Phenolic compounds and antioxidant activity of blackberry, black raspberry and blueberry seed meals

by

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ABSTRACT

Berries are highly valued crops due to their unique flavour, texture, colour and phytochemicals. They are rich in phenolic compounds which have been recognized as having beneficial health effects in humans. Phenolic compounds are present in the free, soluble ester and insoluble-bound forms; these were extracted using four different solvents {methanol–acetone–water (7:7:6, v/v/v), acetone-water (80: 20, v/v), methanol-water (70: 30, v/v), and water}. The insoluble-bound phenolics were procured after alkaline hydrolysis and subsequent extraction into diethyl ether-ethyl acetate. Phenolic extracts of each fraction were separately assayed for their antioxidant activity using several methods, namely oxygen radical absorbance capacity (ORAC), the reducing power capacity, as well as iron (II) chelation capacity, among others. There were significant differences in the total content of phenolics, flavonoids, and anthocyanins between blackberry, black raspberry, and blueberry seed meals. The bound phenolics contributed the highest proportion to the total contents of different classes of phenolics. Furthermore, blackberry seed meals had higher total antioxidant activity compared with black raspberry and blueberry seed meals in all assays employed. High-performance liquid chromatography−diode array detection−electrospray ionization multistage mass spectrometry (HPLC-DAD-ESI-MS^n) was used to identify and quantify the phenolic compounds. Hydroxybenzoic and hydroxycinnamic acids, anthocyanins, flavonols, flavan-3-ols, and proanthocyanidins were identified and quantified in the aforementioned fractions. Extracts were found to contain various levels of phenolic compounds that were specific to each berry seed meal type.

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CHAPTER 1

INTRODUCTION

The consumption of fresh fruits and vegetables has continued to increase, primarily due to a better understanding about the association of their intake with a lower incidence of cardiovascular disease, cancer, degenerative diseases, and other chronic ailments. Oxidative stress which is induced by free radical attack on cellular components by reactive oxygen species (ROS) has a major role in the development of many degenerative diseases. As a result, antioxidants protect against oxidative stress and therefore they are considered important in reducing the initiation and progression of these diseases. Not only endogenous antioxidant systems play a crucial function in combating oxidative stress, but dietary antioxidants are also important (Cortan *et al.,* 1999). Fruits and vegetables are valuable sources of phytochemicals, such as carotenoids, vitamins C and E, folate, phenolic and thiol compounds. The relationship between increased fruit and vegetable intake and lower risk of chronic diseases could be attributed to their antioxidant activity (AA). Unlike animal dietary sources, plant-based diets contain many simple phenolic and polyphenolic compounds that possess significant AA. In plants, the polyphenolic compounds provide several eco-physiological functions, involving chemical and physiological defense responses, and they are varied in their structures and chemical properties. As components in the human diet, phenolic compounds are considered to act as antioxidants directly, or to affect the production or function of other antioxidant compounds in the body (Halvorsen *et al.,* 2002).

Berries such as blackberry (Rubus sp.), black raspberry (Rubus occidentalis), blueberry (Vaccinium corymbosum) are among the most widely consumed fruits in the human diet in fresh and in processed forms such as beverages, yogurts, jellies, and jams. Moreover, berry extracts are commonly used in botanical dietary supplement forms for their possible health benefits. Extensive research, using laboratory animals, has found that berries have anticancer properties. The biological activities of berries are partially ascribed to their high content of a wide range of phytochemicals such as flavonoids (anthocyanins, flavonols, and flavanols), tannins (proanthocyanidins, ellagitannins, and gallotannins), stilbenoids (e.g., resveratrol), phenolic acids (hydroxybenzoic and hydroxycinnamic acid derivatives), and lignans (Seeram, 2006). In epidemiological and clinical studies, these constituents have been associated with improved cardiovascular risk profiles (Bsau *et al.*, 2010). Phenolic compounds exhibit many biologically significant mechanisms of action, such as scavenging or detoxification of reactive oxygen species (ROS), blocking ROS production, impacting cell cycle, suppression of tumors, modulation of signal transduction, apoptosis, detoxifying enzymes and metabolism (Han *et al.*, 2007; and Liu *et al.*, 2004).

In the production of juices, jellies and jams from berries, most processors consider the seeds of the berries to be removed from the berry pulp during processing to enhance the end product. The seeds are commonly used as a source of oils and the leftover is the meal (flour) that is still rich in bioactive ingredients (Helbig *et al.*, 2008). Unlike berry fruits and berry seed oils, berry seed meals have not received much attention as antioxidant sources and this could be attributed to their lack of popularity and lack of commercial applications. Not only berry fruits contain several beneficial compounds but also berry seed meals contain various phenolic compounds and

can be a good source of natural antioxidants. Therefore, berry seed meals were used in this study in order to evaluate them systematically. It would be beneficial, if berry seed meals could be employed as a source of natural food ingredients in enhancing the full utilization of the seeds.

Antioxidant efficiency may be evaluated by several methods such as 2, 2-azino-di-(3-ethylbenzothialozine-sulphonic acid) (ABTS), 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging potential, reducing power (RP), oxygen radical absorbance capacity (ORAC), and iron chelating capacity (Ozgen *et al.*, 2006). Antioxidants can scavenge radicals by single electron transfer (SET) and hydrogen atom transfer (HAT) mechanisms. ABTS and DPPH represent the SET, and ORAC represents the HAT mechanism (Ozgen *et al.,* 2006; Huang *et al.,* 2005). These assays vary in terms of their assay principles and experimental conditions. Because of multiple reaction characteristics and mechanisms, a single assay will not be sufficient to reflect all antioxidants in a mixed or complex system (Li *et al.,* 2009). Therefore, each assay provides an estimate of antioxidant efficiency that is based on the type of method selected and experimental conditions employed. Thus, the use of different methods assists to identify variations in the response of the compounds extracted from plant sources.

The first aim of this study was to determine the total antioxidant capacities and phenolic contents of selected berry seed meals. The second aim of this study was to identify the various phenolic compounds in blackberry, black raspberry, and blueberry seed meals by high-performance liquid chromatography (HPLC).

CHAPTER 2

LITERATURE REVIEW

Oxidizable substrates, such as proteins, lipids, carbohydrates and nucleic acids, constitute the major components of food, whereas antioxidants are found in minor amounts. Antioxidants effectively postpone or suppress the oxidation of these substrates (Halliwell and Gutteridge, 1990). Moreover, antioxidants have stimulated much interest among biochemists and health professionals due to their role in assisting the body to protect itself against damage resulting from reactive oxygen species (ROS) (Shahidi, 1997). Therefore, antioxidant intake can reduce disease risk and health problems (Sen *et al.,* 2010). The present investigation evaluated the antioxidant capacity in the seed meals of blackberry, black raspberry, and blueberry as potential sources of natural antioxidants as little information was available in this area. In this connection, it was important to identify the phytochemicals of blackberry, black raspberry, and blueberry seed meals in order to evaluate how their consumption affects human health as a result of their biological properties. The subsequent sections provide a cursory review to explain the effect of lipid oxidation in food and health, the functions of reactive oxygen species (ROS), and roles of synthetic and natural antioxidants in the control of oxidation.

2.1-Lipid oxidation

Food lipids may be categorized as triacylglycerols, which are found in fat storage depots of plants and animals, and phospholipids, which occur as structural components of the cell membranes. Lipids may become rancid due to oxidation, and this oxidative rancidity is a

primary cause of food spoilage (Shahidi and Zhong, 2010). Food with lipids composed of fatty acids with a higher degree of unsaturation is more susceptible to oxidation. Foodstuffs that contain high amounts of polyunsaturated fatty acids (PUFA) are greatly susceptible to oxidation (Sun *et al.,* 2011).

Lipid oxidation is predominately mediated by ROS. By definition, "a free radical is any chemical species that has the ability to exist independently and possesses one or more unpaired electrons" (Karlsson, 1997). A free radical species containing an oxygen atom is referred to as an oxygen free radical. The oxygen free radicals include superoxide radical anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$) and perhydroxyl radical ($HO_2^{\bullet}$) and are all considered reactive oxygen species (ROS) (Barry, 1995; Halliwell, 1995).

Triplet oxygen (or $^3O_2$) is the ground state of the oxygen molecule containing two unpaired spin electrons with the lowest energy status. In the presence of light, photosensitizers interact with atmospheric oxygen to produce singlet oxygen. Hydroperoxides are generated through a process called photosensitized oxidation, in which the direct reaction of singlet oxygen with unsaturated fatty acids occurs. The primary factor that results in the spoilage of edible oils during processing is singlet oxygen (Bradley and Min, 1992; Frankel, 1998).

2.2-Mechanism of autoxidation

The free radical chain mechanism of autoxidation proceeds through three distinct steps, namely, initiation, propagation, and termination reactions (Figure 2.1) (Shahidi and Zhong, 2010).

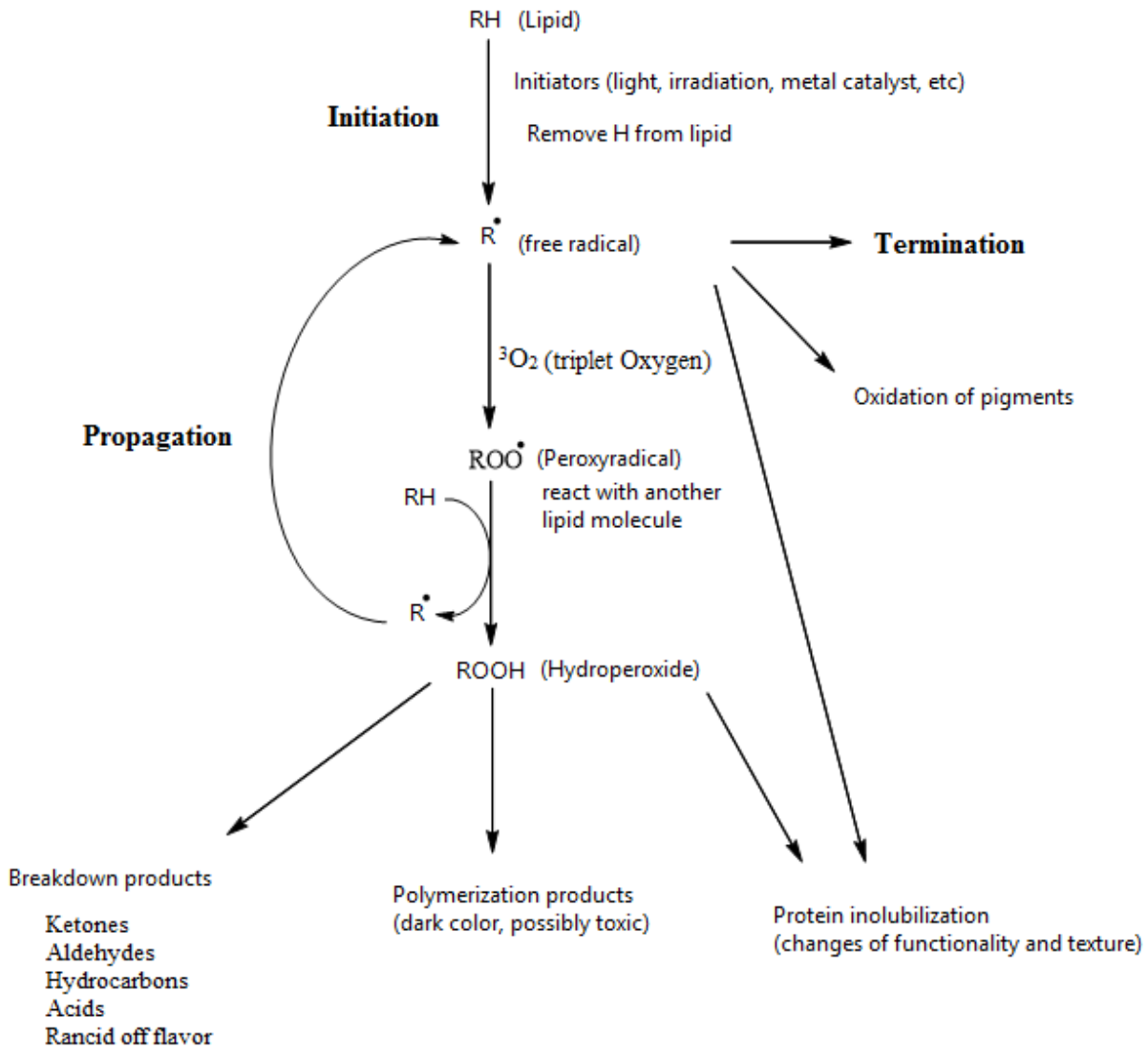


Figure 2.1. General schematic for the autoxidation of lipids containing polyunsaturated fatty acids (RH) and their consequences. Adapted from Shahidi and Zhong (2010).

In the initiation step, hydrogen atoms are abstracted from lipid molecules by a reactive species, such as a hydroxyl radical, which leads to the formation of lipid radicals (Shahidi and Zhong, 2010). Peroxyl radicals (ROO•) are produced from the interaction of a lipid radical, which is highly excited, with triplet oxygen ($^3O_2$) (Bradley and Min, 1992; Frankel, 1998) through a reaction that is commonly catalysed by metal ions *(*Shahidi and Zhong, 2010). After the initiation reaction, propagation reactions, in which the peroxyl radical interacts with another lipid molecule to produce a hydroperoxide and another lipid radical, take place. Then, the produced free radical R• reacts with oxygen to form a peroxyl radical (ROO•), which in turn leads to a self-catalysed, cyclical mechanism (Figure 2.2). In the termination phase, two free radicals combine to form a non-radical product (Bradley and Min, 1992; Frankel, 1998).

Hydroperoxides, which are the main products of oxidation, are very unstable and quickly degrade to secondary compounds, such as aldehydes, ketones, alcohols, acids, and hydrocarbons (Shahidi and Zhong, 2010). These new molecules have unfavourable odours and flavours and are responsible for the development of oxidative rancidity of unsaturated lipids (Bradley and Min, 1992; Frankel, 1998; St. Angelo, 1996).

2.3 Antioxidant effects

Various terms have been employed by different researchers to describe the antioxidant capacity involving the total antioxidant efficiency, effectiveness, action, power, parameter, potential, potency, and activity. Even though antioxidant activity and antioxidant capacity have different meanings, these terms are frequently used interchangeably (MacDonald-Wicks *et al.,* 2006).

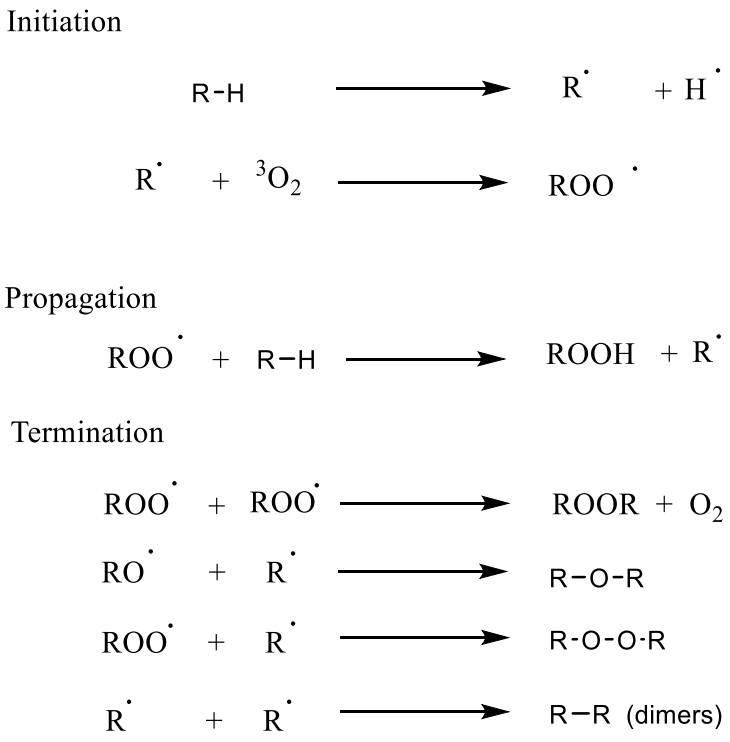


Figure 2.2. Radical chain reactions of lipid oxidation. Adapted from Saldana and Martinez-Monteagudo (2013).

Antioxidants can be categorized, depending on their mechanism of action, into two groups: primary and secondary. The primary antioxidants are known as chain-breaking antioxidants because they can donate a hydrogen atom to lipid radicals and produce lipid derivatives and antioxidant radicals (A˙) (Figure 2.3) (Wanasundara and Shahidi, 2005).

Through different mechanisms, secondary antioxidants provide their antioxidant activity to slow the rate of oxidation reactions. They do not convert free radicals into stable products, unlike primary antioxidants. They act as chelators for prooxidant or catalyst metal ions, offer H to primary antioxidants, decompose hydroperoxide to nonradical species, deactivate singlet oxygen, absorb ultraviolet radiation, or act as oxygen scavengers. The antioxidant activity of primary antioxidants is usually improved by secondary antioxidants (Wanasundara and Shahidi, 2005).

The delocalization of the unpaired electron throughout the aromatic ring can stabilize the formed antioxidant radical. Therefore, these antioxidant radicals are more stable and less reactive compared with the lipid or peroxyl radical (Figure 2.4) (Bradley and Min, 1992; Frankel, 1998).

Due to the apparent extent of the available oxidation initiators, there are several "preventive" antioxidation mechanisms. Different mechanisms, including chelation of transition metals, singlet-oxygen deactivation, enzymatic ROS detoxification, UV filtration, inhibition of prooxidant enzymes, are responsible for the reaction through which secondary antioxidant act and delay the onset of oxidation (Laguerre *et al.,* 2007). Dietary antioxidants often mainly involve radical chain reaction inhibitors, metal chelators, oxidative enzyme inhibitors, and antioxidant enzyme cofactors (Huang *et al.,* 2005).

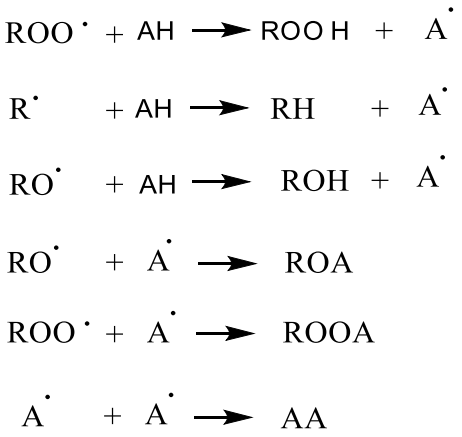


Figure 2.3. Mechanism of action of a primary antioxidant, AH (Wanasundara and Shahidi, 2005).

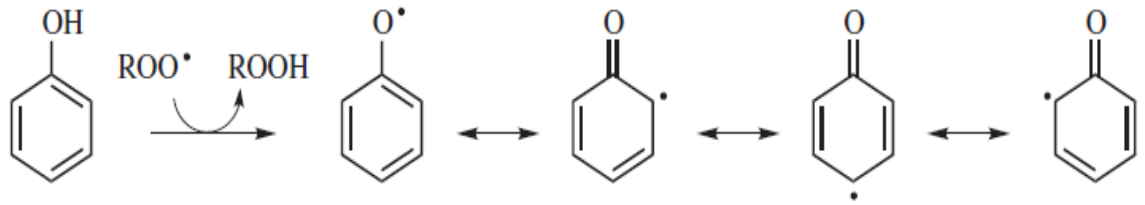


Figure 2.4. Stable resonance hybrids of a phenoxy radical of a phenolic antioxidant. Adapted from Fattah *et al.* (2014).

2.4-Fruit as a source of phenolic compounds

Fruits and vegetables, which are excellent sources of many antioxidants, such as phenolics, including tocopherols, thiols, and carotenoids, exhibit health-promoting effects, such as reducing blood pressure and decreasing incidences of cancer and cardiovascular diseases (Block *et al.,* 1992). Thus, it is recommended that individuals should increase their intake of fruits and vegetables (Cevallos-Casals and Cisneros-Zevallos, 2003). Additionally, the sensory qualities of fruits and vegetables (colour, astringency, bitterness, and flavour) are influenced by phenolics and related enzymes (Herrmann, 1990). Different types of fruits are important sources of phenolic compounds. For instance, cinnamate esters of tartaric acid are generally present in grapes, and flavanone glycosides occur in citrus fruits (Dimitrios, 2006). The most common and noticeable plant phenols are built upon a C6-C3-C6 flavone skeleton in which the three-carbon bridge connecting the two benzene rings is generally cyclised with oxygen (Robards *et al.,* 1999). The next most widespread and diverse flavonoids are the anthocyanins (anthocyanidin glycosides) (Hong and Wrolstad, 1986). Increased attention has been paid to natural antioxidants that occur in plant foods, particularly polyphenols, including flavonoids, which inhibit the production of ROS (Galvano *et al.,* 2004).

Sources of tocopherols, carotenoids and ascorbic acid are widely known, and there is considerable literature associated with their health-promotion function (Dimitrios, 2006). Flavonoids and other plant phenolics, such as phenolic acids, stilbenes, tannins, lignans, and lignin, are substantially distributed throughout plants, e.g., in their leaves, flowering tissues, and woody parts, and these are essential in plants for normal growth and resistance to infection and

injury. However, due to the complex chemical nature of plant phenols and their extensive presence in plant materials, they have not been entirely investigated (Dimitrios, 2006).

2.5-Berries

Berries are fruits with more than one seed within the fruit and are not compartmentalised (Stewart *et al.*, 2007). A variety of flavonoids and phenolic acids that exhibit antioxidant activity is found in berry fruits. Anthocyanins, proanthocyanidins, flavonols, and catechins are the major flavonoids that occur in berries and fruits. Berries generally show high total phenolic contents and high antioxidant activity. Even though the content of phenols in berries with a dark purple colour, such as crowberry (Empetrum nigrum), aronia (chokeberry), bilberry (Vaccinium myrtillus), and whortleberry (Vaccinium uliginosum), is greater (28.7-50.8 mg/g GAE) than that found in the yellowish rowanberries (Sorbus aucuparia) and cloudberries (Rubus chamaemorus) (18.7 and 16.2 mg/g GAE, respectively), there are no marked differences between their antioxidant effectiveness (Kahkonen *et al.*, 1999). The strongly coloured berries contain high amounts of anthocyanins. Moreover, berry wines made from berries with a dark colour, such as bilberries (Vaccinium myrtillus), black currants (Ribes nigrum), cowberries (Empetrum nigrum), cranberries (Vaccinium macrocarpon), and crowberries (Empetrum nigrum), exhibit higher antioxidative efficiency against the oxidation of methyl linoleate (MeLo) in comparison with berries with a pale colour, such as cloudberries (Rebus chamaemorus), red raspberries (Rubus idaeus), and strawberries (Fragaria X ananasso Duch.). Hydroxylated derivatives of the benzoic acid and cinnamic acid families are commonly found in berries and fruits (Heinonen *et al.*, 1998).

Extreme scavenging efficiency against chemically produced superoxide radicals is observed with the extracts of blackberries (Rubus fructicosus), black currants (Ribes nigrum), red currants (Ribes sativum), blueberries (Vaccinium corymbosum), and black raspberries (Rubus occidentalis), and red raspberries (Rubus idaeus) (Constantino *et al.*, 1992). Furthermore, the oxidation of human low-density lipoproteins (LDL) and liposomes is prevented by phenolic extracts of berries, such as blackberries (Rubus fructicosus), red raspberries (Rubus idaeus), sweet cherries (Prunus avium L.), blueberries (Vaccinium corymbosum), and strawberries (Fragaria X ananasso Duch.). In an LDL oxidation system, more powerful antioxidant efficiency was noted with blackberries, red raspberries, sweet cherries, and blueberries than with strawberry (Heinonen *et al.*, 1998). The phenolic compounds in berries have been found to have antioxidant, anticancer, anti-inflammatory, and anti-neurodegenerative properties (Seeram, 2008).

2.6-Phenolic compounds

Phenolic compounds are one of the most common groups of substances and are the products of secondary metabolism in plants. More than 8000 phenolics have been identified, and these play vital activities in the development and growth of plants, work as defence agents against pathogens, parasites, and predators, and contribute to the colour of plants (Dong *et al.*, 2007).

Phenolic compounds have been classified into three main classes*:* phenolic acids, flavonoids, and tannins (Figure 2.5) (Chung *et al.*, 1998). Phenolic acids are derivatives of two basic chemical structures: C6-C1 (benzoic acids) and C6-C3 (cinnamic acids) (Figure 2.6). The differences between the two groups are that the hydroxyl benzoic acids have two carbons less

than the hydroxycinnamic acids in their side chains. The most commonly found hydroxybenzoic acids include gallic, *p*-hydroxybenzoic, protocatechuic, vanillic and syringic acids, whereas among the hydroxycinnamic acids, caffeic, ferulic, *p*-coumaric and sinapic acids can be noted (Bravo, 1998). The number of hydroxyl groups and their esters in the molecules enhances the antioxidant activity of phenolic acids. The antioxidant activities of hydroxylated cinnamic acids are markedly better than those of their benzoic acid counterparts (Cuvelier *et al.,* 1992). Flavonoids are composed of two units: a C6-C3 fragment from cinnamic and a C6 fragment from malonyl-CoA. Carbon-carbon bonds link the polymers of five to seven flavan-3-ol units (catechin) in condensed tannins, also known as proanthocyanidins (Hahn *et al,* 1984; Mehansho *et al.,* 1987; Butler, 1990). However, phenolic compounds possess a wide variety of structures but have some common structural features, including a benzene ring with one or more hydroxyl substituents. These compounds can generally be classified by the number and arrangement of their carbon atoms in the structure of the basic phenolic skeleton and may be as simple as phenols, phenolic acids, coumarins, flavonoids and stilbenes (Figure 2.5) (Hurtado-Fernandez *et al.,* 2010).

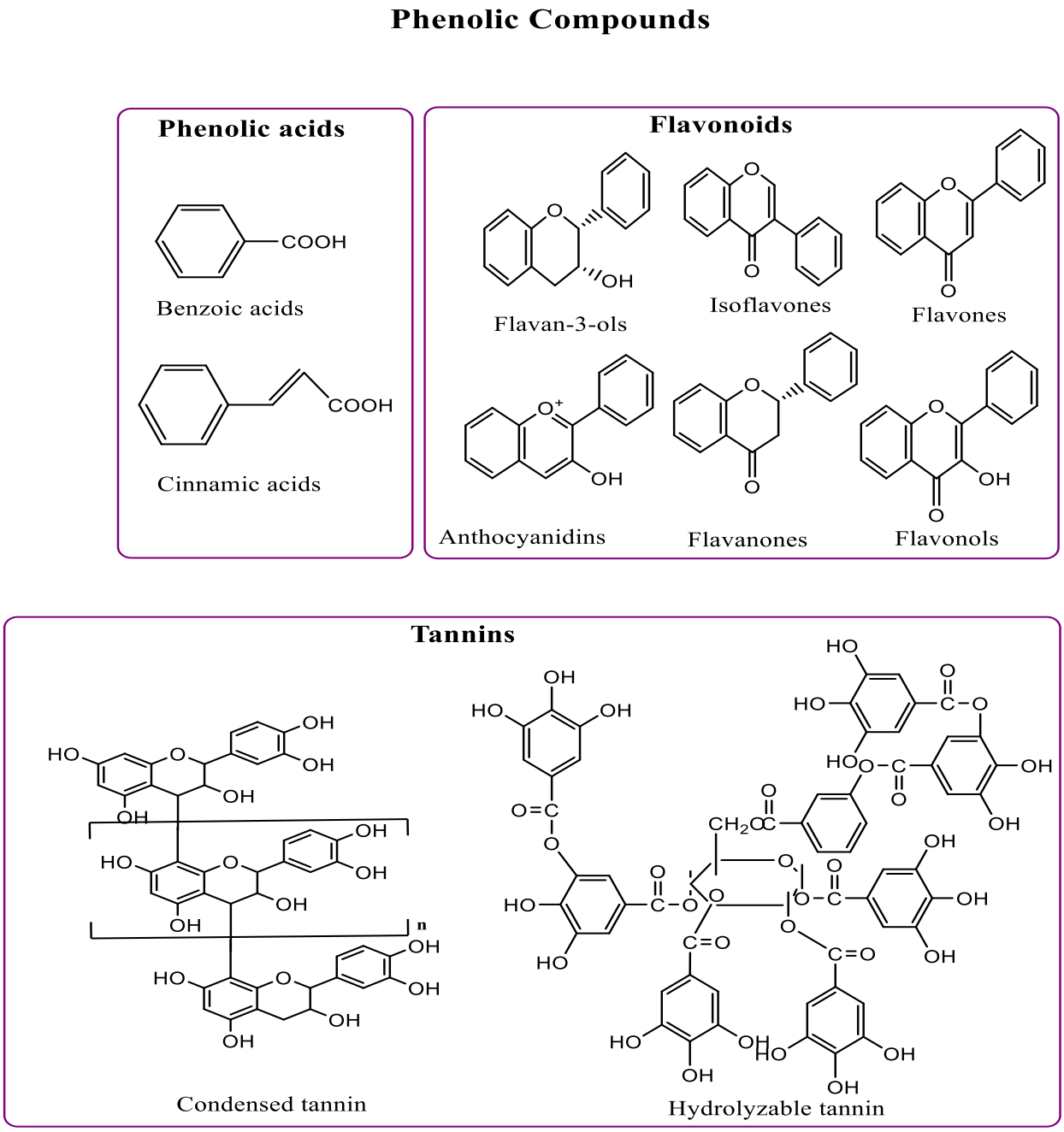


Figure 2.5. Classification of phenolic compounds and representative structures belonging to hydroxybenzoic acids, hydroxycinnamic acids, flavones, isoflavones, flavanones**,** flavonols, flavanols, anthocyanins and tannins. Adapted from Hurtado-Fernandez *et al.* (2010).

2.7-Phenolic acids

Phenolic acids generally carry one benzene ring linked to one or more hydroxyl groups (Crozier *et al.,* 2009). Only a fraction of these compounds occur as "free acids", whereas most are conjugated through ester, ether, or acetal linkages to structural constituents of the plant, such as cellulose, proteins, and lignin, larger polyphenols (flavonoids), smaller organic molecules (e.g., glucose, quinic, malic, or tartaric acids), or other natural products (e.g., terpenes). These bonds result in the generation of a large number of derivatives. Moreover, this variety is a vital factor in the complexity associated with the analysis of phenolic acids (Robbins, 2003). *p*-Hydroxybenzoic, protocatechuic, vanillic, syringic, and gallic acids comprise the hydroxybenzoic acid derivatives, and these commonly occur in their conjugated form. *p*-Coumaric acid, caffeic acid, ferulic acid, sinapic acid, and caffeic acid are the most common hydroxycinnamates (Figure 2.6). These acids often present in the bound form or conjugated with quinic acid, the latter groups are known as chlorogenic acids. Furthermore, ellagic acid and ellagitannins occur abundantly in fruits, such as raspberries, strawberries, pomegranate, blackberries, persimmon, walnuts, hazelnuts, and oak-aged wines (Crozier *et al.,* 2009).

The interest in the analytical examinations of phenolics is also attributed to their contribution to the organoleptic properties (flavour, astringency, and hardness) of foods. Moreover, food manufacturers need to investigate the nature and profile of phenolic acids and their effect on fruit maturation and inhibition of enzymatic browning and their function as food preservatives related to their antioxidant potential ascribed to the reactivity of the hydroxyl moiety in the phenols (Robbins, 2003).

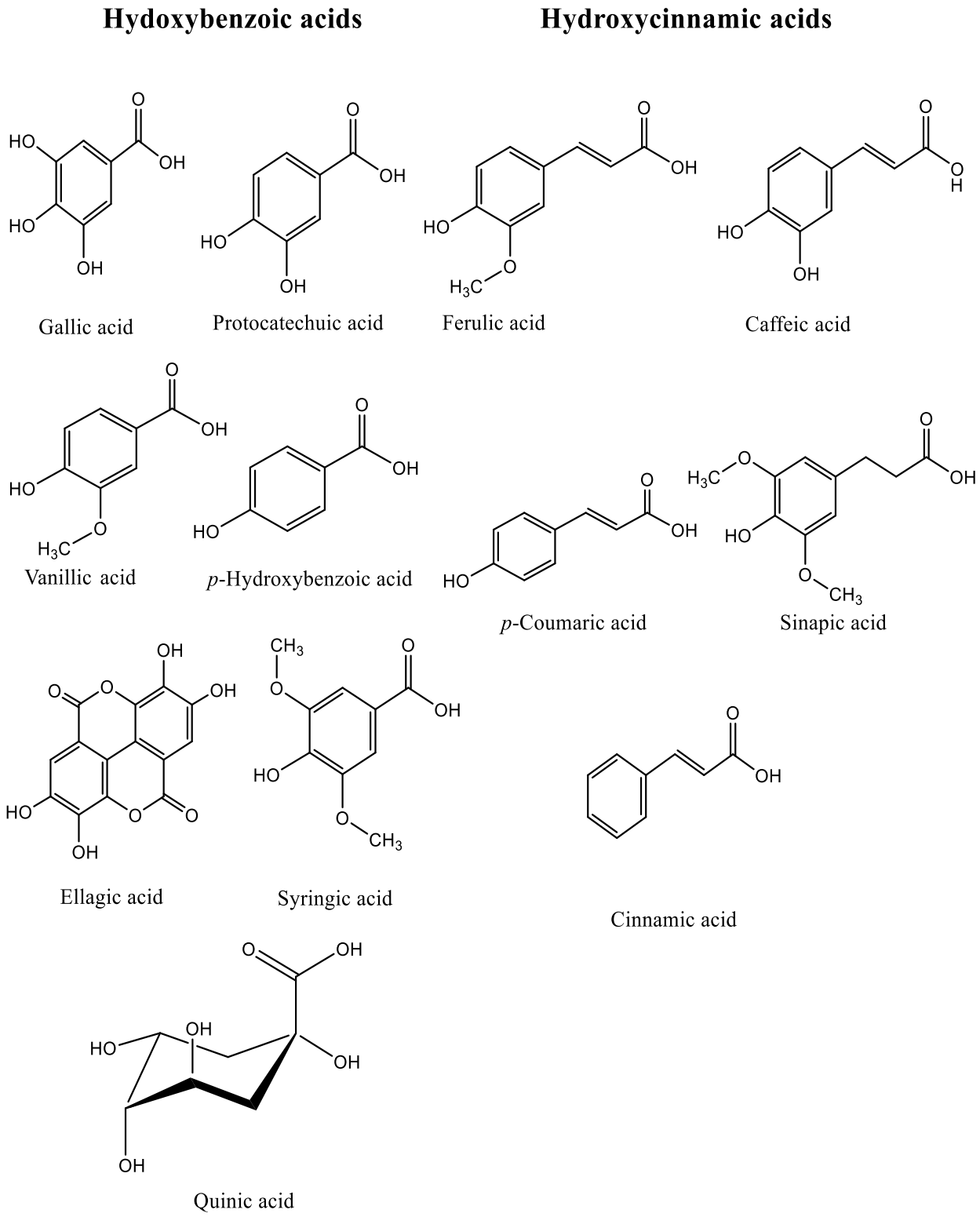


Figure 2.6. Examples of naturally occurring phenolic acids. Adapted from Naczk and Shahidi (2004).

2.8-Flavonoids

2.8.1-Chemical properties of flavonoids

Flavonoids are polyphenolic compounds that occur ubiquitously in nature (Nijveldt *et al*., 2001). Some flavonoids serve as plant pigments and provide colour to fruits and flowers. Depending on their chemical structure, flavonoids are classified into flavonols, flavones, flavanones, isoflavones, flavanols, anthocyanidins, and chalcones. A large number of substitution patterns in the two aromatic rings of the basic structure can be present in nature (Namiki, 1990). They differ in the structure around the heterocyclic oxygen ring, but they all contain a unique C6-C3-C6 carbon skeleton (Figure 2.7) (Clifford and Cuppett, 2000). To date, more than 6000 flavonoids have been identified (Harborne and Williams, 2000). All flavonoids are common products of the 2-phenylchromone parent compound, which possesses three aromatic rings, known as A, B, and C rings, with different degrees of hydroxylation and methoxylation (Clifford and Cuppett, 2000).

2.8.2-Antioxidant activity of flavonoids

In addition to their distribution in plants, flavonoids play essential roles in human health due to their pharmacological effectiveness as radical scavengers with high antioxidant capacities (Cook and Samman, 1996). As dietary components, flavonoids render health-promoting properties both in *vivo* and in *vitro* (Cook and Samman, 1996; Rice-Evans *et al.,* 1996). Based on their chemical structures, the antioxidant activities of flavonoids can be changed. Hydroxylation (associated to the position and number of hydroxyl groups) of the B ring is especially essential for such activity (Namiki, 1990; Nijveldt *et al*., 2001).

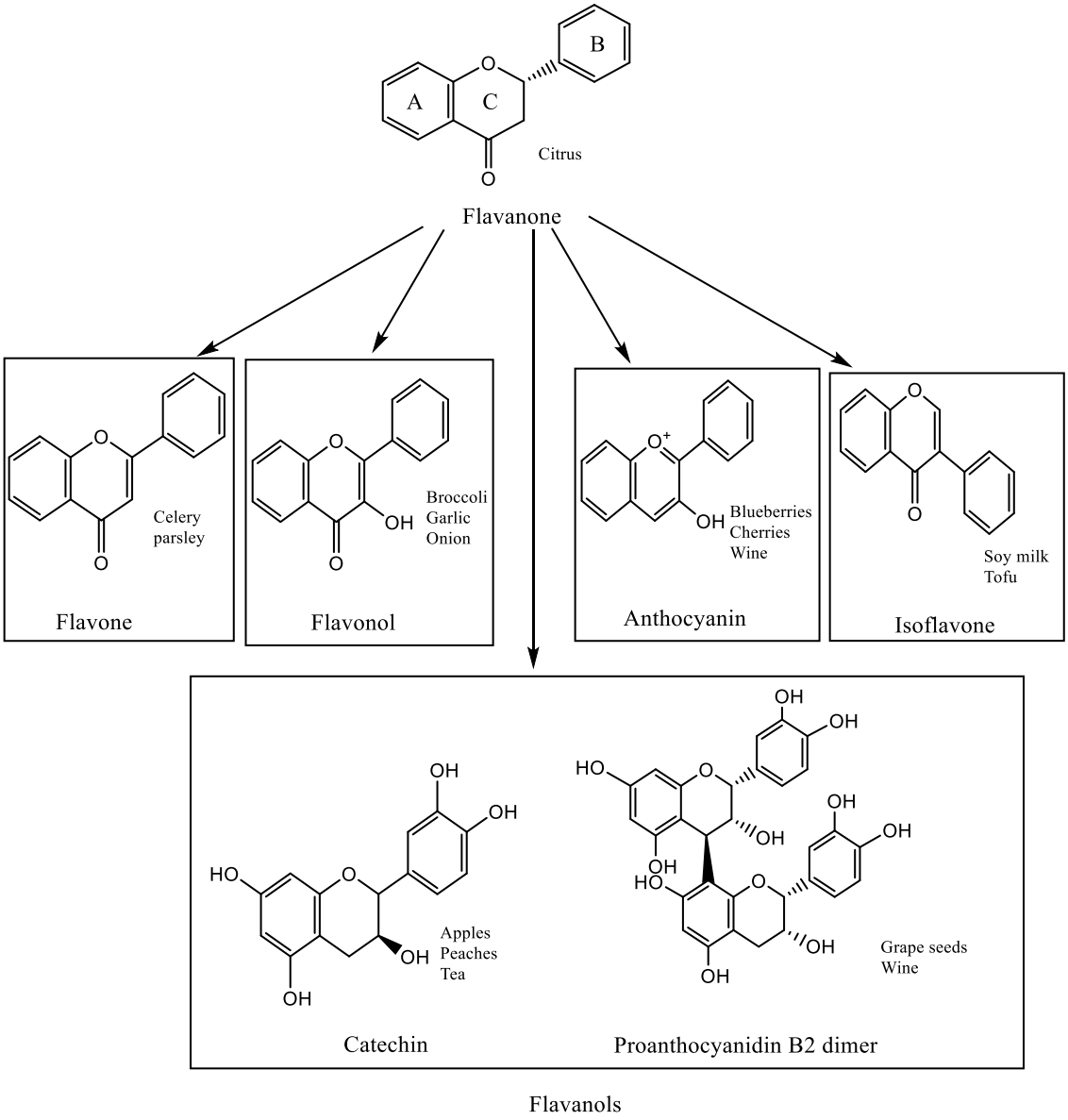


Figure 2.7. Structure and dietary occurrence of the main classes of flavonoids. Adapted from Peterson and Dwyer (1998).

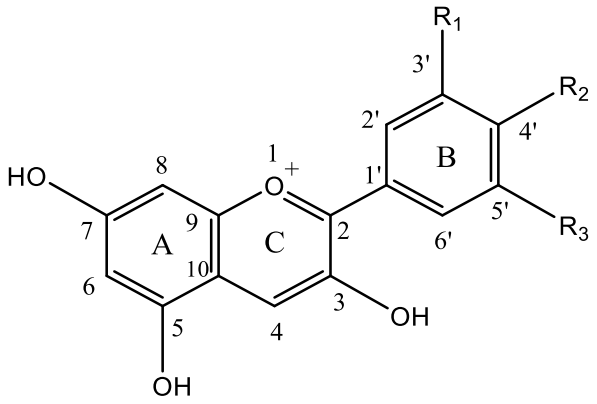
2.9-Anthocyanins

2.9.1-Chemical structure

Anthocyanins (ACNs), which are water-soluble plant pigments, provide the blue, purple, and red colour in different plants. Because a high concentration of anthocyanins provides colour, there is a significant proportion of anthocyanins in the total polyphenol concentration in coloured berries. Anthocyanins are found mainly as glycosides or acyl glycosides of their respective aglycone anthocyanidins (Jakobek *et al.*, 2007). In fresh plant materials, a limited number of aglycones can be found. Seventeen anthocyanidins occur naturally, but only six of these are distributed in plant materials, namely, cyanidin (Cy), delphinidin (Dp), petunidin (Pt), peonidin (Pn), pelargonidin (Pg), and malvidin (Mv) (Figure 2.8). Therefore, there are more than 600 naturally occurring ACNs, and these differ in the following characteristics: (1) the number and location of hydroxyl and methoxyl groups on the basic anthocyanidin structure; (2) the identity, amount, and locations at which sugars are joined; and (3) the range of sugar acylation and identity of the acylating agent.

2.9.2-Antioxidant activity of anthocyanins

In plant physiology, anthocyanins (ACNs) play essential roles in both pollination and seed dispersal, and this feature is ascribed to their strong colour. ACNs are also considered natural colorants in the food industry. Currently, more attention has been concentrated on their potential health effects. ACNs exhibit powerful antioxidant activity and may render a great variety of health benefits through antioxidant or other mechanisms (Kong *et al.*, 2003; Galvano *et al.*, 2004). They have beneficial impacts in the treatment of different diseases and are prescribed as medicines in many countries (Cao *et al.*, 2001).



Anthocyanidin	**R_1**	**R_2**	**R_3**
Pelargonidin (Pg)	H	OH	H
Cyanidin (Cy)	OH	OH	H
Delphinidin (DP)	OH	OH	OH
Peonidin (Pn)	OMe	OH	H
Petunidin (Pt)	OMe	OH	OH
Malvidin (Mv)	OMe	OH	OMe

Figure 2.8. Structures of common anthocyanidins. Adapted from Clifford (2000); Nazak and Shahidi (2004).

2.10-Proanthocyanidins (Tannins)

The specific structure of proanthocyanidins (PACs), which are a type of condensed tannin, can affect their biological activity. Flavanols (or flavan-3-ols) can be present as monomers, such as catechin, epicatechin, gallocatechin, and epigallocatechin, or in their oligomeric and polymeric forms, which are referred to as proanthocyanidins (Robbins *et al.,* 2006; Nandakumar *et al.,* 2008). The flavan-3-ol units (epicatechin or catechin) are most often linked through carbon–carbon bonds from position 4 of one flavanol subunit to position 8, but the C4→C6 bond is also present to a lesser extent (both called B-type). A less common structural feature of PACs is the A-type linkage, which involves a double linkage through an additional ester bond, namely, C2→O7 (Gu *et al.,* 2004). Procyanidins, which are members of the proanthocyanidin family of compounds, consist exclusively of (epi)catechin units and are widely found in plants. In contrast, the less common proanthocyanidins, which contain (-)epiafzelechin and (+)afzelechin or (epi)gallocatechin subunits, are called propelargonidins and prodelphinidins, respectively (Figure 2.9).

The most relevant polyphenolic compounds in berries are anthocyanins, hydrolysable tannins (gallo- and ellagitannins), flavonols, and flavan-3-ols. Flavan-3-ols, which are better known as proanthocyanidins or condensed tannins, are polymers of flavan-3-ols and/or flavan-3, 4-diol mixtures. These compounds give a characteristic astringent or bitter taste to many berries (Howell, 2007).

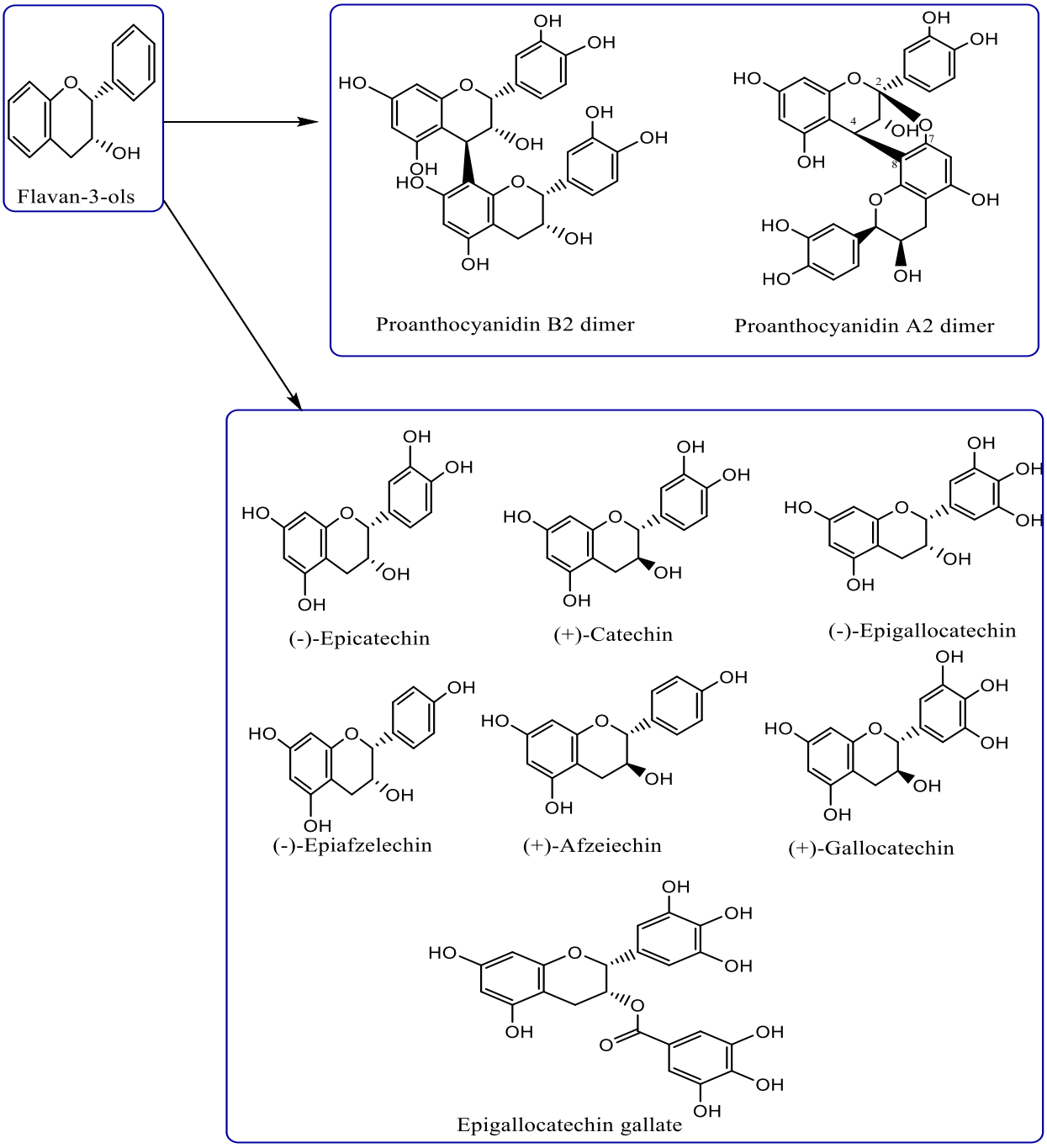


Figure 2.9. Classification of proanthocyanidins. Adapted from Iglesia *et al.* (2010).

2.11-Phenolic compounds in blackberries, raspberries, and blueberries

Blackberries are promoted as a rich source of polyphenols with high antioxidant capacity and radical scavengers with beneficial functions in human health, such as reducing the risk of cancer, cardiovascular disease, and other diseases. Raspberries (*Rubus idaeus* L.) and blackberries (*Rubus fruticosus* L.), which are members of the Rosaceae family, can be eaten fresh or used as ingredients in processed products, such as ice creams, jams, jellies, marmalades, purées, fruit juices, and liquors, among others (Haffner *et al.*, 2002).

Three hydroxycinnamic acids (ferulic, caffeic, and *p*-coumaric acids) and two hydroxybenzoic acids (*p*-hydroxybenzoic and protocatechuic acid) have been identified in all raspberry and blackberry cultivars (Rommel and Wrolstad, 1993a). These hydroxycinnamic and hydroxybenzoic acids are found as glycosides or esters (Rommel and Wrolstad, 1993a). Moreover, flavonols (quercetin, kaempferol, and myricetin) have been found in raspberry and blackberry cultivars, with quercetin being dominant in all cultivars (Turkben *et al.*, 2010). Fruits belonging to the Rubus family (red raspberries and blackberries) also contain catechins, ellagic acid derivatives and high amounts of anthocyanin (Jakobek *et al.*, 2009).

The major anthocyanins in red raspberry have been identified as cyanidin and pelargonidin glycosylated with rutinose and sophorose (Mertz *et al.*, 2007).

Blueberries have drawn considerable attention due to their high antioxidant capacity and high concentration of anthocyanins, mainly in their glycosylated forms, and other phenolic compounds. Therefore, blueberries are one of the most preferable and nutritious choice among

fresh fruits and vegetables (Prior *et al.*, 1998). Blueberry fruit contains four major anthocyanins, namely delphinidin, cyanidin, petunidin, and malvidin. The content of malvidin-based anthocyanins in blueberries is much higher than those of petunidin-, delphinidin-, or cyanidin-based anthocyanins (Wang *et al.*, 2008). Of the secondary plant metabolites found in blueberries, the anthocyanins have received the most attention (Gao and Mazza, 1994; Wu *et al.*, 2006); although flavonols (predominantly quercetin derivatives), phenolic acids (caffeic, chlorogenic, *p*-coumaric and ferulic acid) and proanthocyanidins are also present (Moyer *et al.*, 2002; Taruscio *et al.*, 2004). In addition to anthocyanins, blueberries are also one of the richest sources of chlorogenic acid, quercetin, kaempferol, myricetin, procyanidins, catechin, epicatechin, resveratrol, and vitamin C with antioxidant activity. The composition and content of phenolic compounds in blueberries differ widely, according to the cultivar, the season and the growing location, therefore, it is difficult to provide a good comparison of the existing data in the literature (Giovanelli and Buratt, 2008).

2.12-Extraction of polyphenolics

Phenolics are present in three forms: free, esterified, and insoluble bound. At present, there is no convenient prevalent extraction technique for the extraction of all phenolics or a particular class of phenolic compounds from plant materials (Xu and Chang, 2007). For the extraction of phenolics, various solvents, such as methanol, ethanol, acetone, water, ethyl acetate and, to a lesser extent, propanol, dimethylformamide, and their combinations, are often employed. Selecting a suitable solvent influences the amount and rate of the polyphenols extracted (Xu and Chang, 2007). The lower-molecular-weight polyphenols are better extracted with methanol, whereas aqueous acetone has more powerful capacity to extract the higher-molecular-weight

flavanols (Labarbe *et al.,* 1999). An acidified organic solvent, such as methanol or ethanol, may be used for the extraction of anthocyanin pigments from plant materials. The simultaneous denaturation of the cell membrane and the dissolution and stabilization of the anthocyanins can be achieved using an acidified organic solvent (Naczk and Shahidi, 2004).

The extraction time and temperature affect the recovery of phenolic compounds from plant products. A number of phenolic compounds are easily hydrolysed and oxidized. Low extraction yields of phenolic compounds and a high rate of oxidation of phenolics can be achieved with long extraction times at high temperatures. Thus, it is necessary to choose an efficient extraction technique and to ensure the stability of the phenolic compounds. In addition, other factors, such as the solvent-to-solid ratio and the particle size of the sample, may also impact the extraction of phenolic compounds from plant materials. The yield of phenolics could be improved by increasing the solvent-to-solid ratio (Pinelo *et al.,* 2005).

Free, esterified, and insoluble-bound phenolic compounds were extracted as described by Krygier *et al.* (1982). The highest amount of free and esterified phenolic acids from oilseeds may be obtained using a mixture of methanol–acetone–water (7:7:6, v/v/v) at ambient temperatures. After the free phenolics are extracted with diethyl ether from the acidified aqueous suspension of phenolic extract, the water suspension of the extract can be neutralized with 4 M NaOH under nitrogen to release the esterified phenolic acids. The hydrolysate is then acidified, and the liberated phenolic acids are extracted with diethyl ether. To release the insoluble-bound phenolic acids, the residue is subsequently neutralized with 4 M NaOH under nitrogen following exhaustive extraction with a mixture of methanol–acetone–water.

2.13-Methods for assessment of antioxidant activity

Oxidation in foods and biological systems results in the production of free radicals. Thus, it is essential to search for methods to determine free radical scavenging (Sanchez-Moreno, 2002), and it is important to determine the antioxidant status in biological systems (Halliwell and Gutteridge, 1990). Moreover, the consumption of foods containing antioxidants and the assessment of the real contribution of foods to the antioxidant status in biological systems needs to be evaluated (Namiki, 1990; Jacob, 1995).

Due to the numerous activities of polyphenols and because the main activity is based on the environment and type of antioxidant, it is necessary to develop a protocol that can measure more than one feature. The response of antioxidants to various radical or oxidant sources may be different. For instance, in relation to phenolics, carotenoids are not particularly good quenchers of peroxyl radicals but are the most effective singlet oxygen quenchers. Thus, no individual assay can be used as the most appropriate assay for precisely determining the antioxidant capacity (Prior *et al.*, 2005).

The assays used to determine the antioxidant capacities of foods and biological systems can be divided into two groups. The assays belonging to the first group examine lipid peroxidation; these use a lipid or lipoprotein substrate under standard conditions and evaluate the level of oxidation inhibition. The assays belonging to other group evaluate the free radical scavenging ability, which is the major mechanism through which antioxidants function in foods (Sanchez-Moreno *et al.*, 1999). Various approaches that can be employed to evaluate the antioxidant capacity include scavenging of superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) scavenging,

hypochlorous acid (HOCl) scavenging, hydroxyl radical (HO•) scavenging, and peroxyl radical (ROO•) scavenging. Methods that use azo-compounds are also employed to generate peroxyl radicals, and these include the following methods: TRAP (total radical-trapping antioxidant parameter) and the ORAC (oxygen radical absorbance capacity) methods, the scavenging of radical cation 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS) and TEAC (Trolox equivalent antioxidant capacity) methods, the scavenging of stable DPPH (2,2-diphenyl-1-picrylhydrazyl) radical method and the scavenging of radical cation DMPD (*N*,*N*-dimethyl-*p*-phenylenediamine) method (Sanchez-Moreno, 2002). The main difference among these assays is the quantification approaches. For instance, the ORAC assay applies the area under the kinetic curve approach, the TRAP assay is based on the lag time, and the crocin-bleaching assay employs the initial reaction rate (Huang *et al.* 2005). Thus, the analysis of the total antioxidant capacity should include methods applicable to both lipophilic and hydrophilic antioxidants due to their similarities and differences in both the hydrogen atom transfer (HAT) and electron transfer mechanisms (Karada *et al.,* 2009).

There is a strong desire to standardize measurements of antioxidant activity even though a variety of methods can be used. The search for more specific methods may provide knowledge that could be associated directly to the oxidative rancidity of foods and biological systems (Sanchez-Moreno, 2002). Even though several methods are available to determine antioxidant efficiency, it is essential to use a consistent and rapid method (Krishnaiah *et al.,* 2011). To identify and quantify the antioxidant molecules in plant species, techniques based on spectrophotometry, high-performance liquid chromatography (HPLC), and mass spectrometry (MS) have been established (Covey *et al*., 1986). Kahkonen *et al*. (2001) indicated that there are

several simple methods to extract fruit phenolic compounds (Escribano *et al*., 2006). Thus, the choice of method is very important to ensure that the results are reliable and representative.

CHAPTER 3
MATERIALS AND METHODS

3.1-Materials

Sampling. The seed meal of blackberry (Rubus sp.), black raspberry (Rubus occidentalis), and blueberry var. Jersey (Vaccinium corymbosum) samples were kindly provided by the Fruit Smart Company, Grandview, WA, USA. Samples were packed immediately and stored in vacuum bags and stored in a freezer at -20°C.

Chemicals. Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) was purchased from Acros Organics (Fair Lawn, NJ, USA). Organic solvents and reagents such as methanol, acetone and sodium carbonate were purchased from Fisher Scientific Co. (Nepean, ON). 2,2'-Azobis (2-methylpropionamidine) dihydrochloride (AAPH), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), Folin and Ciocalteau's phenol reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and all phenolic compound standards with a purity of ≥96% were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON). Sodium hydroxide, ferric chloride as well as mono- and dibasic sodium and potassium phosphates, ethylenediaminetetraacetic acid (EDTA), deoxyribonucleic acid (DNA) of pBR 322 (*E.coli* strain RRI) were also purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON).

3.2-Methods

3.2.1-Preparation of samples

The oil from the berry seed meals has been commercially removed but there is still some residue of oil remains in the berry seed meals. Therefore, an extra effort is needed to remove the residual oil so that the results that are obtained in this work are not influenced by the residues of oil in the samples. All samples were defatted by blending the ground material with hexane (1:5, w/v, 5 min) in a Waring blender (Model 33BL73, Waring Products Division, Dynamic Corp of America, New Hartford, CT, USA) at room temperature three times. The resultant extracts were combined and filtered through Whatman #1 filter paper with suction using a Buchner funnel. Defatted samples were air dried for 12 h and vacuum packaged in polyethylene pouches and kept at -20°C until used. The filtrate from the three extractions were combined and desolventised using a rotary evaporator in a previously weighed round bottom flask at -40°C. The resulting oil was weighed and transferred into 10 mL sample vials, purged with nitrogen and stored at -20°C.

3.2.2-Extraction of phenolics

Extraction of phenolic compounds from the defatted seed meals of blackberry, black raspberry, and blueberry were performed according to the method outlined by Krygier *et al.* (1982) and as explained by Naczk and Shahidi (1989) (Figure 3.1). Phenolic compounds found in defatted samples were extracted using methanol–acetone–water (7:7:6, v/v/v), acetone-water (80: 20, v/v), methanol-water (70: 30, v/v), and water, and each was used separately. About 5 g of each sample were extracted with 75 mL of each solvent. These samples were ultrasonicated for 20 min at 30°C, then centrifuged for 5 min at 4000xg. After centrifugation, the upper layers were combined and the extraction operation was repeated twice. The collected supernatants were

examined for free phenolic acids and soluble phenolic esters, and the residue (the lower layer) was stored for evaluation of insoluble-bound phenolics. The organic solvent, methanol–acetone–water (7:7:6, v/v/v), from combined extracts was removed under vacuum at 40°C using a rotary evaporator (Rotavapor model 461, Büchi, Flawil, Switzerland). The supernatant was freeze-dried using a freeze dryer (CoolSafe 55, ScanLaf A/S, Lyngbe, Denmark) and kept at 4°C until use. Before extraction with hexane, the aqueous phase was acidified to pH 2 using 3 M HCl and centrifuged to separate any precipitates. The residue in water was extracted with an equal volume of hexane to remove interfering lipids and other lipid contaminants (Krygier, 1982). Extraction of free phenolic acids was carried out 3 times with an equal volume of diethyl ether-ethyl acetate (1:1, v/v). Diethyl ether-ethyl acetate layer was filtered through anhydrous sodium sulphate using a no. 1 Whatman filter paper and combined, evaporated to dryness, and dissolved into 5 mL of HPLC grade methanol. The esters which are the remaining water phase were hydrolyzed with 4 M NaOH for 4 hours at ambient temperature. The pH of the hydrolysate was adjusted to 2 and the liberated phenolic acids were extracted with diethyl ether-ethyl acetate (1:1, v/v), evaporated to dryness and subsequently dissolved in 5 mL methanol.

To extract insoluble-bound phenolics the solid residue remaining after the first set of extractions was digested with 40 mL of 4 M NaOH and stirred for 4 h. The mixture was then brought to pH 2, centrifuged and the bound phenolics were extracted with diethyl ether- ethyl acetate (1:1, v/v) as explained above.

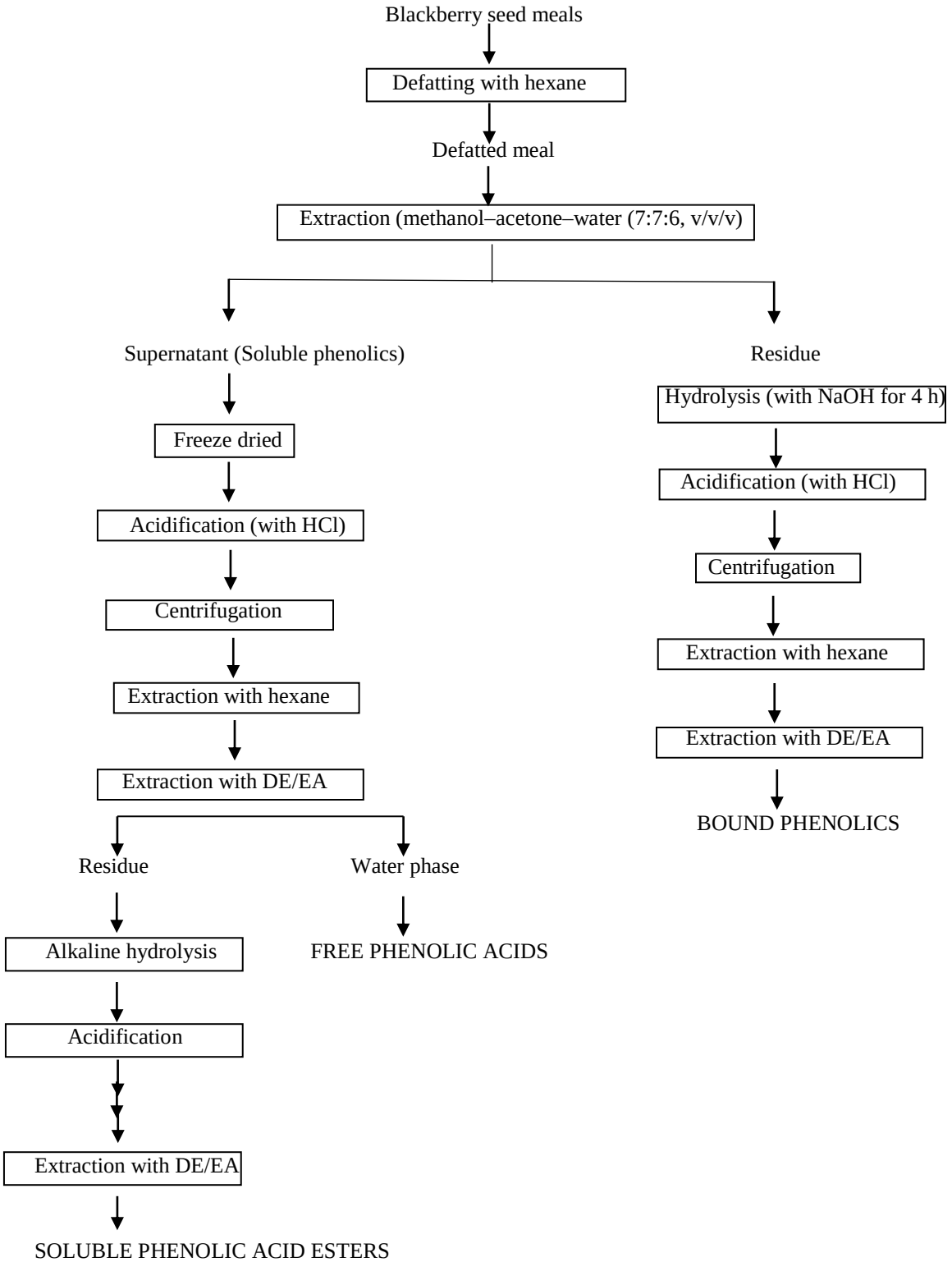


Figure 3.1. Flow diagram of the procedure for extraction of soluble (sum of free and soluble esters), and bound phenolics from berry seed meals. Black raspberry and blueberry seed meals are following the same trend. Diethyl ether-ethyl acetate (DE/EA). Sodium hydroxide (NaOH). Hydrochloric acid (HCl).

3.2.3-Determination of total phenolic content

The total phenolics were estimated by the Folin–Ciocalteu colourimetric method, according to Singleton and Rossi (1965). The Folin-Ciocalteau reagent (0.5 mL) was mixed with 0.5 mL of methanolic extracts in a centrifuge tube. Then, 1 mL of saturated sodium carbonate (75 g/L) was added to each tube, followed by adjusting the total volume to 10 mL with distilled water. The contents were thoroughly mixed by vortexing and allowed to stand for 45 min at ambient temperature (23°C). The mixture was then centrifuged for 5 min at 4000xg (ICE Centra M5, International Equipment Co., Needham Heights, MA, USA). The absorbance of the resulting blue-coloured solution was read at 725 nm. A blank sample for each extract was used for background subtraction. Gallic acid was used as a standard and determination of content of total phenolics in each extract (Appendix A). The total amount of phenolic compounds was expressed as mg of gallic acid equivalents per gram of extract.

3.2.4-Assay for total flavonoid content determination

Total flavonoid content was measured by the aluminum chloride colourimetric assay (Zhishen *et al.,* 1999). Two dilutions were made to determine the total flavonoid content of berry seed meals. Five millilitres of methanol were added to 5g of each berry seed meal extract in order to make the first dilution. Then, from the first dilution, 0.5mL was taken and added to 14.50 mL to make the second dilution. Distilled water (4 mL) was added to 1 mL of extracts or standard solution of quercetin (3, 4, 5 6, 6.5 mg/mL). Then, 0.3 mL of 5% sodium nitrite solution was added. After 5 min, 0.3 mL 10% $AlCl_3$ solution was added. At the 6^{th} min, 2 mL of 1M NaOH solution were added and the volume of the reaction mixture was made to 10 mL with distilled

water. The mixture was thoroughly vortexed and the absorbance of the pink colour developed was measured against a prepared reagent blank at 510 nm. A calibration curve was prepared with quercetin and the results were expressed as mg quercetin equivalents (QE)/g of the original dry material. Samples were analyzed in triplicates and the results were expressed as mean ± standard deviation.

3.2.5-Measurement of oxygen radical absorbance capacity ($ORAC_{FL}$)

The $ORAC_{FL}$ assay was carried out using a Fluostar Optima microplate reader (BMG Labtech, Durham, NC, USA). The internal wells of a 96-well Costar 2650 black plate (Nepean, ON, Canada) were used to perform the ORAC assay. Solutions were prepared in triplicate, in which 20 µL of appropriately diluted extract, blank, or trolox were mixed with 200 µL of 0.11 µM fluorescein solution directly in the microplate and the plate was incubated in FLUOstar at 37°C for 15 min. Subsequently, the machine was programmed to inject 75 µL of AAPH into each well to initiate the reaction. The plate was shaken for 4 s, and the fluorescence was determined and recorded every minute for 60 min with an excitation and emission wavelengths of 485 and 520 nm, respectively. The ORAC values were measured as trolox equivalents per gram sample (μmol TE/ g) using a standard curve prepared with 6.25-100 µM trolox.

3.2.6-Reducing power activity

The reducing power of extracts was determined by the method of Amarowicz *et al.* (1999). Briefly, 1.0 mL of sample or trolox standard was mixed with 2.5 mL of a 0.2 M phosphate buffer, and 2.5 mL of a 1% (w/v) solution of potassium ferricyanide was incubated in a water bath at 50°C for 20 min. Aliquots of a 10% (w/v) trichloroacetic acid (2.5 mL) were added to

the mixture and then centrifuged at 3000 rpm (1650 xg) for 10 min. The upper layer of solution (1 mL) was combined with 2.5 mL of distilled water and 0.5 mL of a 0.1% (w/v) solution of ferric chloride. Only potassium ferricyanide and ferric chloride were prepared in 1 M HCl. The absorbance of the reaction mixture was measured spectrophotometrically at 700 nm and the results were expressed as μmoles trolox equivalent per gram of dried sample. Increased absorbance of the reaction mixture indicates greater reducing power.

3.2.7-Measurement of iron (II) chelation capacity

The capacity of phenolic extracts to chelate ferrous ions was estimated according to the procedure explained by Dinis *et al*, (1994). In brief, 0.4 mL of extracts was added to a solution of 2mM $FeCl_2$ (0.05 mL). The reaction was initiated by addition of 5 mM Ferrozine (0.2 mL), and the total volume was adjusted to 4 mL with distilled water. The mixture was vigorously shaken and left to react at room temperature for 10 min. The absorbance of the reaction mixture was measured spectrophotometrically at 562 nm. Distilled water was used for the control instead of the extract. Appropriate blanks were prepared with 0.4 mL of the sample and 3.6 mL of distilled water for background subtraction. Different concentrations (25-300 mM) of ethylenediaminetetraacetic acid (EDTA) were used to prepare the standard curve. Iron chelation capacities of the extracts were calculated using the following equation:

Fe (II) chelation capacity (%) = (1-Absorbance of sample)* 100/ Absorbance of blank

The results were expressed as micromoles of EDTA equivalents per gram of defatted meal.

3.2.8-Measurement of total antioxidant capacity

Analyses were performed by using the method described by Berg *et al.* (1999) and modified by John and Shahidi (2010). An $ABTS^{\bullet+}$ solution was prepared by mixing 2.5 mM 2,2'-azobis-(2-methylpropionamidine) dihydrochloride (AAPH) with 2.0 mM $ABTS^{\bullet+}$ stock solution in 2.5 mM saline phosphate buffer (pH 7.4, 0.15M sodium chloride) (PBS). The solution was heated for 12 min at 60°C, protected from light by covering in tin foil and stored at room temperature until used. To check $ABTS^{\bullet+}$ formation the absorbance at 734 nm was determined. Because of a gradual decrease in absorbance of the $ABTS^{\bullet+}$, the absorption had to be between 0.35 and 0.40. For measuring antioxidant capacity, 40 µL of the sample were mixed with 1.96 mL of the radical solution. Absorbance was measured at 734 nm after a six min period. The decrease in absorption (734 nm) at 10 s ("fast" reaction) and 6 min ("total" reaction) was used for calculating TEAC. A calibration curve was prepared with various concentrations of trolox. TEAC values were expressed as micromoles of trolox equivalents (TE) per gram of defatted material. TEAC values were determined as follows:

$$\Delta A_{trolox} = \{A_{t=0\ trolox} - A_{t=6\ min\ trolox}\} - \Delta A_{solvent\ (0\text{-}6\ min)}$$

$$\Delta A_{trolox} = m * [trolox]$$

$$TEAC = \{\Delta A_{extract} / m\} * d$$

Where, ΔA= reduction in absorbance, A= absorbance at a given time, m= slope of the standard curve, [trolox] = concentration of trolox, and d= dilution factor.

3.2.9-DPPH radical scavenging capacity (DRSC) using electron paramagentic resonance (EPR)

DRSC assay was performed according to the method described by Madhujith and Shahidi (2007) with slight modification. Two millilitres of 0.18 mM solution of DPPH in methanol were added

to 500 µL of appropriately diluted free, esterified and bound phenolics extracts in methanol. Trolox, which is generally used as a reference antioxidant, was also analyzed in this work. Contents were mixed well, and after 10 min the mixture was passed through the capillary tubing, which guides the sample through the sample cavity of a Bruker e-scan EPR spectrophotometer (Bruker E-scan, Bruker Biospin Co., Billercia, MA, USA). The spectrum was recorded on Bruker E-scan food analyzer (Bruker Biospin Co.). The parameters were set as follows: 5.02 x 10^2 receiver gain, 1.95 G modulation amplitude, 2.621 s sweep time, 8 scans, 100.000 G sweep width, 3495.258 G centre field, 5.12 s time constant, 9.79309 GH_Z microwave frequency, 86.00 kH_Z modulation frequency, 1.86 G modulation amplitude. For quantitative measurements of radical concentration remaining after reaction with the extracts, the method of comparative determination based on the corresponding signal intensity of first-order derivative of absorption curve was used. DRSC of the extracts was calculated using the following equation:

DPPH radical scavenging capacity (%) = 100 – (EPR signal intensity for the medium containing the additive/EPR signal intensity for the control medium) * 100.

From the standard curve plotted for the DRSC of trolox, the scavenging activity of berry seed meal extracts was determined and expressed as µmol trolox equivalent /g berry seed meal extracts.

3.2.10-Antioxidant assay using the β-carotene linoleate model system

The antioxidant activity of berry seed meal extracts was examined by the β-carotene linoleate model system (Jayaprakasha, 2001). A solution of β-carotene was prepared by dissolving 10 mg of β-carotene in 10 mL of chloroform. A portion of this solution (1.2 mL) was transferred into a

100 mL round-bottom flask containing 20 mg linoleic acid and 200 mg of Tween 40. After removal of chloroform under a stream of nitrogen, oxygenated-distilled water were added to the flask and the mixture was stirred vigorously using a magnetic stirring bar. Aliquots (4.5 mL) of this emulsion were transferred into different test tubes containing 0.5 mL of the berry seed meal extracts. The control was prepared by adding 4.5 mL of the emulsion to 0.5 mL of ethanol. The tubes were shaken and incubated at 50°C in a water bath. Once the emulsion was added to each tube, the zero time absorbance was calculated at 470 nm using a spectrophotometer. Absorbance readings were then recorded at 15 min intervals until the control sample had changed colour. A blank, devoid of β-carotene was also prepared (20 mg of linoleic acid + 200 mg Tween 40 + 50 mL oxygenated distilled water) for background subtraction. Antioxidant activity was calculated using the following equation: antioxidant activity (%) = (β-carotene content after 2 h of assay/initial β-carotene content) * 100.

3.2.11-Hydroxyl radical scavenging activity

The capacity of phenolic compounds in scavenging hydroxyl radicals formed by Fenton reaction was examined by electron paramagnetic resonance (EPR) spectroscopy using a slightly modified version of a method previously reported (Wettasinghe, and Shahidi, 1999). The hydroxyl radical was generated through Fe (II)-catalyzed Fenton reaction and spin trapped with 5, 5-dimethyl-1 pyrroline N-oxide (DMPO). The resultant DMPO-OH adduct was detected using an electron paramagnetic resonance (EPR) spectrometer. Extracts (200 µL) were mixed with 200 µL of 10 mM H_2O_2, 300 µL of 17.6 mM DMPO, and 200 µL of 0.1 mM $FeSO_4$. All solutions were prepared in deionized water. After 1 min, the EPR spectrum of the mixture was recorded at 5.02 $\times 10^2$ receiver gain, 1.86 G modulation amplitude, 2.621 s sweep time, 8 scans, 100.000 G sweep

width, 3495.258 G center field, 5.12 s time constant, 9.795 GHz microwave frequency, 86.00 kHz modulation frequency, and 1.86 G modulation amplitude. Hydroxyl radical scavenging capacities of the extracts were calculated using the following equation:

Hydroxyl radical scavenging capacity (%) = 100 – (EPR signal intensity of samples with extracts/ EPR signal intensity for control) * 100

3.2.12-Determination of 2-thiobarbituric acid reactive substances (TBARS) in cooked comminuted fish meat model system.

Fish model systems were prepared as explained by Shahidi and Pegg (1990). In Mason jars, ground salmon (20 g) was mixed with deionized water (10 mL). Fractions of berry seed meal extracts (200 ppm) and 100 ppm catechin**,** were added individually to fish mixture in Mason jars. A sample containing no extract, was run as a control. In a thermostated water bath, the contents were mixed thoroughly and cooked at 80°C for 40 min with occasional stirring with a glass rod. Fish samples were homogenized in a precooled Waring Blender for 30 s after being cooled to room temperature. Homogenized fish samples were transferred into plastic bags, and then stored in a refrigerator at 4°C for 7 to 14 days. Samples were analyzed for TBARS on days 0, 7, and 14.

TBARS were determined using a modified version of the assay explained by Wijeratne *et al.*, (2006). Two grams of each sample were weighed in a centrifuge tube to which 2.5 mL of a 10% (w/v) solution of trichloroacetic acid (TCA) was added and vortexed (Fisher Vortex Genie 2; Fisher Scientific, Nepean, ON) at high speed for 2 min. An aqueous solution (0.02 M) of TBA (2.5 mL) was then added to each centrifuge tube, followed by further vortexing for 30 s. The samples were subsequently centrifuged at 3000xg for 10 min and the supernatants were filtered

through a Whatman No. 3 filter paper. Filtrates were heated in a boiling water bath for 45 min, cooled to room temperature in cold water, and the absorbance of the resultant pink-coloured chromogen read at 532 nm. TBARS values were measured using 1, 1, 3, 3-tetramethoxypropane as a precursor of the malondialdehyde (MDA; 0, 1, 2, 3, 4, and 6 ppm). The TBARS values were then calculated using the standard curve and expressed as milligrammes MDA equivalents per kg sample. Inhibition of TBARS production was determined using the equation: inhibition (%) =100 (1-TBARS value for the treated sample/TBARS value for the control sample).

3.2.13-Effect of berry seed meal extracts on preventing cupric ion induced human low density lipoprotein (LDL) cholesterol peroxidation

To evaluate inhibitory activities of berry seed meal extracts toward human LDL cholesterol oxidation, the assay of Chandrasekara and Shahidi (2011a) was employed. Human LDL cholesterol (in PBS, pH 7.4, with 0.01% EDTA) was dialyzed against 10 mM PBS (pH 7.4, 0.15 M NaCl) for 12 h under nitrogen at 4°C, and EDTA-free LDL was subsequently diluted with PBS to obtain a 0.1 mg/mL. The diluted LDL cholesterol solution (0.8 mL) was mixed with 100 µL of extract (0.125 and 0.5 mg/mL) in an Eppendorf tube. Oxidation of LDL cholesterol was initiated by adding 0.1 mL of 100 µM $CuSO_4$ solution in distilled water. The mixture was incubated at 37°C for 20 h. The initial absorbance (t=0) was read at 234 nm immediately after mixing and conjugated diene (CD) hydroperoxides formed at the end of 22 h were measured. The corrected absorbance at 22 h against 0 h was employed to calculate the percentage inhibition of CD formation using the following equation:

Inhibition of CD formation (%) = $(Abs_{oxidative} - Abs_{sample})/ (Abs_{oxidative} - Abs_{native}) * 100$, where $Abs_{oxidative}$ = absorbance of LDL mixture and distilled water with $CuSO_4$ only, Abs_{sample} =

absorbance of LDL with extract and $CuSO_4$, and Abs_{native} = absorbance of LDL with distilled water.

3.2.14-Inhibition of peroxyl and hydroxyl radical induced supercoiled DNA strand scission

The inhibition activity of the berry seed meal extracts against supercoiled DNA strand scission induced by peroxyl radical was determined according to the method of Chandrasekara and Shahidi, (2011a). Plasmid supercoiled DNA (pBR 322) was dissolved in 0.5 M phosphate buffered saline (PBS) pH 7.4. In an Eppendorf tube, the DNA (5 µL) was added to 5 µL of extract samples and 10 µL of AAPH (7 mM dissolved in PBS), in the order stated for peroxyl radical-induced oxidation. The mixture was mixed well and incubated at 37°C for 1h. At the end of 1 h incubation, the loading dye (consisting 0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol) was added to the sample. The gel was prepared in Tris–acetic acid-EDTA (TAE) buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.5), containing SYBR safe DNA gel stain (5 µL/50 mL of gel). The gel was poured carefully into tray and allowed to set for 30 min. Samples (10 µL) were added into wells carefully and the gel electrophoresis was running at 80 Volt for 2 h. DNA strands were visualized under ultraviolet light. For hydroxyl radical-induced DNA oxidation, 5 µL of test compounds, were added into an Eppendorf tube and the solvent was evaporated under a stream of nitrogen. To each tube, the following reagents were then added in the order stated: 5 µL of PBS (pH 7.4), 5 µL of supercoiled pBR322 DNA, 5 µL of H_2O_2 and 5 µL of $FeSO_4$ were mixed well and incubated at 37°C for 1 h.

The protective effect of extracts was calculated as DNA retention (%) based on the following equation.

DNA retention (%) = (Supercoiled DNA content in sample/Supercoiled DNA content in control)*100

3.2.15-HPLC analysis

In order to identify the predominant phenolic compounds available in the berry seed meal samples, high-performance liquid chromatography−diode array detection−electrospray ionization multistage mass spectrometry (HPLC-DAD-ESI-MS^n) was used (Agilent Technologies, Palo Alto, CA, USA). A slightly modified version of the method described by Maatta *et al.* (2003) was used. Analytical separation of the phenolic compounds and anthocyanins was carried out on Supelcosil LC-18 column (250×4.6 mm inner diameter, 5-μm particles, Supelco, Bellefonte, PA, USA). The column oven temperature was maintained at 25°C. Mobile phases constituted of 0.1% formic acid (A) and acidified water containing 0.1% acetonenitrile (B). The elution conditions were as follows: 0 min, 100% B; 5 min, 90% B; 35 min, 85% B; 45 min, 60% B; 50 min, 60% B; 55 min, 100% B; 65 min, 100% B and then held for 1 min before returning to the initial conditions. The flow rate was 0.5 mL/min and the wavelengths of detection were 280 nm for monitoring phenolic compounds and 520 nm for monitoring anthocyanins. An external standard method using authentic compounds was used to identify the unknown peaks in the samples. Peak identification of unknown compounds of interest in this study was performed by matching the retention times of unknown compounds with external standards. All the peaks detected were tentatively identified by careful studies of the MS and MS–MS spectra and by comparison with literature data (Appendix B).

3.2.16-Statistical analysis

Three different samples of every mix were analyzed, each in triplicates. The results were analyzed using ANOVA and Tukey's test ($p < 0.05$) and SAS software. All values are means ± standard deviation of three samples. Differences at $P<0.05$ were considered statistically significant. Correlation between total phenolic content and antioxidant activity was established by using linear regression analysis at $P<0.05$.

Results and Discussion

4.1-Selection of solvent for the preparation of phenolic extracts

The yield of chemical extraction differs based on the type of solvent (which exhibit varying polarities), pH, extraction time, temperature and chemical compositions of the sample. The solvent and the chemical properties of the sample are two most important factors under the same conditions of time and temperature (Lopez *et al.*, 2011). The amounts (mg gallic acid equivalent/g defatted meal) of the antioxidant extract have been determined for berry seed meals using four different solvents {methanol–acetone–water (7:7:6, v/v/v), acetone-water (80:20, v/v), methanol-water (70:30, v/v), and water}. These solvents were chosen to determine the best solvent that can be used in tests for the evaluation of the antioxidant activity. The results of the total phenolic assays conducted to determine the best solvent among the aforementioned solvent systems showed that the highest phenolic contents (sum of free, esterified and bound phenolics) from blackberry seed meals were obtained using methanol–acetone–water (7:7:6, v/v/v), which afforded a yield of 13.06 mg GAE/g of dry weight of defatted meal, followed by acetone-water (80:20, v/v), methanol-water (70:30, v/v) and water with extract yields of 7.23, 5.59, and 3.11 mg GAE/g of dry weight of defatted meal, respectively. Similarly, for black raspberry seed meals, the highest yield of extract (7.68 mg GAE/g of dry weight of defatted meal) was obtained with methanol–acetone–water (7:7:6, v/v/v), followed by acetone-water (80:20, v/v), methanol-water (70:30, v/v) and water with yields of 4.87, 5.87 and 2.16 mg GAE/g of dry weight of

defatted meal, respectively. The same trend was also found for blueberry seed meals: methanol–acetone–water (7:7:6, v/v/v) showed the highest yield (1.84 mg GAE/g of dry weight of defatted meal), followed by acetone-water (80:20, v/v), methanol-water (70:30, v/v) and water with extract yields of 1.53, 0.77, and 0.58 mg GAE/g of dry weight of defatted meal, respectively. The corresponding values on the basis of defatted berry seed meals are also shown in Table 4.1.

These findings are in agreement with those reported by Kahkonen *et al.* (2001), who obtained a lower extract yield from berry fruits with 100% H_2O compared with that obtained with mixtures of water and organic solvents. Jaroszynska (2003) examined the effect of solvents, namely, diethyl ether, acetone, methanol, chloroform and water, on the recovery of phenolic compounds during extraction from plant materials and noted that the highest recovery was achieved with methanol and acetone. Based on these preliminary studies, the mixture of methanol, acetone, and water was found to be a good extraction solvent for the preparation of phenolic extracts and was therefore used for all subsequent studies. However, acetonic extract appeared to be a common extractant for condensed tannins (proanthocyanidins) in other studies on grape seeds (Liu and white, 2012) and grape skin and seeds (Hernandez-Jimenez *et al.*, 2009). Several studies on berry phenolic compounds and their antioxidant activities have been reported (Simirgiotis *et al.*, 2013; Leon-Gonzalez *et al.*, 2013), and various aqueous solutions of acetone, methanol, and ethanol have also been employed to extract the free phenolic compounds from berries (Tuberoso *et al.*, 2010). However, it is difficult to compare the data within the literature because of the various antioxidant activity evaluation assays and extraction solvents used by the different researchers. In addition, when choosing the most suitable assay, it should be considered that analysis conditions, substrate, and concentration of antioxidants must simulate

real food or biological systems as much as possible. Antioxidants may act by various mechanisms which means employing a method depending on one mechanism may not reflect the true antioxidant capacity.

Table 4.1. Total phenolics of free, esterified, bound phenolics of berry seed meals extracted with different solvents {methanol–acetone–water (7:7:6, v/v/v), acetone-water (80:20, v/v), methanol-water (70:30, v/v), and water}.

Solvents	Total phenolics (mg gallic acid eq/g defatted meal)		
	Free	**Esterified**	**Bound**

Blackberry			
Methanol–acetone–water (7:7:6, v/v/v)	2.23±0.05^{Ca}	2.90±0.15^{Ba}	7.93±0.02^{Aa}
Acetone-water (80:20, v/v)	1.19±0.05^{Cb}	2.45±0.02^{Bc}	3.59±0.08^{Ab}
Methanol-water (70:30, v/v)	0.57±0.06^{Cc}	1.66±0.45^{Bb}	3.36±0.13^{Ab}
Water	0.38±0.03^{Cc}	1.20±0.03^{Bd}	1.53±0.01^{Ac}
Black Raspberry			
Methanol–acetone–water (7:7:6, v/v/v)	1.18±0.10^{Ca}	1.90±0.06^{Ba}	4.60±0.20^{Aa}
Acetone-water (80:20, v/v)	1.28±0.80^{Ba}	1.36±0.09^{Bb}	2.23±0.14^{Ac}
Methanol-water (70:30, v/v)	1.16±0.30^{Ca}	1.49±1.10^{Bb}	3.22±0.48^{Ab}
Water	0.67±0.03^{Cb}	0.37±0.03^{Bc}	1.12±0.01^{Ad}
Blueberry			
Methanol–acetone–water (7:7:6, v/v/v)	0.09±0.12^{Ca}	0.70±0.97^{Ba}	1.05±0.12^{Aa}
Acetone-water (80:20, v/v)	0.08 ±0.03^{Ca}	0.60±0.05^{Ba}	0.85±0.07^{Ab}
Methanol-water (70:30, v/v)	0.05 ±0.01^{Cb}	0.26±0.12^{Bb}	0.46±0.20^{Ac}
Water	0.03±0.06^{Bb}	0.05±0.32^{Bc}	0.50±0.05^{Ac}

Data represent the mean values for each sample ± standard deviations (n = 3). Means followed by the same capital letters within a row are not significantly different ($p > 0.05$). Means followed by the same lower case letters within a column part are not significantly different ($p > 0.05$).

For example, the limitations for determination of hydrophilic antioxidants, the problems occurring in determination of reaction end point, the concern on light sensitivity of initiators or probes, carrying out the analysis in the physiologically irrelevant pH, possible interference from certain food components, and the use of different standards for expressing results may cause difficulties for making comparison. Fruit variety is also a factor influencing antioxidant activity as has been reported in both blueberry (Prior *et al.*, 1998) and apple (Wolfe *et al.*, 2003). The

total antioxidant capacity value should include assays applicable to both lipophilic and hydrophilic antioxidants with regard to similarities and differences of both hydrogen atom transfer (HAT) and single electron transfer (SET) (Karadag *et al.*, 2009).

4.2-Estimation of total phenolic and total flavonoid contents by spectrophotometry

The total phenolics were determined spectrophotometrically using the Folin-Ciocalteu reagent and are expressed as gallic acid equivalents (GAE). The absorbance was read at 725 nm, and the total phenolic content determined using the corresponding calibration curve (2-20 µg/mL). The three readings taken for each sample solution showed accurate and reproducible results, as shown in Table 4.2. The contents of total phenolics in blackberry, black raspberry, and blueberry seed meals were different, with blackberry seed meals being a richer source of total phenolics than black raspberry seed meals (7.30 mg GAE/g of dry weight) and blueberry seed meals (1.84 mg GAE/g of dry weight). This result is in general agreement with the earlier findings reported by Souza *et al.* (2014) who noted that the blackberries had the highest total phenolic content, the red raspberries the intermediate and the blueberries the lowest.

Table 4.2. Total phenolics of free, esterified, bound phenolics of berry seed meals extracted with solvent mixture (methanol–acetone–water (7:7:6, v/v/v)).

Samples	Total phenolics (mg gallic acid eq/g defatted meal)		
	Free	**Esterified**	**Bound**

Blackberry	2.23±0.05^{Ca}	2.90±0.06^{Ba}	7.93±0.20^{Aa}
Black Raspberry	1.18±0.10^{Ca}	1.90±0.15^{Bb}	4.60±0.20^{Ab}
Blueberry	0.09±0.12^{Cc}	0.70±0.97^{Bc}	1.05±0.12^{Ac}

Data represent the mean values for each sample ± standard deviations (n = 3). Means followed by the same capital letters within a row are not significantly different ($p > 0.05$). Means followed by the same lower case letters within a column part are not significantly different ($p > 0.05$).

Therefore, seed meals and the edible portions of fruits could have similar antioxidant activity. Furthermore, the antioxidant assays utilized corresponded to the total phenolic contents, and the results showed that blackberry seed meals had the highest total phenolic content, whereas the

black raspberry seed meals had an intermediate content, and the blueberry seed meals exhibited the lowest content.

The phenolics present include free phenolics, soluble esters and insoluble-bound phenolics (bound to the cell walls). The analysis of blackberry seed meals showed that the insoluble-bound fraction contained more phenolics (7.93±0.20 mg GAE/g of dry weight) compared with the esterified (2.90±0.06 mg GAE/g of dry weight) and the free (2.23±0.05 mg GAE/g of dry weight) fractions. A similar trend was obtained for the other berry seed meals examined in this study. These result agree with the findings of other studies. For example, Yang *et al.* (2009) reported a high contribution (72%) of the insoluble-bound form to the total phenolic content of cashew nut kernels. Therefore, it is significant to include the bound phenolics in the evaluation of total phenolics. It is also significant to note that the insoluble-bound phenolics are very important in the quantification process and a lot of work that have been reported in the literature are missing this important fact and contribution that comes from the insoluble-bound phenolics (Baublis *et al.*,2000).

The phenolic contents in the studied berry seed meals varied greatly and ranged from 0.09 to 7.93 mg GAE/g of dry weight. Khankonen *et al*. (2001) reported that the total phenolic contents of berries were 12.4-50.8 mg GAE/g of dry weight of the extract. These values are higher than the results obtained in the present study: 1.84 mg GAE/g of dry weight for blueberry seed meals, 7.30 mg GAE/g of dry weight for black raspberry seed meals and 13.06 mg GAE/g of dry weight for blackberry seed meals.

The phenolics (including anthocyanins and other flavonoid constituents) in plants are mainly responsible for their antioxidant activity (Dai and Mumper, 2010). The most popular group of polyphenolic constituents in human food and plants are flavonoids, which can prevent coronary heart disease (Gulcuin *et al.*, 2011). The total flavonoid content in blackberry, black raspberry, and blueberry seed meals varies greatly. This result is not surprising as total flavonoid content can vary even within same species. The total flavonoid content was estimated by the aluminium chloride colourimetric assay at 510 nm. Quercetin was chosen as the standard, and the data are expressed as milligrams of quercetin equivalents (QE)/g of dry weight of berry seed meals. The total flavonoid content in this study was found to be 0.01 –2.01 mg of quercetin equivalents (QE)/g of dry weight of berry seed meals, which is not similar to the results reported by Li *et al.* (2013), who reported values of 21.65 of quercetin equivalents/g of dry weight of blueberry pomace. Blackberry seed meals also had a higher level of total flavonoids, namely, 38.59 mg (QE)/g of dry weight. Among the different fractions of blackberry seed meals, the highest level of total flavonoids was obtained in the bound phenolics (2.01 mg of QE/g of dry weight) followed by the esterified phenolics (1.43 mg of QE/g of dry weight), whereas lower levels of quercetin were present as free phenolics (1.04 mg of QE/g of dry weight). As mentioned before, that insoluble bound phenolics must be extracted and evaluated in order to measure a better evaluation of the total flavonoids. Therefore, it is not always significant to include only the soluble bound. Similar results were obtained for black raspberry and blueberry seed meals because the bound phenolics had the highest flavonoid content (1.26 and 0.11 mg of QE/g of dry weight, respectively). Lower flavonoid contents were found in the free phenolics fraction of black raspberry and blueberry seed meals (0.52 and 0.01 mg of QE/g of dry weight, respectively). According to the data from the literature for berries, the flavonoid contents

obtained in this study for blackberry, black raspberry and blueberry seed meals were within the previously reported ranges (Table 4.3) (Samec and Zegarac, 2011).

4.3-Oxygen radical absorbance capacity (ORAC) assay

The oxygen radical absorbance capacity (ORAC) assay measures the capacity of phenolics, as well as that of all other non-phenolic molecules such as sugar, organic acids and fats, to scavenge the (AAPH-derived) peroxyl radicals that characterize this method (Prior *et al.*, 2005). The ORAC assay also measures both the degree of inhibition of peroxyl-radical-induced oxidation and the inhibition time (Davalos *et al*., 2004). In the presence of antioxidants, the fluorescence decay curve exhibits a delay or shift, and the magnitude of the shift is converted into ORAC values through a computer-aided analysis (Figure 4.1) (Kohri *et al.*, 2009). The results (Table 4.4) showed a higher antioxidant capacity of the bound phenolics in blackberry seed meals (32.80 µmol of trolox/g of dry weight) compared with those of the free (6.50 µmol of trolox/g of dry weight) and esterified (20.90 µmol of trolox/g of dry weight) fractions. In the numerous *in vitro* antioxidant assays carried out, the bound phenolic fraction showed a significantly higher antioxidant activity than free and esterified phenolics. Liyana-Pathirana and Shahidi (2006) showed that the content of bound phenolics in the hard and soft wheat samples was significantly higher than that of free and esterified phenolics. Therefore, it is essential to include bound phenolics in studies related to quantification and antioxidant capacity evaluation of berry seed meals. Blueberry seed meals had lower values with a maximum of 8.30 µmol of trolox/g of dry weight, whereas black raspberry seed meals had intermediate values with a maximum of 16.50 µmol of trolox/g of dry weight. Wang and Lin (2000) found that the ORAC values for

Table 4.3. Total flavonoid of free, esterified, bound phenolics of berry seed meals extracted with methanol–acetone–water (7:7:6, v/v/v)).

Samples	Total flavonoids (mg quercetin eq/g sample)		
	Free	Esterified	Bound
Blackberry	1.04 ± 0.06^{Ca}	1.43 ± 0.03^{Ba}	2.01 ± 0.05^{Aa}
Black Raspberry	0.52 ± 0.02^{Cb}	0.95 ± 0.05^{Bb}	1.26 ± 0.12^{Ab}
Blueberry	0.01 ± 0.03^{Bc}	0.06 ± 0.30^{Bc}	0.11 ± 0.06^{Ac}

Data represent the mean values for each sample ± standard deviations (n = 3). Means followed by the same capital letters within a row are not significantly different ($p > 0.05$). Means followed by the same lower case letters within a column part are not significantly different ($p > 0.05$).

Table 4.4. ORAC of free, esterified, bound phenolics of berry seed meals extracted with methanol–acetone–water (7:7:6, v/v/v)).

Samples	ORAC (µmol trolox eq/ g sample)		
	Free	Esterified	Bound
Blackberry	6.50 ± 1.10^{Cb}	20.90 ± 0.03^{Ba}	32.80 ± 1.30^{Aa}
Black Raspberry	14.00 ± 0.40^{Ba}	16.00 ± 0.20^{Ab}	16.50 ± 0.60^{Ab}
Blueberry	3.25 ± 0.07^{Cc}	6.70 ± 0.30^{Bc}	8.30 ± 0.02^{Ac}

Data represent the mean values for each sample ± standard deviations (n = 3). Means followed by the same capital letters within a row are not significantly different ($p > 0.05$). Means followed by the same lower case letters within a column part are not significantly different ($p > 0.05$).

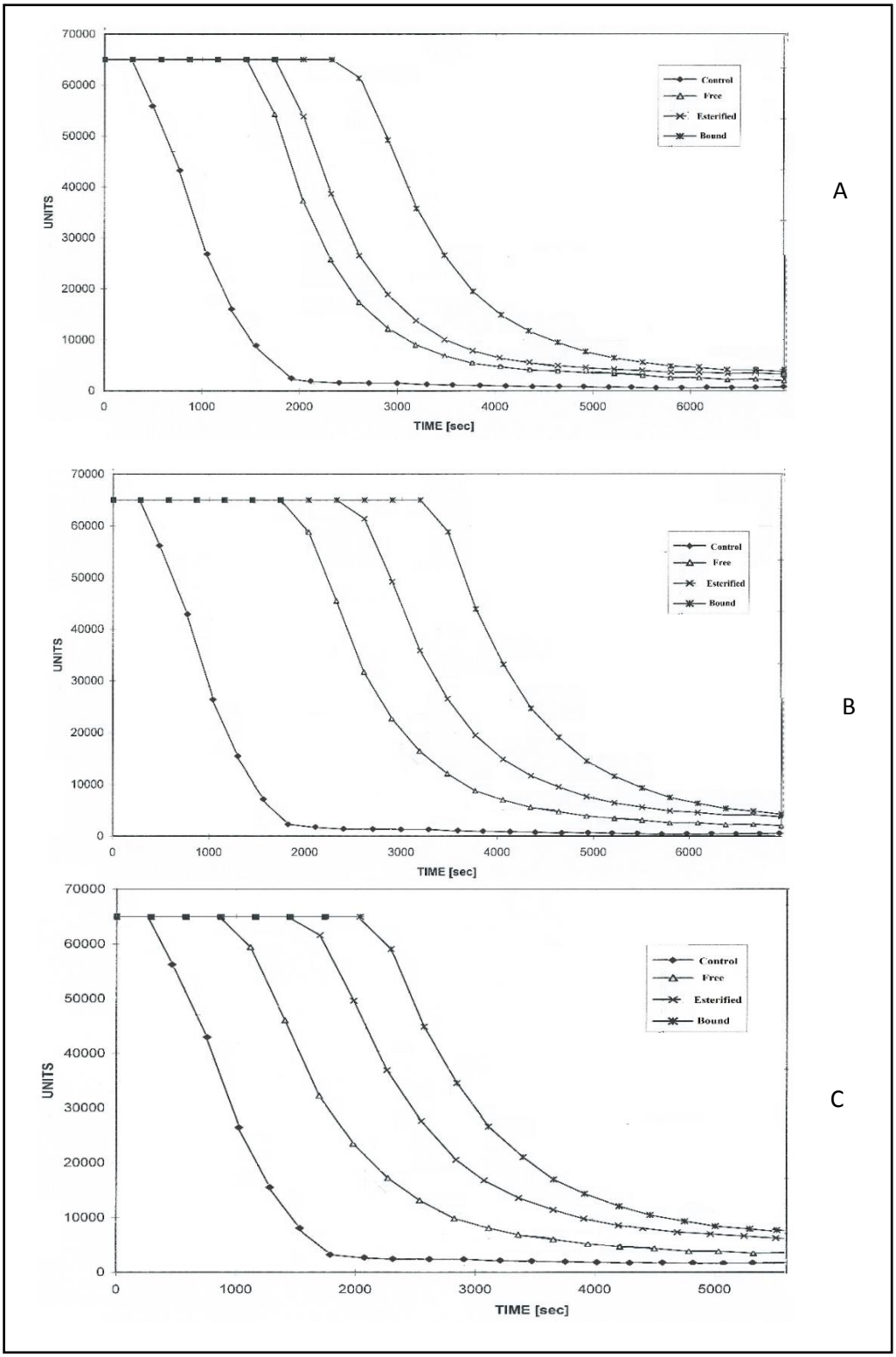


Figure 4.1. Fluorescent decay curves of fluorescein in the presence of (a) blackberry seed meal: control; free; esterified; and; bound phenolics (0.1 mg/ml), (b) black raspberry seed meal: control; free; esterified; and; bound phenolics (0.1 mg/ml), and (c) blueberry seed meal: control; free; esterified; and; bound phenolics (0.1 mg/ml).

blackberries (Rubus sp.) ranged from 13.7 to 28.8 μmol of trolox equivalents/g and 7.8 to 33.7 μmol of trolox /g of dry weight for raspberry fruits (Rubus idaeus L. and Rubus occidentalis L.). In addition, Zheng and Wang (2003) reported that an ORAC value of 22.81 of μmol trolox/g dry weight for blueberries (Vaccinium corymbosum cv. Sierra), which is within the range found in the present study: the ORAC values found for berry seed meals ranged from 8.30 μmol of trolox equivalents/g of dry weight to 32.8 μmol of trolox equivalents/g of dry weight of seed meals.

4.4-Reducing power

The assay for the determination of reducing power follows the reduction of the ferric form to ferrous form. This reduction is highlighted by spectrophotometric measurement of the intensity of Perl's Prussian blue, which depends on the reducing ability of the extract (blackberry seed meals had the highest reducing power and showed the darkest green colour for the test solution). A higher absorbance corresponds to a greater reducing power (Gulcin *et al.*, 2007). The reducing power of a given sample is measured using trolox as the standard, and the absorbance was read at 593 nm. The results are reported as μmoles of trolox equivalents/g of dry weight of berry seed meals. As a result of the effect of the phenolic extracts of berry seed meals as electron donors, the radical chain reactions are terminated by converting free radicals to more stable products; hence, these extracts primarily exhibited considerable reducing power.

Table 4.5 lists the reducing power values obtained for berry seed meals. The blackberry seed meal samples had a higher reducing power (52.20 μmoles of trolox equivalents/g of dry weight) compared with the black raspberry seed meals (32.80 μmoles of trolox equivalents/g of dry

weight) and blueberry seed meals (5.00 µmoles of trolox equivalents/g of dry weight). Therefore, the phenolics found in blackberry seed meals extracts are powerful electron donors and can terminate the radical chain reactions that may otherwise be very damaging. The data obtained in this study correlated well with the content of total phenolics in the investigated materials (r^2=0.95). The reducing powers of berry seed meals were similar to those reported by Amarowicz *et al.* (1999) for crude phenolic extracts of canola hulls and ethanolic extracts of evening primrose seeds. In the present study, the bound phenolics in all berry seed meals showed a very high reducing power compared to the free and esterified phenolics. Jayaprakasha *et al* (2001) found that grape seed (*vitis vinifera*) had good reducing power. The grape seed flavanol/ procynidin compounds may act as reductones by donating electrons and reacting with free-radicals to convert them to more stable products and terminating the free-radical chain reaction (Pin-Der, 1998).

4.5-Iron (II) chelation capacity

Transition metal ions, which may be introduced by processing methods, can contaminate foods. Bivalent ferrous ions play an important role as catalysts of oxidative processes, leading to the formation of superoxide anion radicals and hydroxyl radicals via Fenton reactions (Ebrahimzadeh *et al.*, 2009). Ferrozine can quantitatively chelate iron ions. The red colour of the complex is reduced when the complex formation is interrupted in the presence of chelating agents. The chelating activity of the coexisting chelator can be determined by an assessment of the colour reduction (Ningappa *et al.*, 2008). The deprotonation of phenol forms the phenoxide ion, which possesses a high charge density and can attach to a suitably highly charged cation.

Table 4.5. Reducing power of free, esterified, bound phenolics of berry seed meals extracted with solvent mixture (methanol–acetone–water (7:7:6, v/v/v)).

Samples	Reducing Power (µmoles trolox eq/g sample)		
	Free	**Esterified**	**Bound**
Blackberry	17.60 ± 0.20^{Ba}	18.04 ± 0.42^{Ba}	52.20 ± 0.60^{Aa}
Black Raspberry	6.50 ± 0.03^{Cb}	20.90 ± 1.10^{Ba}	32.80 ± 3.02^{Ab}
Blueberry	2.77 ± 0.13^{Cc}	4.19 ± 0.13^{Ab}	5.00 ± 0.25^{Ac}

Data represent the mean values for each sample ± standard deviations (n = 3). Means followed by the same capital letters within a row are not significantly different ($p > 0.05$). Means followed by the same lower case letters within a column part are not significantly different ($p > 0.05$).

The possible mechanism of Fe^{2+} chelation by phenolics is as follows: phenolics, which possess a number of hydroxyl groups, first lose a proton and then form strong coordination oxygen ion complexes with a ferrous ion (Fernandez *et al.,* 2002). These extracts should be able to form complexes with metals ions and thereby stabilize them. Therefore, they should prevent metal-catalysed initiation and hydroperoxide decomposition reactions. The ability of the berry seed meal extracts to compete with ferrozine for iron ions in the solution was examined due to the significance of metal chelation as an antioxidant property. In the presence of other chelating agents or antioxidants, the complex formation is disrupted, as determined by a reduction in the purple colour of the complexes. As the extract concentration is increased, the amount of iron (II) chelated is also increased (Luo *et al.,* 2011).

The chelation activities of the phenolic extracts from berry seed meals were examined against Fe(II) and reported as EDTA equivalents. The Fe (II) chelation capacities of the free, esterified, and bound phenolics of blackberry seed meals were 63.47, 66.43 and 68.60 µmol of EDTA eq/g of dry weight**,** respectively, whereas the metal chelation capacities of the phenolics in balck raspberry seed meals were 20.50, 22.50 and 26.20 µmol of EDTA eq/g of dry weight, respectively. The same trend was observed in the fractions from blueberry seed meals (10.06, 8.20 and 6.50 µmol of EDTA eq/g of dry weight, respectively). The metal chelation activity of blackberry seed meals was higher than that of the black raspberry and blueberry seed meals. However, the metal chelation effect of the phenolic fractions of the three berry seed meals were not significantly different ($p>0.05$) (Table 4.6). This might be due to the structural features of phenolics that are involved. So, if they are having similar structure in the three groups, then the difference is may not be seen.

Table 4.6. Iron (II) chelation capacity of free, esterified, bound phenolics of berry seed meals extracted with solvent mixture (methanol–acetone–water (7:7:6, v/v/v)).

	Iron (II) chelation capacity (µmol EDTA eq/ g sample)		
Samples	**Free**	**Esterified**	**Bound**
Blackberry	63.47 ± 0.20^{Ca}	66.43 ± 0.01^{Ba}	68.60 ± 0.30^{Aa}
Black Raspberry	20.50 ± 0.01^{Cb}	22.50 ± 0.02^{Bb}	26.20 ± 0.010^{Ab}
Blueberry	6.50 ± 0.01^{Cc}	8.20 ± 0.01^{Bc}	10.06 ± 0.001^{Ac}

Data represent the mean values for each sample ± standard deviations (n = 3). Means followed by the same capital letters within a row are not significantly different ($p > 0.05$). Means followed by the same lower case letters within a column part are not significantly different ($p > 0.05$).

The Fe (II) chelating ability of an antioxidant extract is an important feature used in assessing its antioxidant efficacy because it contributes to the generation of hydroxyl radicals via Fenton's reaction, which in turn attack biomolecules. It could be concluded from the results that the berry seed meal extracts may provide protection against oxidative damage by eliminating iron(II) ions, which may alternatively engage in metal-catalysed hydroperoxide decomposition reactions (Liyana-Pathirana and Shahidi, 2007).

As a control, EDTA showed strong capacity to chelate ferrous ions due to its unique structure. EDTA has two nitrogen atoms and four oxygen atoms bearing a carboxyl moiety, which can chelate ferrous ion in the centre and block formation of the Fe^{2+}–ferrozine comple (Luo *et al.*,, 2011).

4.6-Radical scavenging activity assays, DPPH radical scavenging capacity (DRSC) assay using electron paramagnetic resonance (EPR), and β-carotene linoleic acid method

TEAC, an electron transfer (ET)-based assay, depends on the scavenging ability of the antioxidants toward the nearly stable blue/green 2, 2'-azinobis (3 ethylbenzothiazoline-6 - sulphonic acid) (ABTS) radical and its conversion into a colourless product. In this method, ABTS is oxidized by peroxyl radicals or other oxidants to its radical cation, $ABTS^{\bullet+}$, which presents a deep colour. The antioxidant capacity is measured as the ability of the test compounds to decrease the colour by reacting directly with the $ABTS^{\bullet+}$. The amount of radicals scavenged and therefore the antioxidative activity of the test compound may be reflected by the degree of decolourization (Sharma and Singh, 2013).

The total antioxidant capacity was assayed using the ABTS method, and the results are expressed as the trolox equivalent antioxidant capacity, i.e., mmol of trolox/g of dry weight of berry seed meals. All berry seed meal extracts exhibited various degrees of free radical-scavenging activity. The blackberry seed meals showed the best antioxidant capacity with a high TEAC value of 19.20 µmol of trolox/g of dry weight compared with the other samples. The lowest TEAC value, which showed the weakest antioxidant activity, was obtained with the blueberry seed meals. The antioxidant activity measured by the ABTS assay ranged from 2.20 µmol of trolox/g of dry weight for the free phenolics of black raspberry seed meals to 19.20 µmol of trolox/g of dry weight for bound phenolics of blackberry seed meals. Considering the differences of moisture contents, the result of berry seed meals is markedly less than the result reported by Sariburun *et al.* (2010), who measured the antioxidant activities of red raspberry (*Rubus idaeus* L.) of 5 cultivars (Aksu Kırmızısı, Rubin, Newburgh, Hollanda Boduru, Heritage) and blackberry fruits (*Rubus fruticosus* L.) of 4 cultivars (Bursa 1, Bursa 2, Jumbo, Chester) using the ABTS assay and found that these activities ranged from 64.36 ± 1.73 to 117.07 ± 0.94 µmol of trolox/g of fresh weight. The antioxidant activity was determined by the ABTS assay in 4 blackberry cultivars and the Jumbo cultivar had the highest values of antioxidant activity in all blackberry cultivars. High level of antioxidant activity in Jumbo in all assays as compared to the other blackberry cultivars could be due to its high level of total phenol and total flavonoid contents. In the current study, blackberry seed meals also contain the highest content of total phenol and total flavonoid. Thus, blackberry seed meals may be considered as a good source of antioxidant. It is also clear from Table 4.7 that the TEAC of the bound phenolics from blackberry seed meals was significantly ($p \leq 0.05$) higher than the TEAC values obtained for the bound phenolics in black raspberry and blueberry seed meals. The higher content of bound phenolics in blackberry seed

meals may explain its higher antioxidant activity compared with that of the black raspberry and blueberry seed meals. The result of berry seed meals is in agreement with the results obtained in a previous study, which used the iron chelating capacity method to show that blackberry fruits are the richest source of antioxidants and that blueberry fruits are the poorest (Souza *et al.*, 2014). However, the TEAC value for blueberry seed meals obtained in the present study is markedly different from those published for blueberries (*Vaccinium ashei* cv.) from other locations (Huang *et al.*, 2012). They found that blueberry had the highest antioxidant activity among blueberry, blackberry and strawberry, with corresponding TEAC values of 14.98, 11.48, and 4.44 mmol Trolox/ 100 g dry weight. If there are differences in the composition between fruits, then the different in antioxidant activity due to the differences between seed meals and fruits should be expected.

There is strong correlation between the total antioxidant capacity obtained using the TEAC and the total phenolic content (r^2=0.88). Each point represents the mean of two individual experiments, and each experiment included triplicate measurement. Different studies with berries and cherries have reported relationships between the antioxidant activity and the phenolic compounds (Koca and Karadeniz, 2009; Pantelidise *et al.*, 2007; Wu *et al.*, 2010). In this study, blackberry seed meals were found to contain higher levels of phenolic compounds than black raspberry and blueberry seed meals. Therefore, a higher total phenolic content was found to be associated with a higher antioxidant activity.

To examine the scavenging capacities of antioxidative compounds, the DPPH radical method has been extensively employed in model systems because it is rapid, simple, and easy reproducible

Table 4.7. TEAC of free, esterified, bound phenolics of berry seed meals extracted with solvent mixture (methanol–acetone–water (7:7:6, v/v/v)).

Samples	TEAC (µmoles trolox eq/g sample)		
	Free	**Esterified**	**Bound**
Blackberry	15.90 ± 0.60^{Ba}	16.60 ± 0.04^{Ba}	19.20 ± 1.80^{Aa}
Black Raspberry	2.20 ± 1.60^{Cc}	9.50 ± 0.80^{Bb}	18.04 ± 0.42^{Aa}
Blueberry	3.30 ± 1.20^{Bb}	6.60 ± 0.19^{Bc}	7.20 ± 1.30^{Ab}

Data represent the mean values for each sample ± standard deviations (n = 3). Means followed by the same capital letters within a row are not significantly different ($p > 0.05$). Means followed by the same lower case letters within a column part are not significantly different ($p > 0.05$).

(Barua *et al.,* 2014). The colour of DPPH changes from purple to yellow upon acceptance of an electron. The antioxidant property of the corresponding extracts was estimated by measuring the bleaching of the purple colouration of DPPH at 517 nm (Thambiraj and Paulsamy, 2012). The antioxidant transfers an electron or hydrogen atom to DPPH and thus neutralizes its free radical character and converts it to the corresponding hydrazine, and the degree of discolouration indicates the scavenging activity of berry seed meals (Villano *et al.,* 2007). The results obtained for berry seed meals in this study, which are expressed in terms of trolox equivalents, followed a similar trend, as shown in Table 4.8. The bound phenolics of berry seed meal extracts exhibited the highest DPPH radical scavenging activity, which is expressed as trolox equivalents/g of defatted meal. These results notably indicate that the bound phenolics of berry seed meal extracts are more effective in scavenging free radicals than the esterified and free phenolics. Additionally, the results shown in Figure 4.2 indicate that the blackberry seed meal extract scavenges free radicals more effectively than the black raspberry and blueberry seed meal extracts. Huang *et al.* (2012) found that blueberries have a similar strong scavenging activity against DPPH radicals but a higher total antioxidant capacity than the blackberries, which may be attributable to the higher contents of hydrophilic antioxidants, e.g., anthocyanidins, in blueberries. This difference is due to the variety in the composition of the fruits depending on a series of factors, including the species, variety, cultivation, region, weather conditions, ripeness, time of harvest and storage conditions (Faniadis *et al.,* 2010; Haffner *et al.,* 2002). The three berry seed meals, with the exception of the free phenolic fraction from blueberry seed meals, demonstrated significantly higher scavenging activities than the control. Epicatechins and catechins, which are present in high concentrations in blackberry seed meals, can donate a hydrogen atom to a free radical to neutralise the potential damage. This could be related to the

chemical structure of epicatechin, which has several hydroxyl groups. In addition, the antioxidant efficiency of phenolics can be determined by the basic structural orientation of the compounds, such as how easily a hydrogen atom from a hydroxyl group can be donated to a free radical, and by the capacity of the compounds to support an unpaired electron (Roginsky, 2003). The location of hydroxyl groups is more essential than their number for the antioxidant efficiency of phenolics. For instance, the antioxidant efficiency can be extremely improved when the hydroxyl group is in the ortho position of the B ring, as is observed in catechins (Rice-Evans *et al.,* 1996).

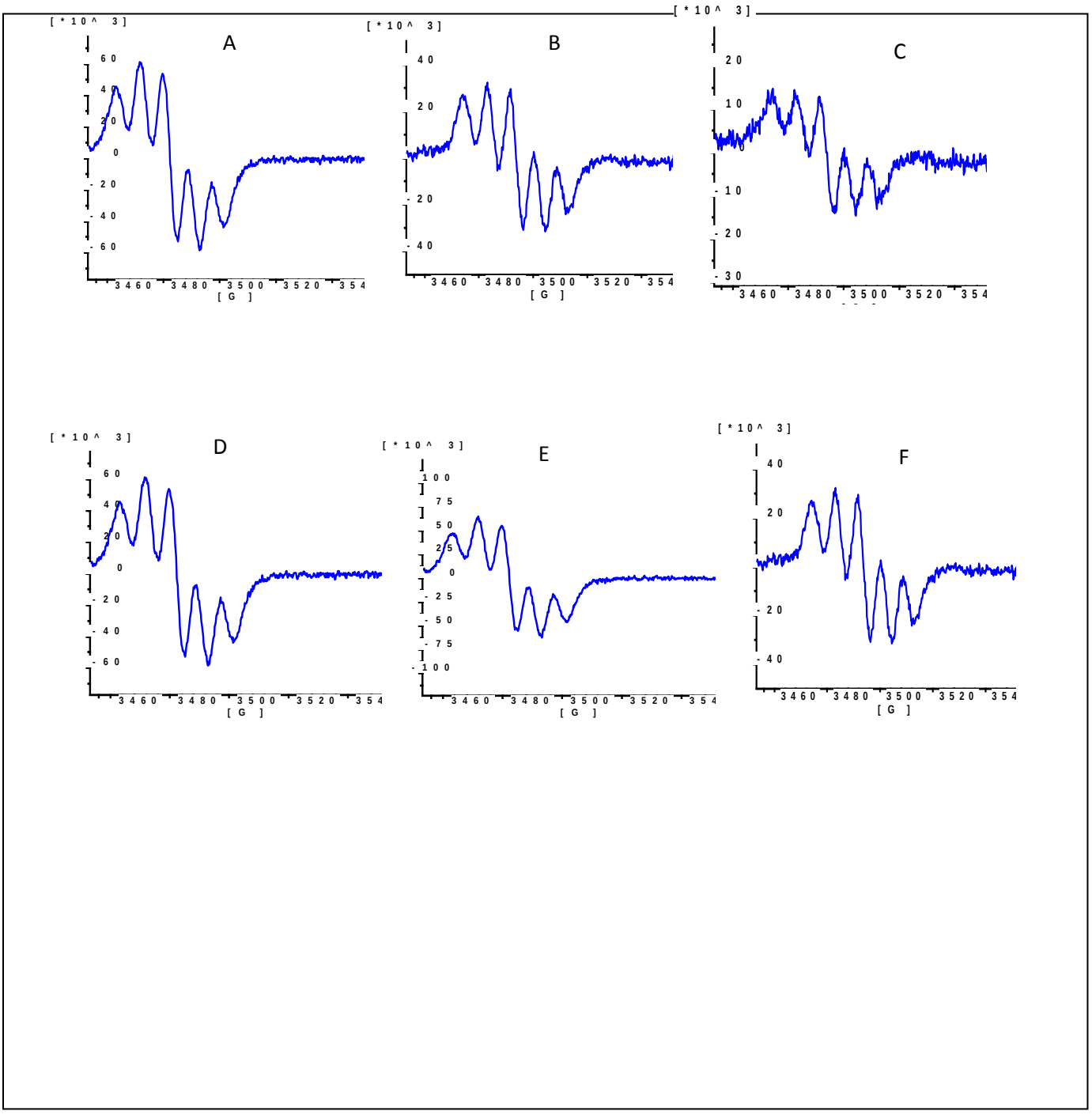
Sariburun *et al.* (2010) reported that the antioxidant activities of raspberry and blackberry measured by the DPPH assay ranged from 64.36 ± 0.98 to 127.59 ± 1.84 µmol of trolox/g of fresh weight and from 90.95 ± 1.04 to 177.11 ± 3.17 µmol of trolox/g of fresh weight, respectively. These values are markedly higher than our results: the values obtained for blackberry, black raspberry, and blueberry seed meals were 54.40, 23.60, and 19.87 µmol of trolox /g of dry weight, respectively. Sariburun *et al.* (2010) also showed that for antioxidant activity there were significant differences between blackberry cultivars and black raspberry cultivars ($P < 0.01$). They found that blackberry cultivars had higher total antioxidant activity than raspberry cultivars in the DPPH assay.

The discolouration of β-carotene has widely been employed to measure the antioxidant activity of plant extracts (Madhujith and Shahidi, 2007; Shahidi *et al.*, 2007; Wijerathne *et al.*, 2006). When antioxidants are employed in various media, they have been found to behave in different manners.

Table 4.8. DPPH scavenging activity of free, esterified, bound phenolics of berry seed meals extracted with solvent mixture (methanol–acetone–water (7:7:6, v/v/v)).

Samples	DPPH scavenging activity (µmol trolox eq/ g sample)		
	Free	**Esterified**	**Bound**
Blackberry	11.70 ± 0.04^{Ca}	14.80 ± 0.25^{Ba}	27.90 ± 0.02^{Aa}
Black Raspberry	4.40 ± 0.10^{Cb}	6.70 ± 0.20^{Bb}	12.50 ± 0.75^{Ab}
Blueberry	3.00 ± 0.50^{Cc}	5.07 ± 0.60^{Bc}	11.80 ± 0.03^{Ac}

Data represent the mean values for each sample ± standard deviations (n = 3). Means followed by the same capital letters within a row are not significantly different ($p > 0.05$). Means followed by the same lower case letters within a column part are not significantly different ($p > 0.05$).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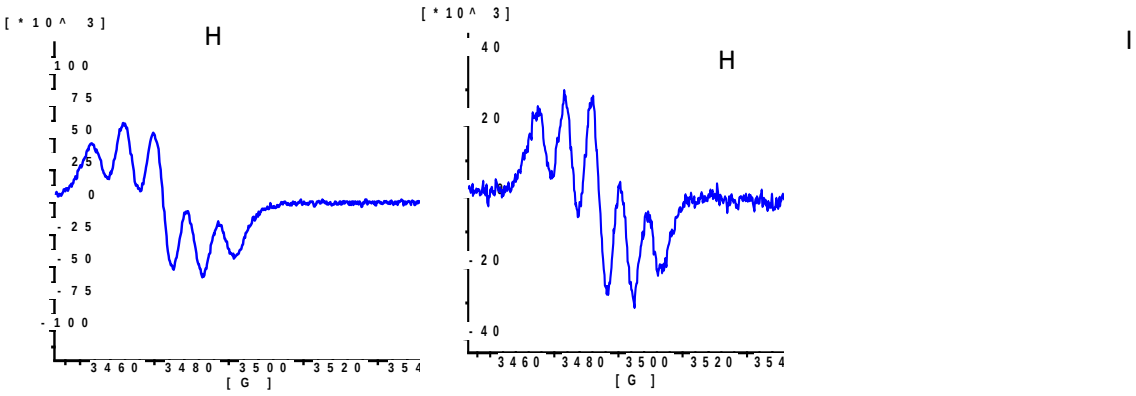
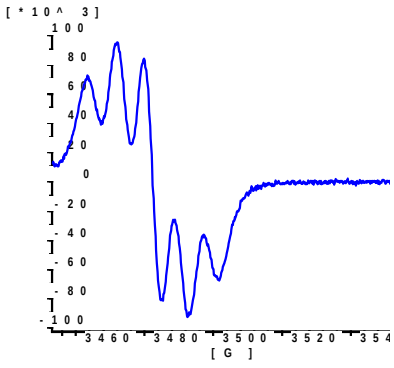


Figure 4.2. ESR spectra of berry seed meal extracts on scavenging of DPPH for blackberry fractions (0.1 mg/ml) (A), free; (B), esterified; and (C), bound fractions (13, 16 and 31 %, respectively); and of black raspberry fractions (0.2 mg/ml) (D), free; (E), esterified; and (F), bound (5, 7 and 14 %, respectively); and of blueberry fractions (0.5 mg/ml) (G), free; (H), esterified; and (E), bound (3, 5 and 12 %, respectively).

Therefore, their efficiency in bulk oil may not certainly reflect that in oil/water emulsions. Thus, it is important to include information on the effectiveness of an antioxidant in oil/water emulsions. In this study, the antioxidant capacity of berry seed meals in an oil-in-water emulsion was determined using a β-carotene/linoleic acid model emulsion system. Monitoring the bleaching of β-carotene reflects the antioxidant capacity of the test compounds (Figure 4.3). The oxidation of linoleic acid in the emulsion results in the decolouration of β-carotene, which is a free radical-mediated phenomenon. The loss of β-carotene during the coupled oxidation of linoleic acid and β-carotene can be minimized in the emulsified aqueous system (Tan and Shahidi, 2013).



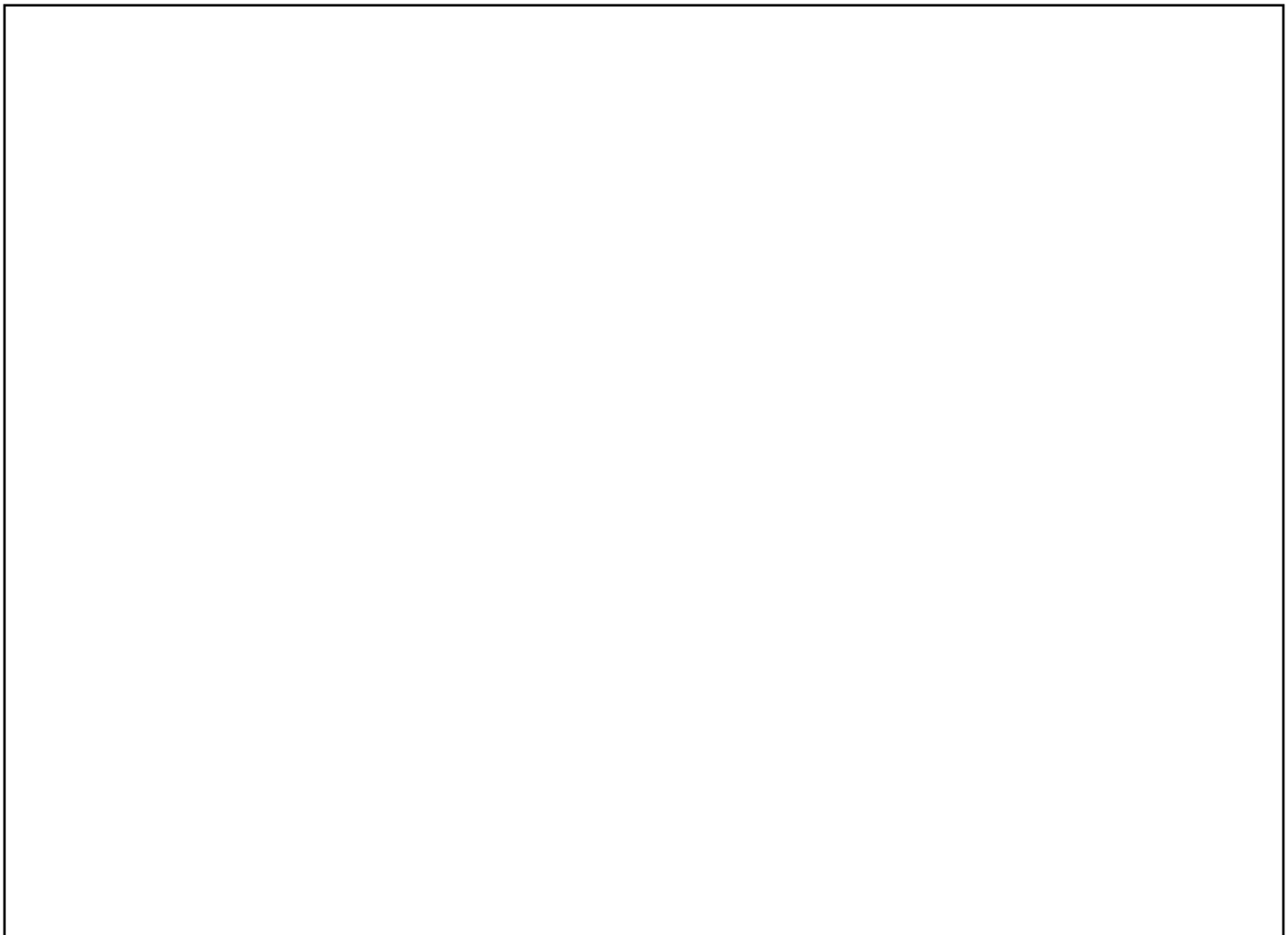
The results show that berry seed meals prevented the bleaching of β-carotene by 3- 22% over the 105-min incubation period. The insoluble bound phenolic fractions of each berry seed meal had

a higher antioxidant activity than the esterified and free fractions. In addition, the blackberry seed meals showed the highest antioxidant activity in the β-carotene/linolate emulsion system (Table 4.9). This finding may be attributed to the high total phenolic content and the high contents of flavonoids, such as catechin, gallocatechin, epicatechin, and procyanidin, detected in the bound fraction. The absorbance of the control where no antioxidants had been added was determined by replacing the sample with ethanol. The control sample oxidized more rapidly than the berry seed meal extracts.

Table 4.9. Inhibitory effect of free, esterified, bound phenolics of berry seed meals extracted with solvent mixture (methanol–acetone–water (7:7:6, v/v/v)) against β-carotene bleaching.

	β-Carotene bleaching Inhibition (%)		
Samples	**Free**	**Esterified**	**Bound**
Blackberry	5.70 ± 0.60^{Ca}	10.00 ± 0.50^{Ba}	22.00 ± 0.32^{Aa}
Black Raspberry	3.90 ± 0.02^{Cb}	8.50 ± 0.19^{Bb}	16.00 ± 0.30^{Ab}
Blueberry	3.80 ± 0.50^{Cb}	7.00 ± 0.10^{Bc}	14.50 ± 1.40^{Ac}

Data represent the mean values for each sample ± standard deviations (n = 3). Means followed by the same capital letters within a row are not significantly different ($p > 0.05$). Means followed by the same lower case letters within a column part are not significantly different ($p > 0.05$). 1 Inhibition (%) calculated at the end of incubation (105 min).



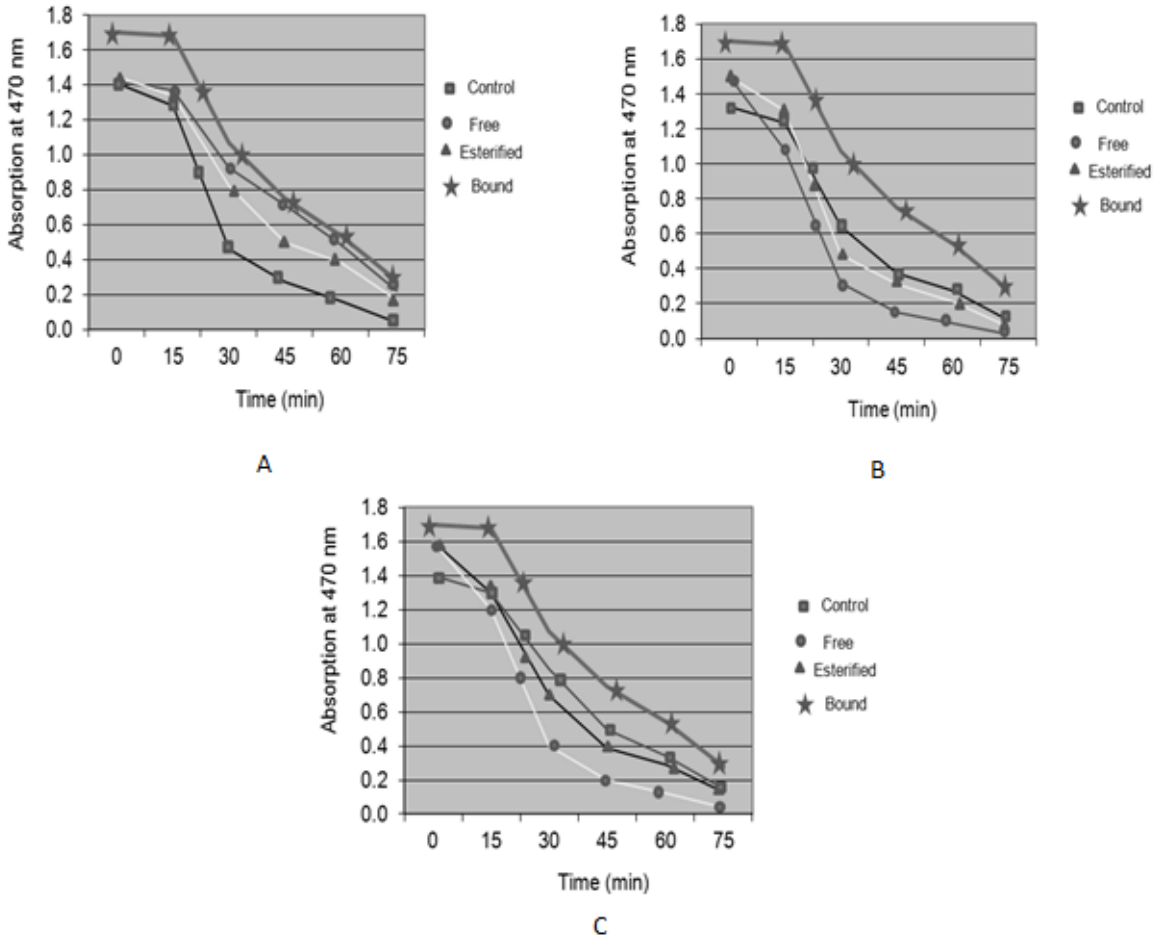


Figure 4.3. Antioxidant activity of (A) blackberry (0.1mg/ml), (B) black raspberry (0.1mg/ml), and (C) blueberry (0.1mg/ml) seeds meal extracts in a β-carotene linoleate model system. Ethanol was used as a control.

Phase partitioning is one of the various factors that affect the ultimate performance of a phenolic compound in a dispersed system (McClements and Decker, 2000). In the oil-in-water emulsion

system, hydrophilic antioxidants, such as phenolic acids, tend to move to the water phase and therefore afford less protection to the oil. In contrast, lipophilic hydroxycinnamic acids are more soluble in the oil phase or oriented in the oil–water interface due to the presence of both hydrophobic aliphatic side chains and hydrophilic hydroxyl groups in their molecule and therefore exhibit higher antioxidant activity than their hydrophilic phenolic acid counterparts (Tan and Shahidi, 2013).

According to Hassimoto *et al.* (2005), the choice of the analytical test, which is specific for only one property, is considered one of the major problems associated with the assessment of the antioxidant activity of biological materials. The three methods used in this study, namely, TEAC, DPPH, and β-carotene methods, presented coherent results for the berry seed meals evaluated. In fact, the ABTS, DPPH, and β-carotene methods all indicated that the blackberry seed meal is a good source of antioxidants. The ordering of berry seed meals based on decreasing antioxidant activity, as determined through the ABTS, DPPH, and β-carotene methods, was the following: Blackberry > Black raspberry > Blueberry.

4.7-Hydroxyl radical scavenging activity of berry seed meal extracts

Hydrogen peroxide in the presence of ferric ions can produce the hydroxyl radical, which is an extremely reactive and biologically toxic species, via the Fenton reaction (Thambiraj and Paulsamy, 2012). The Fenton reaction is very important in biological systems because most cells have some level of iron, copper, or other metals that can catalyse this reaction. The hydroxyl radical passes easily through membranes and cannot be kept out of cells (Markovic *et al.*, 2012).

This reductive cleavage of H_2O_2 is considered the major source of hydroxyl radical in *vivo* and is a main mechanism of biological damage (Thambiraj and Paulsamy, 2012). Therefore, examination of the hydroxyl radical scavenging activity of the berry seed meal extracts is significant. The hydroxyl radical was spin-trapped with DMPO, which results in the formation of a DMPO-OH adduct, a stable spin trap that is detectable by EPR spectroscopy. This allows the easy detection of short-lived hydroxyl radicals (Yen and Chen, 1995). The intensity of the characteristic 1:2:2:1 quarter with a hyperfine coupling constant of 14.9 G was reduced with the addition of the extract due to scavenging of the hydroxyl radical by the extract, chelation of Fe (II) by the extract, or, most probably, a combination of both actions. Figure 4.4 shows the hydroxyl radical scavenging activity of the berry seed meal extracts based on the quenching of the EPR signal by phenolic compounds, which donate a hydrogen atom from their OH group (Markovic *et al.,* 2012). The EPR spectra acquired for all of the extracts indicated the differences in their hydroxyl radical scavenging activities. The results of this study are in agreement with those reported by other researchers, who have shown that polyphenolics from plants or their fractions present direct scavenging activities against hydroxyl radicals (Barua *et al.,* 2014; Wettasinghe and Shahidi, 1999). Chandrasekara and Shahidi (2011b) showed that extracts of whole cashew nut and testa contain a higher amount of flavonoids, such as (+)-catechin, (-)-epicatechin, and epigallocatechin, which may have prooxidative effects at high concentrations, particularly in the presence of transition metal ions. If berry seed meal extracts act as an effective antioxidant, there is likely some constituent within the extract that can be employed as hydrogen atom donors and oxygen scavengers. It has been suggested that flavonoids, such as myricetin and quercetin, are hydroxyl radical scavengers (Yilmaz and Akkaya, 2012). The quenching of the EPR signal results from the donation of a hydrogen atom

by the phenolic compounds in the crude extract to ·OH (Wettasinghe and Shahidi, 1999). It has been observed that the extracts exerted significant scavenging effects on hydroxyl radicals and that these effects increased with an increase in the concentration. The extract from blackberry seed meals exhibited the highest ability to function as a free-radical acceptor and to reduce the formation of the DMPO-HO (2, 2-dimethyl-5-hydroperoxy-1-pyrrolidinyloxyl-DMPO/OOH) adduct. Even at higher concentrations, the blueberry seed meal samples could not significantly minimize the signal intensity, thereby showing their weak efficiency as a source of natural antioxidants (Table 4.10).

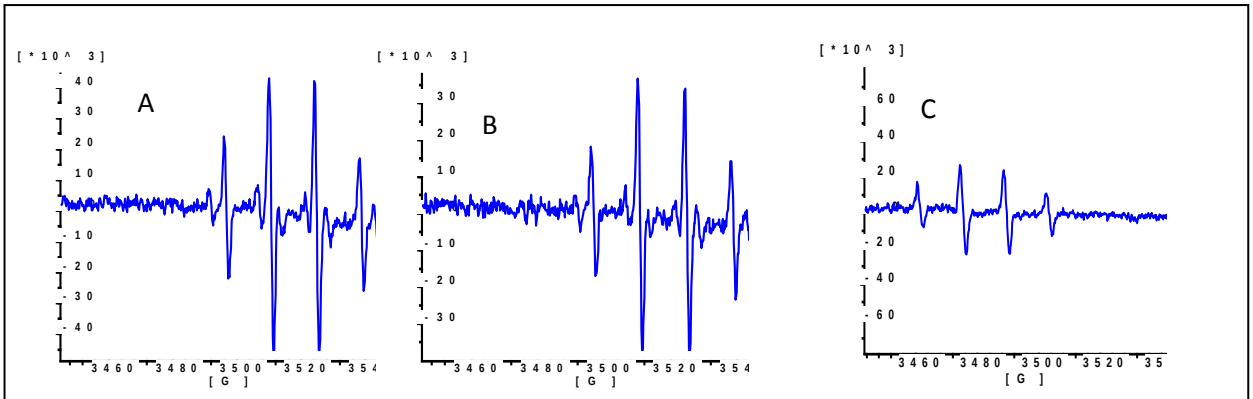
4.8-Inhibition of oxidation in a fish meat model system

Secondary oxidation products serve as quality and flavour indicators of fish products (Shahidi, 1997b). The thiobarbituric acid reactive substances (TBARS) assay offers a measure of the secondary oxidation products, mainly carbonyl compounds, including malondialdehyde (MDA). The TBARS values (Table 4.11) increased slowly during the storage period, and catechin was used as a positive control at 100 ppm. The TBARS of the control increased rapidly during the first week and then remained practically unchanged. However, the TBARS of catechin remained practically unchanged during the entire storage period, whereas the TBARS of the remaining samples increased steadily during the experimental period. On day 0, no significant ($p > 0.05$) difference in efficiency of inhibiting the formation of TBARS was obtained between the phenolic fractions from berry seed meals.

Table 4.10. Hydroxyl radical scavenging activity of free, esterified, bound phenolics of berry seed meals extracted with solvent mixture (methanol–acetone–water (7:7:6, v/v/v)).

Samples	Hydroxyl Radical (mg trolox eq/ g sample)		
	Free	Esterified	Bound
Blackberry	32.60±0.08^{Ca}	38.60±0.85^{Ba}	53.80±0.50^{Aa}
Black Raspberry	2.40±0.30^{Cb}	16.70±1.81^{Bb}	48.30±1.20^{Ab}
Blueberry	1.60±0.56^{Cc}	16.80±0.76^{Bb}	18.60±0.10^{Ac}

Data represent the mean values for each sample ± standard deviations (n = 3). Means followed by the same capital letters within a row are not significantly different ($p > 0.05$). Means followed by the same lower case letters within a column part are not significantly different ($p > 0.05$).



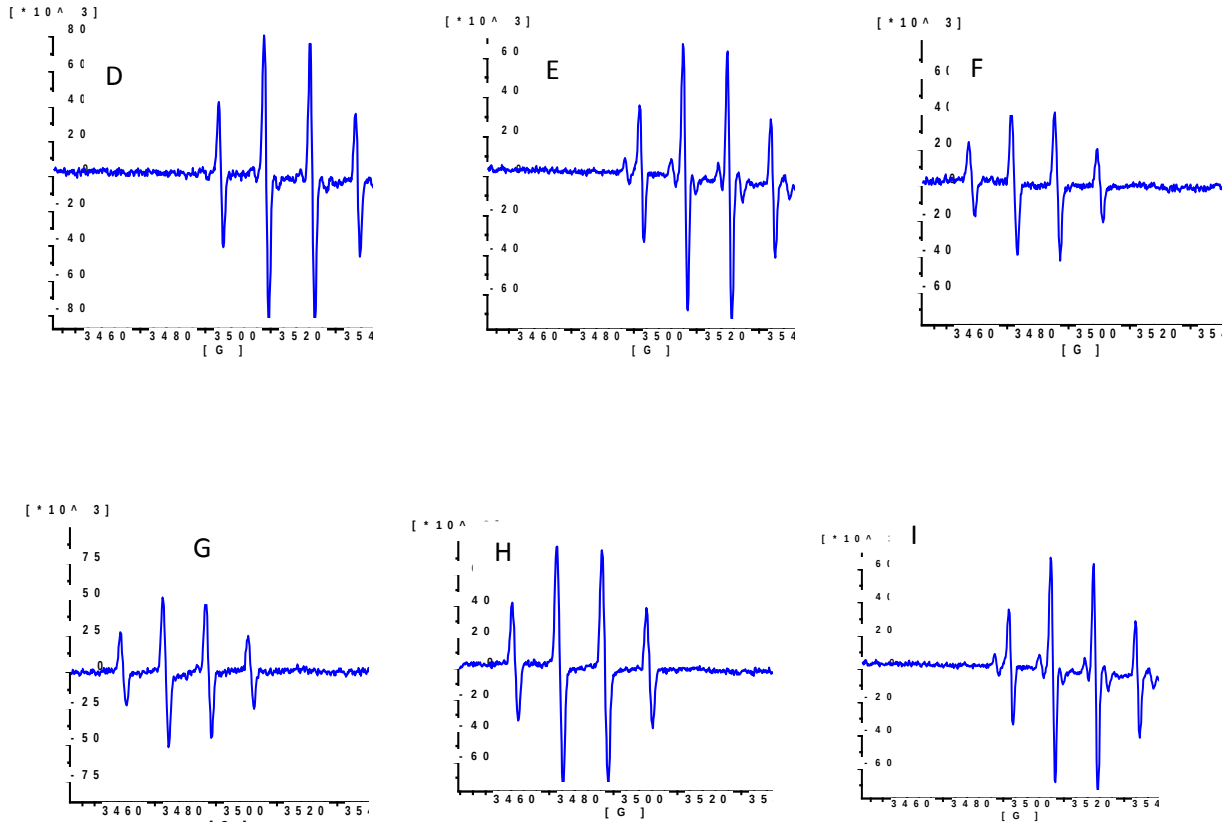


Figure 4.4. Electron spin resonance spectra showing the effect of berry seed meal extracts on scavenging of hydroxyl radical in blackberry fractions (0.1 mg/ml) (A), free; (B), bound; and (C), free; were 36, 43 and 59 %, respectively; for black raspberry fractions (0.3 mg/ml) (D), free; (E), esterified; and (F), bound; were 2, 18 and 53 %, respectively; and for blueberry fractions (0.5 mg/ml) (G), free; (H), esterified; and (E), bound; were 1, 16 and 18 %, respectively.

Table 4.11. TBARS of free, esterified, bound phenolics of berry seed meals extracted with solvent mixture (methanol–acetone–water (7:7:6, v/v/v)) on days 0, 7, and 14 of storage at 4°C.

	TBARS (mg MDA eq/kg fish)		
Samples	**Day 0**	**Day 7**	**Day 14**

Control	3.52 ±0.13[a]	8.81 ±0.08[a]	9.90 ±0.04[a]
Catechin	1.48 ±0.02[b]	5.05 ±0.29[c]	4.15 ±0.07[c]
Blackberry			
Free	1.13 ±0.01[c]	6.50±0.04[b]	7.13±0.36[b]
Esterified	0.81±0.12[c]	3.90±0.05[d]	4.70±0.07[c]
Insoluble-Bound	0.09±0.10[d]	2.32±0.16[d]	3.71±0.75[c]
Black Raspberry			
Free	1.73± 0.08[b]	7.81 ±0.09[a]	8.90 ±0.12[a]
Esterified	0.95 ±0.27[c]	5.60 ±0.37[c]	6.60 ±0.90[b]
Insoluble-Bound	0.52 ±0.30[c]	3.70 ±0.60[d]	4.52 ±0.03[c]
Blueberry			
Free	1.78 ±0.45[b]	8.41 ±0.45[a]	9.75 ±1.14[a]
Esterified	1.60 ±0.20[b]	8.43 ±0.10[a]	8.90 ±0.01[a]
Insoluble-Bound	1.60 ±0.50[b]	8.31 ±0.02[a]	8.39 ±0.30[a]

Data represent the mean values for each sample ± standard deviations (n = 3). Means followed by the same lower case letters within a column part are not significantly different ($p > 0.05$). Catechin concentration was 100 ppm. Berry seed meal extracts concentrations were 200 ppm. Ethanol has been used as a control. MDA, malondialdehyde.

At the end of the seventh day of storage, the bound, esterified, and free phenolics in blackberry seed meals inhibited the formation of TBARS by 63, 53, and 28%, respectively, whereas the black raspberry seed meals inhibited oxidation by 55, 34, and 11%, respectively. In addition, the bound, esterified, and free phenolics in blueberry seed meals inhibited fish meat oxidation by 15,

9, and 1%, respectively. It could be concluded from the results that black berry seed meals were more effective than black raspberry and blueberry seed meals in inhibiting the formation of TBARS. Furthermore, catechin at 100 ppm inhibited TBARS formation by 4%. Therefore, berry seed meals prevented salmon oxidation better than catechin except of the free phenolics in blueberry seed meals.

4.9-Inhibition of oxidation of human LDL cholesterol

Dietary antioxidants are important in the prevention of atherosclerosis and associated cardiovascular diseases due to their ability to inhibit LDL cholesterol oxidation (Chandrasekara and Shahidi, 2011a). It has been shown that the production of fatty streaks or vascular lesions, which accumulate lipids, is caused by the consumption of oxidized LDL by macrophages and smooth muscle cells (Levitan *et al.*, 2010). Because the oxidation of LDL is one of the biomarkers associated with cardiovascular diseases, berries that inhibit the in *vivo* oxidation of LDL are postulated to have potential benefits in disease prevention (Heinonen, 2007).

Because LDL cholesterol molecules possess antioxidant compounds, such as tocopherol, β-carotene and lycopene, the rate of CD formation was slow at the beginning. As soon as the depletion of the internal antioxidants of LDL cholesterol molecules occurs, rapid oxidation is initiated. Table 4. 12 indicates the inhibition effects of berry seed meals at a concentration of 500 ppm against copper-induced human LDL cholesterol oxidation.

Table 4.12. Effect of berry seed meals phenolic extracts in preventing cupric ion induced human low density lipoprotein (LDL) peroxidation (%)

Samples	**0**	**12**	**16**	**22**

Catechin (25 ppm)	34.50 ±0.65^{B}	42.30 ±0.02^{B}	57.10 ±0.30^{B}	65.80 ±0.18^{B}
Catechin (50ppm)	39.95 ±0.14^{A}	48.27 ±0.10^{A}	63.83 ±0.08^{A}	74.15 ±1.30^{A}
Blackberry				
Free	24.80 ±0.10^{Da}	31.30±0.11^{Ea}	42.70±0.05^{Ea}	50.40±0.20^{Ea}
Esterified	21.20±0.13^{Ea}	35.70±0.23^{Da}	47.20±0.01^{Da}	58.90±0.16^{Ca}
Insoluble-Bound	26.30±0.02^{Ca}	37.40±0.90^{Ca}	48.70±0.65^{Ca}	59.90±1.30^{Da}
Black Raspberry				
Free	18.00± 0.58^{Eb}	23.00 ±0.85^{Eb}	42.50±0.43^{Da}	52.00 ±0.15^{Eb}
Esterified	19.50 ±0.05^{Db}	26.70 ±0.07^{Db}	45.05±0.75^{Cb}	55.20 ±0.01^{Db}
Insoluble-Bound	19.80 ±0.20^{Cb}	29.00 ±0.14^{Dc}	42.30±0.37^{Db}	56.70 ±0.10^{Cb}
Blueberry				
Free	17.50 ±0.46^{Dc}	20.40 ±0.10^{Ec}	27.00±0.40^{Eb}	28.00 ±0.85^{Ec}
Esterified	6.40 ±0.20^{Ec}	22.80 ±0.08^{Dc}	31.00±0.64^{Dc}	33.80 ±0.25^{Dc}
Insoluble-Bound	18.50 ±0.46^{Cc}	24.00 ±0.30^{Cc}	37.80±0.97^{Cc}	48.50 ±0.40^{Cc}

Data represent the mean values for each sample ± standard deviations (n = 3). Means followed by the same capital letters within each berry crop are not significantly different ($p > 0.05$). Means followed by the same lower case letters within a column part are not significantly different ($p > 0.05$).

The capacity to eliminate cupric ions from the medium may be the reason underlying the ability of phenolic compounds to prevent copper ion-mediated LDL cholesterol oxidation. Catechin,

which is one of the major flavonoids in berry seed meals, was used as a positive control and showed high inhibitory activity against LDL cholesterol oxidation. It has been reported that red- or purple-coloured berries, such as blueberries and red raspberries, contain anthocyanins, which are among the principle antioxidants in berries (Heinonen, 2007). Despite their lack of effect in bulk lipids, berry anthocyanins are powerful antioxidants in lipid-containing hydrophilic environments, such as emulsified lipids, liposomes, and LDL (Khknen *et al.,* 2003). Because procyanidins can form complexes with proteins, their contribution to the prevention of the oxidation of cholesterol at the concentration of berry seed meal extracts used in the current work could be low or ineffective. Nevertheless, it has been shown in earlier studies that procyanidins are effective antioxidants and prevent LDL cholesterol oxidation in *vitro* (Huh *et al.,* 2014). The contribution of other flavonoids, such as flavonols, and phenolic acids to the antioxidant effect of berries is generally much less significant compared with the activity of anthocyanins and procyanidins (Hukkanen *et al.,* 2006).

Berry seed meals were quite effective in reducing the oxidation of human LDL cholesterol. The inhibition of the oxidation of human LDL cholesterol reaches up to 50 % after 16 hours. Depending on the berry seed meal that is used and the types of the phenolics (free, esterified, and bound), the effect were somewhat different. However, the difference in the effect after 16 hours are not so great for the free, esterified and bound phenolics and all of them are actually vary between about 30 to 60% in different seed meals. In addition, the results of the current study showed that blackberry seed meals were more effective than black raspberry and blueberry seed meals at inhibiting human LDL oxidation ($P < 0.05$). Various factors, such as differences in the solubility and partitioning between the aqueous and lipid phases in the LDL system, may be

responsible for the observed differences. It is known that lipophilic antioxidants, such as tocopherol, offer a higher protection against LDL oxidation than hydrophilic antioxidants (Gruszecki and Strzalka, 2005) because lipophilic antioxidants enter LDL particles, whereas hydrophilic ones act only on the surfaces of the LDL particles and are thus less effective (Lagor and Millar, 2010). The preventive effect of phenolic compounds against the oxidation of LDL cholesterol may increase due to their free radical scavenging activity or metal ion chelation properties. Furthermore, phenolic compounds can protect endogenous antioxidants, such as tocopherols, β-carotene, lycopene and ubiquinol, in the LDL cholesterol molecule or inhibit enzymes, such as xanthine oxidase, involved in the initiation of oxidation or cell-mediated LDL cholesterol oxidation (Chandrasekara and Shahidi, 2011a). The results of this study demonstrate that the phenolic extracts of berry seed meals are involved in the chelation of cupric ions and therefore minimize the metal-stimulated oxidation of LDL cholesterol.

It has been found that the apolipoprotein B moiety of LDL contains specific copper-binding sites. The binding of Cu^{2+} to LDL is important for the initiation of the copper-mediated oxidation of LDL. Phenolic compounds may contribute to the antioxidant capacity by binding to apolipoprotein B of LDL and thus hindering the binding of Cu^{2+} (Liyana-Pathirana and Shahidi, 2005).

4.10-Inhibition of peroxyl and hydroxyl radical induced supercoiled DNA strand scission

The breakage of supercoiled plasmid DNA is induced by the peroxyl radical, which is produced via AAPH. All berry seed meal extracts showed strong ability to prevent peroxyl radical-induced DNA damage in a concentration-dependent manner. An increase in the concentration of antioxidative extracts increased the protective effect against the nicking of supercoiled DNA. The degree of protection against damage is provided as the percentage inhibition. Figures 4.6 (A) and 4.6 (C) present the impact of peroxyl radical on supercoiled DNA, and S and N represent the supercoiled and nicked DNA bands, respectively. When a circular supercoiled DNA suffers a single-strand nick, the constraint is removed and the specific linking difference can vary, giving rise to open circular DNA (Fishman and Patterson, 1998). Lane 1 shows the native supercoiled DNA sample without any additives. The presence of a high-intensity S and the disappearance of N in lane 1 show a high concentration of supercoiled DNA and a low concentration of nicked DNA in the native DNA. Lane 2 presents the results of the incubation of supercoiled DNA with AAPH and PBS. The presence of a high-intensity N and the disappearance of S in lane 2 indicate that the DNA was completely nicked. The presence of a high-intensity S was predominantly found in the insoluble-bound fraction, followed by the esterified and free phenolic fractions in the extracts of berry seed meals. These results show that a higher level of retention of supercoiled DNA was obtained due to the protection provided by increased concentrations of berry seed meals (Table 4.13). In contrast, the presence of a low-intensity N band was mainly found with the free phenolic fractions, whereas the bound phenolics contained the highest-intensity N bands, which indicates that lower concentrations of nicked DNA are obtained with an increase in the level of protection provided by the extracts. The protection percentage provided by the extracts ranged from 48 to 97 % at an extract concentration of 0.10 mg/mL. At this concentration, the blackberry seed meals exhibited the

highest inhibition against DNA scission (90-97 %), whereas the black raspberry seed meals exhibited intermediate inhibition (81-87 %) and the blueberry seed meals exhibited the lowest inhibition (57-78 %). Marked scission of supercoiled DNA was caused by the presence of peroxyl radicals, which are more stable than other oxygen radicals and have the capacity to diffuse relatively far from the site of their formation before they react with a target molecule. In the absence of any antioxidant, the peroxyl radical abstracts a hydrogen atom from the nearby DNA to generate new radicals, which results in the induction of a free radical chain reaction leading to the breakage of DNA molecules.

Berry seed meals were effective at suppressing the hydroxyl radical-induced DNA damage, but the level of protection was lower compared with that against peroxyl radicals. The hydroxyl radical can not only attack purine and pyrimidine bases and the deoxyribose backbone of the DNA molecule but also be added to DNA bases to produce a number of oxidative products. The first potential mechanism for the inhibition of supercoiled DNA scission induced by hydroxyl radicals involves the chelation of ferrous ions. The chelation of ferrous ions is required to initiate and catalyse the decomposition of H_2O_2 or the scavenging of H_2O_2 itself and therefore inhibit the production of hydroxyl radicals. The second mechanism is associated with the ability of phenolic extracts to scavenge hydroxyl radicals generated in the system (Chandrasekara and Shahidi, 2011a). The results obtained in the current study indicated that berry seed meals were effective metal chelators. Moreover, the berry seed meal extracts effectively scavenged H_2O_2 and hydroxyl radicals. The extracts were tested at a concentration of 6 mg/mL. For the analysis of hydroxyl radical-induced DNA oxidation, a higher concentration of the samples was used. The berry seed meal extracts inhibited DNA scission induced by peroxyl radicals more

favourably than that induced by hydroxyl radicals. Madhujith and Shahidi (2007) also showed that phenolic extracts of different barley varieties inhibited DNA scission induced by peroxyl radicals to a higher extent than that induced by hydroxyl radicals. These results further indicate that the phenolic compounds in berry seed meal extracts could be more effective against the scavenging of peroxyl radicals compared to the scavenging of hydroxyl radicals. The supercoiled DNA retention ranged from 48 % to 96 % against hydroxyl radical-induced oxidation in the presence of the berry seed meal extracts. Figures 4.6 (B) and 4.6 (C) depict the effect of hydroxyl radicals on supercoiled DNA, and S and N represent the supercoiled and nicked DNA, respectively. Lane 1 presents the native supercoiled DNA sample without any additives, and lane 2 shows the incubation of supercoiled DNA, radical, and PBS. The presence of a high-intensity N band and the disappearance of the S band in lane 2 indicate that the supercoiled DNA was completely nicked. In general, the total phenolic and total flavonoid contents of these extracts showed that the blackberry seed meals were richer in total phenolics and flavonoids than the black raspberry and blueberry seed meals. Noroozi *et al.* (1998) reported that flavonoids are effective against hydrogen peroxide-induced oxidative DNA damage to human lymphocytes. Therefore, berry seed meals contain higher concentrations of phenolics, which protect against free radicals and offer protection against DNA damage caused by ROS. Overall, the present study demonstrates that berry seed meals inhibit DNA scission to varying degrees (Table 4.13).

Table 4.13. Effect of berry seed meals phenolic extracts in DNA nicking inhibition (%).

Samples	Hydroxyl	Peroxyl
Blackberry		
Free	85.22 ±0.24^{C}	90.80 ±0.02^{C}
Esterified	88.90 ±0.15^{B}	95.43 ±0.72^{B}
Insoluble-Bound	96.70 ±0.01^{A}	97.90 ±0.14^{A}
Black Raspberry		
Free	63.40 ±0.17^{H}	81.50 ±0.01^{F}
Esterified	79.80 ±0.43^{E}	85.80 ±0.02^{E}
Insoluble-Bound	82.60 ±0.07^{D}	87.00 ±0.31^{D}
Blueberry		
Free	48.99 ±0.05^{I}	57.70 ±0.89^{I}
Esterified	64.40 ±0.32^{G}	70.80 ±1.15^{H}
Insoluble-bound	71.00 ±0.51^{F}	78.30 ±0.62^{G}

Data are expressed as means ± SD (n=3) on and extract. Means± SD followed by the same letter, within a column are not significantly different ($p>0.05$).

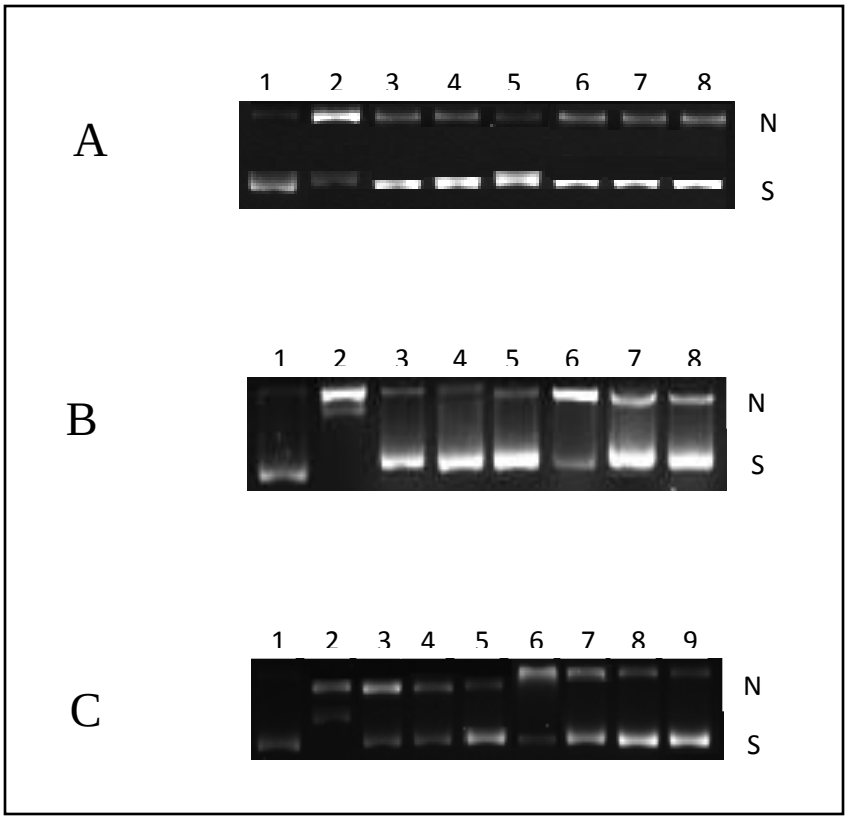


Figure 4.4. Agarose gel electrophoresis of DNA treated with peroxyl (A) and hydroxyl (B) radicals (R) in the presence of blackberry and black raspberry seed meal phenolic extracts at 37 C. Lane 1, DNA blank; lane 2, DNA + R control; lane 3, DNA + R + free phenolics of blackberry seed meals ; lane 4, DNA + R + esterified phenolics of blackberry seed meals; lane 5, DNA + R + bound phenolics of blackberry seed meals; lane 6, DNA + R + free phenolics of black raspberry seed meals; lane 7, DNA + R + esterified phenolics of black raspberry seed meals; and lane 8, DNA + R + bound phenolics of black raspberry seed meals. (C) Agarose gel electrophoresis of DNA treated with hydroxyl radicals (R) in the presence of blueberry seed meals phenolic extracts at 37 C. Lane 1 = DNA + R, control; lane 2 = DNA, blank; lane 3 = DNA + R + free phenolics of blueberry seed meals; lane 4 = DNA + R + esterified phenolics of blueberry seed meals; lane 5 = DNA + R + bound phenolics of blueberry seed meals; Agarose gel electrophoresis of DNA treated with peroxyl radicals (R) in the presence of blueberry seed meals phenolic extracts at 37 C. Lane 6 = DNA + R, control; lane 7 = DNA + R + free phenolics of blueberry seed meals; lane 8 = DNA + R + esterified phenolics of blueberry seed meals; lane 8 = DNA + R + bound phenolics of blueberry seed meals.

4.11-HPLC analysis of anthocyanins, proanthocyanidins, and other phenolics.

The high-performance liquid chromatography (HPLC) analysis of the berry seed meal extracts showed the existence of gallic hexoside, gallic acid, epigallocatechin, procatechuic acid, catechin, caffeic acid, epicatechin, *p*-coumaric acid, epicatechin gallate, quercetin 3-O-glucoronide, peonidin-3-O-glucoside, quercetin pentose, myricetin, quercetin, and proanthocyanidins B1, B2, B3 and B4. These anthocyanins, proanthocyanidins, and phenolics in the blackberry, black raspberry, and blueberry seed meals were determined by high-performance liquid chromatography−diode array detection−electrospray ionization multistage mass spectrometry (HPLC-DAD-ESI-MS^n). Whenever available, reference standards of phenolics were used to substantiate the identification and quantification of the peaks obtained for the berry seed meal extracts. High-performance liquid chromatography (HPLC) has been proven to be the most suitable technique for the separation and characterization of phenolic compounds in plant materials , and this technique has been coupled to diode array detection (DAD) for routine analysis and to mass spectrometry for a more sophisticated identification of phenolic compounds (Gavrilova *et al.*, 2011). Tables 4.14, 4.15, and 4.16 list the identified and quantified compounds based on data obtained for the phenolic extracts of berry seed meals through HPLC-DAD-ESI-MS^n analyses.

Table 4.14. Content of soluble and insoluble-bound polyphenols (µg /g dry weight of seed meal) of blackberry seed meals extracted with two solvent systems: methanol–acetone–water (7:7:6, v/v/v), and acetone (80:20, v/v) using HPLC.

Peak No.	[M-H] (m/z)	Fragments (m/z)	Retention time (min)	Compound	Methanol–acetone–water extracts			Acetone extracts		
					Free	Esterified	Bound	Free	Esterified	Bound
1	331	169	12.70-13.00	Gallic hexoside	37.20	268.30	702.60	29.61	387.83	106.70
2	169	125	15.60	Gallic acid	53.20	241.85	344.86	27.05	235.00	275.10
3	305	151,179,247, 287	16.00-16.10	Epigallocatechin	78.00	137.70	*	22.40	24.50	299.00
4	153	109	20.00-20.40	Protocatechuic acid	48.49	132.90	221.00	14.90	39.93	52.24
5	577	425,405,289	19.90	Procyanidin B1	37.99	40.50	351.09	7.17	19.90	22.06
6	577	425,405,289	22.80-23.20	Procyanidin B3	27.80	19.28	101.65	6.20	26.80	35.70
7	289	245	26.30-26.80	Catechin	6.05	123.90	101.90	6.90	63.80	39.50
8	577	425,405,289	27.30-27.80	Procyanidin B4	20.60	43.41	31.02	-	25.80	49.48
9	577	425,405,289	28.80-29.10	Procyanidin B2	96.80	65.32	-	25.50	70.60	70.80
10	179	135, 167	27.80-28.30	Caffeic acid	52.00	69.60	217.00	20.30	27.00	25.40
11	197	153	32.50	Syringic acid	-	-	-	-	279.00	-
12	289	245	39.20-39.70	Epicatechin	137.20	326.31	115.96	30.60	162.00	283.00

Continued

13	163	119,139	44.60-45.00	*p*-Coumaric acid	37.76	26.90	25.50	*	*	*
14	441	289,169	48.00-48.20	Epicatechin gallate	208.00	861.00	117.20	77.70	-	54.90
15	477	301	50.30	Quercetin 3-O-glucoronide	792.30	646.60	513.00	507.95	236.50	792.05
16	463	301	50.30	Peonidin-3-O-glucoside	244.00	162.40	265.40	128.60	69.40	106.60
17	433	301	49.60-50.10	Quercetin pentose	-	144.00	329.60	46.90	206.70	146.00
18	317	151,179	51.80-52.20	Myricetin	-	-	208.50	-	-	-
19	301	121,179	55.50-56.00	Quercetin	70.80	232.80	207.20	49.40	54.90	68.20

‘*’: Compounds detected but concentration in sample not determined; **‘-‘:** Not detected;

Table 4.15. Content of soluble and insoluble-bound polyphenols (µg/ g dry weight of seed meals) of black raspberry seed meals extracted with two solvent systems: methanol–acetone–water (7:7:6, v/v/v), and acetone (80:20, v/v) using HPLC.

Peak No.	[M-H] (m/z)	Fragments (m/z)	Retention time (min)	Compound	Methanol–acetone–water extracts			Acetone extracts		
					Free	Esterified	Bound	Free	Esterified	Bound
1	331	169	12.70-13.00	Gallic hexoside	161.00	26.80	564.70	26.30	130.80	118.30
2	169	125	15.60	Gallic acid	344.80	53.20	241.85	13.50	89.70	118.30
3	305	151,179,247, 287	16.00-16.10	Epigallocatechin	22.00	86.50	135.90	15.30	22.60	-
4	153	109	20.00-20.40	Protocatechuic acid	10.35	44.00	93.47	9.21	13.06	30.06
5	577	425,405,289	19.90	Procyanidin B1	6.60	41.60	36.00	10.30	5.13	8.00
6	577	425,405,289	22.80-23.20	Procyanidin B3	22.00	63.00	71.40	4.20	8.30	11.60
7	289	245	26.30-26.80	Catechin	6.29	237.80	40.00	8.70	4.30	16.10
8	577	425,405,289	27.30-27.80	Procyanidin B4	7.40	96.70	46.60	-	-	10.60
9	577	425,405,289	28.80-29.10	Procyanidin B2	28.60	108.70	97.50	-	7.00	9.40
10	179	135, 167	27.80-28.30	Caffeic acid	6.30	113.70	157.70	20.30	25.40	27.00

Continued

11	197	153	32.50	Syringic acid	-	-	-	30	*	6.7
12	289	245	39.20-39.70	Epicatechin	6.30	67.50	311.00	71.90	*	10.20
13	163	119,139	44.60-45.00	*p*-Coumaric acid	26.80	483.70	242.80		-	
14	441		48.00-48.20	Epicatechin gallate	40.00	295.00	93.70	51.80	13.49	22.30
15	477	289,169	50.30	Quercetin 3-O-glucoronide	218.50	714.00	1149.00	195.00	68.10	41.80
16	463	301	50.30	Peonidin-3-O-glucoside	47.80	216.00	-	55.80	41.70	44.00
17	433	301	49.60-50.10	Quercetin pentose	32.00	231.00	-	54.50	-	-
18	317	151,179	51.80-52.20	Myricetin	-	176.90	-	28.90	198.80	-
19	301	121,179	55.50-56.00	Quercetin	69.07	159.80	325.80	14.40	97.50	20.60

'*': Compounds detected but concentration in sample not determined; **'-':** Not detected.

Table 4.16. Content of soluble and insoluble-bound polyphenols (µg /g of dry weight of seed meals) of blueberry seed meals extracted with two solvent systems: methanol–acetone–water (7:7:6, v/v/v), and acetone (80:20, v/v) using HPLC**.**

Peak No.	[M-H] (m/z)	Fragments (m/z)	Retention time (min)	Compound	Mixture extracts			Acetone extracts		
					Free	Esterified	Bound	Free	Esterified	Bound
1	331	169	12.70-13.00	Gallic hexoside	46.30	14.70	102.60	13.30	4.60	16.40
2	169	125	15.60	Gallic acid	337.00	79.30	355.80	6.10	7.10	13.80
3	305	151,179,247, 287	16.00-16.10	Epigallocatechin	*	*	40.40	5.20	4.60	*
4	153	109	20.00-20.40	Protocatechuic acid	37.90	19.40	39.70	8.00	34.00	9.80
5	577	425,405,289	19.90	Procyanidin B1	14.30	21.90	24.30	10.50	6.07	11.40
6	577	425,405,289	22.80-23.20	Procyanidin B3	29.90	6.50	45.70	4.70	3.60	18.20
7	289	245	26.30-26.80	Catechin	65.90	12.90	76.60	3.80	4.30	22.70
8	577	425,405,289	27.30-27.80	Procyanidin B4	-	-	-	-	-	-
9	577	425,405,289	28.80-29.10	Procyanidin B2	-	-	-	-	-	-
10	179	135, 167	27.80-28.30	Caffeic acid	51.90	27.00	25.40	20.30	-	44.80

Continued

11	197	153	32.50	Syringic acid	91.00	40.80	93.60	26.00	27.30	55.90
12	289	245	39.20-39.70	Epicatechin	17.00	11.30	7.80	5.60	4.25	8.20
13	163	119,139	44.60-45.00	*p*-Coumaric acid	26.00	57.40	23.80	29.90	30.00	44.00
14	479	317	45.20-45.60	Petunidin 3-galactoside	344.00	61.24	24.55	12.90	13.00	22.00
15	465	303	47.10-47.20	Delphinidin 3-galactoside	-	-	23.14	-	-	-
16	449	317	47.90-48.30	Petunidin 3-arabinoside	93.70	37.60	32.90	9.08	18.80	48.20
17	463	301	48.10-48.60	Peonidin-3-O-glucoside	58.00	18.30	114.13	15.70	17.00	32.00
18	433	301	49.50-49.90	Peonidin 3-(6”-acetoyl) galactoside	116.00	-	115.80	-	-	-
19	433	301	49.60-50.10	Quercetin pentose	18.00	45.70	57.45	12.20	9.80	30.90
20	317	151, 79	51.80-52.20	Myricetin	51.00	27.90	26.25	13.50	12.80	23.60
21	491	287	52.30	Cyanidin 3- (6”-acetoyl) galactoside	75.20	-	47.60	-	-	-

Continued

22	447	257, 285, 327, 401	54.30	Kaempherol hexoside	90.70	24.26	68.40	-	-	-
23	301	121,179	55.50-56.00	Quercetin	26.00	9.10	27.40	12.70	11.00	7.40

‘*’: Compounds detected but concentration in sample not determined; **‘-‘:** Not detected.

The main groups of phenolic compounds found in berries have been reported to be anthocyanins, flavonols, flavanols, gallotannins, proanthocyanidins, and phenolic acids (Seeram, 2006). In this study, the peaks in the chromatograms were mainly classified into hydroxybenzoic and hydroxycinnamic acids, anthocyanins, flavonols, flavan-3-ols, and proanthocyanidins through a comparison of their ultraviolet–visible (UV–Vis) spectra and HPLC retention times with those of the available standards. Figures 4.5, 4.7, and 4.9 show the HPLC -DAD chromatograms and the HPLC ion chromatograms for the acetone extracts, while figures 4.6, 4.8, and 4.10 for the mixture of acetone, methanol, and water extracts. Acetone extracts only used for identification and quantification of proanthocyanidins.

4.11.1-Analysis of phenolic acids

The extracts from the berry seed meals showed great diversity in the phenolic acids present. Five phenolic acids were identified in berry seed meals: gallic acid, protocatechuic acid, caffeic acid, syringic acid, and *p*-coumaric acid. In general, gallic acid (6.39-7.72 µg / 100 g of dry weight) was the major phenolic acid in berry seed meals (Tables 4.14, 4.15, and 4.16). In raspberry seed meals, *p*-coumaric acid was the predominant phenolic acid, comprising 41 % of the total phenolic acids. Black raspberry seed meals contained the highest level of phenolic acids (18.18 µg / 100 g of dry weight), and blueberry seed meals contained the lowest (9.50 µg /100 g of dry weight).

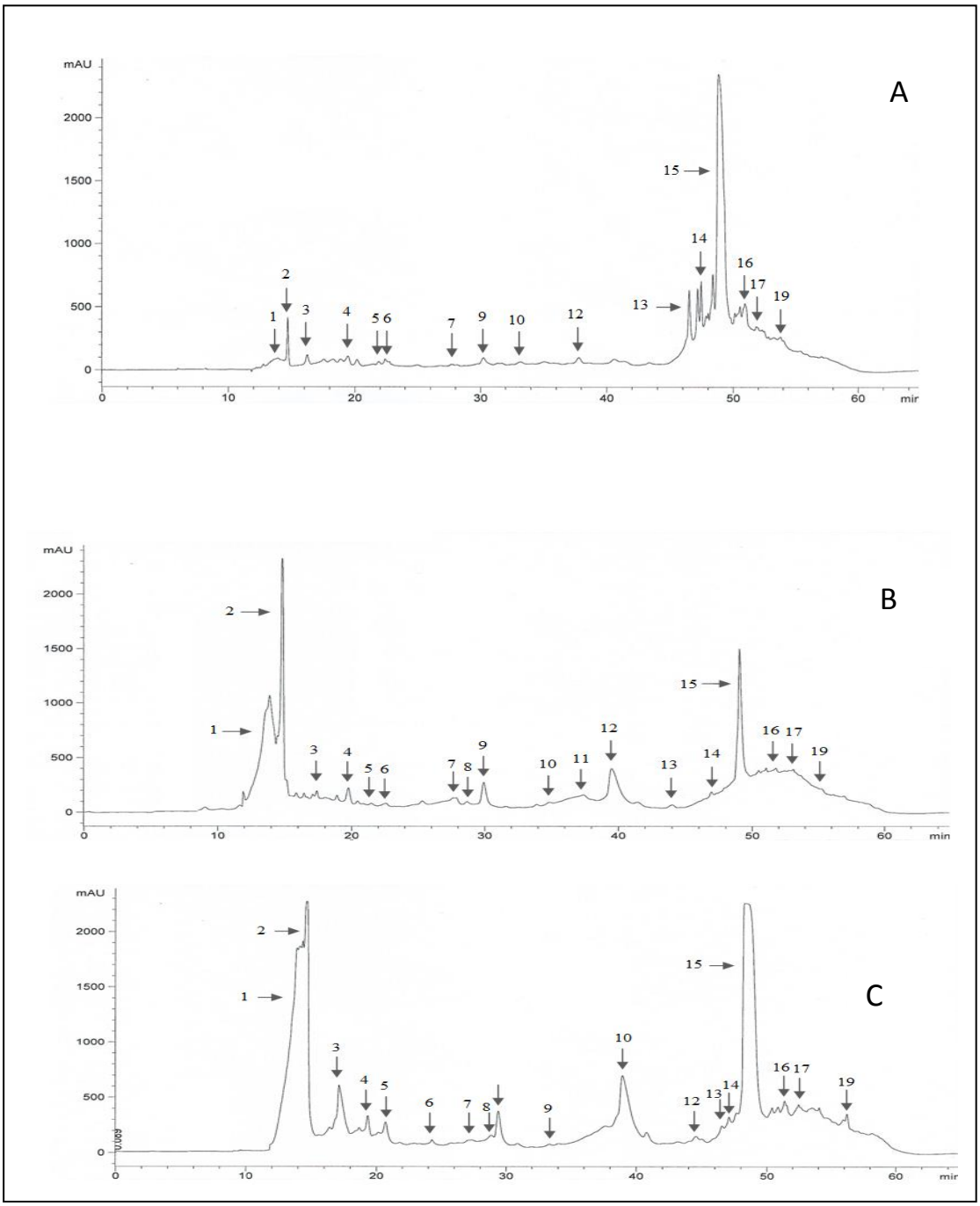


Figure 4.5. HPLC chromatogram at 520 and 280 nm of blackberry seed meal extracted with solvent acetone (80:20, v/v). (A), free; (B), esterified; and (C), and bound: 1, gallic hexoside; 2, gallic acid; 3, epigallocatechin; 4, protocatechuic acid; 5, procyanidin, B1; 6, procyanidin B3; 7, catechin; 8, procyanidin B4; 9, procyanidin B2; 10, caffeic acid; 11, syringic acid; 12, epicatechin; 13, *p*-Coumaric acid; 14, epicatechin gallate; 15, quercetin-3-O-glucoronide; 16, peonidin 3-O-glucoside; 17, quercetin pentose; 18, myricetin; and 19, quercetin. Supelcosil LC-18 column was used (250×4.6 mm inner diameter, 5-µm particles, Supelco, Bellefonte, PA, USA).

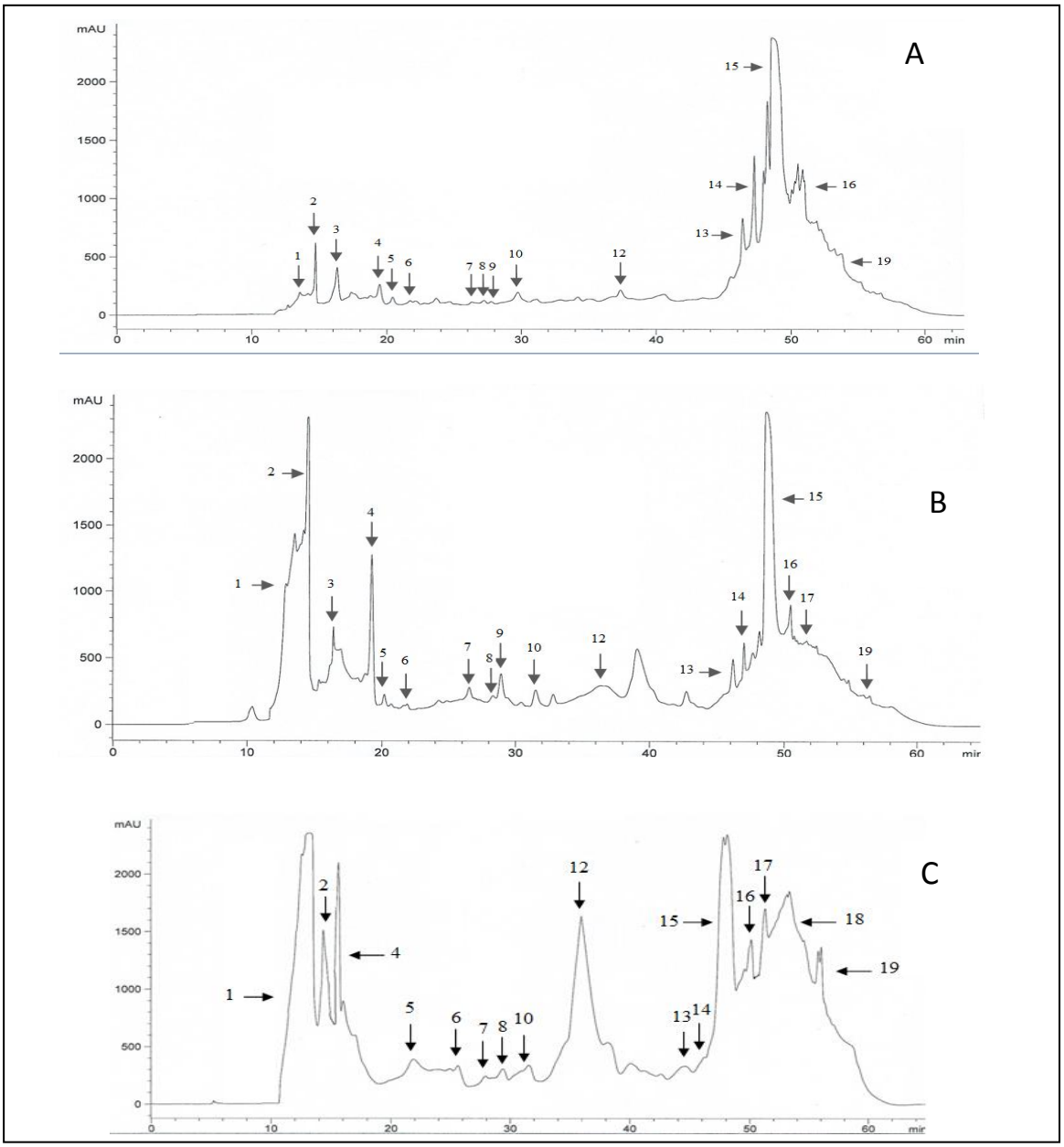


Figure 4.6. HPLC chromatogram at 520 and 280 nm of blackberry seed meals extracted with solvent mixture (methanol–acetone–water (7:7:6, v/v/v)). (A), free, (B), esterified; and (C), bound: 1, gallic hexoside; 2, gallic acid; 3, epigallocatechin; 4, protocatechuic acid; 5, procyanidin, B1; 6, procyanidin B3; 7, catechin; 8, procyanidin B4; 9, procyanidin B2; 10, caffeic acid; 11, syringic acid; 12, epicatechin; 13, *p*-Coumaric acid; 14, epicatechin gallate; 15, quercetin-3-O-glucoronide; 16, peonidin 3-O-glucoside; 17, quercetin pentose; 18, myricetin; and 19, quercetin. Supelcosil LC-18 column was used (250×4.6 mm inner diameter, 5-µm particles, Supelco, Bellefonte, PA, USA).

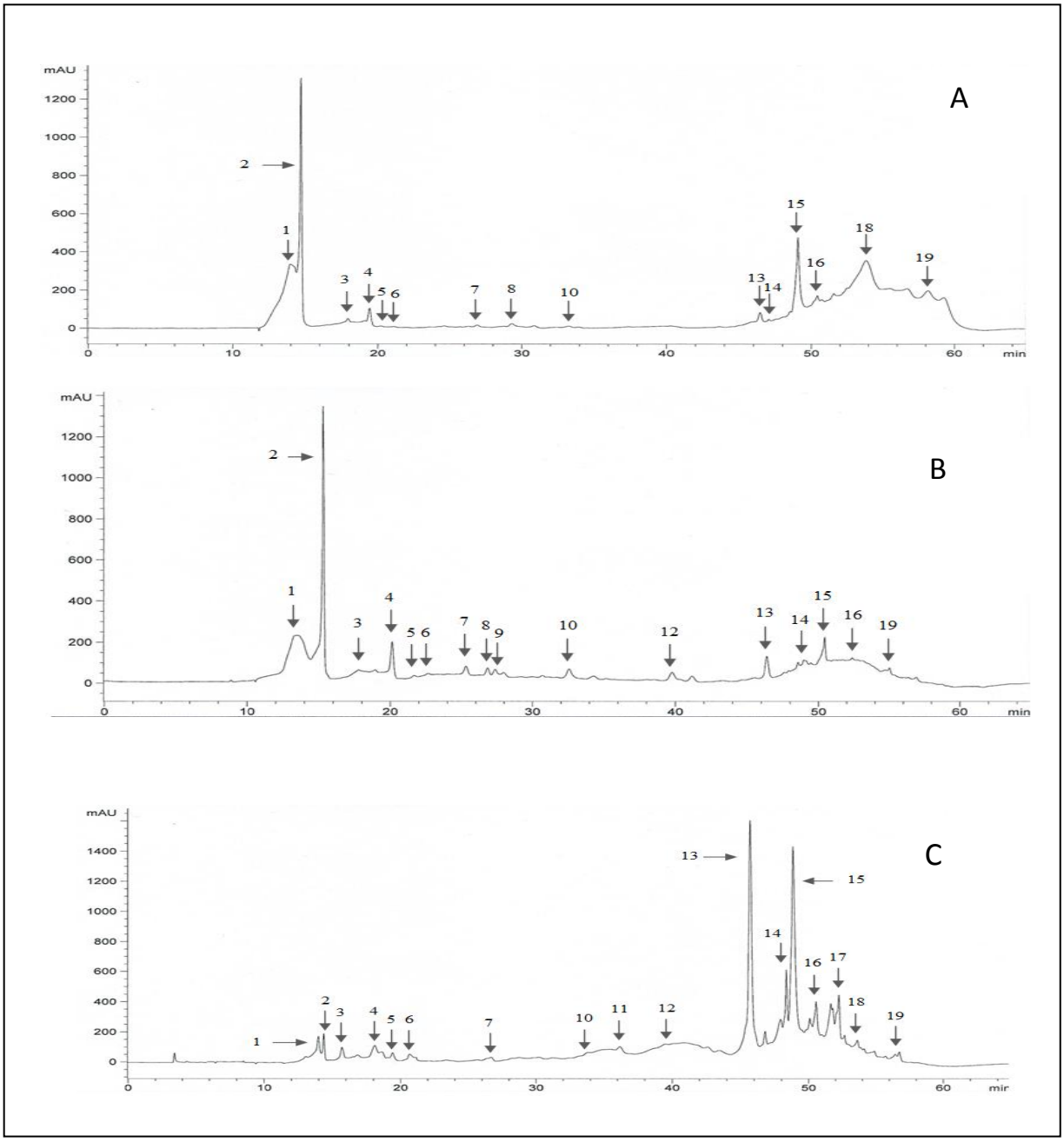


Figure 4.7. HPLC chromatogram at 520 and 280 nm of black raspberry seed meals extracted with solvent acetone (80:20, v/v). (A) free (B) esterified (C) and bound: gallic hexoside; 2, gallic acid; 3, epigallocatechin; 4, protocatechuic acid; 5, procyanidin, B1; 6, procyanidin B3; 7, catechin; 8, procyanidin B4; 9, procyanidin B2; 10, caffeic acid; 11, syringic acid; 12, epicatechin; 13, *p*-Coumaric acid; 14, epicatechin gallate; 15, quercetin-3-O-glucoronide; 16, peonidin 3-O-glucoside; 17, quercetin pentose; 18, myricetin; and 19, quercetin. Supelcosil LC-18 column was used (250×4.6 mm inner diameter, 5-µm particles, Supelco, Bellefonte, PA, USA).

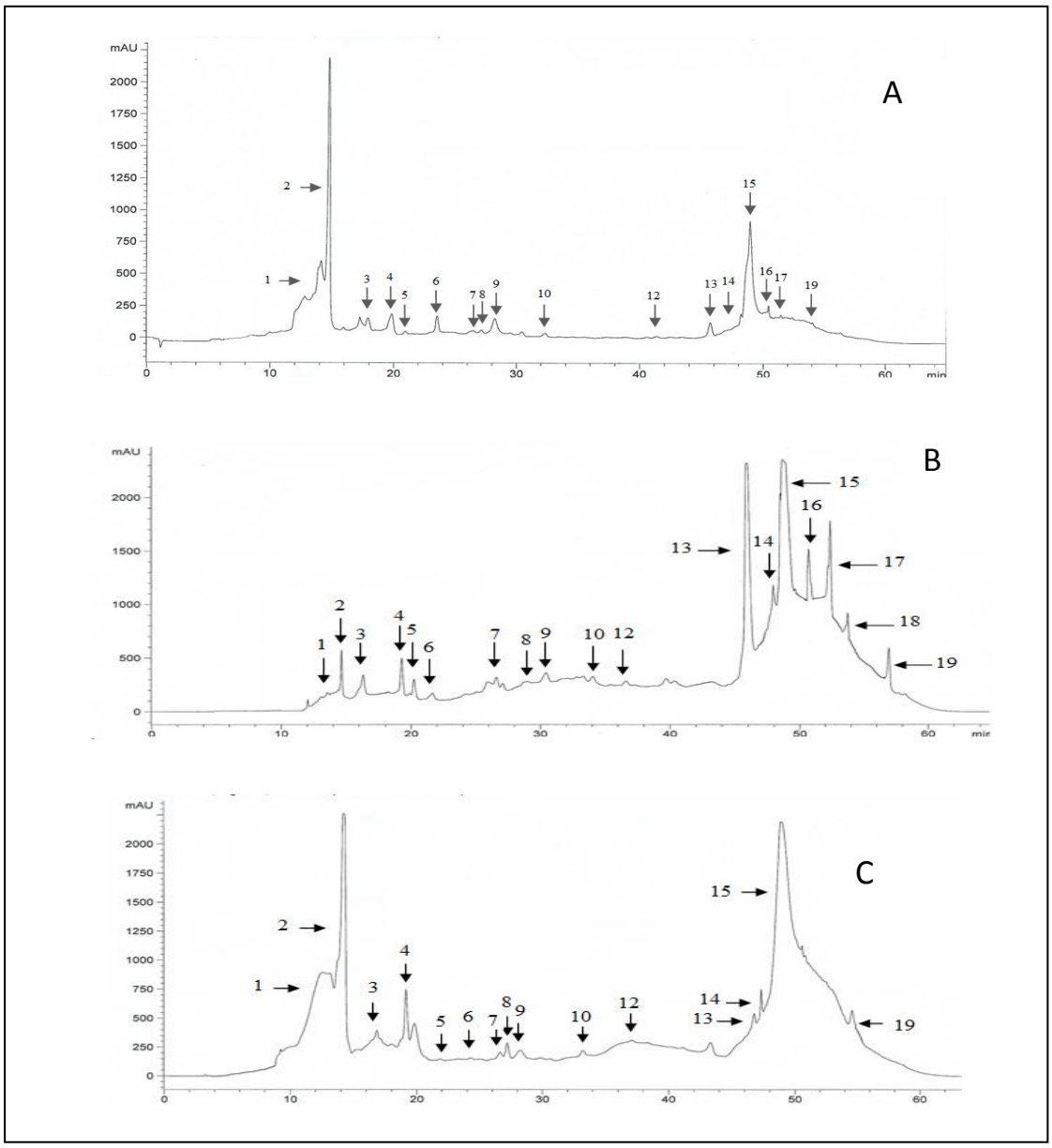


Figure 4.8. HPLC chromatogram at 520 and 280 nm of black raspberry seeds meals extracted with solvent mixture (methanol–acetone–water (7:7:6, v/v/v)). (A) free (B) esterified (C) and bound: 1, gallic hexoside; 2, gallic acid; 3, epigallocatechin; 4, protocatechuic acid; 5, procyanidin, B1; 6, procyanidin B3; 7, catechin; 8, procyanidin B4; 9, procyanidin B2; 10, caffeic acid; 11, syringic acid; 12, epicatechin; 13, *p*-Coumaric acid; 14, epicatechin gallate; 15, quercetin-3-O-glucoronide; 16, peonidin 3-O-glucoside; 17, quercetin pentose; 18, myricetin; and 19, quercetin. Supelcosil LC-18 column was used (250×4.6 mm inner diameter, 5-µm particles, Supelco, Bellefonte, PA, USA).

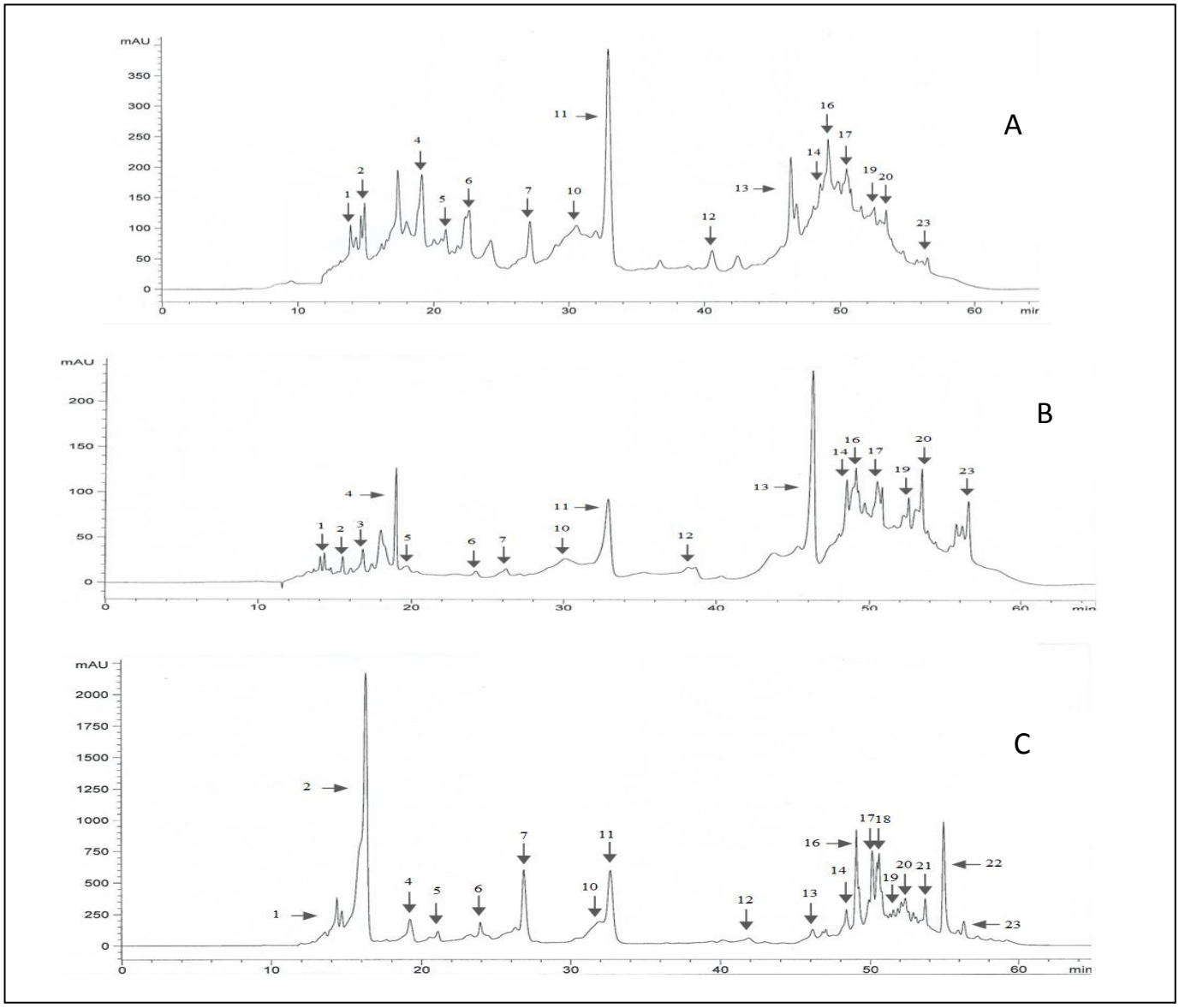


Figure 4.9. HPLC chromatogram at 520 and 280 nm of blueberry seed meals extracted with solvent acetone (80:20, v/v). (A) free (B) esterified (C) and bound : 1- gallic hexoside; 2- gallic acid; 3- epigallocatechin; 4- protocatechuic acid; 5- procyanidin B1; 6- B3; 7- catechin; 8- procyanidin B4; 9- procyanidin B2; 10- caffeic acid; 11- syringic acid; 12- epicatechin; 13- *p*-Coumaric acid; 14- petunidin 3-galactoside; 15- delphinidin 3-galactoside; 16- peonidin 3-O-glucoside; 17- petunidin 3-arabinoside ; 18- peonidin 3-(6”-acetoyl) galactoside; 19- quercetin pentose; 20- myricetin ; 21- cyanidin 3-(6”-acetoyl) galactoside ; 22- kaempherol hexoside ; 23- quercetin. Supelcosil LC-18 column was used (250×4.6 mm inner diameter, 5-µm particles, Supelco, Bellefonte, PA, USA).

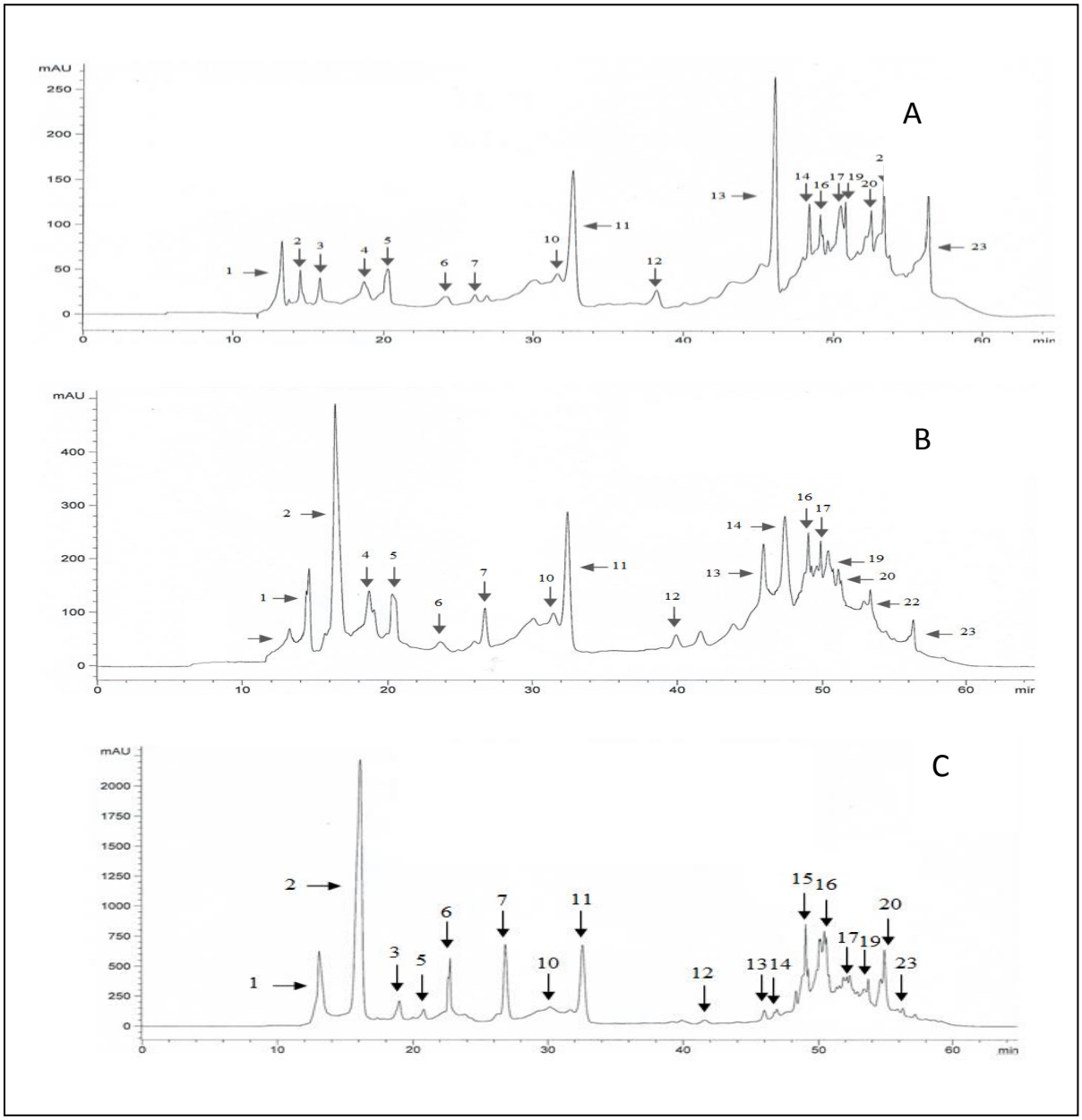


Figure 4.10. HPLC chromatogram at 520 and 280 nm of blueberry seeds meals extracted with solvent mixture (methanol–acetone–water (7:7:6, v/v/v)). (A) free (B) esterified (C) and bound : 1- gallic hexoside; 2- gallic acid; 3- epigallocatechin; 4- protocatechuic acid; 5- procyanidin B1; 6- B3; 7- catechin; 8- procyanidin B4; 9- procyanidin B2; 10- caffeic acid; 11- syringic acid; 12- epicatechin; 13- *p*-Coumaric acid; 14- petunidin 3-galactoside; 15- delphinidin 3-galactoside; 16- peonidin 3-O-glucoside; 17- petunidin 3-arabinoside ; 18- peonidin 3-(6”-acetoyl) galactoside; 19- quercetin pentose; 20-myricetin ; 21- cyanidin 3-(6”-acetoyl) galactoside ; 22- kaempherol hexoside ; 23- quercetin. Supelcosil LC-18 column was used (250×4.6 mm inner diameter, 5-µm particles, Supelco, Bellefonte, PA, USA).

Compared with some other berries, the contents of total phenolics in berry seed meals (29.88–30.41 μg / 100 g of dry weight) are lower than those of rowanberry (103 mg/100 g), chokeberry (96 mg/100 g), blueberry (85 mg/100 g), sweet rowanberry (75 mg/100 g), and saskatoon berry (59 mg/100 g) (Mattila *et al.*, 2006). The marked discrepancies may originate from the different of the type of sample used, ie fruits vs fruit seed meals. Hydroxycinnamic acid derivatives constituted from 22 % (blueberry seed meals) to 56 % (black raspberry seed meals) of the total phenolic acids present in the berry seed meals. The amounts of hydroxycinnamic acid derivatives found in berry seed meals are comparable to those reported for small fruits in the literatures (Gavrilova *et al.,* 2011; Zadernowski, 2005).

Phenolic acids liberated from the bound fraction were the predominant phenolic acids in the berry seed meals. This fraction comprised from 40 % (black raspberry seed meals) to 56 % (blueberry seed meals) of the total phenolic acids present in the berry seed meals. The amount of total phenolics, using HPLC, correlates with the results obtained using the Folin-Ciocalteau assay where the insoluble bound fraction contained the highest amounts of total phenolic acids, followed by esterified and free phenolics. Therefore, most of the antioxidant bioactive compounds in berry seed meals are bound and can survive gastrointestinal digestion to reach the colon intact, where they provide an antioxidant environment (Pérez-Jiménez and Saura-Calixto, 2005). These antioxidant bioactive compounds are in the insoluble form and bound to cell wall materials (Adom *et al.,* 2003). The bound phenolics survive upper gastrointestinal digestion, and finally reach the colon because the cell wall materials are difficult to digest. The fiber is fermented in the colon and some of the bioactive compounds which have antioxidant activity are

liberated (Vitaglione *et al.*, 2008). Thus, bound antioxidant phenolic acids might act along the whole length of the digestive tract by trapping oxidative compounds. Hydroxycinnamic acids constituted from 5 % (blueberry seed meals) to 22 % (black raspberry seed meals) of the phenolic acids identified in this fraction. Free phenolic acids were the minor fraction of phenolic acids, constituting only from 12 % (blackberry seed meals) to 57 % (blueberry seed meals) of the total phenolic acids present in these berry seed meals. Therefore, the fraction of free phenolic acids may promote significant effects against free radical damage. The comparative analyses of the phenolic acids in the three phenolic fractions have revealed lower amounts of free phenolic acids compared with the other two fractions. It has been reported that phenolic acids in their free forms are very rarely present in plants, whereas the majority of phenolic acids are present in their bound forms (Zuo *et al.,* 2002; Robbins, 2003).

4.11.2- Analysis of anthocyanins

The individual anthocyanins were identified by comparing their mass spectral data and retention times with published data (Wu and Prior, 2005). Berries are rich in anthocyanins, compounds that provide pigmentation to fruits and serve as natural antioxidants. However, the amount of anthocyanins in berry seed meals is very minimum. The only anthocyanin identified in the blackberry and black raspberry seed meals was peonidin-3-O-glucoside. The HPLC data confirmed the results that there are more total procyanidins and less total anthocyanins in the blackberry and black raspberry seed meals. However, the blueberry seed meals contain a diversity of anthocyanins compared with blackberry and black raspberry seed meals. Of the common fruits and vegetables, blueberries are considered to possess the highest content of

anthocyanidins (Juranic and Zizak, 2005). However, many factors, such as genes, soil type, light, temperature, and agronomic conditions, influence the anthocyanin composition in plants (Naczk and Shahidi, 2004; and Lee, 2005). Boss *et al.* (2008) found that some genes were expressed in all grapes, even where little or no anthocyanins accumulated, but expression of the gene encoding a UDP glucose-flavonoid 3-O-glucosyl transferase (UFGT) was only detected in coloured grapes that synthesised anthocyanins. Southern and northern analysis of the white grapes indicated that the UFGT gene was present but was not expressed. Therefore, the lack of anthocyanins in white-skinned varieties correlates with a lack of expression of the UFGT gene (Boss *et al.,* 2008). Fracassetti *et al.* (2013) indicated that anthocyanins are labile in nature and susceptible to deterioration during processing and storage. Huang *et al.* (2012) also detected higher levels of anthocyanidins in blueberries (Vaccinium ashei cv.) than in blackberries (Rubus laciniatus cv.) and strawberries (Fragaria x ananassa cv.). In the present study, the blueberry seed meals were found to contain petunidin 3-galactoside (15 %), delphinidin 3-galactoside (2 %), petunidin 3-arabinoside (19 %), peonidin-3-O-glucoside (22 %), peonidin 3-(6”-acetyl) galactoside (26 %), and cyanidin 3-(6”-acetyl) galactoside (14 %) of total anthocyanins content. Similar findings were obtained in another study, which found that blueberries contain cyanidin-3-galactoside, delphinidin-3-galatoside, petunidin-3-galactoside, petunidin-3-glucoside, petunidin-3-arabinoside, and peonidin-3-galactoside (Seeram, 2006).

4.11.3- Analysis of flavonols

Flavonols were found in all berry seed meals. Quercetin was the major flavonol in berry seed meals (71%), and a significant content of myricetin was measured in some fraction of the berry seed meals (28%). In contrast, Rebello *et al.* (2013) showed that the flavonol profile of Violeta

grape skin as dominated by myricetin-based flavonols (74%) whereas the second most important type of flavonols was based on quercetin (20%). The content and distribution of flavonols in the berry seed meals found were as follows: 7.19 µg / 100 g dry weight (7 %) in blackberry seed meals, 7.31 µg / 100 g dry weight (8 %) in black raspberry seed meals, and 1.67 µg / 100 g dry weight (1 %) in blueberry seed meals. In this study, kaempferol was not found, which is in agreement with the results reported by Gavrilova *et al.* (2011). Kaempferol hexoside was identified as a kaempferol derivative due to its UV spectra and MS fragmentation ions at *m*/*z* 285 in negative- and positive-mode MS. Kaempferol hexoside was only found in blueberry seed meals (1.83 µg / 100 g dry weight (52 %)).

4.11.4- Analysis of quercetin derivatives

Two quercetin derivatives were detected in berry seed meals: quercetin-3-O-glucuronide and quercetin pentose. Quercetin-3-O-glucuronide had a characteristic peak with (tR) 48.90 min and a λmax value of 280 nm. This compound had an m/z 477, and its fragmentation yielded a quercetin ion at m/z 301. Quercetin pentose was identified as $[M \_ H]^-$ at m/z 433 and a λmax value of 280 nm. This compound had an m/z 433, and its fragmentation yielded a quercetin ion at m/z 301. The data presented in (Tables 4.14, 4.15, and 4.16) showed a high content of flavonol derivatives, whereas the other compounds, such as phenolic acids, accounted for 16 % of the black raspberry seed meal dry weight and 31 % of the blackberry seed meal dry weight. The flavonol derivatives were the most prevalent phenolic group present in the blackberry and black raspberry seed meals (26 and 40 % by dry weight, respectively). Among the identified flavonol derivatives, the most important compound was quercetin-3-O-glucuronide (21 % for

blackberry raspberry seed meals and 35 % for blackberry seed meals). These compounds are typical to berries of the Rubus family. This result is similar to the findings reported by Oszmianski *et al.* (2011), who found that quercetin derivatives were the most predominant phenolic group found in black currant leaf extract and constituted 11 % of black currant leaf extract. Additionally, Gavrilova *et al.* (2011) indicated that quercetin derivatives are the dominant flavonols in blueberries and red currants. Only quercetin pentose was identified in blueberry seed meals (4 % of dry weight), but quercetin-3-O-glucuronide was not detected.

4.11.5-Identification of flavan-3-ols

Two solvents have been used to determine the best solvent for the extraction of proanthocyanidins: acetone (80:20, v/v) and methanol–acetone–water (7:7:6, v/v/v). Bonoli *et al.* (2004) found that aqueous acetone selectively improved the catechin and proanthocyanidin extraction yield. However, the ethanol and methanol extractions enabled the recovery of considerable amounts of catechins, proanthocyanidins, and hydrolysable tannins (Bonoli *et al.*, 2004). In this study, methanol–acetone–water (7:7:6, v/v/v) showed the highest extraction capacity for proanthocyanidins and catechins. Proanthocyanidins were measured as epicatechin equivalents. Because there is variation in the molar absorbances due to the chain length and type of bond, the best available standards for procyanidins are the original isolates of A-type and B-type procyanidins (Rohr *et al.*, 2000). Proanthocyanidins composition differs between berry compartments: total content is usually higher in seeds while polymer size is much larger in skin (Prieur *et al.*, 1994; Souquet *et al.*, 1996).

The monomeric of flavan-3-ol derivatives are (+)-catechin, (-)-epicatechin, (-)-epicatechin-3-O-gallate, epigallocatechin and epigallocatechin-3-O-gallate. To produce dimers, trimers, tetramers, pentamers and longer chain polymers, the monomers of flavanol-3-ol are condensed (Rebello *et al.,* 2013). Four flavan-3-ol monomers and four B-type dimers were generally found in the different fractions of analyzed berry seed meals, showing different concentrations and distribution within these fractions. Berries are known to have catechin and epicatechin at different amounts depending on the *Rubus* species (Arts *et al.,* 2000). Flavan-3-ol monomers in blackberry and black raspberry seed meals were mainly epicatechin gallate, followed by epicatechin, catechin and epigallocatechin. In contrast, epicatechin gallate was not detected in blueberry seed meals and the major flavan-3-ol monomers identified in blueberry seed meals were catechin. Therefore, catechin and epicatechin were detected in the berry seed meals (11.81 % for blackberry seed meals, 12 % for black raspberry seed meals, and 2.74 % for blueberry seed meals of total flavonoids). As could be expected, the berry seed meals were an important source of proanthocyanidins, their content ranging from 6.50 to 1149.00 μg/ g of defatted berry seed extracts (catechin equivalent). With regard to flavan-3-ol dimers, procyanidins B2 and B4 are the major flavonols detected in the blackberry and black raspberry seed meals. This result is in agreement with the findings from previous studies, which found only primary B-type forms in other bluish-black coloured *Vaccinium* species (Schmidt *et al.,* 2004). In contrast, procyanidins B2 and B4 were not detected in blueberry seed meals. The aforementioned results suggest that berry seed meals accounted for a relevant amount of bitter compounds (flavan-3-ols of low molecular weight). It is recommended to avoid the transfer of bitter compounds from grape to wine during winemaking, that is, long macerations (Rebello *et al.,* 2013). Blackberry seed meals exhibited higher antioxidant activity than black raspberry and blueberry seed meals, and this

difference is attributed to their proanthocyanidins, namely, procyanidins B2 and B4. The blueberry seed meals presented the lowest concentration of proanthocyanidins. Hosseinian *et al.* (2007) showed that raspberries contain the highest content of total flavanols compared with strawberry, saskatoon berry, chokecherry, and seabuckthorn and that wild blueberry presented the lowest content. The bound phenolic fractions of the berry seed meal samples were present at higher concentrations compared with the free and esterified form. Therefore, the insoluble-bound fractions of berry seed meals have the best antioxidant activity.

CHAPTER 5

CONCLUISION

5.1-Summary of the Work

A complete profile of phenolic distribution, including free, ester, and bound forms in blackberry (Rubus sp.), black raspberry (Rubus occidentalis), and blueberry var. Jersey (Vaccinium corymbosum) seed meals were examined and discussed in this systematic investigation. It is very important to develop and select a reliable and robust assay for accurately measuring antioxidant activity of natural antioxidants. The present study demonstrated that berry seed meals are rich sources of natural antioxidants with high free radical scavenging capacity. The high antioxidant value indicated the potential of these berry seed meals for use as nutraceutical and functional food ingredients. The contents and distribution of phenolic compounds varied among the fractions. Antioxidants can protect against the damage induced by free radicals acting at various levels. The results of this study indicated that the insoluble-bound form of phenolics was generally a major contributor to the highest antioxidant activity of the berry seed meals, and this due to their high content of bioactive compounds. This is very important finding because most of the literature they have only reported on the soluble phenolics and they have ignore the insoluble bound ones. Moreover, the results of present experiment also revealed high quality of antioxidants in blackberry seed meals, as proved by all the assays conducted. Therefore, the berry seed meals exhibited good antioxidant capacity mostly because of the existence of these special phenolic compounds. The dominant phenolic compounds identified in this study were phenolic acids, catechins (flavanols), and proanthocyanidins (condensed tannins). Only flavan-

3-ol monomers and dimers were generally found in the various fractions of analyzed berry seed meals. All these factors contribute to the significance of the study conducted.

5.2-Future Work

The hope that antioxidants can inhibit or relieve several pathological conditions requires reconsideration. Research efforts should continue to focus on the elucidation of mechanisms of action at the cellular and molecular levels. Because extrapolations cannot be made between *in vitro* and *in vivo* systems, further studies should be designed to investigate the cancer-preventive potential of berry seed meals in animal models and human subjects.

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Appendix A

Standard calibration curves

The standard calibration curve was created by plotting a known concentration of gallic acid for determining total phenolics and quercetin for total flavonoids. Trolox also can be used as a standard to measure antioxidant capacity using a number of antioxidant assays, including: DPPH radical scavenging assay, ABTS decolourization assay, oxygen radical absorbance capacity (ORAC), reducing power activity, as shown in Figures A.1,2,3,4,5, and 6, respectively.

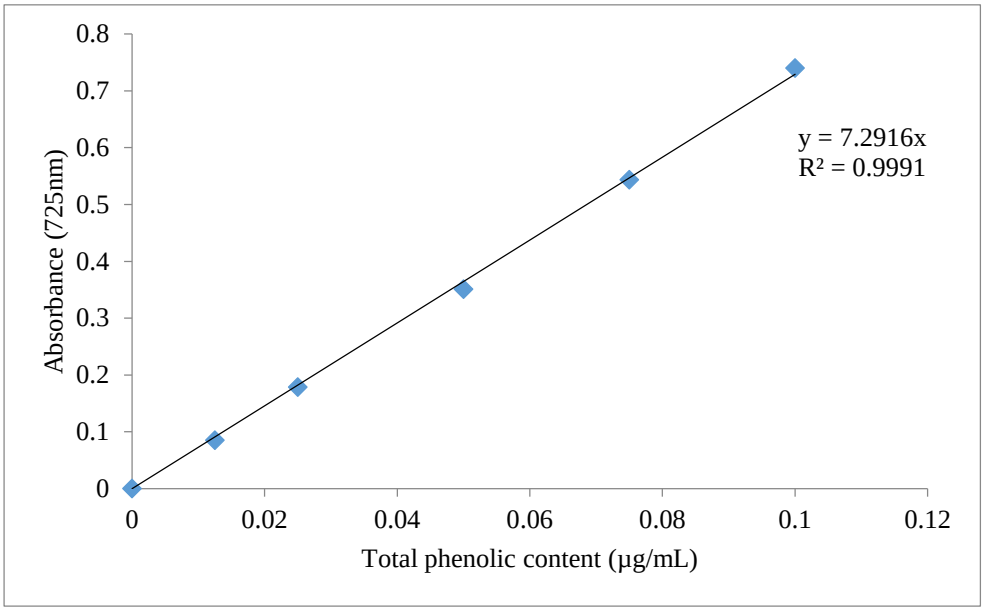

Figure A.1. Folin-Ciocalteu calibration curve for gallic acid

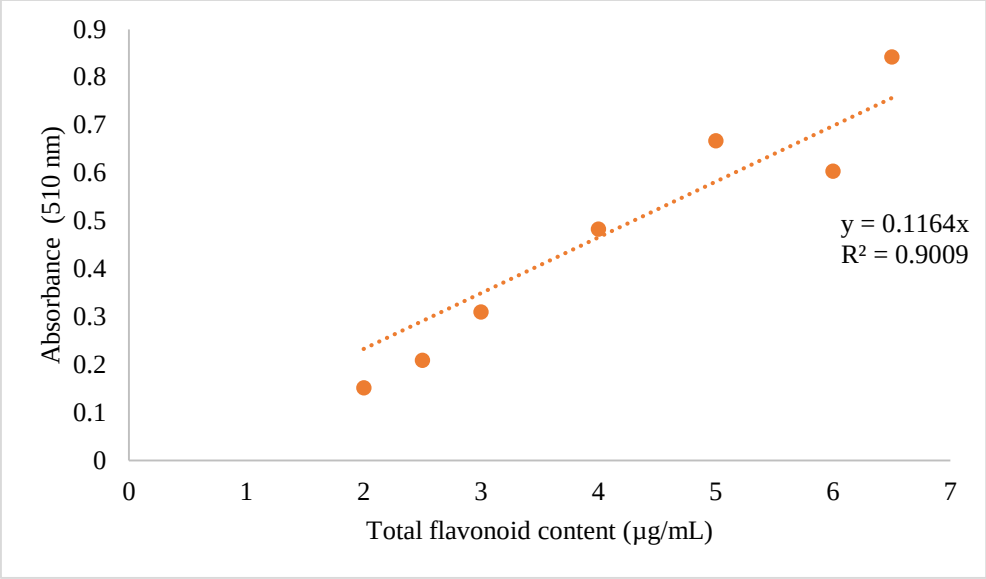

Figure A.2. Total flavonoid calibration curve

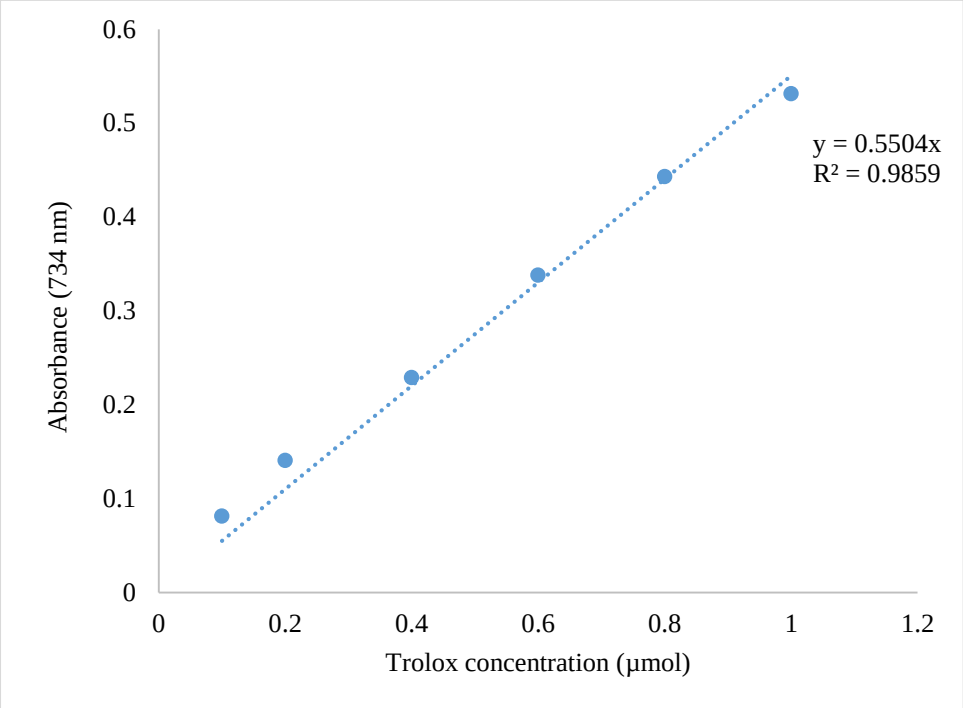

Figure A.3. Trolox calibration curve (TEAC)

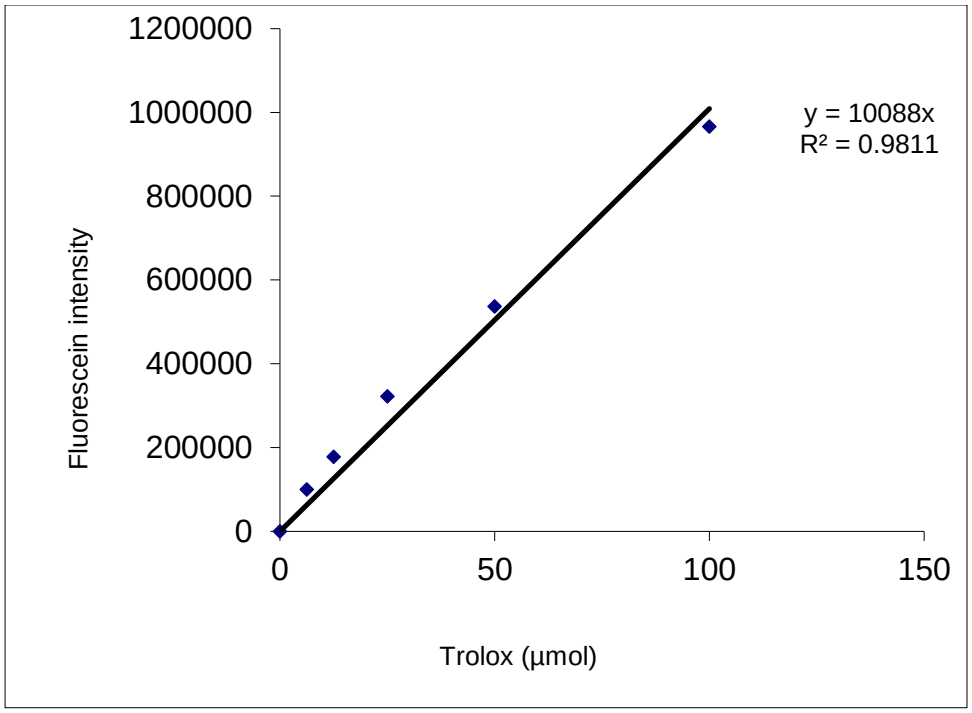

Figure A.4. ORAC calibration curve

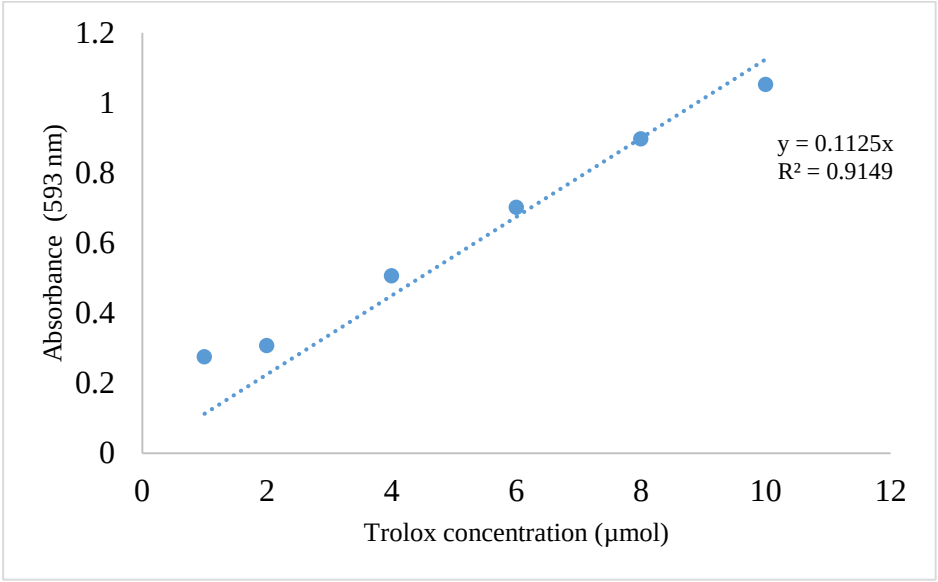

Figure A.5. Reducing power calibration curve

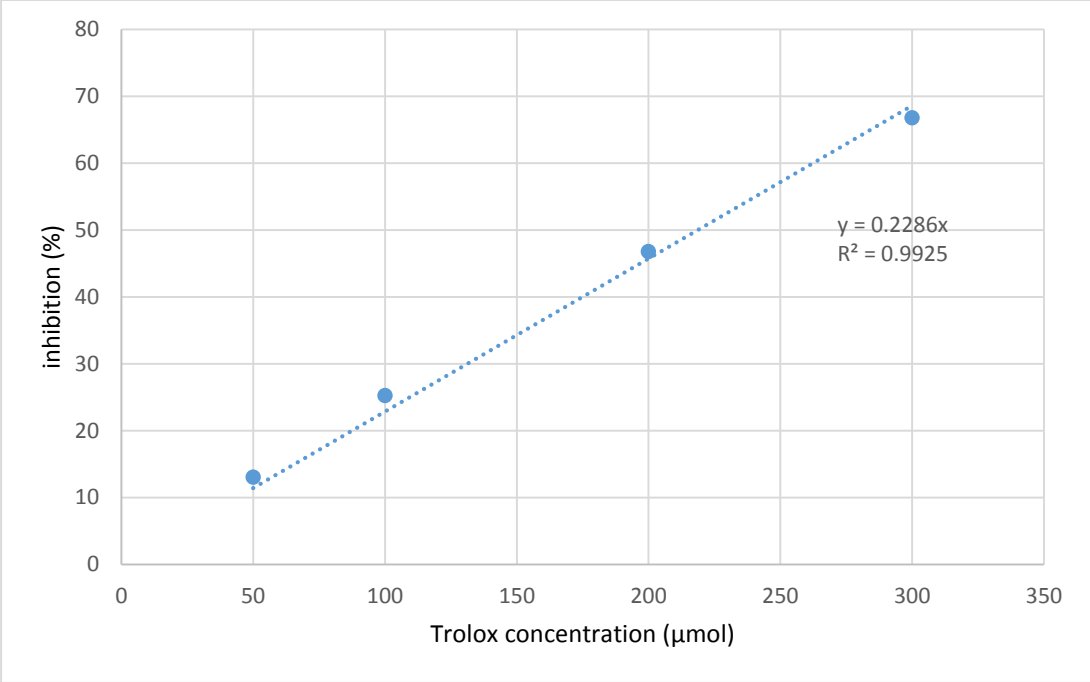

Figure A.6. Trolox calibration curve (DPPH-EPR)

Appendix B

Full MS and MS/MS spectra obtained in positive and negative ion mode for peaks tentatively identified in berry seed meals.

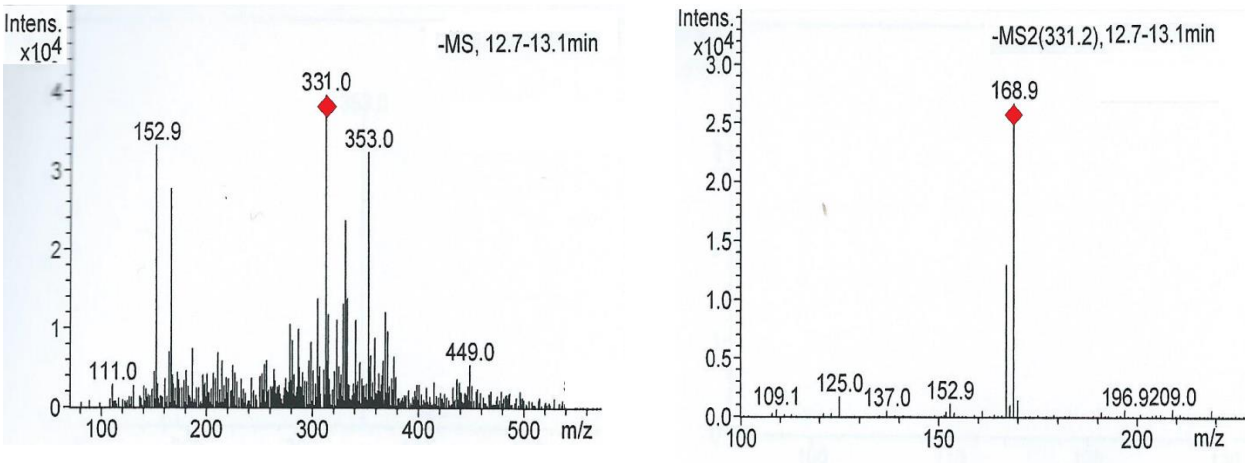

Figure B.1. ESI MS^- fragmentation pattern of gallic hexoside (Rt = 12.7-13. min)

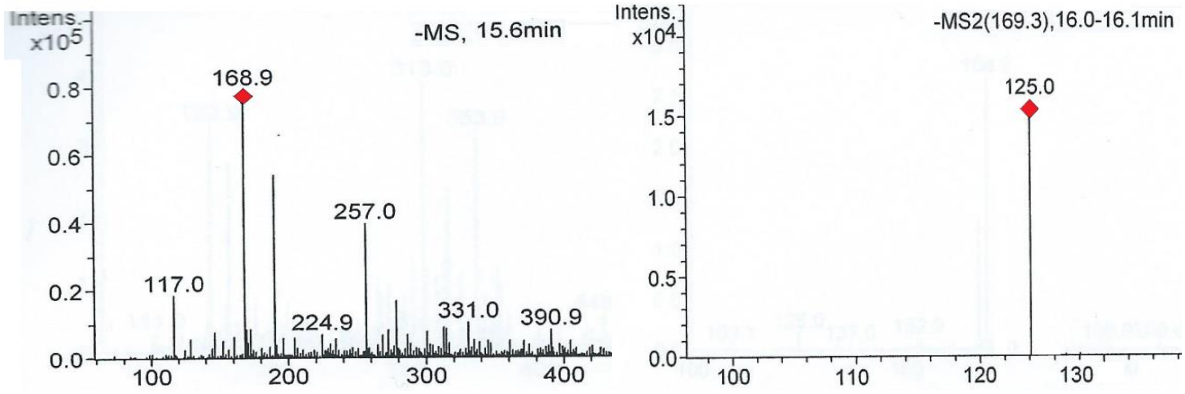

Figure B.2. ESI MS^- fragmentation pattern of gallic acid (Rt = 15.6 min)

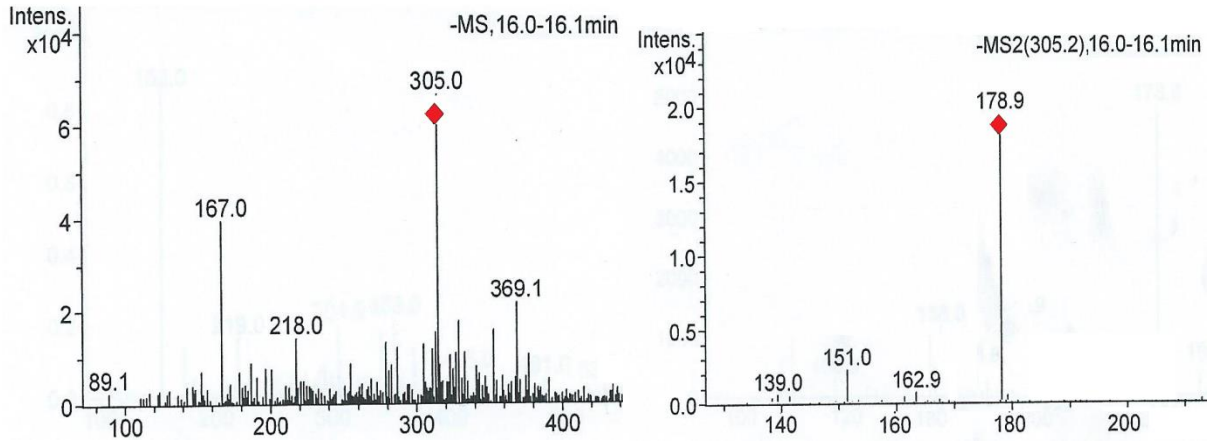

Figure B.3. ESI MS^- fragmentation pattern of epigallocatechin (Rt = 16.0-16.1 min)

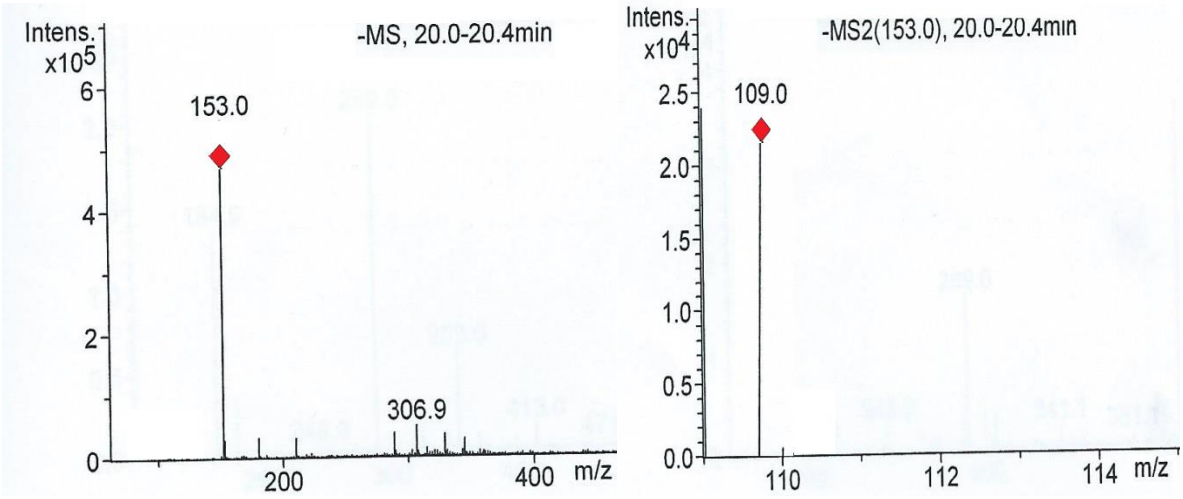

Figure B.4. ESI MS^- fragmentation pattern of protocatechuic acid (Rt = 20-20.4 min)

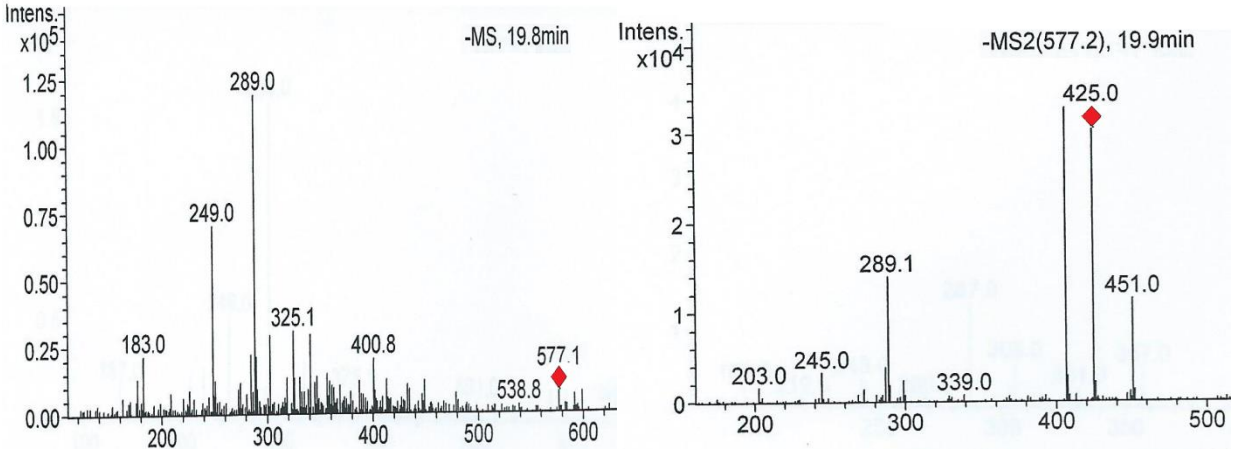

Figure B.5. ESI MS^- fragmentation pattern of procyanidin B1 (Rt = 19.9 min)

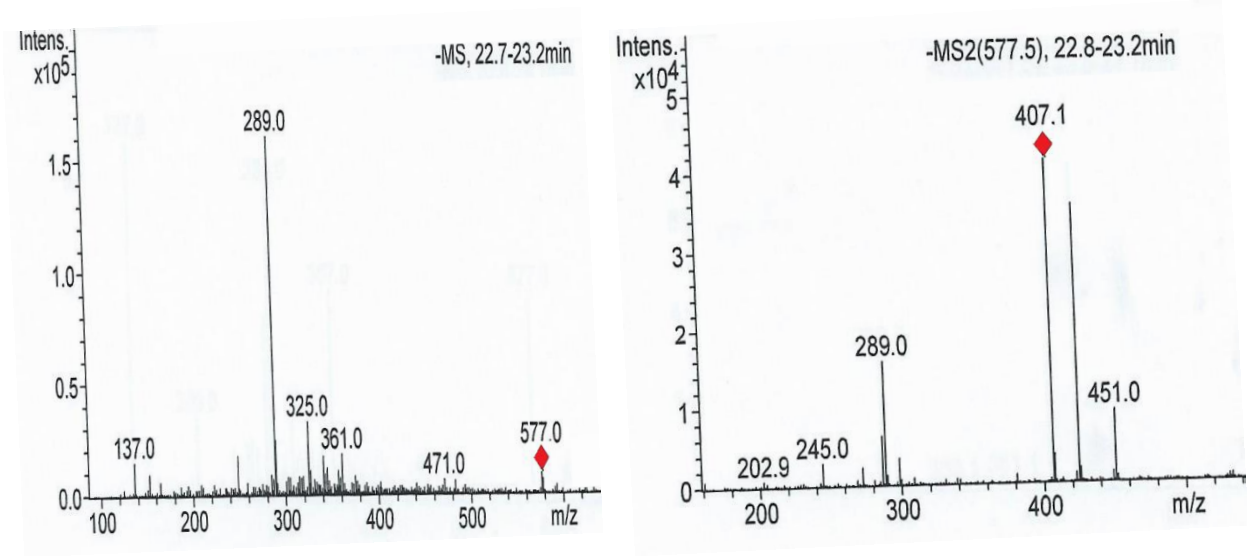

Figure B.6. ESI MS^- fragmentation pattern of procyanidin B3 (Rt = 22.8-23.2 min)

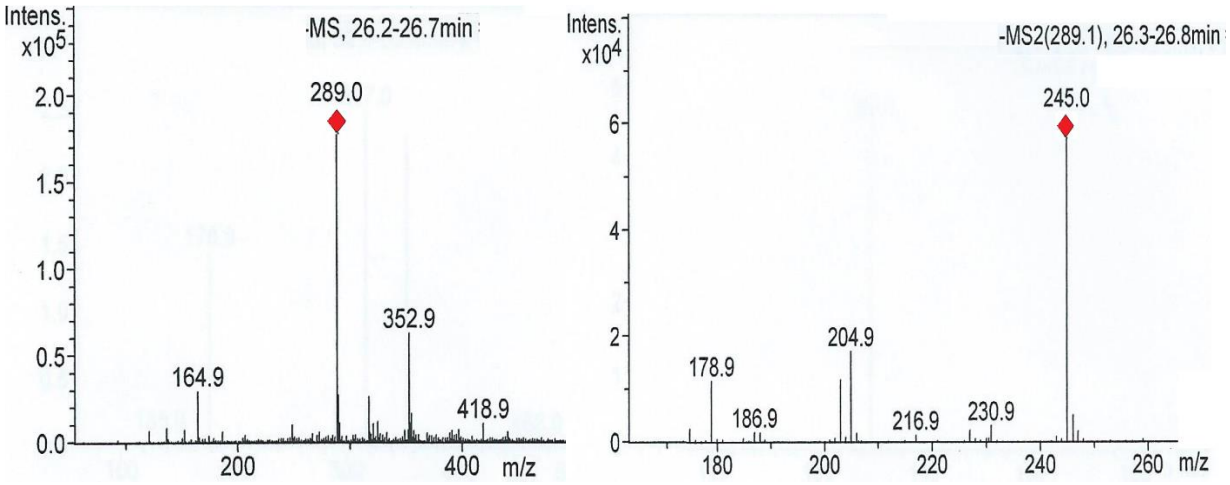

Figure B.7. ESI MS^- fragmentation pattern of catechin (Rt = 26.3-26.8 min)

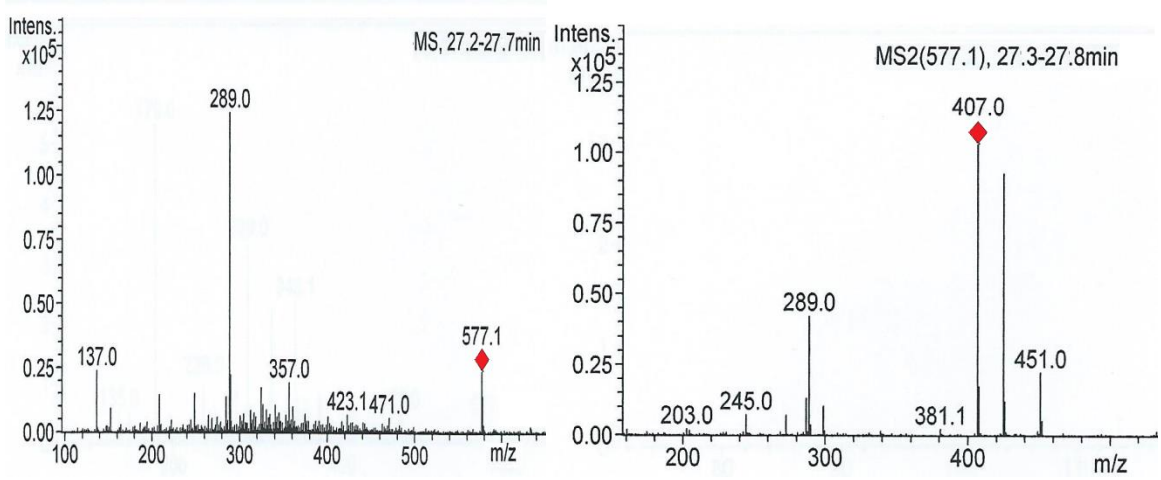

Figure B.8. ESI MS^- fragmentation pattern of procyanidin B4 (Rt = 27.3-27.8 min)

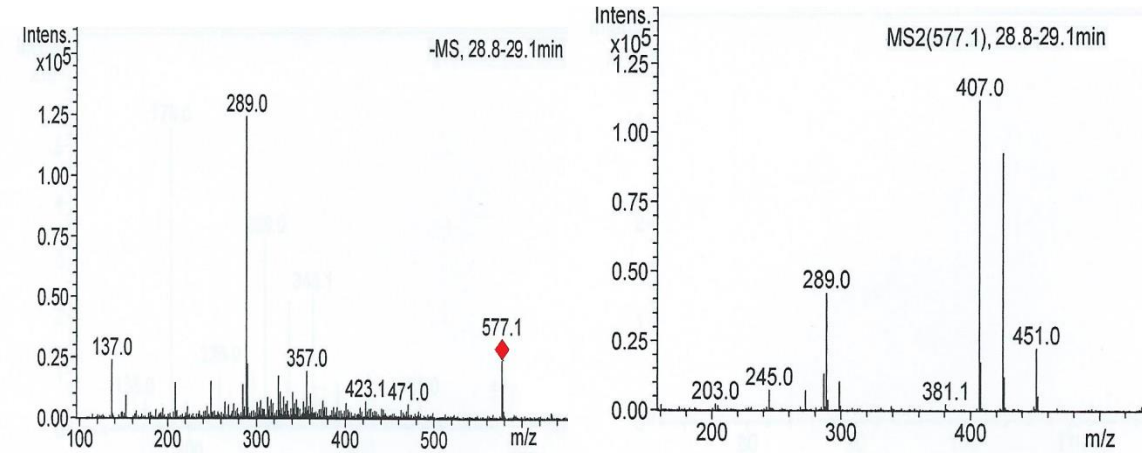

Figure B.9. ESI MS^- fragmentation pattern of procyanidin B2 (Rt = 28.8-29.1 min)

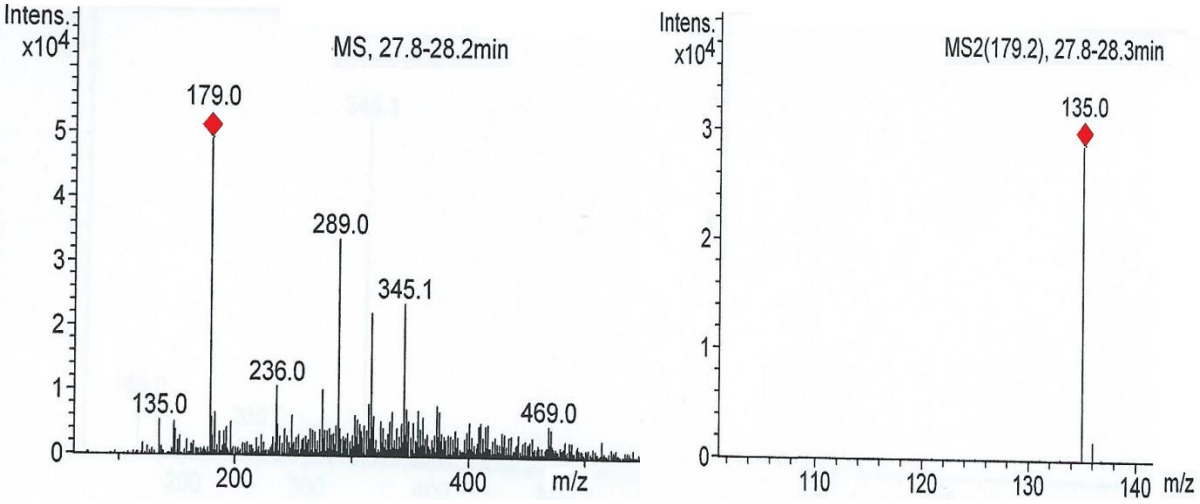

Figure B.10. ESI MS^- fragmentation pattern of caffeic acid (Rt = 27.8-28.3 min)

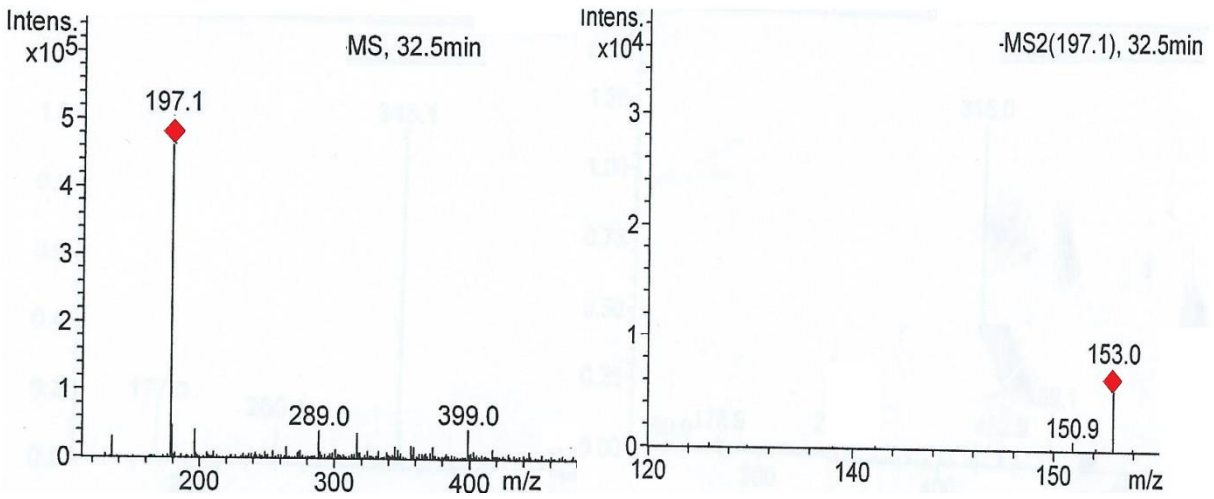

Figure B.11. ESI MS^- fragmentation pattern of syringic acid (Rt = 32.5 min)

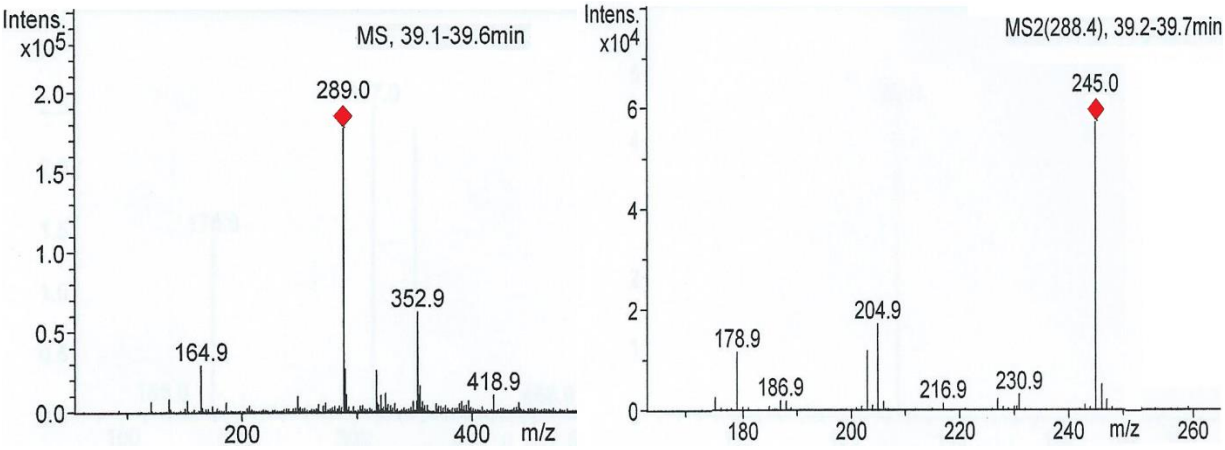

Figure B.12. ESI MS^- fragmentation pattern of epicatechin (Rt = 39.2-39.7 min)

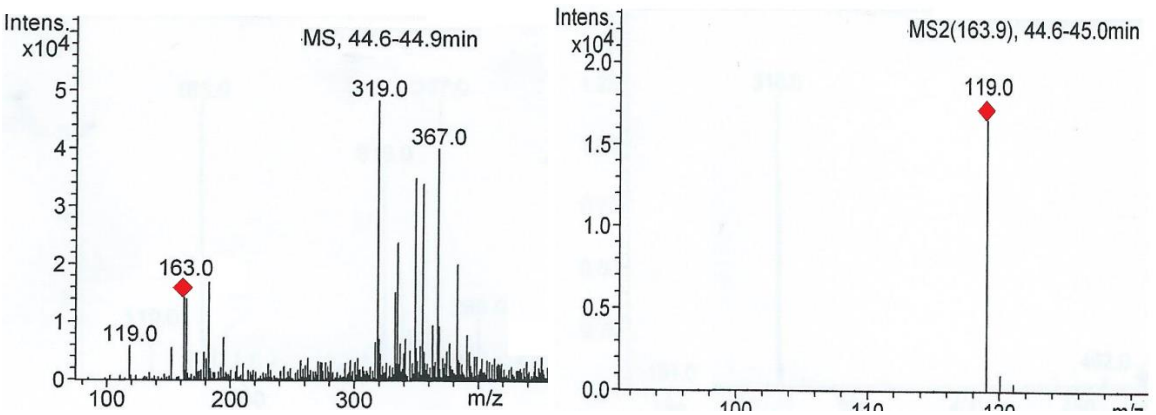

Figure B.13. ESI MS^- fragmentation pattern of *p*-Coumaric acid (Rt = 44.6-45 min)

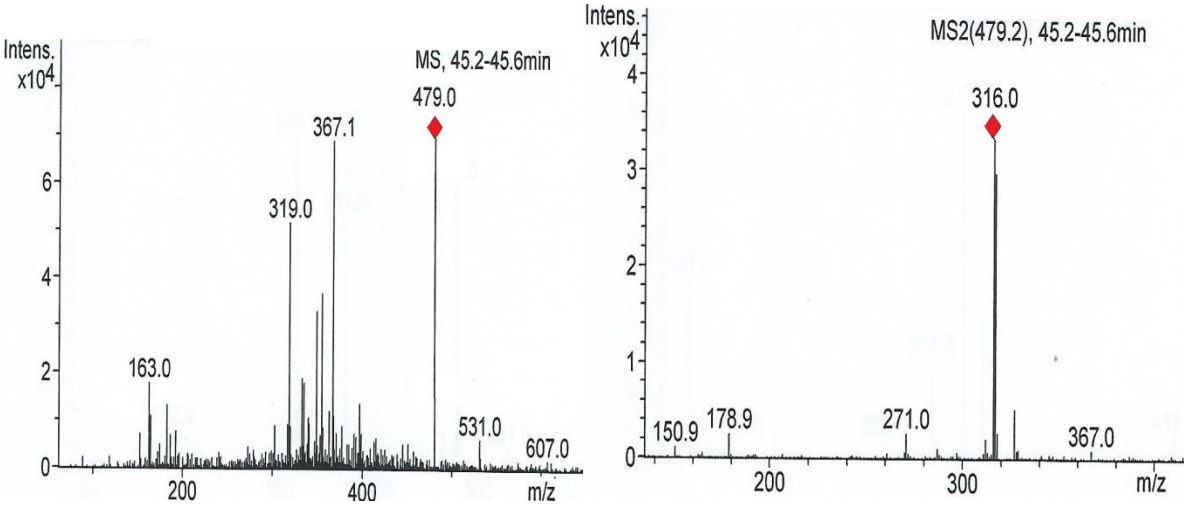

Figure B.14. ESI MS^- fragmentation pattern of petunidin 3-galactoside (Rt = 45.2-45.6 min)

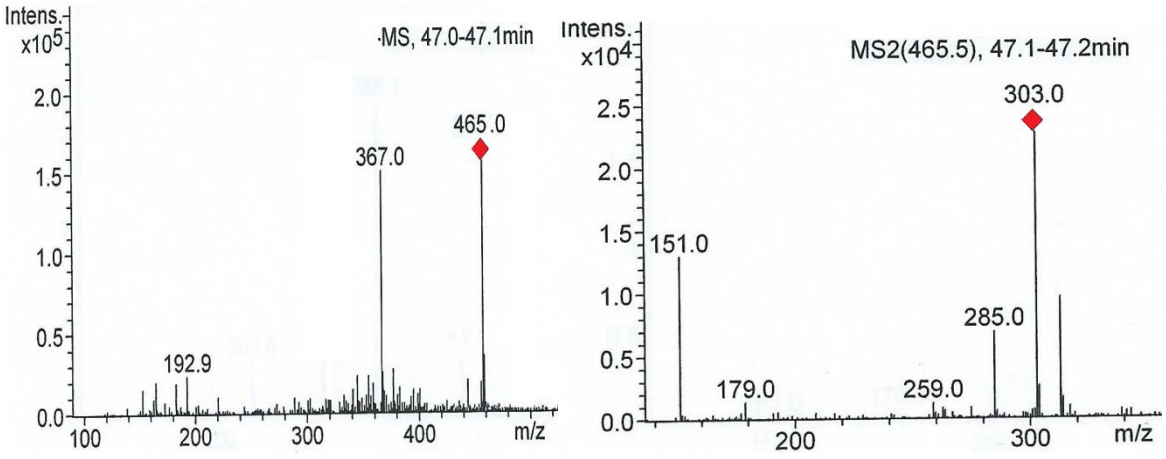

Figure B.15. ESI MS$^-$ fragmentation pattern of delphinidin 3-galactoside (Rt = 47.1-47.2 min)

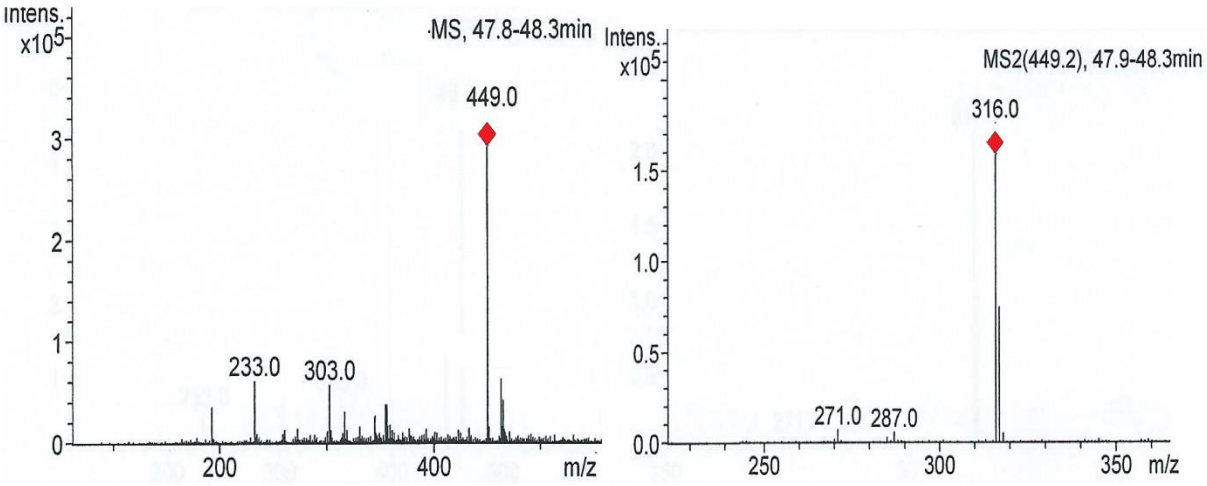

Figure B.16. ESI MS$^-$ fragmentation pattern of petunidin 3-arabinoside (Rt = 47.9-48.3 min)

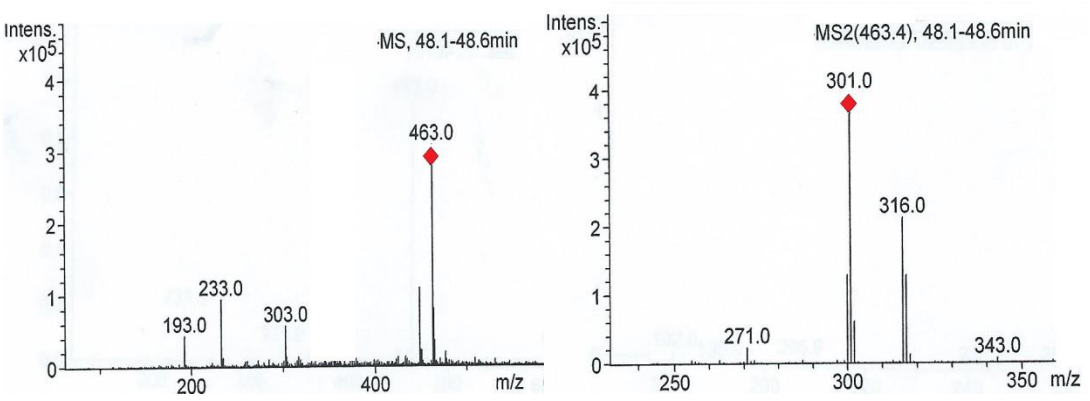

Figure B.17. ESI MS$^-$ fragmentation pattern of peonidin 3-O-glucoside (Rt = 48.1-48.6 min)

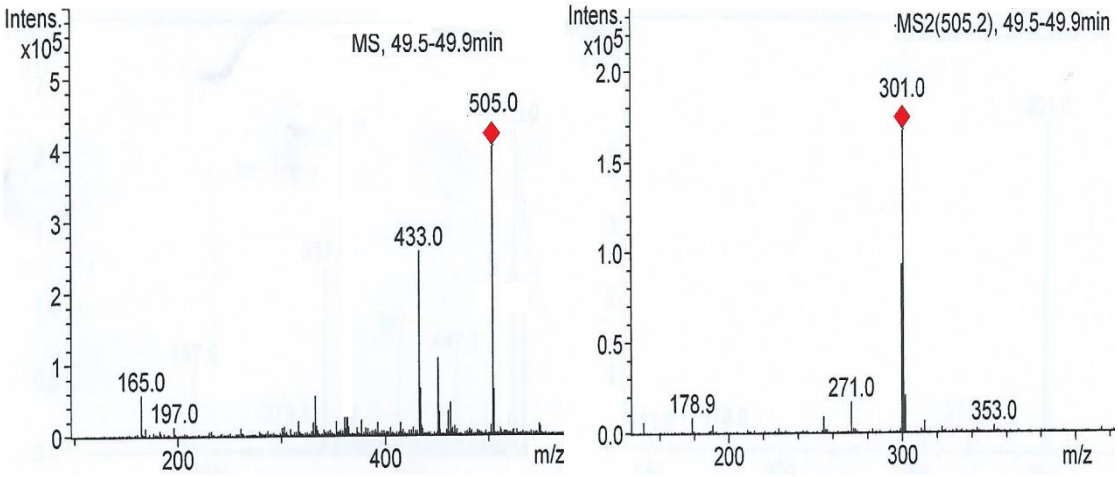

Figure B.18. ESI MS$^-$ fragmentation pattern of peonidin 3-(6”acetoyl) galactoside (Rt = 49.5-49.9 min)

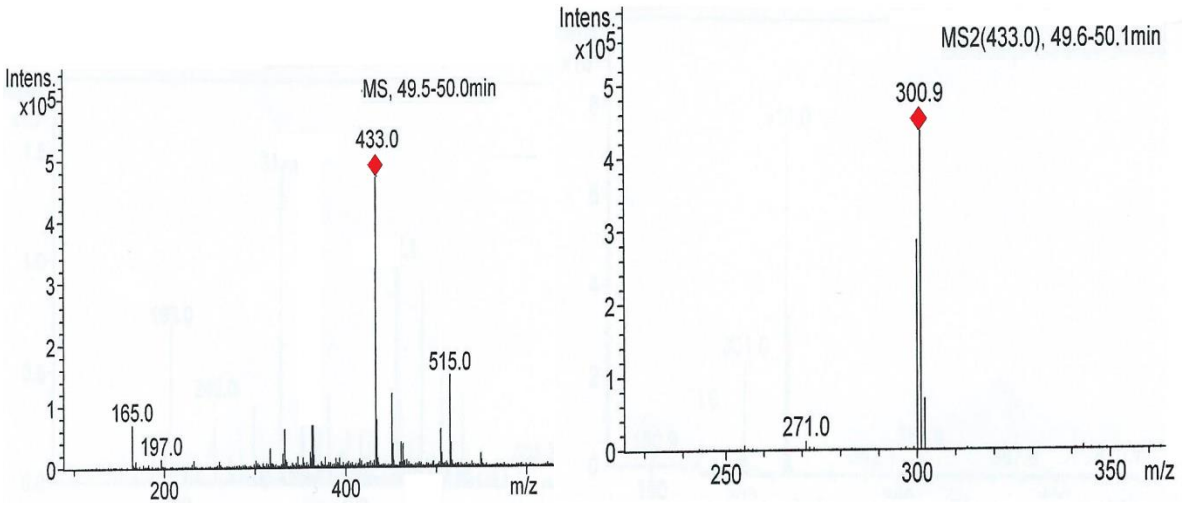

Figure B.19. ESI MS^- fragmentation pattern of quercetin pentose (Rt = 49.6-50.1 min)

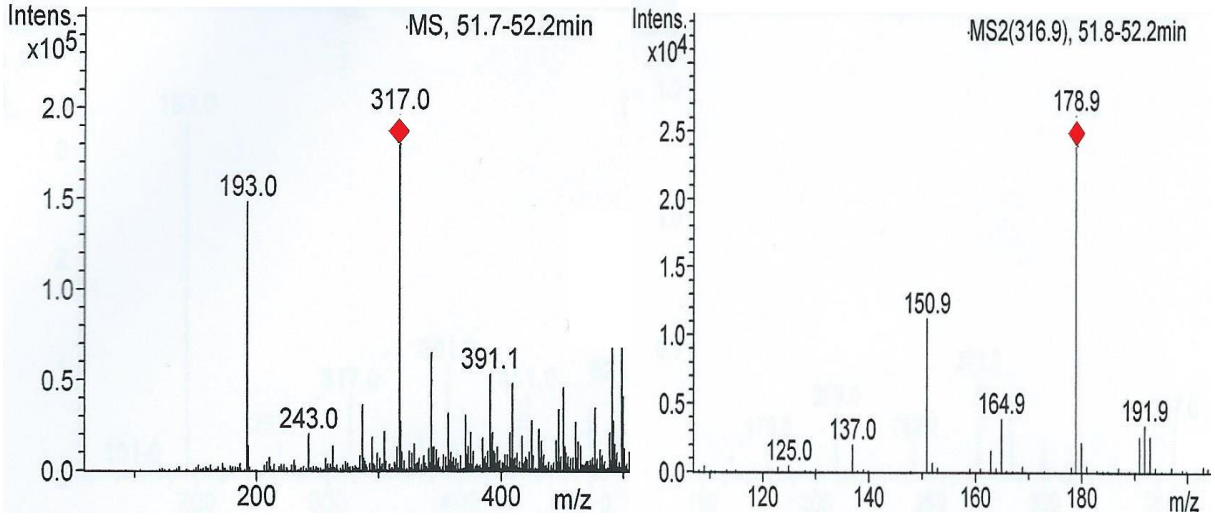

Figure B.20. ESI MS^- fragmentation pattern of myricetin (Rt = 51.8-52.2 min)

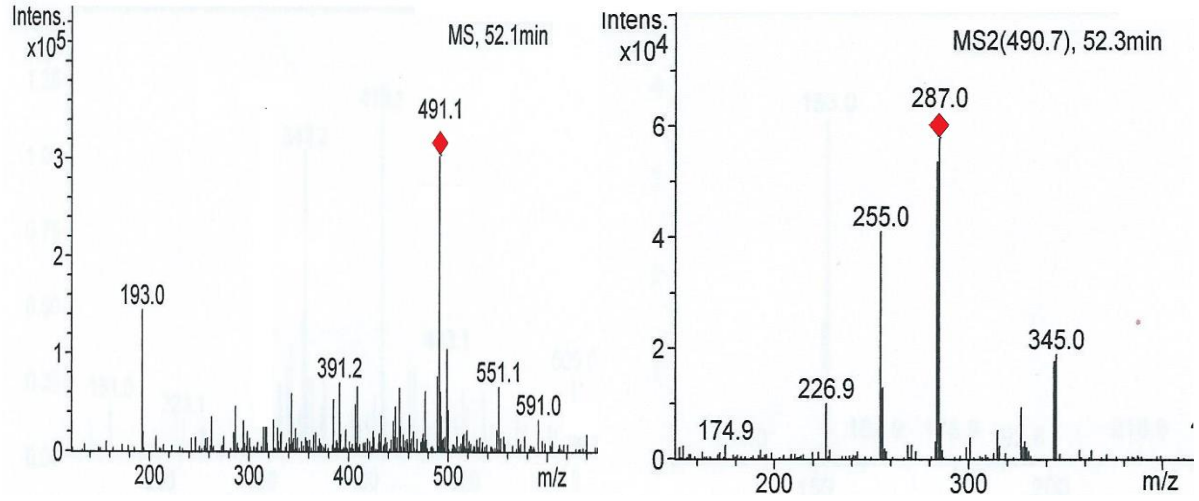

Figure B.21. ESI MS$^-$ fragmentation pattern of cyaniding 3-(6"acetoyl) galactoside (Rt = 52.3 min)

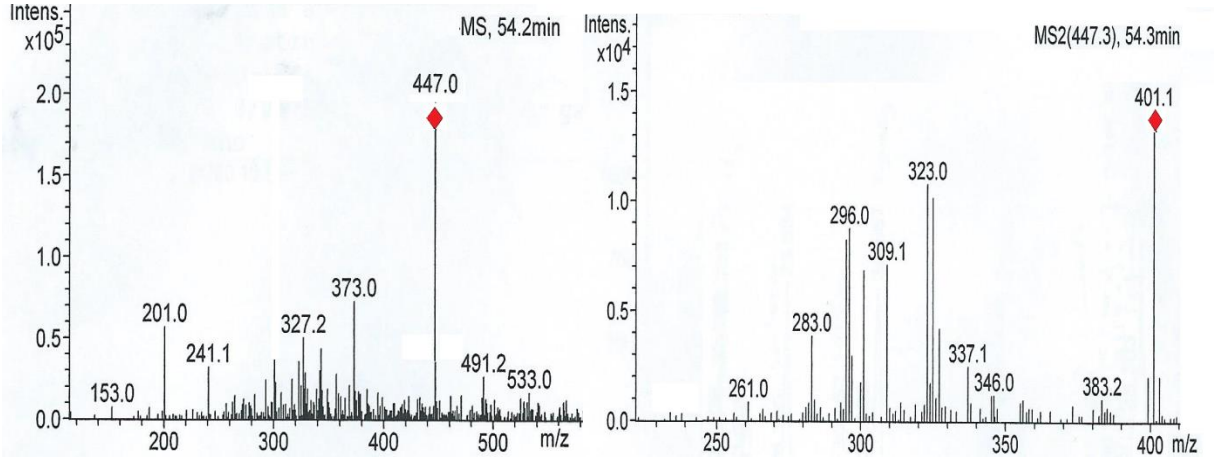

Figure B.22. ESI MS$^-$ fragmentation pattern of kaempherol hexoside (Rt = 54.3 min)

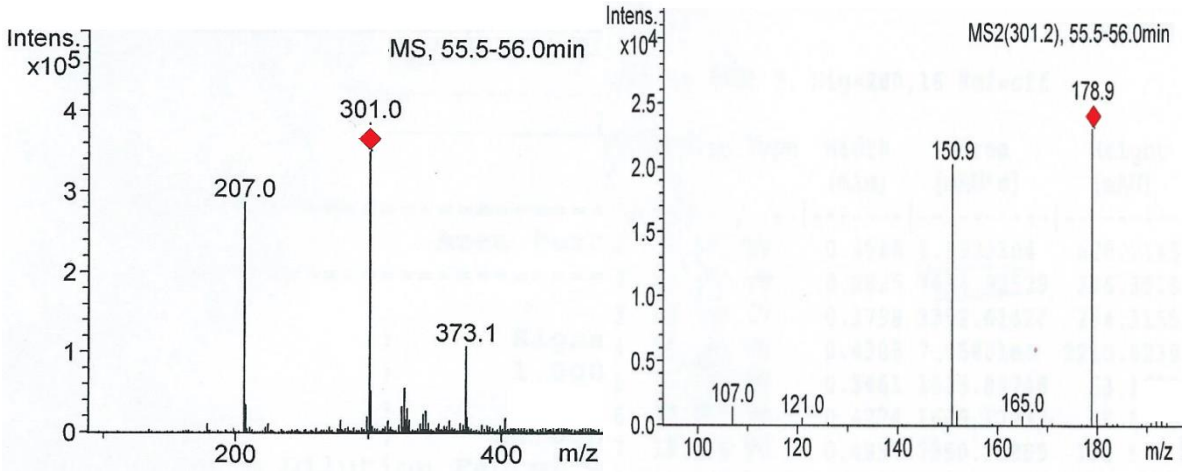

Figure B.23. ESI MS^- fragmentation pattern of quercetin (Rt = 55.5-56 min)

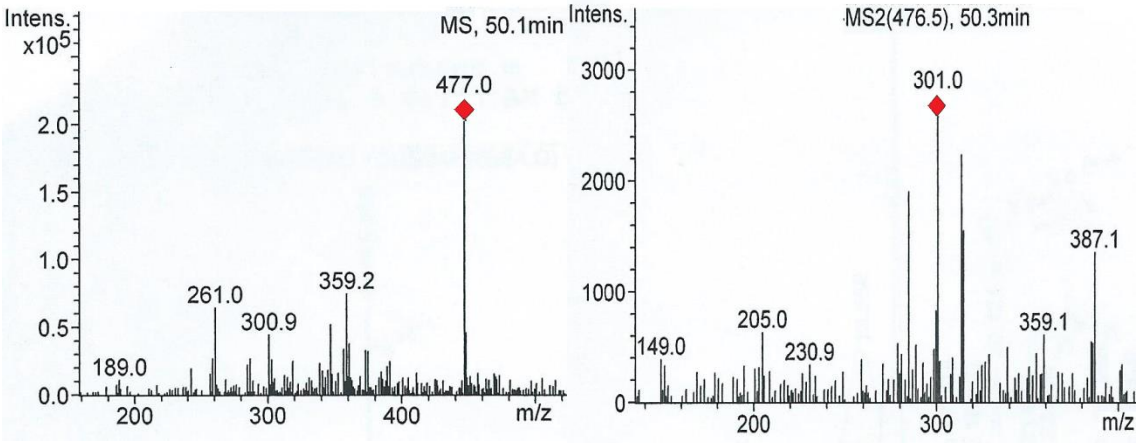

Figure B.24. ESI MS^- fragmentation pattern of quercetin -3-o-glucoronide (Rt = 50.3 min)

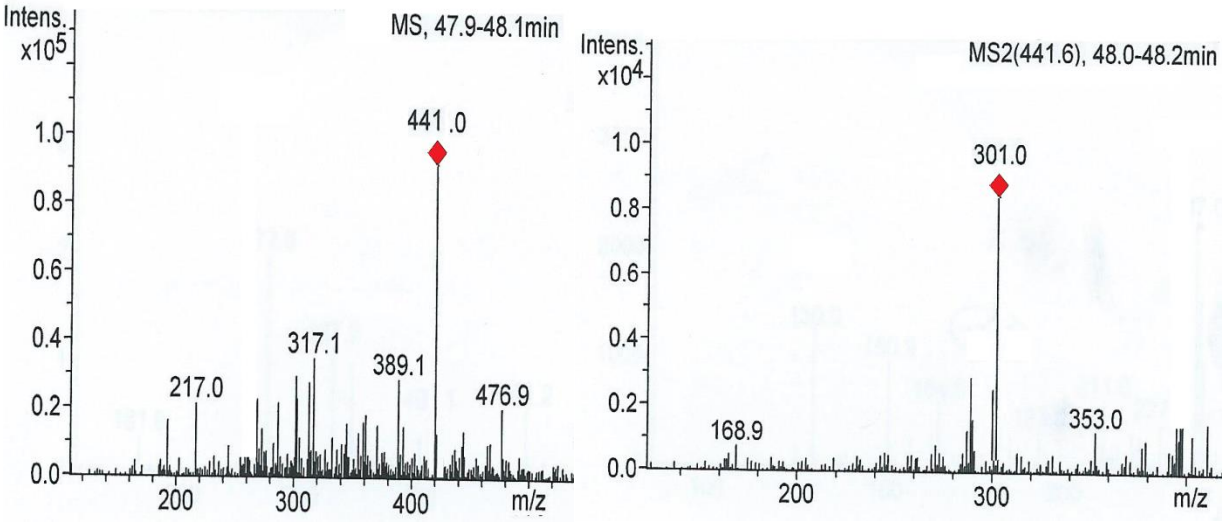

Figure B.25. ESI MS^- fragmentation pattern of epicatechin gallate (Rt = 48-48.2 min)