglucinol] (F-3-34 and 43, *m/z* 683). See **Table 6.3** for formal designations

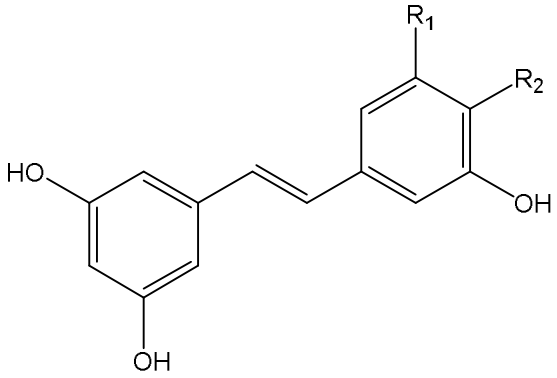
Figure 6.4 Tentative structures and fragmentation schemes of (**A**) PAC A-type dimer [(E)C→A→a novel Flavan-3-ol] (F-3-39, *m/z* 589); (**B**) Prorobinetidin A-type dimer [(E)C→A→C-methyl (Epi)robinetinidol] (F-3-56, 62, 64 and 68, *m/z* 589); and (**C**) biflavonoid [Eriodictyol→C-methyl (Epi)robinetinidol] (F-3-48, 57 and 67, *m/z* 587). See **Table 6.3** for formal designations.



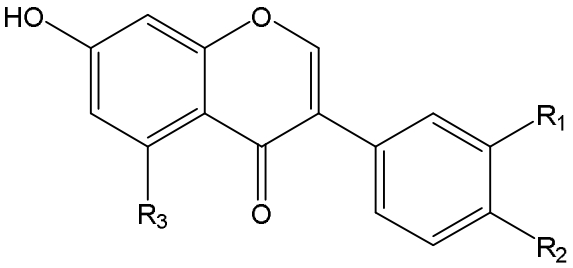


Hydroxybenzoic acids	R_1	R_2	R_3
p-Hydroxybenzoic acid	H	H	OH
Protocatechuic acid	OH	H	OH
Ethyl protocatechuate	OH	H	OCH_2CH_3
Vanillic acid	OCH_3	H	OH
Methyl gallate	OH	OH	OCH_3

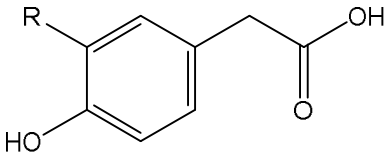
Hydroxycinnamic acids	R_1
p-Coumaric acid	H
Caffeic acid	OH
Ferulic acid	OCH_3



Stilbenes	R_1	R_2
trans-Piceatannol	H	OH
trans-3,3',5,5'-tetrahydroxy-4'-methoxystilbene	OH	OCH_3

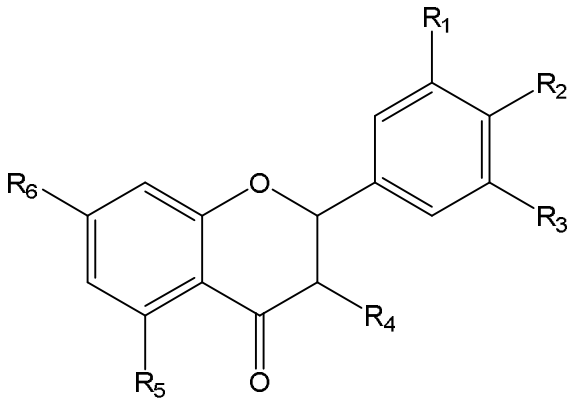


Isoflavone	R_1	R_2	R_3
Biochanin A	H	OCH_3	OH
Pratensein	OH	OCH_3	OH

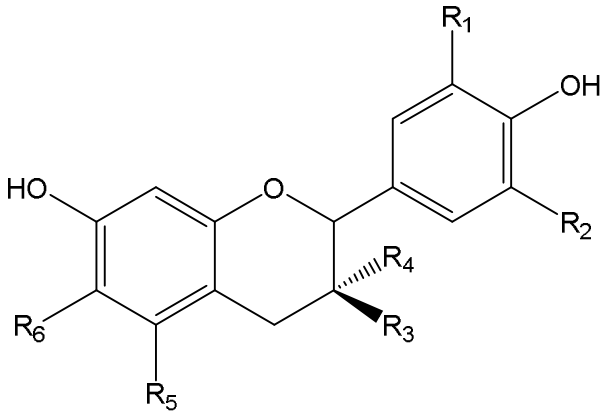


Hydroxyphenylacetic acid	R
3,4-Dihydroxyphenylacetic acid (DOPAC)	H
Homovanillic acid	OCH_3

Figure 6.1

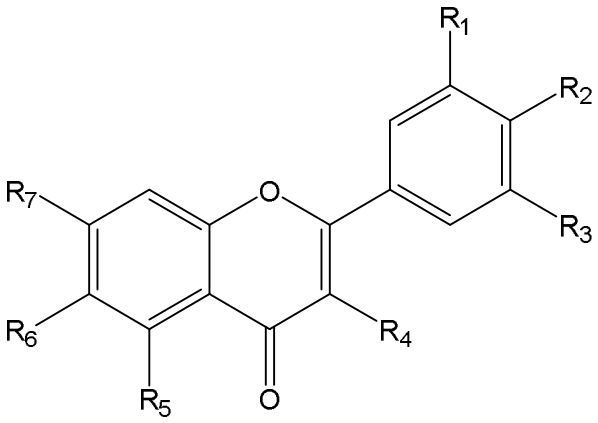


Flavanonol	R_1	R_2	R_3	R_4	R_5	R_6
Taxifolin	OH	OH	H	OH	OH	OH
Flavanones						
Isosakuranetin	H	OCH_3	H	H	OH	OH
Hesperetin	OH	OCH_3	H	H	OH	OH
Homoeriodictyol	OCH_3	OH	H	H	OH	OH



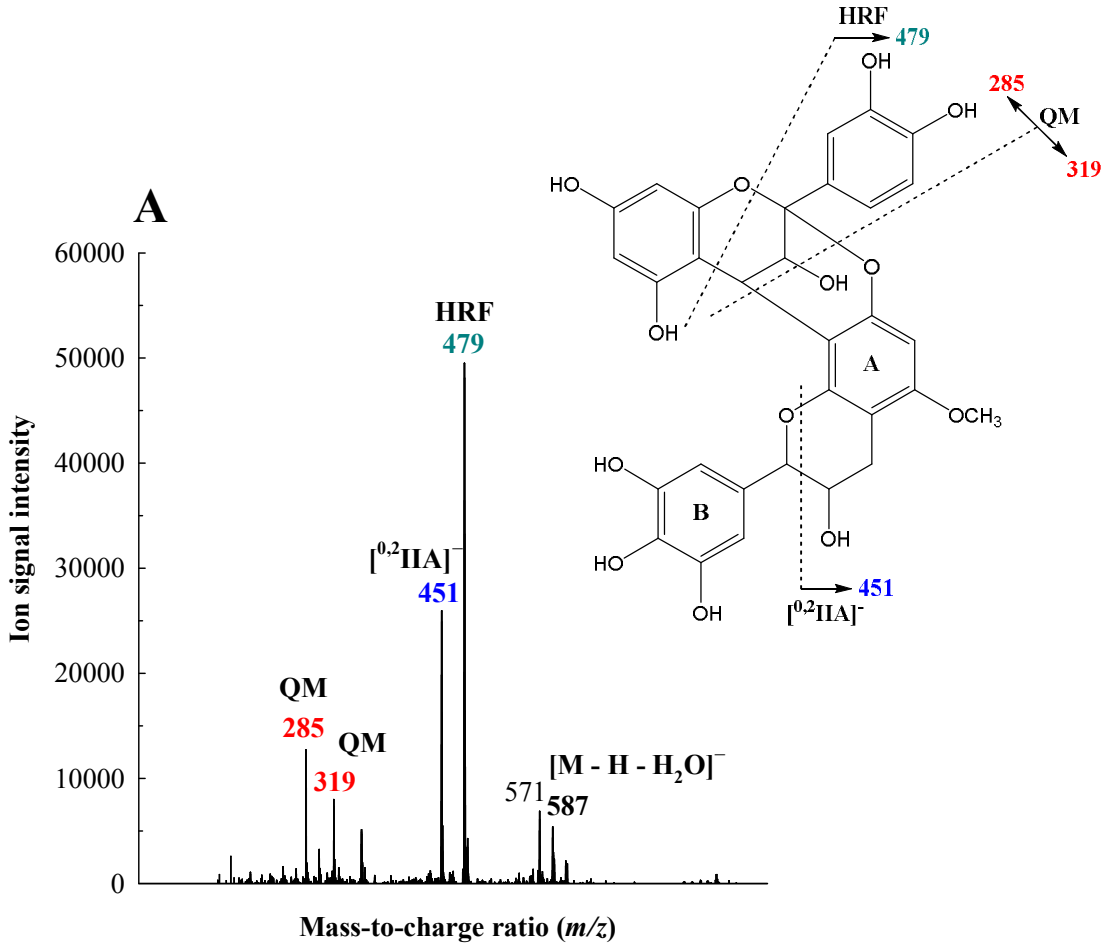
Flavan-3-ols	R_1	R_2	R_3	R_4	R_5	R_6
Catechin	OH	H	OH	H	OH	H
Epicatechin	OH	H	H	OH	OH	H
Afzelechin	H	H	OH	H	OH	H
Epiafzelechin	H	H	H	OH	OH	H
Gallocatechin	OH	OH	OH	H	OH	H
Epigallocatechin	OH	OH	H	OH	OH	H
5-*O*-methyl Gallocatechin	OH	OH	OH	H	OCH_3	H
5-*O*-methyl Epigallocatechin	OH	OH	H	OH	OCH_3	H
C-methyl Robinetinidol	OH	OH	OH	H	H	CH_3
C-methyl Epirobinetinidol	OH	OH	H	OH	H	CH_3

Figure 6.1 Continued



Flavone	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Luteolin	OH	OH	H	H	OH	H	OH
Diosmetin	OH	OCH_3	H	H	OH	H	OH
Genkwanin	H	OH	H	H	OH	H	OCH_3
4',3',5,6-tetrahydroxy-7-methoxyflavone	OH	OH	H	H	OH	OH	OCH_3
3',5,6-trihydroxy-4',7-dimethoxyflavone	OH	OCH_3	H	H	OH	OH	OCH_3
Flavonol							
Quercetin	OH	OH	H	OH	OH	H	OH
Isoharmnetin	OCH_3	OH	H	OH	OH	H	OH
Kaempferol	H	OH	H	OH	OH	H	OH
Fisetin	OH	OH	H	OH	H	H	OH
Robinetin	OH	OH	OH	OH	H	H	OH
Robinetin 3',4',5'-trimethyl ether	OCH_3	OCH_3	OCH_3	OH	H	H	OH
Myricetin	OH	OH	OH	OH	OH	H	OH

Figure 6.1 Continued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30000
20000
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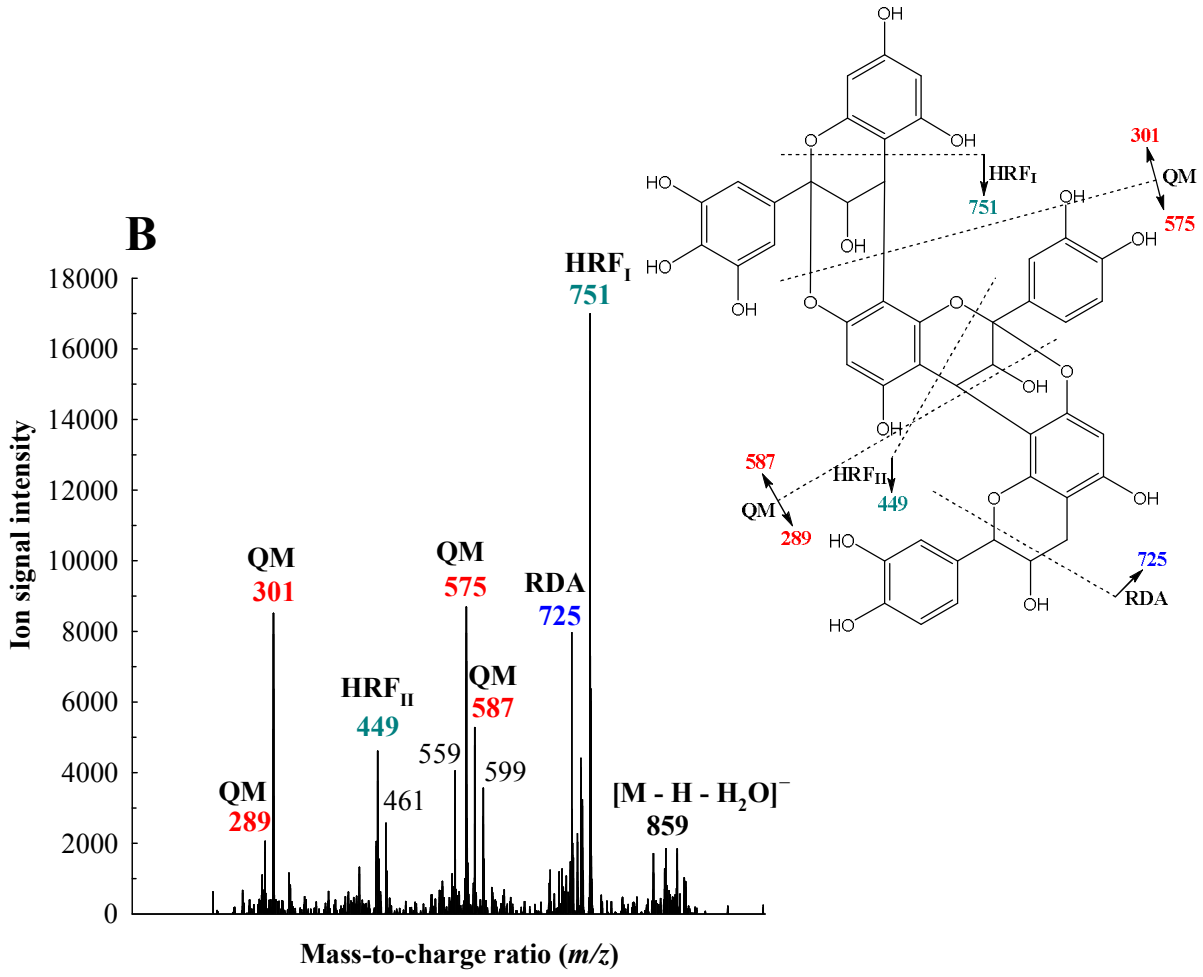
QM
285
QM
319
571
[M - H - H2O]-
587
Mass-to-charge ratio (m/z)
HRF
479
285
QM
319
HO
OH
OH
O
OH
O
A
OCH3
HO
B
HO
OH
OH
451
[0,2IIA]-

Figure 6.2 (A)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12000
10000
8000
6000
4000
2000
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QM
301
QM
289
HRF$_{II}$
449
461
QM
575
559
QM
587
599
RDA
725
HRF$_I$
751
[M - H - H$_2$O]$^-$
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QM
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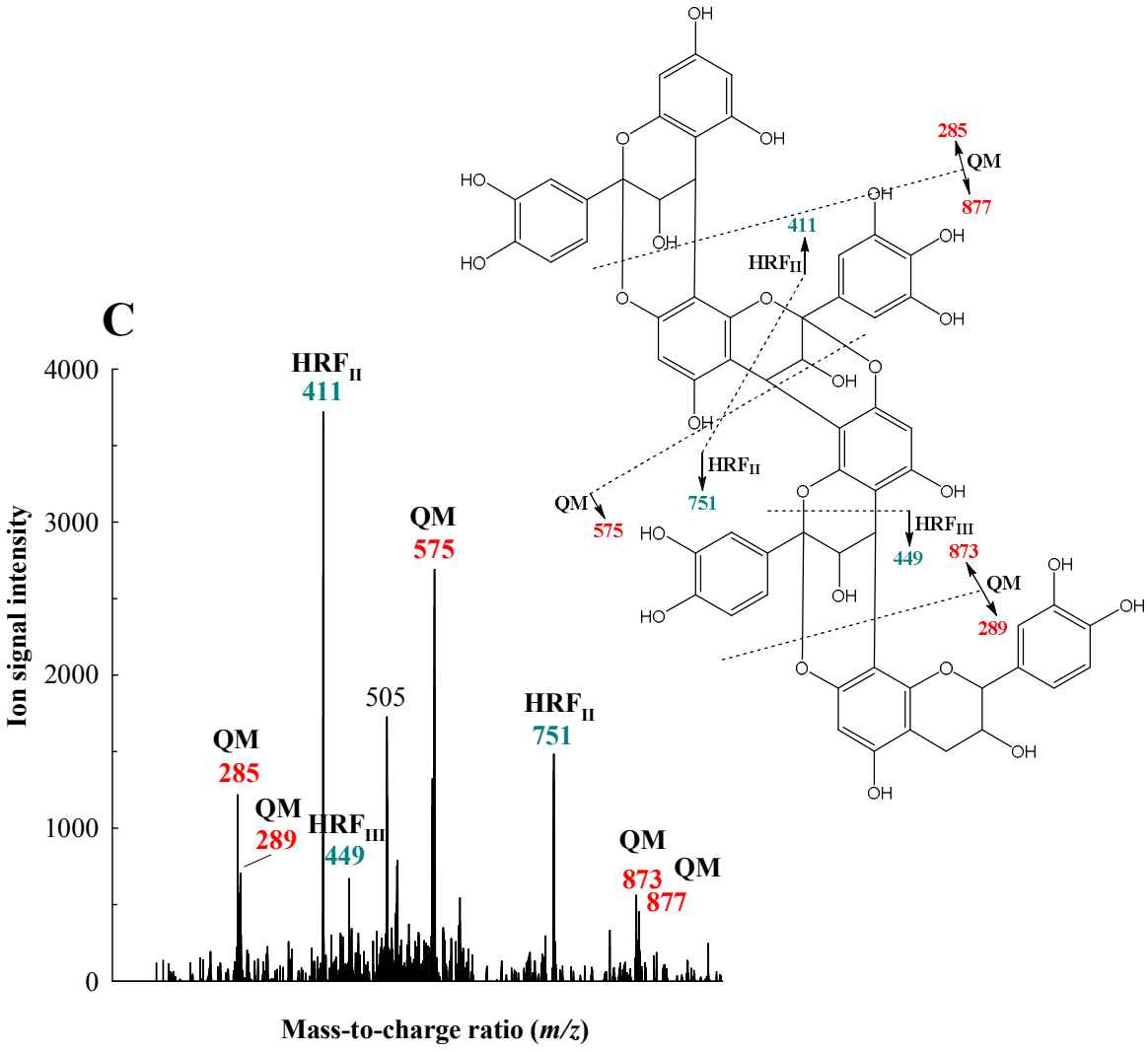
587
QM
289
HRF$_{II}$
449
725
RDA

Figure 6.2 (B)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Mass-to-charge ratio (m/z)
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2000
1000
0
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QM
575
505
HRF II
751
QM
285
QM
289
HRF III
449
QM
873
QM
877
285
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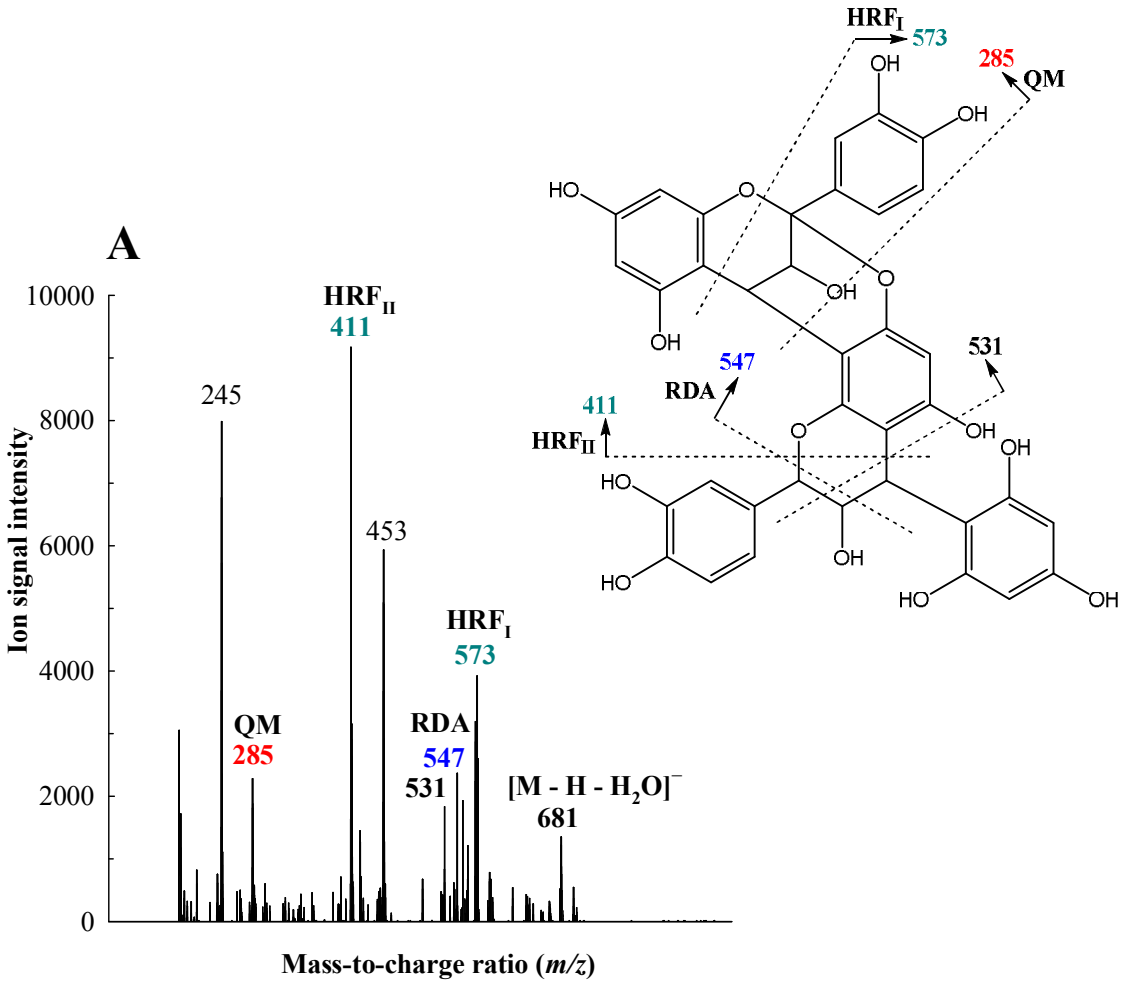
QM
575
HRF III
449
873
QM
289

Figure 6.2 (C)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Ion signal intensity
Mass-to-charge ratio (m/z)
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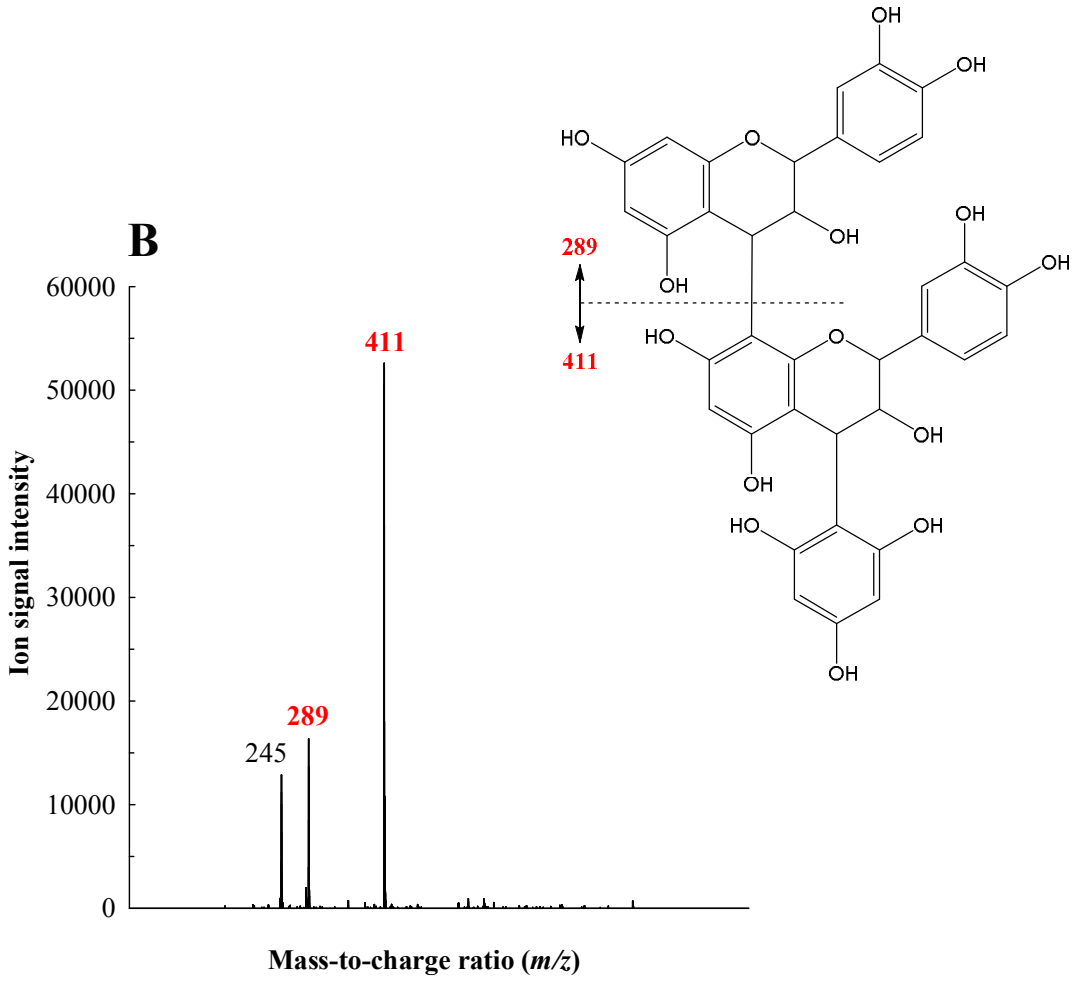
6000
4000
2000
0
245
HRF$_{II}$
411
453
HRF$_{I}$
573
QM
285
RDA
547
531
[M - H - H$_2$O]$^-$
681
HRF$_I$
573
285
QM
531
547
RDA
411
HRF$_{II}$
OH
HO
O

Figure 6.3 (A)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Mass-to-charge ratio (m/z)
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411
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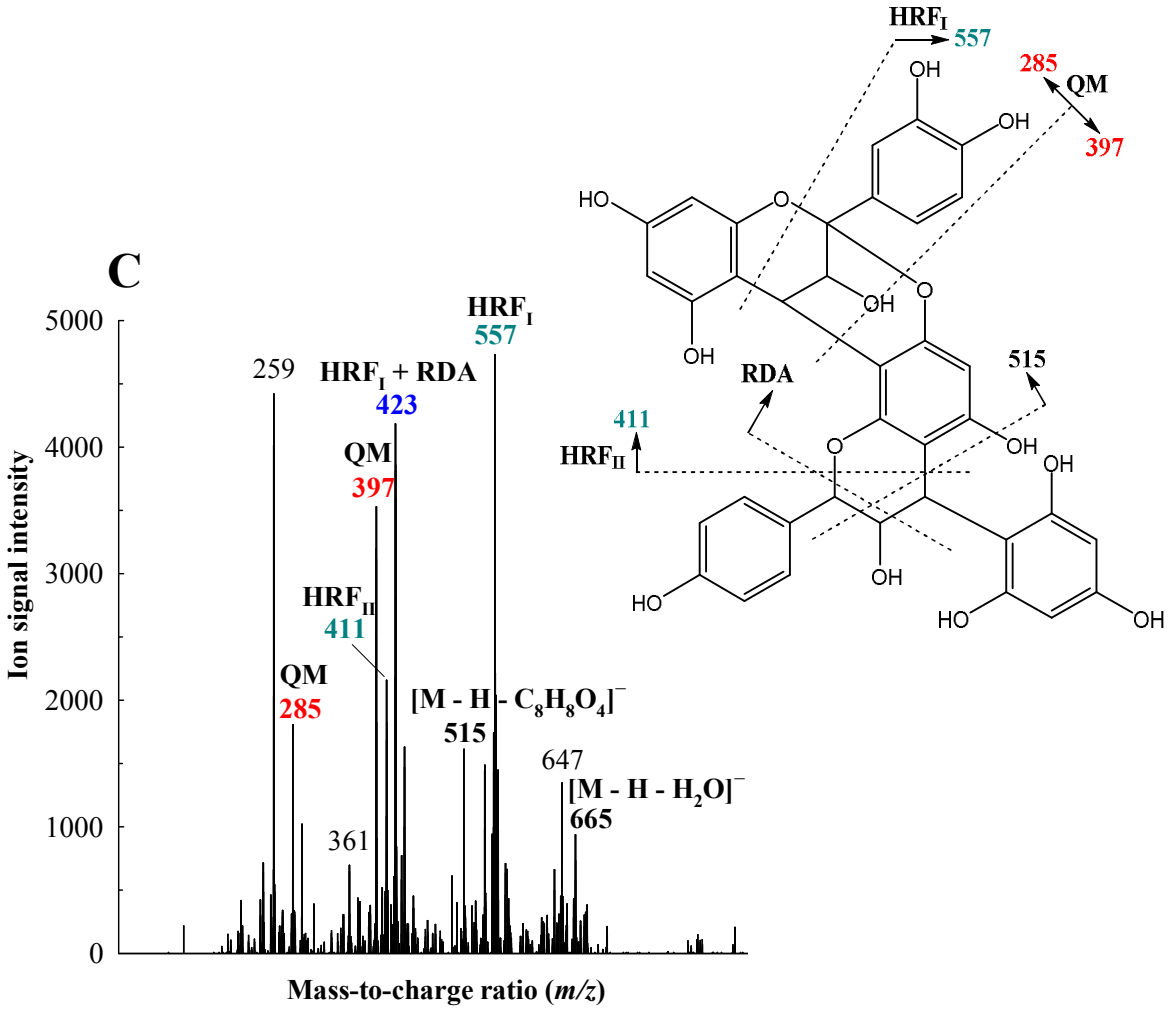
HO
O

Figure 6.3 (B


C
Ion signal intensity
Mass-to-charge ratio (m/z)
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4000
3000
2000
1000
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HRF_I + RDA
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557
QM
397
HRF_II
411
QM
285
[M - H - C8H8O4]⁻
515
647
[M - H - H2O]⁻
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361
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285
QM
397
RDA
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HRF_II
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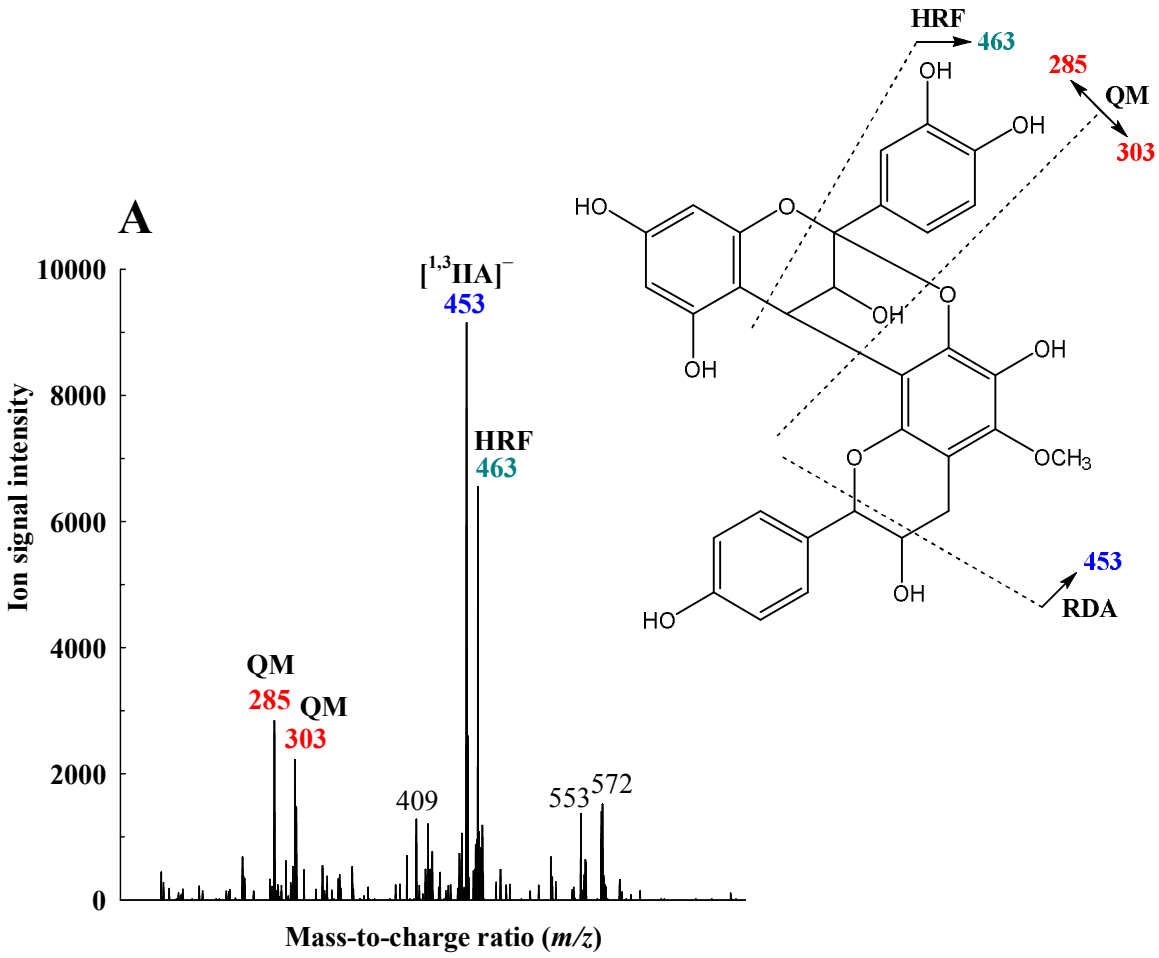
HO
O

Figure 6.3 (C)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2000
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HRF
463
QM
285
QM
303
409
553
572
Mass-to-charge ratio (m/z)
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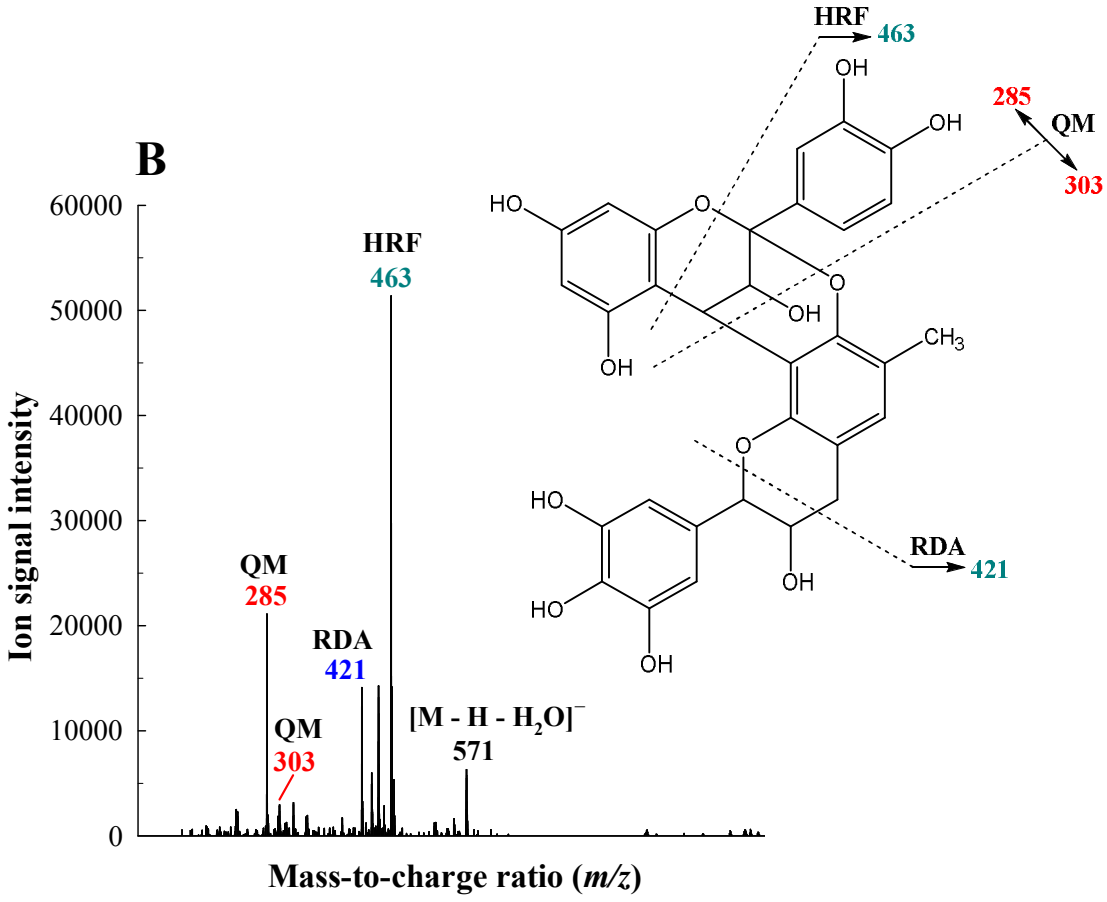
463
285
QM
303
453
RDA
HO
OH
OCH3

Figure 6.4 (A)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Ion signal intensity
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40000
30000
20000
10000
0
HRF
463
QM
285
RDA
421
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303
[M - H - H2O]−
571
Mass-to-charge ratio (m/z)
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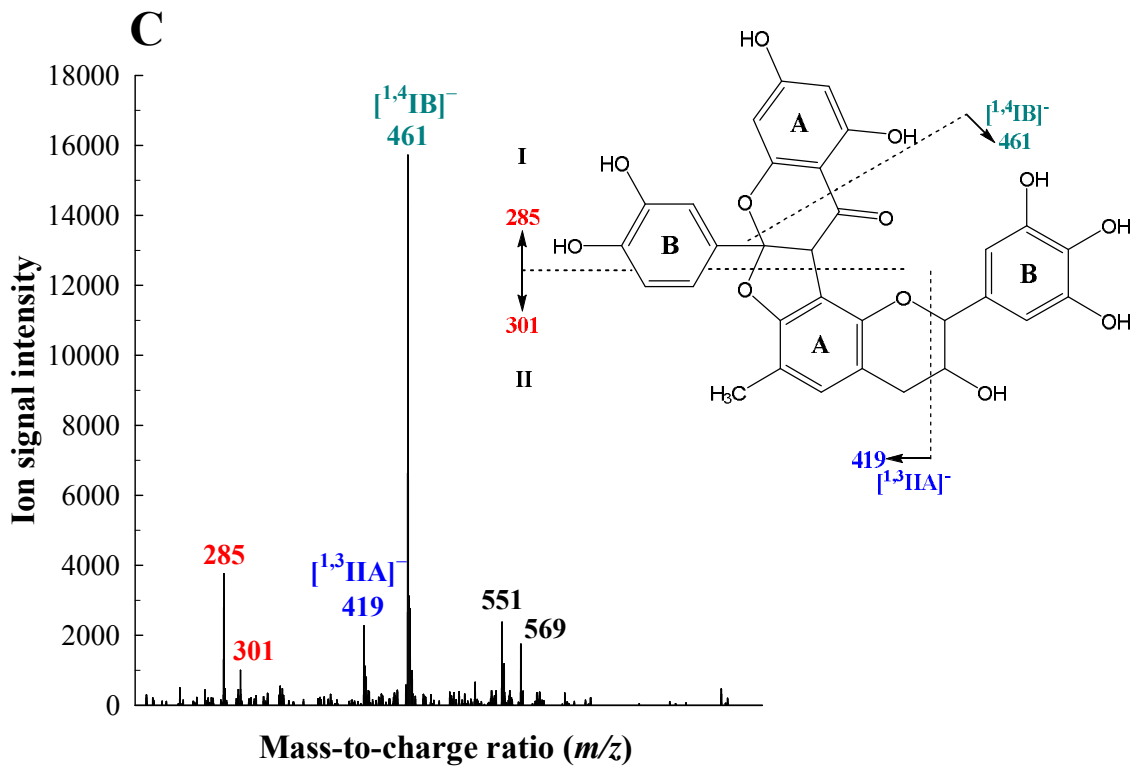
463
285
QM
303
RDA
421
OH
HO
O
CH3

Figure 6.4 (B)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14000
12000
10000
8000
6000
4000
2000
0
[1,4IB]−
461
285
301
[1,3IIA]−
419
551
569
I
II
HO
A
OH
[1,4IB]-
461
HO
B
O
O
OH
OH
B
O
A
H3C
OH
419
[1,3IIA]-

Figure 6.4 (C)

CHAPTER 7

SUMMARY AND CONCLUSIONS

This research provides new insight in the contents and diversity of phenolic compounds in PS, as well as the utilization of PS phenolics via their incorporation in peanut butters. A large number and variety of phenolic compounds were separated, determined and quantified in PS by HPLC-ESI-MS^n, including PACs (monomers to nonamers), free phenolic acids and their esters, stilbenes, flavonoids, and biflavonoids. Overall, PS contain significantly more PACs compared to free phenolic compounds. *p*-Coumaroyl derivatives account for roughly ¾ of the non-PAC phenolics found in PS. This study is the first to report on the presence of new PACs (*i.e.*, PACs containing an additional phloroglucinol unit), selected biflavonoids (*e.g.*, morelloflavone, homoeriodictyol (C3)→eriodictyol (C8)), and *p*-coumaroyltartaric acid derivatives (*e.g.*, *p*-coumaroyltartaric acid etsters of phenolic acids, stilbenes, vitamin B_3) in PS. Although further analytical techniques are required for unequivocal identifications, we believe that the findings reported here will greatly enrich the phenolics database of PS. PS provide an abundant and inexpensive source of natural antioxidants, especially *p*-coumaroyl species and PACs.

Simultaneous grinding of PS with table sugar can produce powders with desirable grind sizes (< 300 μm) of an appropriate range for incorporation into peanut butters. Addition of ground PS also impacts the physical properties of the resultant peanut butters with the extent depending on the type of skins used. In general, dry-blanched PS resulted in the least overall change in physical properties of the fabricated peanut butters at a given level of PS incorporation. Further, these peanut butters also possess greater levels of potentially-healthful phenolic

compounds and DF. Even though dry-blanched PS exhibited the greatest enhancement, all four PS types effectively increased the phenolics content, antioxidant capacity, and the fiber content of the formulated peanut butters in a concentration-dependent manner, while maintaining the US FDA's standard of identity for peanut butter. Moreover, the improved TPCs in the PS-fortified peanut butters were largely attributed to the PACs, as demonstrated by the DMAC assay and NP-HPLC/ESI-MS. Thus, given a similar price structure as nonfortified peanut butters, it is expected that these formulations hold great promise as a functional food. The findings of this study have paved the way for utilization of a low-valued industrial by-product (*i.e.*, PS) to be an ingredient in functional food formulations that can improve an existing product and allow for the diversification of brands already available in the market.

INDEX OF ABBREVIATIONS

A: an A-type bond in PACs with both (C4→C8) and (C2→O→C7) linkages or (C4→C6) and (C2→O→C7) linkages

$^{ij}A^{-}$: A^{-} is a product ion in the negative-ion mode consisting of the intact A-ring of a flavonoid, while the superscripts *i* and *j* denote the positions in the C-ring where two C–C bonds have been ruptured

AAPH: 2,2′-azobis(2-amidinopropane)dihydrochloride

$ABTS^{+\bullet}$: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation

ANOVA: analysis of variance

$ArO^{\bullet}$: phenoxy radical

ArOH: phenolic antioxidants

AUC: the area under the kinetic curve

B: a B-type bond in PACs, which can be (C4→C8) or (C4→C6) linkage

$^{ij}B^{-}$: B^{-} is a product ion in the negative-ion mode consisting of the intact B-ring of a flavonoid, while the superscripts *i* and *j* indicate the positions in the C-ring where two C–C bonds have been ruptured

DAD: diode array detection

DMAC: dimethylaminocinnamaldehyde

DF: dietary fiber

DP: degree of polymerization

DPPH$^{\bullet}$: 2,2-diphenyl-1-picrylhydrazyl radical

d.w.: dry weight

(E)Afz: (epi)afzelechin

(E)C: (epi)catechin

(E)CG: (epi)catechin gallate

EDTA: ethylenediamine tetraacetic acid

(E)GC: (epi)gallocatechin

(E)GCG: (epi)gallocatechin gallate

eq.: equivalents

ET: electron transfer

FDA: U.S. Food and Drug Administration

Fe(II)–TPTZ: ferrous tripyridyltriazine

Fe(III)–TPTZ: ferric-tripyridyltriazine

FL: fluorescein (3′6′-dihydroxy-spiro[isobenzofuran-1[3H],9′[9H]-xanthen]-3-one)

FRAP: ferric-reducing antioxidant power

f.w.: fresh weight

GC: gallocatechin

HAT: hydrogen atom transfer

HILIC: hydrophilic interaction liquid chromatography

H_2O_2: hydrogen peroxide

HOCl: hypochlorous acid

HRF: heterocyclic ring fission

IDF: Insoluble dietary fiber

$[M - H]^-$: molecular ion in the negative-ion mode of mass spectrometry; *m/z*: mass-to- charge ratio

$[M_E]^-$: quinone methide fission of the molecular ion at the extension (E) unit

$[M_T]^-$: quinone methide fission of the molecular ion at the terminal (T)

NADH: nicotinamide adenine dinucleotide

NADPH: nicotinamide adenine dinucleotide phosphate

NP-HPLC/ESI-MS^n: normal-phase high-performance liquid chromatography-electrospray ionization mass spectrometry

$^1O_2$: singlet oxygen

$O_2^{\bullet -}$: superoxide radical anion

$^{\bullet}OH$: hydroxyl radical

ORAC: oxygen radical absorbance capacity

PAC: proanthocyanidin

PAL: phenylalanine ammonia lyase

PS: peanut skins

QM: quinone methide

RDA: retro-Diels-Alder

$RO^{\bullet}$: alkoxyl radical

$ROO^{\bullet}$: peroxyl radical

ROOH: hydroperoxide

ROS: reactive oxygen species

RP-HPLC/ESI-MS^n: reversed-phase high-performance liquid chromatography-electrospray ionization mass spectrometry

RT: retention time

sh: shoulder

SOD: superoxide dismutase

SDF: soluble dietary fiber

TAC: total antioxidant capacity

TAL: tyrosine ammonia lyase

TEAC: trolox equivalent antioxidant capacity

TDF: total dietary fiber

TPAC: total proanthocyanidins

TPC: total phenolics content

TPTZ: 2,4,6-tri(2-pyridyl)-*s*-triazine

Trolox: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

UV: ultraviolet

UV-Vis: ultraviolet visible

USDA: U.S. Department of Agriculture

λ_{em}: emission spectra

λ_{ex}: excitation spectra

λ_{max}: maximum wavelength